Handbook of Experimental Pharmacology 200

Bertil B. Fredholm
Editor

Methylxanthines

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Dedication to Klaus Starke

This volume of the *Handbook of Experimental Pharmacology* was one of the last that Klaus Starke initiated as Editor-in-Chief. We dedicate this 200th volume in the series to him.

Klaus was born in 1937. Since his father was an apothecary, it was natural for him to study pharmacy, but he also graduated in medicine. He combined the two, and devoted himself to pharmacology, where he has left a very important mark. Despite calls from many other universities, he remained in Freiburg from 1977. He was one of the scientists who simultaneously realized that neurotransmitters can regulate their own release via presynaptic receptors. This discovery has been extremely important in our understanding of the fine-tuning of neuronal activity. The pharmacology of these presynaptic receptors was recently summarized in volume 184 of the handbook, which was edited by Klaus together with Thomas Südhof. Klaus is deeply respected internationally for his solid science. He is a member of the Academia Europea, the Heidelberger Akademie der Wissenschaften, and the National German Academy "Deutsche Akademie der Naturforscher Leopoldina." He received the Ernst Jung-Preis and the Wilhelm Feldberg-Preis.

In addition to his scientific accomplishments, Klaus has influenced and shaped German pharmacology by his teaching and his dedicated work on numerous committees and in grant-awarding agencies. He has also been deeply involved in many pharmacology journals. The extreme care he took with each manuscript submitted to *Naunyn-Schmiedeberg's Archives of Pharmacology* during his period as managing editor is very memorable to all of us who benefited from his penetrating, but benevolent editing. This devoted attention to both detail and strategic goals was brought by him to the *Handbook of Experimental Pharmacology*. He had, for example, several excellent suggestions on how to organize this volume and he even suggested that we include two poems. One of them was used in the introduction to this volume. The other is printed below. It shows his interests in cultural activities outside pharmacology.

Given the care with which Klaus always dealt with everything, it is with some trepidation that we submit this volume to his scrutiny.

F. Hofmann

Bertil Fredholm
Volume Editor

Franz Hofmann
Editor-in-Chief

For lo! the Board with Cups and Spoons is crown'd,
The Berries crackle, and the Mill turns round;
On shining Altars of *Japan* they raise
The silver Lamp, and fiery Spirits blaze:
From silver Spouts the grateful Liquors glide,
And *China's* earth receives the smoking Tyde.
At once they gratify their Scent and Taste,
While frequent Cups prolong the rich Repast…
Coffee (which makes the Politician wise,
And see through all things with his half-shut Eyes)
Sent up in Vapours to the *Baron's* Brain
New Stratagems, the radiant Lock to gain.

Alexander Pope (1688–1744) *The Rape of the Lock*

Preface

Methylxanthines are doubtless the most widely consumed of all pharmacologically active agents. The reason for this is, of course, that caffeine-containing beverages are consumed on a daily basis by the majority of humans. The human use of coffee and tea was limited until surprisingly recently. Now the global use means that coffee and tea are very important products commercially. Indeed, the sale of tea and coffee has been an important source of national income and for a long time provided the main source of income of the greatest nation in the world at the time, China.

Methylxanthines are found in several plants, from many parts of the world. Coffee beans were probably discovered in Africa, tea leaves in East Asia, mate and cocoa in South America, but it is also found in some 100 other plant species. To make these compounds the plants have developed sophisticated enzymatic machinery. The reason for the investment in methylxanthine synthesis is possibly because methylxanthines can act as a chemical defense, and hence because methylxanthines can have toxic effects. Caffeine is taken up well and distributed throughout the body and elimination depends on a series of enzymatic steps. These differ between species and ages of the same species, including man.

At the beginning of human use of both coffee and tea, the focus was on the medicinal effects, which were both lauded as beneficial and deplored as being detrimental. Now the major interest is perhaps in the public health consequences of the widespread use. Over the years, considerable effort has been spent in population studies to elucidate the risks of caffeine use. One of the surprising things in recent years has been the realization that the evidence for health benefits in, e.g., Parkinson's disease and type II diabetes, has been easier to document than that for possible detrimental effects in, e.g., cardiovascular disease. There are also some possibilities to use methylxanthines or derivatives as drugs. While this is good news, the bad news is that we are still not clear how these effects are brought about. There have been concerns that caffeine may be a major reproductive hazard, but provided that women limit their intake, this may not be a real concern.

Methylxanthines were early shown to cause muscle contractions in high doses, an effect we now know is due to mobilization of intracellular caffeine. In somewhat lower doses, caffeine and theophylline were found to prevent the enzymatic hydrolysis of cyclic AMP. At still lower doses, they block the actions of adenosine at its receptors. All these actions, and some others, contribute to give methylxanthines a complex pharmacological profile, where utmost care must be taken with dosing.

In this volume of the *Handbook of Experimental Pharmacology*, well-known experts describe the facts alluded to above in detail with a focus on caffeine and theophylline. A special chapter is devoted to theobromine, an active component of chocolate, the actions of which are less well characterized. We also present the pharmacology of one xanthine derivative, propentofylline, as an example of a xanthine that has gone through extensive development for a novel therapeutic area.

The powerful effects caffeine exerts on the nervous system are covered. The ability of methylxanthines to influence the physiological processes involved in sleep and the pathophysiological processes involved in pain are described as largely secondary to adenosine antagonism. Methylxanthines can provoke epileptic seizures, and prevent neurodegenerative disease, but the possible mechanisms, involving actions on one or more adenosine receptors, on both neuronal and nonneuronal cells have not yet been fully elucidated. There are interesting therapeutic possibilities, and novel xanthine derivates are being examined. The fact that caffeine-containing beverages have so rapidly established themselves in a variety of cultural settings raises the possibility that caffeine may actually be a dependence-producing drug. Indeed, there are important interactions with some of the neural systems involved in dependence, but caffeine is not a typical drug of addiction, despite the fact that in the famous coffee cantata of Bach (see below) the heroine is almost willing to forego the pleasures of sex for coffee.

It has also been well known for a long time that caffeine (and some of its metabolites) can influence respiration and can be used to treat asthma, that there are increases in cardiac activity and blood pressure, and that methylxanthines have marked renal effects. In all these instances, a major explanation for the effects is blockade of the actions of endogenous adenosine. This is also the reason why methylxanthines can influence cells of the immune system, an action with therapeutic implications, which has been realized for a much shorter time. By contrast, there is evidence that the metabolic effects of coffee and tea may not be entirely explained by adenosine receptor blockade, or by the caffeine content for that matter.

It has been a pleasure to work with world experts in a common effort to produce an up-to-date and authoritative account of the pharmacology of methylxanthines. We have aimed to give more than just a description of facts or findings, and instead to present ideas, concepts, and open questions.

> "Ei! wie schmeckt der Coffee süße,
> Lieblicher als tausend Küsse,
> Milder als Muskatenwein.
> Coffee, Coffee muß ich haben,
> Und wenn jemand mich will laben,
> Ach, so schenkt mir Coffee ein!"
> Lieschens Aria (fourth movement) from Bach's Coffee Cantata BWV 211, "Schweigt stille, plaudert nicht."

Stockholm Bertil Fredholm

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Notes on the History of Caffeine Use

Bertil B. Fredholm

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Abstract As behooves something so deeply entrenched in culture, the historical origins of the use of methylxanthines are unknown and dressed in myth. This is true for coffee as well as tea, and for both it is interesting to note that their common use is really very recent. For coffee we know that its use became more widespread in the fifteenth and sixteenth centuries, and in Europe this occurred in the eighteenth and nineteenth centuries. The use of tea became more common during the Ming Dynasty in China and during the eighteenth century in Britain. Coffee was mostly an upper-class drink in Arabia, and remained a relative luxury in Europe until quite recently. The use of other methylxanthine-containing beverages, such as maté, is even less well known. It is interesting to note that before these drinks were commonly used on a daily basis they were used for medicinal purposes, indicating that their pharmacological actions had long been noted.

Keywords Cocoa · Coffee · Maté · Tea

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1 Mythological Origins[1]

The coffee bush grows wild in many parts of Africa and it may also have been indigenous in Arabia. There is, however, little solid evidence that people had knowledge that the coffee bush could yield a useful drink before Islamic times. Solid evidence is replaced by good mythological stories. One myth is that the archangel Gabriel offered coffee brewed in heaven to Muhammad when he was overcome by sleepiness. A sip of this heavenly coffee was sufficient to make him so vigorous that he could "unhorse 40 men and make 40 women happy." A more often told myth is about an Ethiopian (sometimes Arabian) goatherd, Kaldi, who observed that his goats became very agitated, indeed dancing, when they ate the berries of a certain bush. Having tried them himself, he informed the abbot of a local (perhaps Sufi) monastery, and from there the conquest of the earth was supposed to start. The story of Kaldi appears to occur first in a book by a Maronite professor of oriental languages, Antoine Faustus Nairon, but local sources for the legend are lacking (Nairon 1617). There are also other stories involving the role of the civet cat in spreading the coffee bush, which has been exploited to market very, very expensive kopi luwak coffee (Mair and Hoh 2009), where beans have passed through the gastrointestinal tract of the animal. In a manuscript published in Paris in 1699 by Antoine Galland, the so-called Abd-el-Kadir manuscript, a sheik Omar is credited with the discovery in AD 1258 during his banishment to a mountainous region near Ousab. There he detected a bush with white flowers and red berries and being famished, he cooked a soup of the berries and leaves and was very invigorated. The brew was also able to heal all illness in the surrounding villages.

The history of tea is similarly clouded in myth and national pride (Mair and Hoh 2009) – the Chinese maintain that tea has "always" been part of Chinese culture. A variant of "always" is the story that the nonhistorical second emperor Shen Nung discovered tea when tea leaves blew into his cup of hot water in 2737 BC. The credibility of this is reduced by the "fact" that this emperor was born with the head of a bull and body of a man: he spoke after 3 days, walked within a week, and could plow a field at age three. Many Chinese associate the widespread use of tea with the introduction and spread of Buddhism. According to one account, a Buddhist monk named Gan Lu brought tea back with him when he returned from a pilgrimage to India during the first century. He is supposed to have planted seven "fairy tea trees." They are still shown to tourists on Mt Mengding in Sichuan.

Another, pretty grim, story says tea sprang from the eyelids of Bodhidharma, the first patriarch of Zen. He had sailed from India to China but after he arrived he merely sat down facing a wall at the Shaolin Temple and did not stir for 9 years. Unsurprisingly, the determined saint once drowsed off, so far forgetting himself that his eyes closed momentarily. Without hesitation he sliced off his eyelids to make sure they would never

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[1] The author is heavily indebted to some excellent earlier treatises and the reader is referred to these for references (Ukers 1922; Weinberg and Bealer 2001; Elgklou 1993; Mair and Hoh 2009).

again close and interrupt his wakefulness. Where they fell the compassionate deity Quan Yin caused tea plants to grow to serve Bodhidharma and all who came after him as an aid on the path to enlightenment. The story may have gained particular popularity in Japan, because the Japanese characters for tea leaf and eyelid are the same.

2 Early History of Coffee

It seems clear that coffee beans were first eaten as such; later they were ground up and mixed with fat paste as a stimulating travel snack. Only around AD 1000 did infusions with boiling water start to be used. The word "coffee" derives from *qahva* (or *qahwah*), which is simply a word denoting a drink made from plants. From the initial cultures in Ethiopia, cultivation of coffee bushes soon came to be dominated by Yemen. The city of Mocha became a center – and its name came to denote the drink. Now coffee is grown in 50 different countries around the world (Ukers 1922; Weinberg and Bealer 2001; Elgklou 1993).

Although the Arabs cultivated the plants and prepared drinks from coffee beans, it was only by the fourteenth century that the process of roasting was discovered. And only when this happened did the use of coffee rapidly spread in the Arab world. In the Muslim world the need for a social drink was filled by coffee and the beverage was consumed both at home and in coffee bars. The fact that these coffee houses developed into independent intellectual centers was perceived as a threat to the authorities, and sometimes they were forced to close. Already in the sixteenth century health arguments were used, for example, when Kair Bey, the governor of Mecca, prohibited the use of coffee in 1511. The coffee bars started in the mid sixteenth century in Constantinople, and soon became very popular. They were called *quaweh khaneh* and developed into centers for cultural and intellectual activity. For that reason they also got the name *mekteb-i-irfan* ("the school of the wise"). In Turkey the use of coffee tended to decrease the use of opiates and was promoted by most officials. Since the Arabs controlled a vast territory at the time, the use of coffee spread to Spain, North Africa, India, Turkey, and the Balkans. The permeation into popular culture is revealed by its common use among women, such that a failure to provide sufficient coffee for the wife (or wives) was reason for dissolution of a marriage.

The use of coffee was described in travelogues. The first may have been that of Leonhard Rauwolf (1535–1596) from Augsburg, who published an account in 1582–1583. One particularly well-known description was in *De Plantis Aegypti liber* (Venice, 1592) by Prospero Alpini (1553–1617). He was born in the republic of Venice. After a time in the army, he went to Padua to study medicine, and settled as a physician outside Paduan territory. He traveled to Egypt in 1580 as physician to the Venetian consul in Cairo. He is reputed to have deduced that "the female date-trees or palms do not bear fruit unless the branches of the male and female plants are mixed together; or, as is generally done, unless the dust found in the male sheath or male flowers is sprinkled over the female flowers." He returned to Padua and became professor of botany. Although *De Plantis Aegypti liber* is his best-known work, an earlier work, *De Medicina Egyptiorum* (Venice, 1591), that describes Egyptian medical practices is said to contain the first account of the coffee plant published in Europe. He used the word *caova* to describe the drink.

Another influential writer was Pietro della Valle (1586–1652), who learned Arabic and traveled widely in Turkey, Egypt, Mesopotamia, and Persia. His name for the drink was *cahve*. He was well connected with the Vatican and this argues that it is probably incorrect that Pope Clemens VIII in 1605 (10 years before della Valle) was given coffee by members of the Curia who wanted him to ban this Muslim drink. He is reputed to have said: “This satanic drink is in truth so good that it would be a pity if only nonbelievers were allowed to drink it. We will fool Satan and baptize it so that it becomes a Christian drink, with no danger for the soul.”

According to one story, coffee was introduced to Europe in the seventeenth century. It is said that after their defeat at the gates of Vienna in 1683, the Turks left a large amount of coffee behind, and that the Viennese learned to prepare it and served it with half-moon-shaped cakes. It is, however, well established that coffee was introduced to Europe by Venetian merchants in early 1615. About 10 years later they had learned to roast and grind the imported green beans. The popularity of coffee was promoted by *botteghe del caffé* (coffee shops, originally called *botteghe delle acque e dei giacci*, because all kinds of drinks made with water and ice were served). They were modeled on the Arab establishments and also developed into gradually more sophisticated coffee establishments such as Caffé alla Venezia Trionfante, which opened in 1720. Here was the start of the typical café with newspapers and reviews that developed into cultural and commercial centers. By the mid eighteenth century Venice had more than 200 such establishments. The first Viennese establishment was established by Georg Kolshnitzky (according to legend using the Turkish leftover coffee sacks). In Paris, Café Procope, in rue de l′Ancienne Comédie, was opened in 1686 by the Sicilian Francesco Procopio dei Coltelli. It still exists and was given a name that alluded to hidden secrets in high places by alluding to the Byzantine historian Procopius, whose secret history had just been discovered and published for the first time ever in 1623. The café was a meeting place of the chic elite especially after Comédie Française opened across the street a few years later. It was a famous meeting place of the encyclopedists and the early revolutionaries, but also the literary elite after the political restoration and late nineteenth century met there. The first coffee house in London was apparently opened even earlier, in 1652.

Venice also became the transit port for shipment of coffee to other parts of Europe. By the end of the seventeenth century, the use of coffee in Europe was widespread. For some time the Arab countries maintained their monopoly of supply, but in the seventeenth century plants were smuggled to India and to Amsterdam and from there to the Dutch colonies in the East Indies. However, coffee had been grown outside the Arab countries and Ethiopia before that as small coffee plantations (and there had also been tea plantations of course) founded on Ceylon by the Portuguese in the early sixteenth century. By the beginning of the eighteenth century, Java and Sumatra were the main suppliers. The quality of the

Indonesian coffee was, however, for long inferior to that of the Yemenite coffee – at least if we are to believe Voltaire, who spoke of the ". . . mauvais café de Batavia et des iles" and explicitly preferred the Mocha coffee. One celebrated description of coffee farming is by Karen Blixen (pen name Isak Dinesen), whose book *Out of Africa* (Dinesen 1937) was filmed (albeit not following the book very closely) with Meryl Streep playing the author and Robert Redford playing her husband, Bror. The book starts, "I had a farm in Africa at the foot of the Ngong Hills...," and in it she describes both ups and downs and the hard labor involved: "Coffee-growing is a long job. It does not all come out as you imagine, when, yourself young and hopeful, in the streaming rain, you carry the boxes of your shining young coffee plants from the nurseries...patiently, awaiting coming bounties" (Dinesen 1937; Lorenzetti and Lorenzetti 1999).

The spread of producers allowed coffee to become a mass product. It is culturally very much entrenched and it is relevant that the culture associated with the coffee house has been the subject of much intense research (Ellis 2007). It is beyond the scope of this historical sketch to outline the development of the coffee business as we now see it (Ukers 1922; Weinberg and Bealer 2001; Prendergast 1999). Suffice it to say here that it is remarkable that coffee has in a few hundred years become such an important part of everyday culture throughout the world that it has become one of the commercially most important traded commodities.

3 Early History of Tea

It is commonly stated that tea use was common in China for thousands of years before Christ. This is difficult to verify and there can be no one simple explanation for China's nationwide adoption of the tea habit. Tea is (probably – but it could be other infusions) referred to in old texts from the first centuries BC. A clear unambiguous reference to the tea plant occurs only from AD 750. Alerting and mood-elevating effects are referred to in early literature. Indeed, this early literature mainly refers to the use of tea for medicinal purposes (Mair and Hoh 2009). However, by the Tang Dynasty, and particular Emperor Tai-tsung (627–649), the cultural and ceremonial aspects took a larger part. During this dynasty the famous *Chá Ching* (*The Classic of Tea*) was written as a manual of tea connoisseurship (Lu 1974) by Lu Yu. This developed into a ceremonial use of tea that incorporated aspects of both Taoism and Confucianism. Tea fell somewhat out of favor as a drink during the years of the Mongol Yuan Dynasty and it does not figure in Marco Polo's descriptions of court life[2] (but he did describe how important taxes on tea were for the national economy). Tea clearly was of major importance in trade – in particular

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[2] This has, however, also been interpreted as evidence that Marco Polo never visited the imperial court.

the trade of horses for tea, which represented a major part of the foreign/defense policy of China (Mair and Hoh 2009).

Tea use increased in popularity under the Chinese Ming Dynasty (1368–1644), which represented a return to power of the Han people, and it was in this period that tea began to be brewed by steeping cured loose leaves in boiling water. Because it was at this time that the tea was first tried by Europeans, it was this method of making tea that became popular in the West and remains so to this day. Also under the Ming Dynasty different types of teas, including fermented black teas, unfermented green teas, and the semifermented variety that is now known as oolong, were developed. During the Ming Dynasty there were tea houses that apparently functioned much as the coffee houses in Arab countries (Mair and Hoh 2009).

Tea was introduced to Japan around 800 BC and it came with all the ceremonial and quasi-religious overtones. However, it took many centuries until tea use became more popular, and it coincided in time with the spread of the Zen variant of Buddhism. The monk Myoan Eisai is credited with the popularization, including the demonstration for the shogun that tea can help you sober up a bit after too much sake. In Japan the institutions of the tea house and tea garden, as well as the Tea Ceremony, reached full development through a series of Zen monks (Mair and Hoh 2009). Of particular importance was Sen No Rikyu (1522–1591), Tea Master of the powerful political leader Hideyoshi, who incorporated the essence of Zen into the Tea Ceremony, and it is in the form he developed that the Way of Tea (*chado*) is practiced through the Tea Ceremony to this day. This classical ceremony is based on powdered green tea that is intensely whisked. By the mid sixteenth century traditions based on “boiled tea,” *sencha*, using intact tea leaves were established (Mair and Hoh 2009). A well-known description of tea use in Japan (with notes on the rest of the world) was written by Okakuzo Kakuzo (2000).

Tea arrived in Europe about the same time as coffee did. The first green tea leaves from China were brought to Amsterdam by the Dutch East India Company and tea was drunk in France by 1636. Tea in Russia was first offered by China as a gift to Czar Michael I in 1618. Tea appeared in Germany by 1657. In Britain tea was apparently first publicly distributed in the 1650s by Thomas Garraway and it was within the confines of his coffee house. Whereas tea never became very popular in mainland Europe (except Russia), by 1730 the use of tea in England had passed the use of coffee. The reason for this could be the strong position of the British East India Company. Despite the fact that tea is an indigenous Indian plant, culture of tea plants began in earnest only after the British rule. In particular, the teas producing the Darjeeling variety were from Chinese plants planted during British rule. The native Assam tea was also being cultivated more systematically, and cultivation was transferred to Ceylon.

Like the Arabs in the case of coffee, the Chinese wanted to monopolize tea trade, and, conversely, many wanted to get plants and grow them. One well-known story is how the Swedish naturalist Carl von Linné (1707–1778) attempted to get tea plants for his botanical garden in Uppsala. The idea was to test if tea could be grown locally and therefore improve the national economy. He made a deal with the Swedish East India Company to have one of his students travel with it and bring home a plant. The student, Pehr Osbeck, managed to collect numerous plants and bring them home, but the one tea plant brought on board that survived most of the sea voyage during the first trip was knocked overboard when the crew fired a salute. He lost a plant on his second trip during a storm off the Cape of Good Hope. The third effort was a plant brought by a director of the East India Company directly to von Linné, but it soon proved not to be the proper plant at all but another species of *Camellia*. The fourth one was the proper thing, but was deemed so valuable that it was left in the commander's safe room – where it was devoured by rats. Finally, one of two plants brought as potted seeds was brought by the wife of the commander of the ship herself after the first one had been destroyed in transit. Finally, in October 1763 von Linné was the first person in Europe to have a tea plant of his own (Mair and Hoh 2009). The plants gradually died and by 1781 only one remained. By now tea is widely used throughout the world (but not very much in Sweden).

Even though coffee was the first methylxanthine-containing drink associated with Muslim countries[3], tea has subsequently become the dominant drink in these parts of the world (Mair and Hoh 2009). In the UK the first such drink was again coffee, but by the nineteenth century there was a switch to tea; in the USA the opposite development is seen. In each of these cases there are interesting political overtones (Mair and Hoh 2009).

4 The History of Cocoa and Maté

These two methylxanthine-containing beverages have Latin American or Central American origins. Cocoa beans were used and the tree was cultured by the Olmec people (1500–400 BC), and was later used by the Maya culture. The Maya recorded their use in writing. After them Toltec and Aztec cultures used it, and via the latter the Spaniards (Hernán Cortés, 1485–1547) came in contact with the drink and its proper preparation. The huge consumption of chocolate by the Montezuma court was recorded by the historiographer Bernal Diaz del Castillo (Ukers 1922; Coe and Coe 1996).

In all probability the cocoa seeds brought by Columbus to King Ferdinand in 1502 were the first plant products containing methylxanthines brought to Europe, and the Spanish royalty was thus prepared when Cortes wrote a exuberant description calling cacao the "drink of goods." Cortés returned to the Americas to set up cocoa plantations for King Charles V in Haiti, Trinidad, and Fernando Po (Weinberg and Bealer 2001; Coe and Coe 1996). The Indians probably mixed vanilla with the cocoa to reduce the bitter taste. In Europe sugar in different forms was added as were milk products. Now, of course, cocoa products are eaten as much as they are drunk (Coe and Coe 1996).

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[3] However, tea export from China to muslim countries had been important for a long time.

Maté is prepared from the leaves of a holly (*Ilex paraguariensis*) harvested in pre-Hispanic times along the Paraná–Paraguay river system. It has oval, dark green leaves that are six to eight inches long and white flowers. As with tea, the best product is from young, unopened leaflets. The history of this use is clouded in mystery, but the use is often associated with the Guarani Indians, who have a myth of the maté being a present from a shaman to a traveling chief. It is clear that the Spanish invaders rapidly adopted the practice and saw economic possibilities. Jesuits developed maté into a plantation crop and before 1700 the use had spread along the Andes and the Rio de la Plata. However, the use has never spread much beyond South America.

5 Methylxanthines and Health

As already noted, in the earliest history of methylxanthine use the medicinal effects were important (Ukers 1922; Weinberg and Bealer 2001; Mair and Hoh 2009). For example, in India, where tea is indigenous, tea was long used for medicinal purposes, but its social use was limited. Just as there were overblown claims for the wholesome effects of the drinks made with coffee, tea, or cocoa, there were claims about the negative health consequences. A case in point is an apocryphal experiment of Gustaf III (1746–1792). This Swedish king was reported to be convinced that coffee was dangerous and in order to prove his opinion he is said to have given two condemned twins an alternative to death: they should take part in a long-term experiment. One twin was to drink coffee, the other tea. Reportedly, the experiment was ended when the tea-drinking twin died at age 83. Unfortunately much of the early literature of the health effects is of the same standard and the present author is not a great believer in the usefulness of mining old literature for clues about medicinal herbs in general. Only by the mid nineteenth century did the reports of health effects get a sufficiently scientific basis to be taken seriously. Some of the early literature is cited in relevant chapters of this volume. One very clear example is the reports of the usefulness of coffee in asthma (Salter 1860). There were also early correct reports on the negative effects of high doses of caffeine (Cole 1833). An attitude representative of much later literature is represented by the “discoverer” of homeopathy, Samuel Hahnemann (1755–1843), who found the alerting effects of caffeine to be very beneficial, but warned against its use because it involved the disruption of a natural balance (Hahnemann 1803).

6 The Early Science of Methylxanthines

It was early realized that the effects of coffee were due to some active principle. Prompted by Goethe, the young amateur chemist Friedrich Ferdinand Runge (1795–1865) was able to identify in a rather pure form a substance we call caffeine (Weinberg and Bealer 2001). The chemistry of caffeine and its relatives was clarified by the great Hermann Emil Fischer (1852–1919) in a series of studies that were explicitly cited in his Nobel prize nomination and lecture in 1902. The study of caffeine was also his first major effort, published when he was 29–30 years of age (Fischer 1881a, b, 1882). Using oxidation with moist chlorine, he found that caffeine had a similar heterocyclic skeleton as uric acid. He then found that it was a trimethylxanthine, but he struggled for some time to clarify the structure of xanthine. However, it was only when Fischer turned to a synthetic approach to structure that he finally correctly realized that the xanthine structure proposed by Ludwig Medicus (1847–1915) prior to Fischer was the correct one (Fischer 1897).

Among the dimethylxanthines, theobromine was first identified in 1841. The name derives from the name of the cocoa plant and refers to the name given to it by Cortés, “the food of Gods” (*Theobroma*). Fischer discovered a synthesis in 1882. Theophylline was identified as a minor component in tea in 1888, and a synthetic pathway was devised by Fischer, who also established the structure. Already at the beginning of the twentieth century theophylline was introduced in the clinic, first as a diuretic, later in the treatment of asthma. The third dimethylxanthine, paraxanthine, is not a major component in plants but is the major metabolite of caffeine in several species, including man.

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Distribution, Biosynthesis and Catabolism of Methylxanthines in Plants

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Abstract Methylxanthines and methyluric acids are purine alkaloids that are synthesized in quantity in a limited number of plant species, including tea, coffee and cacao. This review summarizes the pathways, enzymes and related genes of caffeine biosynthesis. The main biosynthetic pathway is a sequence consisting of xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine. Catabolism of caffeine starts with its conversion to theophylline. Typically, this reaction is very slow in caffeine-accumulating plants. Finally, the ecological roles of caffeine and the production of decaffeinated coffee plants are discussed.

Keywords Biosynthesis · Caffeine · Catabolism · Coffee · *N*-Methyltransferase · Tea · Theobromine

1 Introduction

Methylxanthines and methyluric acids (Fig. 1) are secondary plant metabolites derived from purine nucleotides (Ashihara and Crozier 1999a). The most well known methylxanthines are caffeine (1,3,7-trimethylxanthine) and theobromine (3,7-dimethylxanthine), which occur in tea, coffee, cacao and a number of other non-alcoholic beverages of plant origin. Caffeine was isolated from tea and coffee in the early 1820s, but the main biosynthetic and catabolic pathways of caffeine were not fully established until recently, when highly purified caffeine synthase was obtained from tea leaves and a gene encoding the enzyme was cloned (Kato et al. 1999; Kato et al. 2000). In this chapter, the distribution, biosynthesis and catabolism of methylxanthines in plants are described. Furthermore, the roles of methylxanthines *in planta* and production of decaffeinated coffee plants are summarized.

2 Distribution of Methylxanthines in Plants

Methylxanthines have been found in nearly 100 species in 13 orders of the plant kingdom (Ashihara and Suzuki 2004; Ashihara and Crozier 1999a). Compared with other plant alkaloids, such as nicotine, morphine and strychnine, purine alkaloids are distributed widely throughout the plant kingdom although accumulation of high concentrations is restricted to a limited number of species, including *Coffea*

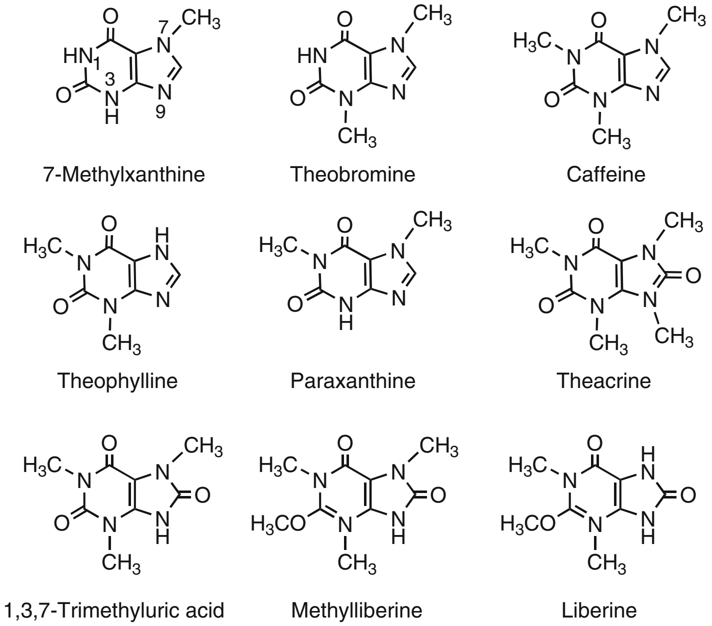

Fig. 1 Structures of purine alkaloids present in plants constituting methylxanthines (7-methylxanthine, theobromine, caffeine, theophylline and paraxanthine) and methyluric acids (theacrine, 1,3,7-trimethyluric acid, methylliberine and liberine)

arabica (coffee), *Camellia sinensis* (tea) and *Theobroma cacao* (cacao). All caffeine-containing plants, except *Scilla maritima*, belong to the Dicotyledoneae. In some species the main methylxanthine is theobromine or methyluric acids, including theacrine (1,3,7,9-tetramethyluric acid), rather than caffeine (Ashihara and Crozier 1999a).

2.1 *Coffee and Related* Coffea *Plants*

The caffeine content of seeds of different *Coffea* species varies from 0.4 to 2.4% dry weight (Mazzafera and Carvalho 1992). Green beans (as opposed to roasted beans, which are used to prepare the beverage) of current commercially cultivated coffee plants contain substantial quantities of caffeine; arabica coffee (*Coffea arabica*) beans usually contain 1.2–1.4% caffeine (Charrier and Berthaud 1975), while robusta coffee (*Coffea canephora*) contains 1.2–3.3% caffeine (Charrier and Berthaud 1975; Mazzafera and Carvalho 1992). There are also several wild coffee species where the green beans contain either no caffeine or extremely low levels of caffeine. Such low-caffeine species include *Mascarocoffea* sp. and *Coffea eugenioides* (Mazzafera and Carvalho 1992; Rakotomalala et al. 1992; Campa et al. 2005).

Caffeine is distributed mainly in the leaves and cotyledons of *Coffea arabica* seedlings, at concentrations ranging from 0.8 to 1.9% dry weight. Essentially, there is no caffeine in roots or in the older brown parts of the shoot (Zheng and Ashihara 2004). Mature leaves of *Coffea liberica*, *Coffea dewevrei* and *Coffea abeokutae* contain the methyluric acids theacrine, liberine [*O*(2),1,9-trimethyluric acid] and methylliberine [*O*(2),1,7,9-tetramethyluric acid] (Fig. 1) (Baumann et al. 1976; Petermann and Baumann 1983). Examples of the purine alkaloid content in the seeds of *Coffea* species are illustrated in Fig. 2a.

2.2 Tea and Related Camellia *Plants*

The caffeine content of young leaves of first flush shoots of *Camellia sinensis*, *Camellia assamica* and *Camellia taliensis* is 2–3% of dry weight, while the level in *Camellia kissi* is less than 0.02%. Unusually, theobromine is the predominant purine alkaloid (5.0–6.8%) in young leaves of a Chinese tea, kekecha (cocoa tea) (*Camellia ptilophylla*) (Ye et al. 1997), and *Camellia irrawadiensis* (less than 0.8%) (Nagata and Sakai 1985). Theacrine and caffeine are the major purine alkaloids in the leaves of another Chinese tea called "kucha" (*Camellia assamica* var. *kucha*). The endogenous levels of theacrine and caffeine in expanding buds and young leaves of kucha are approximately 2.8 and 0.6–2.7%, respectively (Zheng et al. 2002). Some examples of the purine alkaloid content of the leaves of *Camellia* species are shown in Fig. 2b.

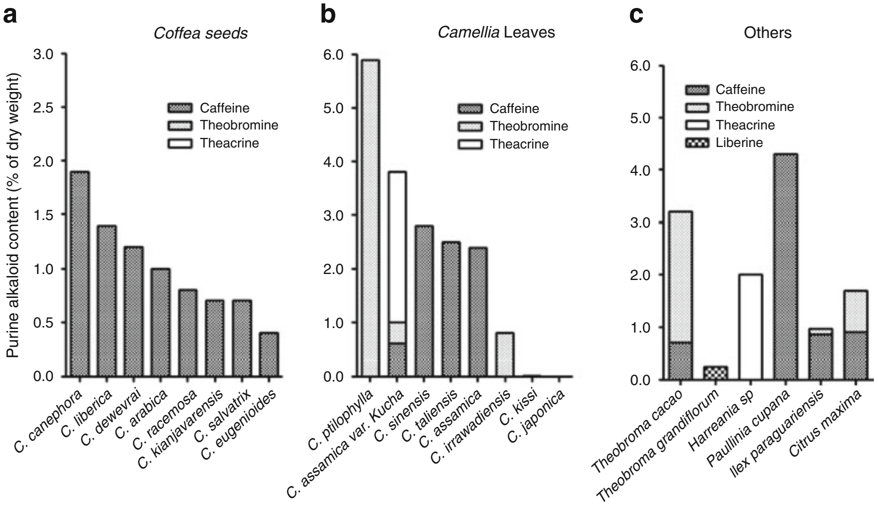

Fig. 2 The methylxanthine and methyluric acid content of selected plant species. **a** Leaves of *Camellia* species, **b** seeds of *Coffea* species and **c** seeds of *Theobroma cacao* (cacao), *Theobroma grandiflorum* (cupu), *Herrania* sp. and *Paullinia cupana* (guarana), leaves of *Ilex paraguariensis* (maté) and anthers of *Citrus maxima* (pomelo). Values were obtained from references cited in the text

2.3 Cacao and Related Theobroma *and* Herrania *Plants*

Theobromine is the dominant purine alkaloid in seeds of cacao (*Theobroma cacao*). The cotyledons of mature beans contain 2.2–2.7% on a dry weight basis and 0.6–0.8% caffeine, while shells contain 0.6–0.7% theobromine and 0.5–0.6% caffeine (Senanayake and Wijesekera 1971). Examination of several cacao genotypes representing the three horticultural races Criollo, Forastero and Trinitario revealed considerable variations in the purine alkaloid content of the seed, with slightly higher levels found within the Criollo types (Hammerstone et al. 1994). Roasted seeds of *Theobroma cacao* are used to make cocoa and chocolate products (Duthie and Crozier 2003).

Cupu (*Theobroma grandiflorum*) contains 0.25% liberine in cotyledons and 0.08% in the nut shells (Baumann and Wanner 1980). Hammerstone et al. (1994) reported that theacrine is the principal purine alkaloid in seeds of 11 species of *Theobroma* and nine species of *Herrania*. Quantitative data on purine alkaloid levels in *Theobroma* and *Herrania* species are presented in Fig. 2c.

2.4 Maté, Guarana and Other Species

Maté (*Ilex paraguariensis*) leaves are used to make a beverage that is consumed widely in rural areas of Argentina, Paraguay and Brazil. Young maté leaves contain caffeine (0.8–0.9%), theobromine (0.08–0.16%) and theophylline (less than 0.02%). Methylxanthines have been detected in *Paullinia cupana* (guarana), *Paullinia yoco*, *Paullinia pachycarpa*, *Cola* species and *Citrus* species (Baumann et al. 1995; Kretschmar and Baumann 1999; Weckerle et al. 2003). In seeds of guarana, caffeine is located mainly in the cotyledons (4.3%) and testa (1.6%). Citrus flowers can accumulate up to 0.17% methylxanthines on a fresh weight basis; caffeine is the main methylxanthine, but theophylline is also present. Trace quantities of caffeine have also found in the nectar of citrus flowers (Weckerle et al. 2003). Quantitative data of selected samples are shown in Fig. 2c.

3 Methylxanthine Biosynthesis in Plants

Methylxanthines are formed from purine nucleotides in plants. Historically, there have been a number of proposals on the pathways involved in such conversions (see Ashihara and Crozier 1999a). However, data from studies on in situ metabolism of labelled precursors, as well as enzymes and genes have established that the main caffeine biosynthetic pathway is a four-step sequence consisting of three methylations and one nucleosidase reaction starting with xanthosine acting as the initial substrate (Fig. 3). Although the information has been obtained mainly from coffee

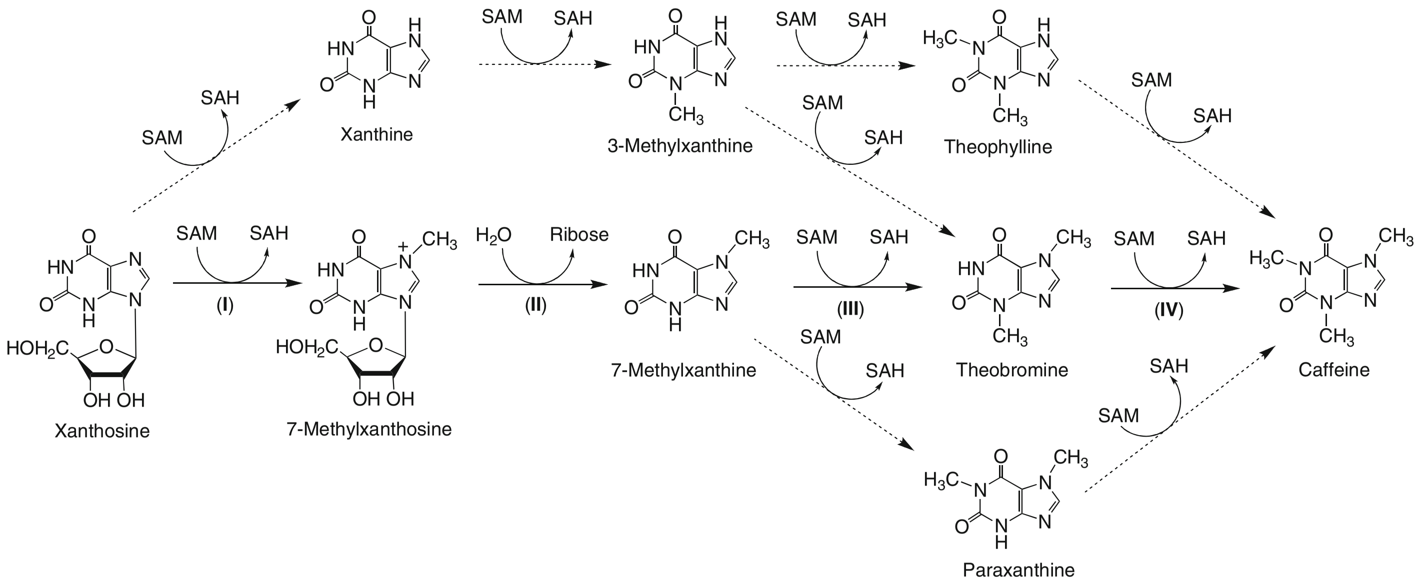

Fig. 3 The biosynthetic pathways of caffeine from xanthosine. The major pathway consists of four steps from I to IV. The enzymes involved are as follows: 7-methylxanthosine synthase (EC 2.1.1.158) (I and II); *N*-methylnucleosidase (EC 3.2.2.25) (II); theobromine synthase (EC 2.1.1.159) (III); caffeine synthase (EC 2.1.1.160) (III and IV). Minor pathways, shown with *dotted arrows*, may occur because of the broad substrate specificities of the *N*-methyltransferases. *SAM S*-adenosyl-L-methionine, *SAH S*-adenosyl-L-homocysteine

(*Coffea arabica*) and tea (*Camellia sinensis*), the available evidence suggests that the pathway is essentially the same in other methylxanthine-forming plants (Ashihara et al. 1998; Zheng et al. 2002; Koyama et al. 2003).

3.1 Formation of 7-Methylxanthine

The formation of monomethylxanthine in the main caffeine biosynthetic pathway is initiated by the conversion of xanthosine to 7-methylxanthosine (Fig. 3). This reaction is catalysed by 7-methylxanthosine synthase (xanthosine 7*N*-methyltransferase, EC 2.1.1.158). The genes encoding 7-methylxanthosine synthase, *CmXRS1* (AB034699) and *CaXMT* (AB048793), were isolated from *Coffea arabica* (Mizuno et al. 2003a; Uefuji et al. 2003). The second step involves a nucleosidase which catalyses the hydrolysis of 7-methylxanthosine. It was thought that *N*-methylnucleosidase (EC 3.2.2.25), which occurs in tea leaves, participates in this reaction (Negishi et al. 1988), but structural studies on coffee 7-methylxanthosine synthase suggested that the methyl transfer and nucleoside cleavage may be coupled and catalysed by a single enzyme (McCarthy and McCarthy 2007).

3.2 Formation of Theobromine

The third step in the caffeine biosynthesis pathways is also catalysed by *S*-adenosyl-L-methionine (SAM)-dependent *N*-methyltransferase(s). Highly purified caffeine synthase (EC 2.1.1.160) obtained from young tea leaves has broad substrate specificity and catalyses the two-step conversion of 7-methylxanthine to caffeine via theobromine (Kato et al. 1999). This enzyme is distinct from the *N*-methyltransferase that catalyses the first methylation step in the caffeine pathway. The isolated complementary DNA from young tea leaves, termed *TCS1* (AB031280), consists of 1,438 base pairs and encodes a protein of 369 amino acids (Kato et al. 2000). The function of *TCS2* (AB031281), which occurs as a paralogous gene to *TCS1* in the tea genome, has not yet been determined (Yoneyama et al. 2006). Plural genes encoding *N*-methyltransferases which have different substrate specificities have been isolated from coffee plants. *CCS1* (AB086414), *CtCS*7 (AB086415) and *CaDXMT*1 (AB084125) are caffeine synthase genes (Mizuno et al. 2003a; Uefuji et al. 2003). The recombinant caffeine synthases (EC 2.1.1.160) can utilize paraxanthine, theobromine and 7-methylxanthine as substrates. *CTS1* (AB034700), *CTS2* (AB054841), *CaMXMT1* (AB048794) and *CaMXMT2* (AB084126) were identified as genes encoding theobromine synthase (Mizuno et al. 2001; Ogawa et al. 2001). The activity of the recombinant theobromine synthase (EC 2.1.1.159) is specific for the conversion of 7-methylxanthine to theobromine.

Theobromine synthase, but not the dual-functional caffeine synthase, appears to participate principally in theobromine synthesis in theobromine-accumulating plants, such as *Theobroma cacao*, *Camellia ptilophylla* and *Camellia irrawadiensis* (Yoneyama et al. 2006).

3.3 Conversion of Theobromine to Caffeine

Conversion of theobromine to caffeine is performed by the dual-functional caffeine synthase discussed already. The methylation of N1 of 7-methylxanthine by caffeine synthase is much slower than that of N3, and as a consequence, theobromine is temporally accumulated in caffeine-synthesizing tissues. This is the final step in the main caffeine biosynthesis pathway, i.e., xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine.

To date, three caffeine synthase genes have been identified in coffee plants (Mizuno et al. 2003b; Uefuji et al. 2003). Expression profiles of these genes in different organs are variable and the kinetic properties of each recombinant enzyme, such as k_m values, are different. Therefore, the enzymes participating in caffeine biosynthesis in organs and at different stages of growth may vary.

In addition to the main caffeine biosynthesis pathway, various minor routes may also operate (Fig. 3) which are mainly dependent upon the broad specificities of the *N*-methyltransferases, especially caffeine synthase. For example, caffeine synthase catalyses the synthesis of 3-methylxanthine from xanthine. Paraxanthine is synthesized from 7-methylxanthine. However, little accumulation of these compounds occurs in plant tissues. 3-Methylxanthine may be catabolized to xanthine, and paraxanthine appears to be immediately converted to caffeine. Paraxanthine is the most active substrate of caffeine synthase, but only limited amounts of paraxanthine accumulate in plant tissues, because the N1-methylation of 7-methylxanthine is very slow (Ashihara et al. 2008).

3.4 Formation of Methyluric Acids

Formation of methyluric acids occurs in a limited number of plant species. As noted in Sect. 2.2, theacrine is found in kucha leaves in high concentrations (Zheng et al. 2002). Radiolabelled feeding experiments, indicate that theacrine is synthesized from caffeine. Conversion of caffeine to theacrine probably occurs by successive oxidation and methylation steps with 1,3,7-trimethyluric acid acting as the intermediate. Leaves of *Coffea dewevrei*, *Coffea liberica* and *Coffea abeokutae* convert caffeine to liberine probably via theacrine and methylliberine (Petermann and Baumann 1983).

3.5 Supply of Xanthosine for Caffeine Biosynthesis

Xanthosine, the initial substrate of purine alkaloid synthesis, is supplied by at least four different pathways: de novo purine biosynthesis (de novo route), degradation of adenine nucleotides (AMP route), the SAM cycle (SAM route) and guanine nucleotides (GMP route) (Fig. 4).

3.5.1 De Novo Route

Like mammals, plants synthesize purine nucleotides by de novo and salvage pathways (Ashihara and Crozier 1999a; Moffatt and Ashihara 2002; Stasolla et al. 2003), although some sections of the pathways are unique to plants. Utilization of IMP, formed by the de novo purine biosynthetic pathway, for caffeine biosynthesis was demonstrated in young tea leaves using ^{15}N-glycine and ^{14}C-labelled precursors and inhibitors of de novo purine biosynthesis (Ito and Ashihara 1999). Xanthosine is produced by an IMP → XMP → xanthosine pathway. IMP dehydrogenase (EC 1.1.1.205) and 5′-nucleotidase (EC 3.1.3.5) catalyse these reactions. Ribavirin, an inhibitor of IMP dehydrogenase, reduces the rate of caffeine biosynthesis in tea and coffee plants (Keya et al. 2003).

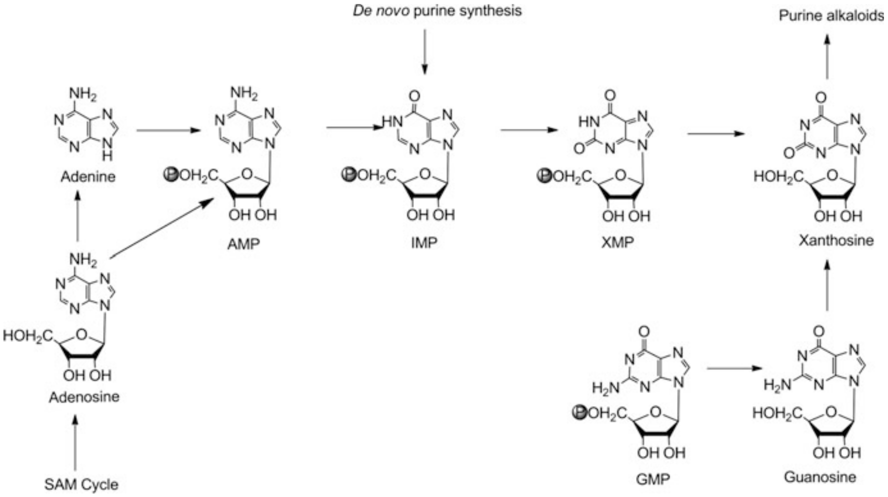

Fig. 4 Formation of xanthosine for caffeine biosynthesis from purine nucleotides and SAM. Xanthosine is produced via at least four routes: from IMP originating from de novo purine synthesis (de novo route), from the cellular adenine nucleotide pool (AMP route), from adenosine released from the SAM cycle (SAM route), and from the guanine nucleotide pool (GMP route)

3.5.2 AMP Route

A portion of the xanthosine used for caffeine biosynthesis is derived from the adenine and guanine nucleotide pools which are produced by the de novo and salvage pathways. There are several potential pathways for xanthosine synthesis from AMP, although the AMP → IMP → XMP → xanthosine route is likely to predominate. All three enzymes involved in the conversion have been detected in tea leaves (Koshiishi et al. 2001).

3.5.3 SAM Route

The SAM route is a variation of the AMP route. SAM is the methyl donor for various methylation reactions in the caffeine biosynthetic pathway. In the process, SAM is converted to *S*-adenosyl-L-homocysteine (SAH), which is then hydrolysed to homocysteine and adenosine. Homocysteine is recycled via the SAM cycle to replenish SAM levels, and adenosine released from the cycle is converted to AMP and utilized for caffeine biosynthesis by the AMP route. Since 3 moles of SAH are produced via the SAM cycle for each mole of caffeine that is synthesized, in theory this pathway has the capacity to be the sole source of both the purine skeleton and the methyl groups required for caffeine biosynthesis in young tea leaves (Koshiishi et al. 2001).

3.5.4 GMP Route

Xanthosine utilized for caffeine biosynthesis is also produced from guanine nucleotides by a GMP → guanosine → xanthosine pathway. 5′-Nucleotidase (EC 3.1.3.5) and guanosine deaminase (EC 3.5.4.15) participate in this conversion (Negishi et al. 1994).

4 *N*-Methyltransferases Involved in Methylxanthine Biosynthesis

4.1 Gene Expression in Coffee and Tea Plants

Expression of genes involved in caffeine biosynthesis has been demonstrated in young leaves, flower buds and developing endosperm of *Coffea arabica* (Mizuno et al. 2003a, b). The expression of *CmXRS1*, *CTS2* and *CCS1*, which encode 7-methylxanthosine synthase, theobromine synthase and caffeine synthase, respectively, was examined. Transcripts of *CmXRS1* and *CCS1* were observed in all organs, but the highest level was found in developing endosperm. Significant expression of *CTS2* was found only in flower buds. The patterns of expression of *CmXRS1* and *CCS1* were synchronized. During development of *Coffea arabica* fruits, the transcripts of *CmXRS1* and *CCS1* are present in every stage of growth except in fully ripened tissues. The pattern of expression of these genes during growth is roughly related to the in situ synthesis of caffeine from adenine nucleotides, although exceptions were found in the very early and the later stages of fruit growth. Since the level of *CTS2* transcripts encoding theobromine synthase is very low in fruits, the alternative *CCS1* gene encoding the dual-functional caffeine synthase may be operative for the last two steps of caffeine biosynthesis. In developing *Coffea arabica* fruits, the levels of transcripts of *CmXRS1* and *CCS1* are higher in seeds than in pericarp. Native caffeine synthase (3*N*-methyltransferase) activity is distributed in both organs in a similar manner. Therefore, caffeine accumulating in ripened coffee seeds appears to be synthesized within the developing seeds and is not transported from pericarp (Koshiro et al. 2006).

In *Camellia sinensis*, expression of *TCS1* encoding caffeine synthase is higher in young leaves than in mature leaves, stems or roots (Li et al. 2008). This is consistent with the fact that biosynthetic activity of caffeine occurs mainly in young leaves (Ashihara and Kubota 1986). Recent studies using *Camellia sinensis* tissue culture indicate that the expression of *TCS1*, and possibly the unidentified gene encoding 7-methylxanthosine synthase, represents the principal control mechanism for caffeine biosynthesis. Although increased caffeine content was observed when cultures were grown in media containing paraxanthine, addition of adenosine, guanosine or hypoxanthine did not have a similar impact. Thus, neither the supply of non-methylated purine precursors nor the availability of SAM appears to be an important factor in the regulation of caffeine biosynthesis (Li et al. 2008; Deng et al. 2008).

4.2 Evolutionary Relationship of Caffeine Synthase and Related Enzymes

Figure 5 shows the amino acid sequences of caffeine synthase and related enzymes. There are four highly conserved regions: motif A, motif B′, motif C and the YFFF region in the amino acid sequence of the caffeine synthase family (Kato and Mizuno 2004). Three conserved motifs, A, B and C, of the binding site of the methyl donor of SAM have been reported in the majority of plant SAM-dependent *O*-methyltransferases (Joshi and Chiang 1998). The motif B′ and YFFF region contains many hydrophobic amino acids which are specific to the motif B′ methyltransferase family. Most members of this newly characterized motif B′ methyltransferase family catalyse the formation of small and volatile methyl esters by using SAM as a methyl donor and substrates with a carboxyl group as the methyl acceptor. Members of this family include salicylic acid carboxyl methyltransferase (SAMT) (Ross et al. 1999),

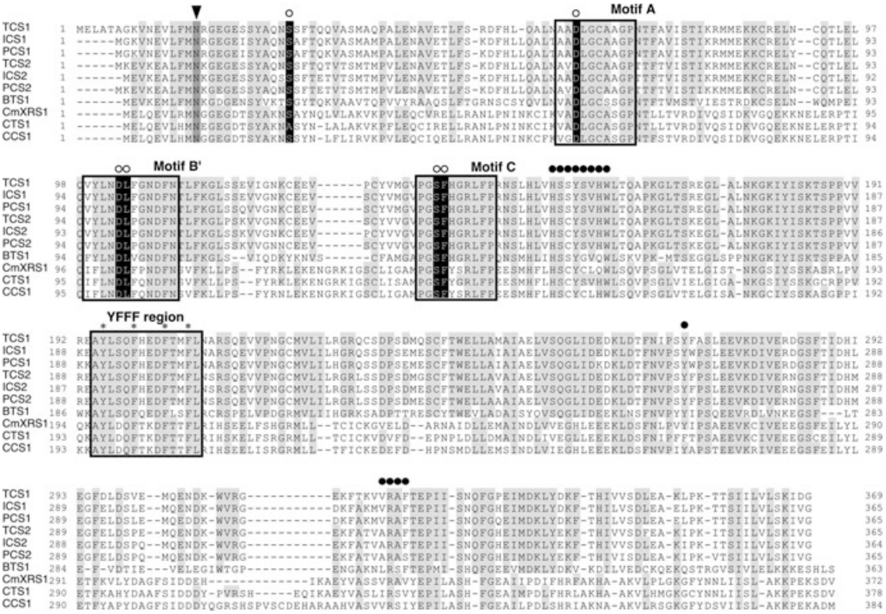

Fig. 5 Comparison of the amino acid sequences of caffeine synthases and its related enzymes. Alignment of the amino acid sequences for TCS1 and TCS2 from tea, ICS1 and ICS2 from *Camellia irrawadiensis*, PCS1 and PCS2 from *Camellia ptilophylla*, BTS1 from cocoa, and CmXRS1, CTS1 and CCS1 from coffee is indicated. *Shaded boxes* represent conserved amino acid residues, and *dashes* represent gaps that have been inserted for optimal alignment. The proposed SAM-binding motifs (A, B′ and C) and the conserved "YFFF region" are shown by *open boxes* (Mizuno et al. 2003a). *Asterisks* indicate tyrosine (Y) or phenylalanine (F) residues in the region. The nominated amino acids in substrate binding are indicated by *closed circles*, and additional active site residues are indicated by *arrowheads* (Zubieta et al. 2003). The sources of the sequences are as follows: TCS1, AB031280 (Kato et al 2000); TCS2, AB031281; BTS1, AB096699; PCS1, AB207817; PCS2, AB207818; ICS1, AB056108; ICS2, AB207816 (Yoneyama et al. 2006); CmXRS1, AB034699 (Mizuno et al 2003b); CTS1, AB034700 (Mizuno et al 2001); CCS1, AB086414 (Mizuno et al 2003a). (Adapted from Yoneyama et al. 2006)

benzoic acid carboxyl methyltransferase (BAMT) (Dudareva et al. 2000), jasmonic acid carboxyl methyltransferase (JAMT) (Seo et al. 2001), farnesoic acid carboxyl methyltransferase (FAMT) (Yang et al. 2006), indole-3-acetic acid methyltransferase (IAMT) (Zhao et al. 2008), gibberellic acid methyltransferase (GAMT) (Varbanova et al. 2007) and loganic acid carboxyl methyltransferase (LAMT) (Murata et al. 2008). The motif B′ methyltransferase family is also referred to as the SABATH family, based on the initial letters of the names of the substrates (D'Auria et al. 2003). Crystallographic data on SAMT from *Clarkia breweri* suggest that members of this family exist as dimers in solution (Zubieta et al. 2003). Further structural analysis of 7-methylxanthosine synthase and caffeine synthase from *Coffea canephora* also revealed a dimeric structure (McCarthy and McCarthy 2007).

Amino acid sequences of the caffeine synthase family derived from coffee are more than 80% homologous but share only 40% homology with caffeine synthase

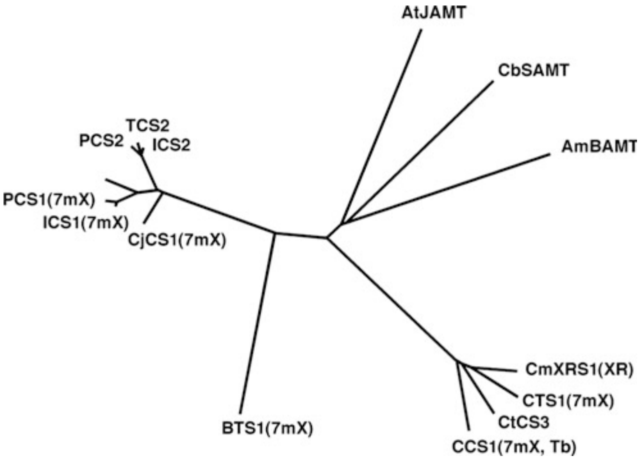

Fig. 6 Evolutionary relationship of caffeine synthase and its related enzymes. Substrates of the enzymes involved in caffeine synthesis are shown in *parentheses*. The substrates of TCS2, ICS2, PCS2 and CtCS3 are not known. The sources of the sequences are as follows: CmXRS1, AB034699 (Mizuno et al. 2003b); CTS1, AB034700 (Mizuno et al. 2001); CtCS3, AB054842 (Mizuno et al. 2003a); CCS1 AB086414 (Mizuno et al. 2003a); CbSAMT, AF133053 (Ross et al. 1999); AtJAMT, AY008434 (Seo et al. 2001); BTS1, AB096699 (Yoneyama et al. 2006); and AmBAMT, AF198492 (Dudareva et al. 2000). The unrooted tree was created by using ClustalW through application of the neighbour-joining method (Thompson et al. 1994). (Adapted from Ishida et al. 2009)

from tea. There is a similar homology between SAMT from *Clarkia breweri* and caffeine synthase from tea and coffee plants. That is to say, the amino acid sequences share a high degree of sequence identity within the same genus.

Figure 6 shows the phylogenetic tree analysis of the motif B′ methyltransferase family. This implies that the caffeine biosynthetic pathways in coffee, tea and cacao might have evolved in parallel with one another, consistent with different catalytic properties of the enzymes involved. Recently, Ishida et al. (2009) reported the occurrence of theobromine synthase genes in purine alkaloid-free species of *Camellia*. This represents additional evidence that monophyletic genes occur in *Camellia* plants.

5 Catabolism of Methylxanthines in Plants

5.1 Conversion of Caffeine to Theophylline

Limited amounts of caffeine are very slowly degraded with the removal of the three methyl groups, resulting in the formation of xanthine in almost all caffeine-forming plant species (Fig. 7). Catabolism of caffeine has been studied using ^{14}C-labelled

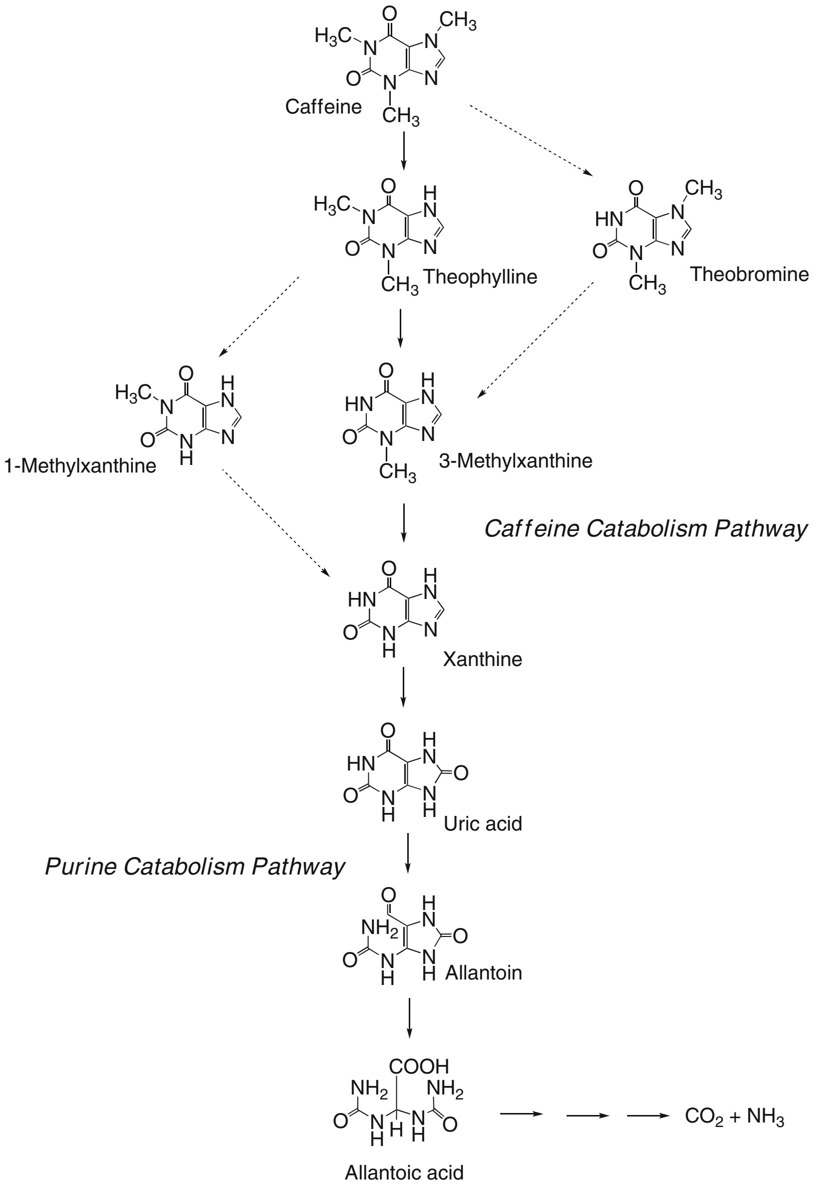

Fig. 7 Catabolic pathways of caffeine and theobromine. Caffeine is catabolized mainly to xanthine via theophylline and 3-methylxanthine. Theobromine is catabolized to xanthine via 3-methylxanthine. Xanthine is further degraded to CO_2 and NH_3 by the conventional oxidative purine catabolic pathway. *Dotted arrows* indicate minor routes

caffeine (Ashihara et al. 1997; Ashihara et al. 1996; Mazzafera 2004; Suzuki and Waller 1984). Caffeine catabolism usually begins with its conversion to theophylline catalysed by *N*7-demethylase. This conversion is the rate-limiting step in purine alkaloid catabolism and provides a ready explanation for the high concentration of endogenous caffeine in species such as *Coffea arabica* and *Camellia sinensis*. The involvement of the P450-dependent monooxygenase activity for this reaction has been proposed (Huber and Baumann 1998; Mazzafera 2004), although the activity of this enzyme has not yet been demonstrated. In leaves of *Coffea eugenioides*, which contain low levels of caffeine, [8-^{14}C]caffeine is catabolized rapidly primarily by the main caffeine catabolic pathway via theophylline. This suggests that the low caffeine accumulation in *Coffea eugenioides* is a consequence of rapid degradation of caffeine, perhaps accompanied by a slow rate of caffeine biosynthesis (Ashihara and Crozier 1999b).

5.2 Metabolism of Theophylline

In caffeine-producing plants such as tea, coffee and maté, [8-^{14}C]theophylline is catabolized rapidly (Ito et al. 1997). The main route of theophylline degradation in higher plants involves a theophylline → 3-methylxanthine → xanthine → uric acid → allantoin → allantoic acid → CO_2 + NH_3 pathway (Fig. 7). In contrast, theophylline is catabolized at extremely low levels in non-methylxanthine-forming plants. Higher plants do not convert [8-^{14}C]theophylline to either 1-methyluric acid or 1,3-dimethyluric acid, which are the main catabolites of theophylline in mammals (Scheline 1991). In tea and maté, large amounts of [8-^{14}C]theophylline are also converted to theobromine and caffeine via a theophylline → 3-methylxanthine → theobromine → caffeine salvage pathway (Ito et al. 1997).

5.3 Catabolism of Theobromine

In contrast to theophylline, theobromine is a precursor, as opposed to a catabolite, of caffeine. However, degradation of theobromine has been observed in mature leaves (Koyama et al. 2003) and pericarp of the theobromine-accumulating plant *Theobroma cacao* (Zheng et al. 2004). Theobromine was degraded to CO_2 via 3-methylxanthine, xanthine and allantoic acid (Fig. 7). Although conversion of caffeine to theobromine was detected in *Theobroma cacao*, caffeine was catabolized principally to CO_2 via theophylline, which is the same degradation pathway that operates in *Coffea arabica* and *Camellia sinensis*.

6 Ecological Roles of Purine Alkaloids

The physiological role of purine alkaloids *in planta* is largely unknown. It appears not to act as a nitrogen reserve since considerable amounts remain in leaves after abscission. There are two hypotheses concerning the ecological roles of caffeine in plants.

6.1 Chemical Defence Theory

The chemical defence theory proposes that the high concentrations of caffeine in young leaves, fruits and flower buds of species such as tea and coffee act as a defence to protect young soft tissues from pathogens and herbivores. It has been shown that spraying tomato leaves with caffeine deters feeding by tobacco hornworms, while treatment of cabbage leaves and orchids with caffeine acts as a neurotoxin and kills or repels slugs and snails (Hollingsworth et al. 2003). This work has now been extended and convincing evidence for the chemical defence theory has recently been obtained with transgenic caffeine-producing tobacco plants (Kim et al. 2006; Uefuji et al. 2005).

6.2 Allelopathy Theory

The allelopathic or autotoxic function theory proposes that caffeine in seed coats and falling leaves is released into the soil to inhibit germination of seeds around the parent plants (Anaya et al. 2006). In caffeine-synthesizing cells, caffeine accumulates in vacuoles, so caffeine does not impact on cellular metabolism. Exogenously applied caffeine does, however, inhibit various aspects of metabolism in the cells. Although there is experimental evidence from laboratory studies to support this proposal, it is unclear to what extent caffeine is involved in allelopathy in natural ecosystems, especially as soil bacteria such as *Pseudomonas putida* can degrade methylxanthines (Hohnloser et al. 1980; Gluck and Lingens 1988).

7 Production of Decaffeinated Coffee

Demand for decaffeinated coffee has increased gradually since the early 1970s. Worldwide sales of “decaf” have achieved a 12% share of the market, estimated to be worth more than US $4 billion (Heilmann 2001). Modern methods of decaffeination, such as supercritical fluid extraction with carbon dioxide, may have minimal effect on the organoleptic quality of the beverage if carried out correctly (Vitzthum 2005). Nevertheless, coffee cultivars combining high cup quality with a low caffeine content may provide a superior, less expensive and ecofriendly alternative to meet the demand for decaffeinated coffee.

7.1 Production by Breeding

Silvarolla et al. (2004) discovered naturally decaffeinated mutant plants in the progeny of *Coffea arabica* accessions from Ethiopia. Three of these Ethiopian mutant plants were almost completely free of caffeine. The seeds of those plants had low caffeine content (mean caffeine content 0.076% dry weight), but significant amounts of theobromine (about 0.61%), another methylxanthine which is capable of causing physiological effects similar to those of caffeine (Eteng et al. 1997). It would, therefore, appear to be worth searching for mutant plants with a low theobromine and caffeine content.

Recently, Nagai et al. (2008) produced a new low-caffeine hybrid coffee which is a tetraploid interspecific hybrid developed in Madagascar from *Coffea eugenioides*, *Coffea canephora* and *Coffea arabica*. Green beans of selected hybrids contain 0.37% caffeine and no detectable theobromine. Low caffeine accumulation is due mainly to the low biosynthetic activity of purine alkaloids, possibly the extremely weak *N*-methyltransferase reactions in caffeine biosynthesis.

7.2 Production by Genetic Engineering

Attempts to use genetic engineering to produce transgenic caffeine-deficient coffee have to date had only limited success. Low-caffeine-containing transgenic *Coffea canephora* plants have been produced but the caffeine content of the leaves was variable, depending on the line; the most notable example yielded a reduction of up to 70% (Ogita et al. 2003; Ogita et al. 2004). Coffee produced from beans of *Coffea arabica* has a flavour superior to that of robusta coffee but as yet caffeine-deficient transgenic arabica beans have not been produced. When this is achieved, because of the substantial market for decaffeinated coffee, it is likely to have major commercial implications.

8 Summary and Perspectives

The major route to caffeine in higher plants is a xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine pathway. The precursors of caffeine are derived from purine nucleotides. The rate of caffeine biosynthesis appears to be regulated primarily by the induction and repression of *N*-methyltransferases, especially 7-methylxanthosine synthase. The first paper on the cloning of caffeine synthase from tea appeared in 2000. Since then there has been a veritable explosion of research that has led to the successful cloning of a number of *N*-methyltransferase-encoding genes from coffee. The rate-limiting step in the caffeine biosynthetic pathway, the initial conversion of xanthosine to 7-methylxanthosine, is catalysed by 7-methylxanthosine synthase, and the encoding gene for this *N*-methyltransferase has been isolated from coffee. Although funding from industry has been very limited to non-existent, much of the extensive interest in this research has been fuelled by the possibilities of using genetic engineering to obtain transgenic, low-caffeine-containing coffee and tea that could be used to produce “natural” decaffeinated beverages. Although transgenic *Coffea canephora* seedlings with a 70% reduced caffeine content have been obtained, there is as yet no information on the caffeine content of beans produced by these plants. The real breakthrough in commercial terms will come with the production of transgenic caffeine-deficient *Coffea arabica* beans.

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Pharmacokinetics and Metabolism of Natural Methylxanthines in Animal and Man

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Contents

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Abstract Caffeine, theophylline, theobromine, and paraxanthine administered to animals and humans distribute in all body fluids and cross all biological membranes. They do not accumulate in organs or tissues and are extensively metabolized by the liver, with less than 2% of caffeine administered excreted unchanged in human urine. Dose-independent and dose-dependent pharmacokinetics of caffeine and other dimethylxanthines may be observed and explained by saturation of metabolic pathways and impaired elimination due to the immaturity of hepatic enzyme and liver diseases. While gender and menstrual cycle have little effect on their elimination, decreased clearance is seen in women using oral contraceptives and during pregnancy. Obesity, physical exercise, diseases, and particularly smoking and the interactions of drugs affect their elimination owing to either stimulation or inhibition of CYP1A2. Their metabolic pathways exhibit important quantitative and qualitative differences in animal species and man. Chronic ingestion or restriction of caffeine intake in man has a small effect on their disposition, but dietary constituents, including broccoli and herbal tea, as well as alcohol were shown to modify their plasma pharmacokinetics. Using molar ratios of metabolites in plasma and/or urine, phenotyping of various enzyme activities, such as cytochrome monooxygenases, N-acetylation, 8-hydroxylation, and xanthine oxidase, has become a valuable tool to identify polymorphisms and to understand individual variations and potential associations with health risks in epidemiological surveys.

Keywords Absorption · Age · Alcohol · Bioavailability · Caffeine · Cytochromes · Diet · Diseases · Distribution · Drugs · Excretion · Gender · Hormones · Interactions · Metabolism · Obesity · Paraxanthine · Pharmacokinetics · Physical exercise · Smoking · Theobromine · Theophylline

Abbreviations

1,3,7DAU	6-Amino-5(*N*-formylmethylamino)-1,3-dimethyluracil
1,3,7TMU	1,3,7-Trimethyluric acid
1,3DMU	1,3-Dimethyluric acid
1,7DAU	6-Amino-5-(*N*-formylmethylamino)-3-methyluracil
1,7DMU	1,7-Dimethyluric acid
1MU	1-Methyluric acid
1MX	1-Methylxanthine
3,7DAU	6-Amino-5-(*N*-formylmethylamino)-1-methyluracil

3,7DMU	3,7-Dimethyluric acid
3MU	3-Methyluric acid
3MX	3-Methylxanthine
7MU	7-Methyluric acid
7MX	7-Methylxanthine
AAMU	5-Acetylamino-6-amino-3-methyluracil
AFMU	5-Acetylamino-6-formylamino-3-methyluracil
AUC	Area under the concentration versus time curve
$C_{\max}$	Peak plasma concentration
CYP	Cytochrome P450
GSH	Glutathione
IBW	Ideal body weight
K_a	Absorption rate constant
K_e	Elimination rate constant
K_m	Michaelis–Menten constant
NAT2	*N*-Acetyltransferase 2
TBW	Total body weight
$T_{\max}$	Time to reach the peak plasma concentration

1 Introduction

Caffeine metabolism and pharmacokinetics have been reported in several reviews and monographs (Arnaud and Welsch 1982; Arnaud 1984, 1987, 1988, 1993a, b, 1998; Anonymous 1991). Reviews on theophylline (Arnaud and Welsch 1982; Hendeles et al. 1986; Anonymous 1991) were published because of its wide application as an active component of a variety of over-the-counter pharmaceutical products and drugs. A few reviews have been published on theobromine (Tarka 1982; Anonymous 1991), but there is no review on paraxanthine, perhaps because it can be found in plants only in trace amounts and is identified in human urine (Salomon 1883). This chapter highlights some of the present knowledge, including the most recently published studies on these four methylxanthines.

2 Caffeine

2.1 Absorption

Absorption and bioavailability of caffeine were generally similar between humans, dogs, rabbits, rats, and mice (Walton et al. 2001). In animals and man, absorption is *characterized* by rapid and complete gastrointestinal absorption (Arnaud 1976;

Arnaud and Welsch 1980a; Yesair et al. 1984). In man, 99% of the administered dose was absorbed in 45 min (Blanchard and Sawers 1983a), mainly from the small intestine but also 20% from the stomach (Chvasta and Cooke 1971). The absolute bioavailability of caffeine (5 mg/kg) in healthy adult male volunteers showed a rapid oral absorption with the time to reach peak the peak plasma concentration ($T_{\max}$) of 29.8 ± 8.1 min and a peak plasma concentration ($C_{\max}$) of 10.0 ± 1.0 μg/mL. From comparison of the caffeine area under the concentration versus time curve (AUC) after intravenous and oral doses, a complete absolute bioavailability of caffeine was demonstrated (Blanchard and Sawers 1983a). Caffeine pharmacokinetics were independent of the route of administration as shown by superimposable plasma concentration curves, suggesting that there is no important hepatic first-pass effect. Caffeine absorption from food and beverages does not seem to be dependent on age, gender, genetics, and disease or consumption of drugs, alcohol, and nicotine. Caffeine absorption from cola and chocolate was delayed, with $T_{\max}$ of 1.5–2 h instead of 0.5 h for a capsule and the $C_{\max}$ values were 1.57 for cola, 1.50 for chocolate, and 2.05 μg/mL for the capsule (Mumford et al. 1996). Pharmacokinetics of caffeine (100 mg) and its dimethylxanthine metabolites studied after inhalation in heroin users and compared with intravenous and oral administration in healthy volunteers showed a rapid and effective absorption after inhalation with an approximate bioavailability of inhaled caffeine of 60% in experienced smokers (Zandvliet et al. 2005). The efficacy of percutaneous caffeine absorption has been demonstrated in premature infants treated for neonatal apnea (Morisot et al. 1990).

2.2 Distribution

Caffeine enters the intracellular tissue water and is found in all body fluids – plasma, cerebrospinal fluid, saliva, bile, semen, breast milk, and umbilical cord blood as well – as in all tissue organs. There is no long-term accumulation of caffeine or its metabolites in the body as seen by whole-animal autoradiography using radiolabeled caffeine (Arnaud 1976).

2.2.1 Tissues

The tissue distribution 1 h after intravenous injection of caffeine into rabbits showed that the caffeine tissue-to-blood concentration ratio was approximately 1.0 with concentrations of 3.32 ± 0.47 μg/g. Exceptions included fat (0.79 ± 0.21 μg/g), adrenals (1.96 ± 0.36 μg/g), liver (4.88 ± 0.48 μg/g), and bile (8.97 ± 0.701 μg/g), in which the ratios were 0.2, 0.6, 1.5, and 2.7, respectively (Beach et al. 1985). Microdialysis applied with simultaneous subcutaneous infusion of caffeine and theophylline (20 mg/kg) to measure their concentrations in blood, adipose tissue, muscle, and liver of rats showed that caffeine was found to be evenly distributed in an in vitro test (Ståhle et al. 1991). While there was no difference between caffeine and theophylline for in vitro recovery, the in vivo recovery of theophylline was significantly less than the recovery of caffeine in brain, liver, muscle, and adipose tissue and this difference was significantly larger in the brain than in other tissues (Ståhle 1991).

There is no blood–brain barrier limiting the passage of caffeine through tissues. Therefore, from mother to fetus and to the embryo, an equilibrium can be continuously maintained. The disposition of caffeine and its metabolites, theophylline, theobromine, and paraxanthine, in the 20-day fetal and adult brains following a single maternal dose of 5 or 25 mg/kg caffeine showed that fetal and adult caffeine AUC values did not differ between the brain and plasma at either dose (Wilkinson and Pollard 1993). The brain-to-plasma ratio was close to 1 for a dose of 100 mg/kg and was lower for 10 mg/kg (0.6–0.7) and changed with time for 1 mg/kg, from 0.8 up to 1.9 after 4 h. When the dose of caffeine administered orally in the rat changed by 10 or 100 times, the AUC changed by 22 and 385 times in brain (Latini et al. 1978). Caffeine was found to be evenly distributed with a free concentration of approximately 120 μM and the rate of penetration into brain extracellular space was higher for caffeine than for theophylline (Ståhle et al. 1991). The pharmacokinetics of caffeine in the blood and cerebrospinal fluid were similar (Vickroy et al. 2008).

Similarly, caffeine is readily distributed to the fetus (Kimmel et al. 1984). The amniotic fluid to maternal plasma concentration ratio was higher for caffeine than for its major metabolite, paraxanthine, throughout gestation, and increased near term for both compounds. Both compounds distributed nearly homogeneously to fluids and tissues of the 29-day fetus, with mean fetal-to-maternal concentration ratios of 0.7 for paraxanthine and 0.9 for caffeine. The free fraction of caffeine was constant during gestation (about 0.8), while that of paraxanthine increased from 0.25 to 0.4 (Dorrbecker et al. 1988a). Caffeine is also readily excreted in milk (Gilbert et al. 1985), but there are significantly lower concentrations of caffeine and dimethylxanthine metabolites in milk when compared with serum in rabbits (McNamara et al. 1992), while in milk of lactating dairy cows the caffeine concentration was similar to the serum concentration 1.5–24 h after caffeine administration (DeGraves et al. 1995).

The situation in man is similar. After oral or intravenous doses of 5–8 mg/kg, mean plasma concentrations of 8–10 mg/L are observed. The caffeine plasma concentrations then decrease more rapidly than those of its metabolite paraxanthine, so in spite of important interindividual differences, paraxanthine concentrations become higher than those of caffeine within 8–10 h after administration. A good correlation was observed between the concentrations of caffeine in serum and in saliva, so noninvasive salivary measurements may be used for determination of caffeine pharmacokinetics in man (Scott et al. 1984). The caffeine concentrations in saliva were 65–85% of those in plasma (Callahan et al. 1982). After a 200 mg caffeine oral load in healthy adults, the saliva concentrations of caffeine, paraxanthine, and theophylline were lower than the plasma concentrations ($P < 0.001$), whereas the theobromine concentrations in plasma and saliva were similar. The saliva concentrations of these methylxanthines were higher than the free plasma concentrations ($P < 0.001$) (Rodopoulos and Norman 1996). The median AUC value for caffeine measured from saliva was 72% of that from serum, with variations of 56–95% between individuals (Spigset et al. 1999a).

In newborn infants, similar levels of caffeine concentration were found in plasma and cerebrospinal fluid (Anonymous 1991). Caffeine and its metabolites were detected in cerebrospinal fluid of patients with severe traumatic brain injury and increased concentration was associated with significant favorable outcomes (Sachse et al. 2008).

Urinary and umbilical cord blood analyses of caffeine have been correlated with reported intake throughout pregnancy ($P < 0.0001$) (Grosso et al. 2008). Ex vivo perfusion of the human placenta showed that caffeine crossed the placenta by passive diffusion (Mose et al. 2008) and analyses of human fetal gonads found that the caffeine concentrations were the same as in plasma (Anonymous 1991; Arnaud 1993a).

Transcutaneous collection allowing quantitation of caffeine that diffuses directly through the skin from within the body in healthy volunteers taking caffeine orally showed that the amount of caffeine collected was linearly related to the plasma AUC. Increased sweating carried out on one arm of each subject maintained at 40°C to induce local sweating showed a larger contribution to transdermal collection (40%) in the first 5 h and much less (14%) after 10 h (Conner et al. 1991). Caffeine, paraxanthine, and theobromine measured from transdermal sweat patches that continuously collected and stored analytes lost through the skin showed caffeine and paraxanthine accumulated at comparable rates, while theobromine accumulated more slowly (Delahunty and Schoendorfer 1998).

Values of the milk-to-serum concentration ratio of 0.52 and 0.81 were found in breast milk. As the binding of caffeine to constituents of serum and whole breast milk was 25.1 and only 3.2%, respectively, it was suggested that all the binding in breast milk was accounted for by the butterfat content (Arnaud 1993a).

2.3 Excretion

In both animals and man, renal excretion dominates. The metabolic disposition of [1-Me-^{14}C]caffeine studied and compared in the rat, the mouse, and the Chinese hamster showed no interspecies differences in urinary excretion of radioactivity, with 67–70% of the administered dose recovered (Arnaud 1985) and after the administration of [8-^{14}C]caffeine to various mouse strains, 73–89% of the dose was recovered in urine (Arnaud et al. 1989). In rabbits, after the administration of [2-^{14}C]caffeine, 82% of the administered radioactivity was recovered in urine (Beach et al. 1985). After oral administration of [2-^{14}C]caffeine and [1-Me-^{14}C] caffeine in rats, fecal excretion amounted to 2–7% of the administered dose. Most of the fecal excretion corresponded to caffeine metabolites secreted from enterohepatic cycling with intestinal and biliary secretion (Arnaud 1976). With [1-Me-^{14}C]caffeine administered to rats, mice, and Chinese hamsters, no interspecies differences appeared in fecal excretion of caffeine metabolites and 3–6% of the administered dose was recovered (Arnaud 1985). After the administration of [8-^{14}C]caffeine to various mouse strains, 7–12% of the dose was recovered in feces (Arnaud et al. 1989). After oral administration of radiolabeled caffeine in man, the 48-h fecal excretion amounted to 2–5% of the dose. The products identified in the feces of human volunteers were 1,7-dimethyluric acid (1,7DMU), 1-methyluric acid (1MU), 1,3-dimethyluric acid (1,3DMU), 1,3,7-trimethyluric acid (1,3,7TMU), and caffeine, which amounted to 44, 38, 14, 6, and 2% of fecal radioactivity, respectively (Callahan et al. 1982).

Caffeine and its dimethylxanthine primary metabolites are extensively reabsorbed in the renal tubule and their renal clearances were highly urine flow dependent, so urinary excretion varied with urine output. About 70% of the administered oral dose of caffeine (7.5 mg/kg) was recovered in urine (Tang-Liu et al. 1983) but less than 2% of caffeine was excreted unchanged in the urine. This low caffeine urine excretion (0.5–2%) is explained by a 98% renal tubular reabsorption. For higher caffeine intake (1 g, 10–12 cups of coffee), the recovery of caffeine in urine was from 0.74 to 0.91% of the dose and the urinary concentration was 14 mg/L. A good correlation was found between urinary and plasma caffeine concentrations (Birkett and Miners 1991).

2.4 Pharmacokinetics

Important pharmacokinetic differences have been reported between animal species, making the extrapolation between species difficult. In most studies performed in animal species, dose-independent pharmacokinetics for caffeine were reported and analyzed according to a one-compartment open model, while at higher doses applied in toxicology, dose-dependent pharmacokinetics were observed with lower plasma clearances. When the dose of caffeine administered orally to rats changed by 10 or 100 times, the AUC by changed by 45 and 746 times in plasma (Latini et al. 1978). These dose-dependent kinetics effects reported in animals can be explained by a saturation of metabolic transformation of caffeine (Bortolotti et al. 1985; Arnaud 1993a, b). Linear or nonlinear caffeine pharmacokinetics may be observed depending on the route and the rate of administration (Lau et al. 1995). The systemic clearance of total caffeine was 3.83 ± 1.94 and 1.14 ± 0.80 mL/min/kg and the unbound systemic clearance was 5.09 ± 2.60 and 1.41 ± 0.71 mL/min/kg in rabbit adults and the pups, respectively. A significant decreased caffeine clearance in the pups is thus demonstrated when compared with the adults (McNamara et al. 1992).

There is minimal (Yesair et al. 1984) or no first-pass metabolism for caffeine in human and the caffeine elimination is a first-order process in healthy human (Arnaud 1993a) and is described by a one-compartment open model system in the dose range of intake of 2–10 mg/kg observed in the population (Blanchard and Sawers 1983a; Newton et al. 1981; Bonati et al. 1982). Dose-dependent kinetics were observed when caffeine plasma levels were higher than 30 mg/L in the case of acute intoxication in an infant (Jarboe et al. 1986), but in adult subjects some metabolic transformations can be saturated in lower dose range of 1–4 mg/kg, particularly demethylation into paraxanthine, which is selectively catalyzed by CYP1A2 (Tang-Liu et al. 1983; Cheng et al. 1990; Denaro et al. 1990; Arnaud and Enslen 1992). To explain why epidemiology studies reported a nonlinear dose response between coffee consumption and health risks, the presence of a dose-dependent metabolism of caffeine was studied. Under chronic dosing conditions, healthy subjects received a placebo, a low dose of caffeine (4.2 mg/kg/day caffeine), or a high dose of caffeine (12 mg/kg/day caffeine) in decaffeinated coffee and in six divided doses spaced throughout the day. Caffeine clearance fell from 0.118 L/h/kg (placebo treatment) to 0.069 L/h/kg (low dose; $P < 0.005$) and to 0.54 L/h/kg (high dose; $P < 0.001$). The formation and metabolite clearances of paraxanthine, the major primary metabolite of caffeine, also decreased when comparing the low and high doses ($P < 0.05$). These results suggest that caffeine metabolism is dose-dependent, resulting in nonlinear elimination (Denaro et al. 1990). When caffeine clearance was determined on separate occasions using a single oral caffeine (70-, 200-, and 300-mg) dose, caffeine exhibited dose-dependent pharmacokinetics, particularly in subjects who showed high initial clearance with the lowest dose of caffeine. This significant decrease in caffeine clearance with increasing dose from 70 to 300 mg ($P < 0.01$) indicated a saturable caffeine metabolism in the dose range tested (Cheng et al. 1990). In addition to the dose, the plasma kinetics of caffeine can be influenced by the presence of food in the stomach and gastric emptying (Brachtel and Richter 1988). Both genetic and environmental factors are suggested as an explanation for the larger variability of caffeine clearance (Nagel et al. 1990). Fluid intake may also modify renal clearance and thus affect caffeine pharmacokinetics (Trang et al. 1985). Chronovariation in caffeine elimination appears to be small (−25 to 16%) in most of subjects (Levy et al. 1984). Measurements of caffeine clearance, acetylation phenotype, and urinary molar ratios of metabolites [5-acetylamino-6-formylamino-3-methyluracil (AFMU) plus 1-methylxanthine (1MX) plus 1MU to1,7DMU] were not changed when caffeine was given orally at 10 a.m. and at 10 p.m. (Hashiguchi et al. 1992). Surprisingly, sleep deprivation in healthy subjects receiving 2.1, 4.3, or 8.6 mg/kg caffeine showed a significantly ($P < 0.05$) and disproportional increase in the dose-normalized caffeine AUC. Clearance and the paraxanthine-to-caffeine ratio were significantly decreased with increasing dose, suggesting that under severe sleep deprivation caffeine exhibited dose-dependent pharmacokinetics (Kamimori et al. 1995).

After a single dose of caffeine (4 mg/kg) peak plasma concentrations were observed at 1–2 h with half-lives of 2.5–5 h (Anonymous 1991; Arnaud 1993a). Larger variations of caffeine plasma half-lives from 2.3 to 9.9 h were reported, indicating substantial intersubject variability in its elimination (Blanchard and Sawers 1983a). The half-lives of theophylline and theobromine (6.2–7.2 h) were significantly longer than those of caffeine and paraxanthine (4.1–3.1 h) (Lelo et al. 1986a). A peak serum level of 13.5 ± 2.9 mg/L for caffeine occurred 1 h after the administration and was delayed 1 h later for theophylline when caffeine (10 mg/kg) and theophylline (5 mg/kg) were given orally to asthmatic young patients. The half-life of caffeine was 3.9 ± 1.4 h and was shorter than the half-lives of theophylline with a twofold higher dosage level (Becker et al. 1984). After oral administration, the total plasma clearance of caffeine was similar to that of paraxanthine (2.07–2.20 mL/min/kg) and approximately twofold higher than the total plasma clearances of theophylline and theobromine (0.93–1.20 mL/min/kg). The unbound plasma clearances of caffeine and paraxanthine were also similar in magnitude (3.11–4.14 mL/min/kg) and also higher than those of theophylline and theobromine (1.61–1.39 mL/min/kg) (Lelo et al. 1986a). In nonsmoking subjects, the mean partial clearance of caffeine to paraxanthine was approximately eightfold and 23-fold greater than that to theobromine and theophylline, respectively, confirming earlier reports that paraxanthine is the major metabolite of caffeine in humans (Lelo et al. 1986b). At the steady state, the volume of distribution of theophylline (0.44 L/kg) was lower than that of the other methylxanthines (0.63–0.72 L/kg) and the unbound volumes of distribution of theophylline and theobromine (0.79 L/kg) were lower than the unbound volume of distribution of caffeine (1.06 L/kg), which was similar to that of paraxanthine (Lelo et al. 1986a).

2.5 *Metabolism*

To assess the validity of the interspecies toxicokinetics of caffeine, theobromine, theophylline, and paraxanthine, absorption, bioavailability, and the route of excretion were generally similar between humans and dogs, rabbits, rats, and mice but there were interspecies differences in the route of metabolism, and the enzymes involved in this process (Walton et al. 2001). CYP1A2, which has been detected only in the liver, and accounts for about 15% of the total cytochromes P450 (CYPs) in the human liver, where its protein content corresponds to 12.7 ± 6.2% of total CYP (Shimada et al. 1994), is responsible for more than 90% of caffeine clearance. The large interindividual variability of CYP1A2 activity influences the disposition of a substrate such as caffeine (Landi et al. 1999) and these variations may be due to factors such as gender, race, genetic polymorphisms, and exposure to inducers (Rasmussen et al. 2002). The molar ratios of metabolites of caffeine used as an index of CYP1A2 activity in populations are distributed according to bimodal or trimodal distributions, and normal or unimodal distributions have also been suggested (Landi et al. 1999). At least two distinct liver CYP enzymes with differing substrate affinities have the potential to catalyze caffeine N-demethylations and C8-hydroxylations in vitro but at the low concentrations routinely encountered in vivo, participation by the high-affinity site is expected to predominate (Campbell et al. 1987a). In vivo and in vitro evidence suggests that CYPs involved in the demethylation pathways are distinct from isozymes involved in the hydroxylation pathways, but these different isozymes seem to be under common regulatory control (Robson 1992).

The ratios of urinary concentrations of AFMU to 1MX or AFMU to 1MX plus 1MU (Grant et al. 1984) or the corresponding ratios with the complete conversion of AFMU into 5-acetylamino-6-amino-3-methyluracil (AAMU) (Tang et al. 1986; Kilbane et al. 1990) give markers of acetylator status in man. In addition, the ratio of 1MU to 1MX represents an index of xanthine oxidase, that of 1,7DMU to paraxanthine represents an index of microsomal 8-hydroxylation, that of AFMU plus 1MX plus 1MU to paraxanthine represents an index of microsomal 7-demethylation, and the caffeine metabolic ratio, AFMU plus 1MX plus 1MU to 1,7DMU, reflects microsomal 3-demethylation and also systemic caffeine clearance as well as polycyclic aromatic hydrocarbon-inducible CYP activity (Arnaud and Enslen 1992; Campbell et al. 1987a, b). The molar ratio of paraxanthine to caffeine in urine taken 3–4 h after caffeine administration was proposed as an alternative to evaluate hepatic CYP1A2 activity (Kadlubar et al. 1990). The ratio of paraxanthine to caffeine or the ratio of paraxanthine plus 1,7DMU to caffeine, has been used as an indicator of CYP1A2 activity and the AFMU-to-1MX ratio indicated *N*-acetyltransferase 2 (NAT2) activity; both appear to be polymorphically distributed in human populations with slow and rapid phenotypes (Butler et al. 1992). These ratios have been tested and validated (Spigset et al. 1999a; Butler et al. 1992; Tang et al. 1994; Carrillo et al. 2000; Doude van Troostwijk et al. 2003b; Derby et al. 2008) but a more detailed analysis of the literature is beyond the scope of this review. No association was found between acetylation activity and sex; race; age; education; smoking; physical activity; weight; consumption of coffee, alcohol, red meat, processed meat, and cruciferous vegetables; or use of estrogens, after taking the genotype into account (Le Marchand et al. 1996). Drug cocktails have been developed for simultaneous phenotyping of CYP1A2, CYP2A6, CYP2C9, CYP2E1, CYP2C19, CYP2D6, CYP3A, NAT2, and xanthine oxidase (Streetman et al. 2000; Zhu et al. 2001; Christensen et al. 2003; Fuhr et al. 2007; Ryu et al. 2007).

CYP1A2 was responsible for caffeine 3-demethylation and paraxanthine 7-demethylation and may catalyze virtually all reactions related to caffeine and its metabolites. Caffeine biotransformation by CYP1A2 averaged 81.5% for paraxanthine, 10.8% for theobromine, and 5.4% for theophylline, while CYP2E1 had major influences on the formation of theophylline and theobromine (Gu et al. 1992). Whereas CYP1A2 accounts for the high-affinity component of all three human hepatic caffeine N-demethylations, CYP2E1 appears to be the main enzyme involved in the low-affinity components of caffeine N1- and N7-demethylation, while 8-hydroxylation of caffeine was suggested to be catalyzed predominantly by a CYP3A isoform (Tassaneeyakul et al. 1994). CYP2D6-Met also had high intrinsic clearance and catalyzed caffeine demethylation and 8-hydroxylation. CYP2E1 played a less important role in vitro and CYP3A4, which predominantly catalyzed 8-hydroxylation, may contribute significantly to the in vivo formation of 1,3,7TMU, owing to its high abundance in human liver. Thus at least four CYP isoforms are involved in caffeine metabolism at 3 mmol/L caffeine concentration, but at concentrations below 0.1 mmol/L, CYP1A2 and CYP1A1 are the most important isoenzymes (Ha et al. 1996).

In humans no gender differences in caffeine metabolism were observed from urinary metabolite patterns or metabolite ratios (Grant et al. 1983), although higher activity of CYP1A2 has been shown in men than in women (Landi et al. 1999). This general conclusion is supported by other studies (Vistisen et al. 1992; Campbell et al. 1987b; Kall and Clausen 1995; Rasmussen et al. 2002; Chung et al. 2000; Ghotbi et al. 2007; Begas et al. 2007; Djordjevic et al. 2008). During pregnancy, the excretion of 1MX and of 1MU were increased (Scott et al. 1986). This observation is in agreement with a caffeine study showing a significantly increased hydroxylation activity during pregnancy. Late pregnancy was also characterized by a decrease in CYP1A2, xanthine oxidase, and acetyltransferase activities (Bologa et al. 1991). In nonsmoking pregnant women and in smoking women using oral contraceptives, the caffeine metabolic ratio was reduced by 29 and 20%, respectively, compared with a control group, demonstrating an inhibition of CYP1A2 (Vistisen et al. 1991). Metabolic ratios for the CYP1A2 index during early, middle, and late pregnancy were significantly lower than the ratio after delivery ($P < 0.0001$). A lower metabolic ratio for NAT2 was also observed during pregnancy ($P < 0.01$) but there was no significant difference in the metabolic ratios for xanthine oxidase during pregnancy and after delivery (Tsutsumi et al. 2001). Oral contraceptive users had lower ($P < 0.05$) ratios of paraxanthine 7-demethylation to 8-hydroxylation products than women not taking oral contraceptives (Campbell et al. 1987b). Upon administration of oral contraceptives, the urinary excretion of caffeine, paraxanthine, and 1,7DMU was increased at the expense of 1MX, 1MU, and the acetylated metabolites AFMU and AAMU. A 33% decrease of the caffeine metabolic ratio was reported in women using oral contraceptives (Kalow and Tang 1991a).

The caffeine ratio AFMU plus 1MU plus 1MX to 1,7DMU in a 6-h urine sample was significantly higher in women not taking oral contraceptives compared with women taking oral contraceptives, thus confirming that CYP1A2 is inhibited by oral contraceptives (Rasmussen et al. 2002). As a marker of CYP1A2 activity, the plasma caffeine-to-paraxanthine ratio was 2.8 times higher ($P < 0.001$) in the oral contraceptive (ethinylestradiol) users than in the control subjects, suggesting an inhibition of CYP1A2 activities (Granfors et al. 2005).

Analysis of caffeine metabolites revealed two interethnic variations, one pertaining to the acetylation polymorphism and the other consisting of a difference in paraxanthine excretion, which might indicate an ethnic difference in renal function (Kalow 1986). A nonsignificant higher proportion of rapid acetylator was observed in the Oriental compared with the European population and a 6.3-fold range variation was observed (Kalow and Tang 1991a). The NAT2 activity showed a typically bimodal distribution with 47% fast acetylators and 53% slow acetylators, consistent with a Danish population (Vistisen et al. 1992). However, only 11.0% of Japanese men and women residents of Kyushu were slow acetylators (Saruwatari et al. 2002). With use of the urinary caffeine metabolic ratio AFMU to 1X (less than 0.6) to classify subjects as slow acetylators, a prevalence of this phenotype of 92.2 and 74.5% was noted in two studies in a population of Minnesota Hmong, but a significant discordance between phenotype and genotype was identified

(Straka et al. 2006). The combined low-risk phenotype (slow CYP1A2/rapid NAT2) was more common in blacks than in whites (25 vs. 15%, $P < 0.02$), but there were no significant racial differences in slow and rapid CYP1A2 phenotypes, and in the combined slow NAT2/rapid CYP1A2 phenotype (Muscat et al. 2008). The ratios reflecting CYP1A2 activities were described as log-normal-distributed (Vistisen et al. 1992). CYP1A2 activity was not normally distributed in subjects from Arkansas, Italy, and China and appeared trimodal with arbitrary designation of slow, intermediate, and rapid phenotypes, which ranged from 12–13% slow, 51–67% intermediate, and 20–37% rapid (Butler et al. 1992). Slow and intermediate CYP1A2 metabolizers represent about 50% of Caucasians, while their frequency in Japanese subjects seems to be much lower (Landi et al. 1999). The distribution of CYP1A2 measured with the plasma paraxanthine-to-caffeine ratio in a Chinese population showed a 16-fold variation of CYP1A2 activity and a coefficient of variation of 62.9%. Nonnormal CYP1A2 activity ($P < 0.001$) with a bimodal distribution ($P < 0.01$) was observed. The percentage of poor metabolizers was 5.24% in this Chinese population (Ou-Yang et al. 2000). The metabolic ratio for CYP1A2 was not polymorphic in Japanese subjects and decreased 1,7DMU formation from caffeine in poor metabolizers of CYP2A6 appeared to affect the metabolic ratio used for the assessment of CYP1A2 activity (Saruwatari et al. 2002). CYP1A2 enzyme activity determined using the 4-h plasma paraxanthine-to-caffeine ratio was 1.54-fold higher in Swedes than in Koreans ($P < 0.0001$) despite them having the same CYP1A2 genotype, smoking habit, and oral contraceptive use. Four known (CYP1A2*1A, CYP1A2*1D, CYP1A2*1F, and CYP1A2*1L) and two novel (CYP1A2*1V and CYP1A2*1W) haplotypes were found (Ghotbi et al. 2007). The mean CYP2A6 activity measured by the caffeine metabolite ratio (1,7DMU to paraxanthine) was significantly lower in Japanese Americans than in native Hawaiians ($P = 0.001$ and $P < 0.0001$, respectively) or whites ($P < 0.0001$) (Derby et al. 2008). The xanthine oxidase index was not different between Chinese and European populations and showed a 1.7-fold range variation (Kalow and Tang 1991a). The ratios reflecting xanthine oxidase activities were normally distributed (Vistisen et al. 1992). Low xanthine oxidase activities exist in a Japanese population corresponding to 11% of the subjects with a mean urinary uric acid concentration 53% lower than that of the other subjects ($P < 0.0001$) (Saruwatari et al. 2002).

As noted in Sect. 2.5, drug intake is expected to alter caffeine metabolism when competitive inhibition or induction of the relevant enzymes is observed. Such interactions can involve smoking and Chinese herbal medicines, but St John's wort, garlic oil, *Panax ginseng*, and *Ginkgo biloba* showed no effect on CYP1A2 activity measured from the paraxanthine-to-caffeine serum ratio (Gurley et al. 2002). As expected, allopurinol treatment caused a specific, dose-dependent inhibition of the conversion of the caffeine metabolite 1MX to 1MU, thus validating an in vivo index of xanthine oxidase activity in man (Grant et al. 1986; Lelo et al. 1989). The proton pump inhibitor omeprazole induces hepatic CYP1A2 activity, as shown by the increased N3-demethylation of [3-Me-^{13}C]caffeine. In extensive metabolizers there was a 8–17% CYP1A2 induction after administration of

40 mg omeprazole and a 25–32% increase ($P < 0.002$) was observed with 120 mg/day. In poor metabolizers a higher increase of 40–41% was observed and there was a good correlation between the caffeine breath test and plasma caffeine clearance (Rost and Roots 1994).

2.6 Sources of Variation in Caffeine Pharmacokinetics and Metabolism

Caffeine metabolism is affected by genetic determinants, age, pregnancy, diet, and lifestyle such as smoking, environmental factors, medications, including contraceptive use, and disease states.

2.6.1 Age

The pharmacokinetics of caffeine studied in young dogs aged 1 day, and 7, 14, and 30–45 days and adult dogs showed that the plasma elimination half-life was 47.5 ± 5.35 h in 1-day-old puppies, as opposed to 6.66 ± 0.85 h in adult dogs. A rapid decrease in plasma half-lives occurred during the first 2 weeks of life and at about 14 days of age the caffeine plasma half-life was similar to that of adults. The volume of distribution was greatest and the total body clearance was smaller in the 1-day-old dogs (Warszawski et al. 1977). The time needed to reach the plateau of the cumulative excretion of radioactivity in the urine decreased with age. All these results are consistent with the slow plasma elimination of caffeine in the newborn as compared with the adult (Warszawski et al. 1982). The elimination of caffeine is impaired in neonates because of their immature metabolizing hepatic enzyme systems (Pons et al. 1988) and plasma half-lives of 65–103 h in neonates have been reported, decreasing rapidly to 14.4 h in 3–5-month-old infants, 2.6 h in 5–6-month-old infants, and 3–6 h in adults and the elderly. The clearance of 31 mL/kg/h in 1-month-old infants increases to a maximum of 331 mL/kg/h in 5–6-month-old infants, and is 155 mL/kg/h in adult subjects. A mean distribution volume of 0.7 L/kg (0.5–0.8 L/kg) was found in newborn infants, adult subjects, or aged subjects. The pharmacokinetics of caffeine in healthy young men aged 20.5 ± 2.0 years and in healthy elderly men aged 71.2 ± 3.9 years showed that $T_{\max}$, $C_{\max}$, and caffeine bioavailability were essentially identical. The apparent volume of distribution was significantly lower but the larger clearances and the greater elimination rate constant in the elderly subjects were not significant because of the wide intersubject variability as shown in the caffeine half-lives ranging from 2.27 to 9.87 h. In this study, pharmacokinetic parameters of caffeine were similar in young and elderly men (Blanchard and Sawers 1983b). The renal clearance of caffeine calculated following both oral and intravenous doses of caffeine in young and elderly, healthy human volunteers showed a highly statistically significant positive correlation ($P < 0.001$) between the renal clearance of both unbound and total clearance of caffeine and the mean urine flow rate (Blanchard and Sawers

1983c). Thus, the comparative pharmacokinetics in the young and elderly shows no significant differences in half-life, suggesting that aging does not alter caffeine elimination in contrast to the rat model, where an age-dependent increase of caffeine half-life has been observed.

2.6.2 Gender and Hormones

The caffeine apparent volume of distribution and the caffeine elimination rate constant were influenced by the different modes of maternal caffeine ingestion during the premating and pregnant periods (Nakazawa et al. 1985). The disposition of caffeine given as single oral dose of 5 and 25 mg/kg to 20-day pregnant and nonpregnant rats showed a significantly longer plasma half-life in the pregnant than in the nonpregnant rats for the highest dose, while the elimination rate was similar at the lowest dose (Abdi et al. 1993). In pregnant rabbits, the pharmacokinetics of caffeine received by continuous intravenous infusion through 29 days of gestation exhibited increased plasma concentrations of caffeine and its major metabolite paraxanthine in the last half of gestation. Rabbits exhibited caffeine AUC at 29 days of gestation that were 85–165% greater than those observed before mating, suggesting that the elimination of caffeine is diminished in late gestation in the rabbit (Dorrbecker et al. 1988a).

Similarly, the caffeine half-life was prolonged during the last trimester in pregnant women and returned to the prepregnant value a few weeks after they had given birth (Arnaud 1993a). From a cohort study of normal third-trimester pregnancies with significant "high"(H) and "low"(L) long-term maternal caffeine ingestion ($P < 0.0002$), it was shown that the maternal serum caffeine levels in group H were significantly higher ($P < 0.05$) at each week of gestation than those in group L and increased until 36 weeks ($P < 0.0039$) but did not increase significantly in group L until 40 weeks (Devoe et al. 1993). The half-life of caffeine increases during pregnancy, reaching 11.5–18 h by the end of pregnancy, leading to an accumulation with regular daily consumption as neither the fetus nor the placenta can metabolize caffeine (Grosso and Bracken 2005). Comparisons of the follicular and luteal phases revealed that systemic clearance of caffeine was slower in the luteal phase, an effect related to the proximity to onset of menstruation and to levels of progesterone although the half-life did not differ (Lane et al. 1992). All pharmacokinetic parameters were similar between women taking no oral contraceptives and men except for the volume of distribution, which was significantly larger in the women ($P < 0.05$) (Patwardhan et al. 1980), and gender had no significant effect on caffeine pharmacokinetics (McLean and Graham 2002). Oral contraceptive use has been shown to double the caffeine half-life (Abernethy and Todd 1985; Patwardhan et al. 1980; Arnaud 1993a). As compared with women taking no oral contraceptives, the half-life of caffeine was significantly prolonged in women taking oral contraceptives from 6.2 ± 1.6 to 10.7 ± 3.0 h ($P < 0.001$), showing impaired elimination of caffeine. Women taking oral contraceptives had a significantly lower total plasma clearance (0.79 ± 0.21 vs. 1.3 ± 0.35 mL/min/kg) and free clearance (1.12 ± 0.28 vs. 1.97 ± 0.57 mL/min/kg) than women not taking oral contraceptives, while the volumes of distribution and plasma binding were similar in both groups (Patwardhan et al. 1980). Oral contraceptive steroids increased twofold the residence time of caffeine in young women. The effect was already observed during the first cycle 2 weeks after starting to take oral contraceptive steroids and was slightly increased in the second cycle, after 6 weeks on oral contraceptive steroids (Rietveld et al. 1984). The effect of chronic administration of low-dose estrogen-containing (less than 50 μg estrogen) oral contraceptives on the pharmacokinetics of caffeine confirmed that the elimination half-life of caffeine was prolonged to 7.88 h versus 5.37 h in the controls, as a result of impairment of the plasma clearance of caffeine (1.05 vs. 1.75 mL/min/kg, respectively), with no change in the apparent volume of distribution (Abernethy and Todd 1985). The plasma caffeine clearances and elimination half-lives after ingestion of a guarana-containing supplement were lower (0.34 ± 0.01 vs. 0.99 ± 0.41 mL/min kg) and longer (15.5 ± 0.3 vs. 5.6 ± 1.7 h), respectively, in subjects taking oral contraceptives (Haller et al. 2002).

2.6.3 Physical Exercise

The effect of moderate exercise (30% of maximum O_2 uptake) on the kinetics of caffeine in healthy volunteers showed that exercise significantly raised $C_{\max}$ and reduced both the half-life and the volume of distribution (Collomp et al. 1991), but other studies show minimal effects on caffeine pharmacokinetics (Kamimori et al. 1987; McLean and Graham 2002).

2.6.4 Obesity

Obesity increases the apparent volume of distribution (69.9 ± 5.9 vs. 43.6 ± 2.8 L; $P < 0.001$), with no significant change in clearance and a trend toward a prolonged elimination half-life (Abernethy et al. 1985). At rest, obese subjects (more than 30% body fat) had a significantly higher absorption rate constant (K_a 0.0757 vs. 0.0397/min), a lower elimination rate constant (K_e 0.0027 vs. 0.0045/min), and a longer serum half-life (4.37 vs. 2.59 h) in comparison with lean subjects. In exercise as well as at rest, lean and obese subjects had a large difference in the volume of distribution, 43.2 versus 101 L in exercise and 54.1 versus 103 L at rest. Exercise consistently resulted in a decrease in caffeine $C_{\max}$ and AUC in obese subjects (Kamimori et al. 1987). In severely obese subjects, the caffeine half-life and oral clearance rate were not altered significantly, but it was confirmed that obese individuals exhibited an increased volume of distribution and this volume was decreased with weight reduction. The effect was more important in females and it was suggested that the caffeine distribution was incomplete into the adipose tissue representing 70–80% excess of body weight in obese subjects (Caraco et al. 1995). After oral administration of caffeine as coffee in obese subjects (body mass index

28.01 ± 0.92) and control subjects, there was no significant difference in the caffeine and theobromine levels in saliva but significantly lower levels of theophylline ($P < 0.05$) and higher levels of paraxanthine ($P < 0.01$) were found in obese subjects, suggesting that obesity alters caffeine metabolism and modifies the urinary metabolite concentration ratios used as indexes of enzyme activities (Bracco et al. 1995).

2.6.5 Drugs

Given the *major* role of the liver in the metabolism *of caffeine and many* drugs*, a few examples of these interactions are described from a very large literature.* Clinical studies have reported frequent drug interactions leading to impaired caffeine elimination and decreased clearance both for caffeine and for its metabolites (Lelo et al. 1989). Traditional medicine as well as supplements prepared from plant extracts may affect caffeine pharmacokinetics. With use of caffeine as a probe drug, the effect of sodium tanshinone IIA sulfonate, a water-soluble derivative of the Chinese medicine Danshen, on the activity of CYP1A2 in humans has been tested on healthy volunteers. CYP1A2 activity monitored by the ratio of paraxanthine to caffeine at 6 h in plasma significantly increased by 41.1%, the AUC of caffeine significantly decreased by 13.3%, and the AUC of paraxanthine significantly increased by 17.4% (Chen et al. 2009a). After administration of St John's wort (Esbericum capsules; 240 mg/day of extract, 3.5 mg hyperforin) or a placebo, no statistically significant differences of the primary kinetic parameter, the AUC of caffeine and paraxanthine, between the placebo group and the St John's wort group were observed (Arold et al. 2005). Interaction between the selective serotonin reuptake inhibitor fluvoxamine (50–100 mg/day) and caffeine (200 mg orally) in healthy volunteers showed a decreased total clearance of caffeine from 107 to 21 mL/min and an increased half-life from 5 to 31 h. The N3-, N1-, and N7-demethylation clearance of caffeine decreased from 46 to 9 mL/min, from 21 to 9 mL/min, and from 14 to 6 mL/min, respectively (Jeppesen et al. 1996). However, fluvoxamine (50 mg/day orally) disposition studied in healthy nonsmoking male volunteers who also received caffeine (200 mg orally) showed no significant correlations between caffeine and fluvoxamine clearance or between the paraxanthine-to-caffeine ratio in serum 6 h after caffeine intake and fluvoxamine oral clearance (Spigset et al. 1999b), in contrast to previous in vitro (Brøsen et al. 1993) and in vivo (Sperber 1991; Jeppesen et al. 1996) studies. Other drugs that may interfere include the antipsychotic drug clozapine (Doude van Troostwijk et al. 2003a), the anti-inflammatory drugs idrocilamide (Brazier et al. 1980a) and rofecoxib (Backman et al. 2006), and tacrine (Fontana et al. 1998). Caffeine metabolism has been shown to be inhibited by quinolone antibiotics. In vitro tests ranked the likelihood of these interactions as follows: enoxacin, 74.9%; ciprofloxacin, 70.4%; nalidixic acid, 66.6%; pipemidic acid, 59.3%; norfloxacin, 55.7%; lomefloxacin, 23.4%; pefloxacin, 22.0%; amifloxacin, 21.4%; difloxacin, 21.3%; ofloxacin, 11.8%; temafloxacin, 10.0%; fleroxacin, no effect. In vivo studies showed that the likelihood of an interaction with caffeine is as follows: enoxacin > ciprofloxacin = norfloxacin > ofloxacin = lomefloxacin (Fuhr et al. 1992). Among fluoroquinolones, enoxacin and to a lesser extent ciprofloxacin and pefloxacin inhibit the metabolic clearance of caffeine (Kinzig-Schippers et al. 1999; Granfors et al. 2004) and it was suggested to use noninteracting quinolones such as ofloxacin and norfloxacin.

Other antidepressants and drugs for the management of anxiety disorders such as venlafaxine, alprazolam, zolpidem, and trimethadione as well as the wakefulness-promoting agent armodafinil did not significantly alter the pharmacokinetics of caffeine and its metabolites (Amchin et al. 1999; Schmider et al. 1999; Cysneiros et al. 2007; Tanaka et al. 1993; Darwish et al. 2008).

2.6.6 Disease

Several animal models of liver disease show reduced total body clearance of caffeine (Tanaka et al. 1992a, 1995; Schaad et al. 1995; Kokwaro et al. 1993). Similarly, humans with several types of liver disease, including cirrhosis (Wietholtz et al. 1981), noncirrhotic, chronic hepatitis B or C, and subjects with cirrhosis, showed a highly significant reduction in plasma clearance correlating with the severity of the disease ($P < 0.001$) (Park et al. 2003; Tanaka et al. 1992b; Scott et al. 1989). The reduced plasma disappearance rate of caffeine in cirrhotics was related to the delayed formation of paraxanthine (Holstege et al. 1989; Jodynis-Liebert et al. 2004). Chronic consumption of alcohol leading to cirrhosis was shown to increase the caffeine half-life up to 50–160 h (Statland and Demas 1980; Desmond et al. 1980; Renner et al. 1984; Scott et al. 1988). A study performed in patients with decompensated type I and type II diabetes mellitus showed that the caffeine half-life, apparent clearance and distribution volume, and paraxanthine-to-caffeine ratio for the CYP1A2 index were similar to those of controls (Zysset and Wietholtz 1991; Matzke et al. 2000).

2.6.7 Smoking

Although some studies showed no effect (Oliveto et al. 1991), most studies found that caffeine clearance was stimulated by smoking (Brown and Benowitz 1989; Parsons and Neims 1978; Wietholtz et al. 1981; Arnaud and Welsch 1982; Kotake et al. 1982; Caraco et al. 1995; Zevin and Benowitz 1999; Bchir et al. 2006). Cigarette smoking nearly doubles the rate of caffeine metabolism owing to the enzyme-inducing effects of polycyclic aromatic hydrocarbons, known to increase liver enzyme activity (Kalow and Tang 1991b; Parsons and Neims 1978). Multivariate analysis revealed that with disease state, smoking ($P < 0.001$) was a significant predictor of the caffeine breath test, thus showing it to be a valid indicator of plasma caffeine clearance and hepatic function (Park et al. 2003). Cigarette smoking increases the elimination of caffeine, whereas cessation of cigarette smoking significantly reduces caffeine clearance (Murphy et al. 1988) and changes the pattern of caffeine metabolism (Brown et al. 1988). The time-course changes of CYP1A2 activity measured from the paraxanthine-to-caffeine ratio in plasma after cessation of smoking in heavy smokers showed that the initial caffeine clearance decreased significantly ($P < 0.01$) by 36.1% and the apparent half-life of the CYP1A2 activity decrease was 38.6 h (Faber and Fuhr 2004).

2.6.8 Diet and Alcohol

During the treatment of neonatal apnea, formula-fed infants, compared with breastfed infants, show a nearly threefold increase in the clearance of caffeine. In HepG2 cells, messenger RNA and protein expression of CYP1A1/CYP1A2 were significantly induced by cow-milk-based formula, but not by human milk. The enhanced in vitro CYP1A expression via an AhR-mediated pathway by infant formula but not human milk provides a potential mechanistic basis for the increased caffeine elimination in formula-fed infants (Xu et al. 2005). The caffeine elimination rate constant was low 2 weeks after birth and displayed a significant positive linear correlation with age ($P < 0.001$). A significantly greater elimination rate constant was observed in formula-fed than in breast-fed infants ($P < 0.001$). This occurred concomitantly with a significant increase in the levels of urinary paraxanthine and 1MX ($P < 0.001$), suggesting increased CYP1A2 activity in formula-fed infants. The urinary molar ratio of paraxanthine plus 1MX to caffeine and age strongly predicted the caffeine elimination rate constant ($P < 0.001$) irrespective of feeding type (Blake et al. 2006).

The influence of nutritional status was investigated in elderly institutionalized patients with either malnutrition or adequate nutrition. The plasma paraxanthine-to-caffeine metabolic ratio was similar in both groups and was not correlated to the body mass index, serum albumin, or renal clearance (Hamon-Vilcot et al. 2004). Daily consumption of at least three cups of coffee significantly increased CYP1A2 enzyme activity (Djordjevic et al. 2008). These results confirmed the findings of a previous study showing that multiple ingestions of dietary caffeine (two to seven cups of coffee) in healthy subjects increased the theophylline serum concentrations given as a single oral dose when compared after deprivation of dietary caffeine. The theophylline half-life was prolonged by 32% ($P < 0.01$) and the total body clearance was reduced by 23% ($P < 0.001$) (Sato et al. 1993). When subjects resumed coffee drinking, interindividual variations preclude a clear answer about the time period required for deinduction to occur. However, regular caffeine intake in high doses for 1 week failed to alter caffeine pharmacokinetics (George et al. 1986).

Grapefruit juice beverage consumption decreased the oral clearance of caffeine by 23% and prolonged its half-life by 31% (Fuhr et al. 1993, 1995). The pharmacokinetics of caffeine and its metabolite paraxanthine were affected by the flavonoid quercetin as shown by their significantly decreased AUC (16%) and decreased urinary excretion of paraxanthine (32%) and 1MX (156%), while urinary excretion of 1,7DMU and of 1MU were both increased by 90% (Chen et al. 2009b).

Alcohol intake in amounts commonly consumed significantly prolonged the caffeine half-life by 72% ($P < 0.005$) and diminished the caffeine clearance by 36% ($P < 0.0005$) (George et al. 1986) ,while the AUC for caffeine was significantly higher when caffeine was administered with 0.8 g/kg alcohol (Azcona et al. 1995).

2.7 Metabolites and Metabolic Pathway

Metabolites specific to animal species were identified in urine, such as trimethylallantoin (Arnaud et al. 1986a), sulfur-containing metabolites (Kamei et al. 1975) and *N*-methylurea and *N*,*N*′-dimethylurea (Arnaud 1976), and may be produced by the intestinal flora. These sulfur-containing metabolites of caffeine were detected in the urine of the horse, rabbit, rat, and mouse and were isolated and identified as α-[7-(1,3-dimethylxanthinyl)]methyl methyl sulfoxide, while two other new metabolites were isolated from the urine of the mouse and identified as α-[7-(1,3-dimethylxanthinyl)]methyl methyl sulfide and α-[7-(1,3-dimethylxanthinyl)] methyl methyl sulfone, respectively (Kamei et al. 1975). Bacterial degradation through C8 oxidation results in the formation of 1,3,7TMU, which is further degraded to trimethylallantoin, *N*,*N*′-methylurea and *N*-methylurea (Madyastha and Sridhar 1998). In urine; a larger fraction of 6-amino-5(*N*-formylmethylamino)-1,3-dimethyluracil (1,3,7DAU) was excreted in rat (30%) in contrast with monkey and man (2%) (Latini et al. 1981). When [Me-^{14}C]-1,3,7DAU was administered orally or intravenously to rats, no further metabolites could be found (Arnaud et al. 1983). After identification and quantification in rat urine of 1,3,8-trimethylallantoin (1–14%) (Rao et al. 1973; Arnaud 1976), its formation from caffeine was demonstrated in rat liver slices and all N-demethylation, oxidation to uric acids, and formation of uracil derivatives were also demonstrated in vitro (Arnaud et al. 1986a). In rat liver slices only primary metabolites were detected and N1-demethylation was the most important pathway, with theobromine representing 51% of total dimethylxanthines produced and 1,3,7DAU was an important metabolite, corresponding to 9.7% of total caffeine metabolites (Bienvenu et al. 1990). In mice strains paraxanthine glucuronide was identified, a metabolite not found in other animal species and in humans (Arnaud et al. 1989). In beagle dogs, the most important metabolic pathway of caffeine (2.8% of the dose) was the 7-methyl demethylation to theophylline (8% with paraxanthine) with further metabolism to 1,3DMU (13%), 3-methylxanthine (3MX) (21%), and 1MU (8%) excreted in urine. Minor metabolites were theobromine (5%), 1,3,7TMU (2.5%), 1,7DMU (2%), 1MX (1%), and 7-methyluric acid (7MU) (2.5%) (Aldridge and Neims 1979). In 2-day-old puppies, urinary caffeine metabolites derived, respectively, from paraxanthine, theophylline, and theobromine accounted for 42, 33 and 14%. Between 2 and 22 days of age, this metabolic pattern changed, with metabolites derived from theophylline increasing from 33 to 82% (Aldridge and Neims 1980). The metabolism of [2-^{14}C]caffeine (4 mg/kg intravenously) studied in rabbits showed that the major urinary metabolites were 1MX (22%), 1MU (19%), 7-methylxanthine (7MX) (16%), and paraxanthine (14%), with other minor metabolites such as 3-methyluric acid (3MU) (4.4%), theobromine (4.0%), 1,7DMU (3.9%), 3MX (3.8%), 1,3DMU (2.7%), 1,3,7TMU (2.0%), and theophylline (1.6%), while the uracil derivative AAMU amounted to 4.9% (Beach et al. 1985). After caffeine administration, similar hepatic capacity to clear caffeine was observed, but 7-demethylation was the preferred pathway in sheep and 3-demethylation in cattle, suggesting different species-specific expression of the CYP1A subfamily (Danielson and Golsteyn 1996).

Caffeine metabolism in humans includes multiple and separate pathways with demethylation to dimethylxanthines and monomethylxanthines, C8 oxidation of these methylxanthines into methylurates, and ring opening yielding substituted uracil derivatives. The reverse biotransformation of theophylline to caffeine was first shown in infants but later also in adult subjects. From the metabolic pathways of caffeine (Fig. 1) it is apparent that each metabolite may be derived from more than one precursor and assessment of caffeine demethylations from urinary metabolite profiles is not accurate (Lelo et al. 1986b). In Fig. 2 the various ratios of metabolites that have been used to measure activities of enzymes involved in caffeine metabolism are shown, particularly CYP1A2, NAT2, and xanthine oxidase. The analysis of urinary caffeine metabolites in man shows the presence of uracil derivatives produced from caffeine, 1,3,7DAU (Arnaud and Welsch 1980a), from theobromine, 6-amino-5-(*N*-formylmethylamino)-1-methyluracil (3,7DAU) (Arnaud and Welsch 1979a, 1980a), and from paraxanthine, 6-amino-5-(*N*-formylmethylamino)-3-methyluracil (1,7DAU) (Arnaud and Welsch 1980a). The amount of 1,3,7DAU found in the urine of adult subjects is about 1% of the administered dose, while its excretion increased in the urine of a premature infant in the case of caffeine overload (Gorodischer et al. 1986a). In this study, the 1,3,7DAU identified in urine was of neonatal and not of maternal origin as it was not present in the urine from the infant obtained prior to administration of caffeine. The acetylated uracil derivative AAMU detected in man (Callahan et al. 1982) has not been identified in animal species. Its precursor was detected, isolated, purified, and identified as AFMU, a structure confirmed by chemical synthesis (Tang et al. 1983). AFMU was unstable in the presence of dilute base and/or methanol, giving rise to a deformylated compound, AAMU, which was reported in the literature as a major metabolite of caffeine in man (Arnaud 1980; Arnaud and Welsch 1980a; Callahan et al. 1982). The production and excretion rates of AAMU and AFMU were shown to be related to the acetylation polymorphism (Grant et al. 1983) with a bimodal distribution of the general population into fast and slow acetylators. Paraxanthine is the precursor of AFMU, which accounts for 67% of paraxanthine clearance. The rate of AFMU production and clearance approximates and changes according to the rates for the production of 1MX and 1MU (Yesair et al. 1984), suggesting that its formation occurs through a common precursor of AFMU and 1MX. This intermediate has not yet been identified. A major difference between humans and rats is the total excretion of caffeine and metabolites without demethylation, which amounts to 5 and 42% of the dose administered, respectively (Arnaud and Welsch 1980a; Arnaud 1985). From the analyses of urine metabolites

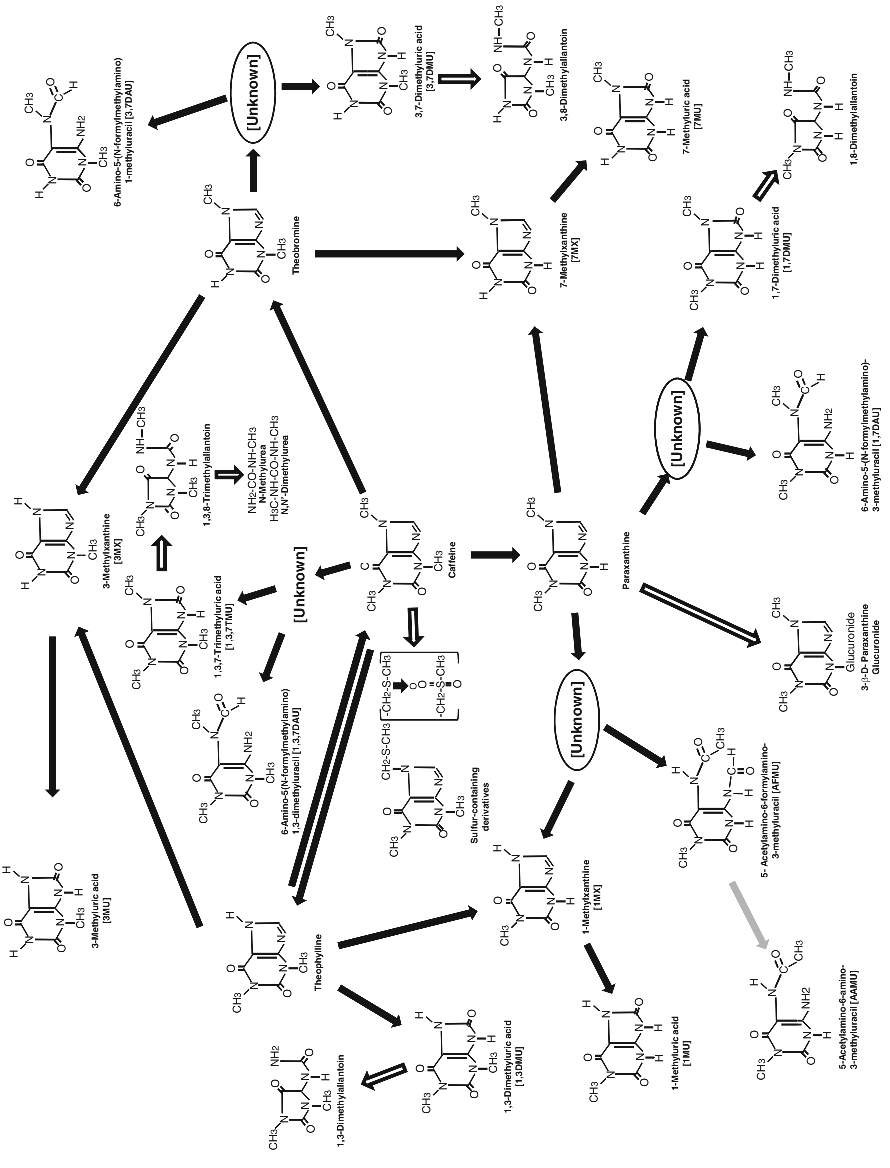

Fig. 1 Metabolic pathways of caffeine, theophylline, theobromine, and paraxanthine in human (➡) and animals (⇨)

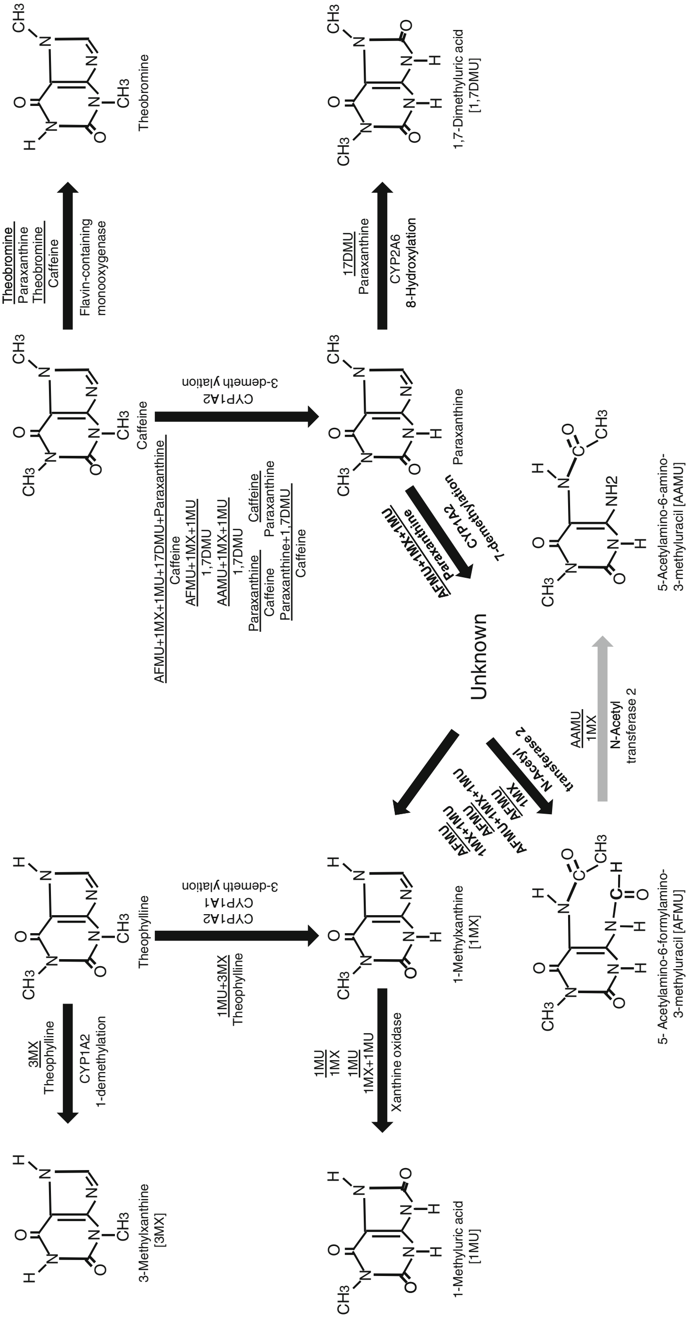

Fig. 2 Metabolite molar ratios measured in urine and plasma used as indexes of enzyme activities

in humans, the quantitative importance of the metabolic pathways through paraxanthine (72%) was the greatest followed by theobromine (20%) and theophylline (8%) (Arnaud and Welsch 1980a, 1982). These results were confirmed where 78 ± 11% of the excreted metabolites were metabolized through the paraxanthine pathway, 14 ± 8% through theobromine, and 9 ± 4% through theophylline. However, the plasma AUC for dimethylxanthines underestimates the formation of paraxanthine, overestimates the formation of theobromine, and gives a similar formation for theophylline from caffeine, when compared from the urinary metabolites (Rodopoulos and Norman 1996). From the plasma AUC of caffeine and each dimethylxanthine, the mean fractional conversion of caffeine to paraxanthine, theobromine, and theophylline was 79.6 ± 21.0, 10.8 ± 2.4, and 3.7 ± 1.3%, respectively (Lelo et al. 1986b). Another study found that paraxanthine accounted for 63 ± 13% of the dimethylxanthines in plasma, theobromine 27 ± 15%, and theophylline 10 ± 2.6% (Rodopoulos and Norman 1996). For demethylation, 3-demethylation represents 52% of all metabolites, 7-demethylation 35%, and 1-demethylation 13% (Arnaud and Welsch 1980a; Yesair et al. 1984). When demethylation pathways are considered from plasma AUC results, paraxanthine, theobromine, and theophylline accounted for 83.9 ± 5.4, 12.1 ± 4.1, and 4.0 ± 1.4%, respectively, of the caffeine demethylations (Lelo et al. 1986b). From clearance values of caffeine and its primary metabolites, it was calculated that approximately 37% of a caffeine dose was biotransformed to paraxanthine (Tang-Liu et al. 1983), a lower value when compared with excreted metabolite. To quantify the total demethylation process, the administration of [1,3,7-Me-^{13}C] caffeine to volunteers and continuous collection of expired $^{13}CO_2$ showed that 21–26% of the total ^{13}C administered was recovered in expired CO_2 over 24 h, corresponding to a mean percentage of demethylation for each methyl group (Arnaud et al. 1980). The quantitative urinary excretion of caffeine metabolites in man and in various animal species, expressed as the percentage of the administered dose, is shown in Table 1. However, large individual variations in urinary metabolite excretion have been reported and the caffeine metabolites recovered, expressed as the percentage of the dose, in young and elderly men showed significantly higher excretion in the elderly for 1,7DMU ($P < 0.05$), 1MU ($P < 0.03$), and 7MU ($P < 0.03$) for both oral and intravenous administration, but lower urine recoveries were observed in young men (Blanchard et al. 1985).

3 Theophylline

3.1 *Absorption*

Theophylline transfer across rat jejunum in vitro showed that its clearance was directly proportional to the fraction unionized at various pH values (Perry et al. 1984), and there were small but nonsignificant differences in absorptive capacity between the

Table 1 Urinary excretion of caffeine, theophylline, theobromine, and paraxanthine metabolites in human and animals

	Caffeine		Theophylline		Theobromine		Paraxanthine	
	Human	Animals	Human	Animals	Human	Animals	Human	Animals
Caffeine	1.2	2^{a}, 3^{b}, 0.9^{c}, 4.5^{d}						
Theophylline	1	0.7^{a}, 6^{b}, 1.6^{c}, $12.5{*}^{d}$	16	38^{b}				
Theobromine	2	4^{a}, 8^{b}, 4^{c}, 7.5^{d}			20	26^{a}, 53^{b}, 19.5^{c}, 50^{d}		
Paraxanthine	6.5	14.5^{a}, 12.5^{b}, $14.5^{c,*d}$					11	52^{b}
Trimethyluric acid	1.4	4^{a}, 8^{b}, 2^{c}, 4^{d}						
Trimethylallantoin		0.4^{a}, 7^{b}						
5-Acetylamino-6-formylamino-3-methyluracil	16	–					20	
5-Acetylamino-6-amino-3-methyluracil	–	5^{c}						
6-Amino-5-[*N*-formylmethylamino]-1,3-dimethyluracil	1.2	9.5^{a}, 21^{b}						
1,7-Dimethyluric acid	6	6.3^{a}, 5^{b}, 4^{c}, 3^{d}					17	7^{b}
6-Amino-5[*N*-formylmethylamino]-3-methyluracil	2.5	1.4^{a}, 2.5^{b}					5	7^{b}
1,3-Dimethyluric acid	2.6	7.5^{a}, 4^{b}, 2.5^{c}, 20^{d}	47	38^{b}				
6-Amino-5[*N*-formylmethylamino]-1-methyluracil	2	5^{a}, 6^{b}			12	44^{a}, 29^{b}, 14^{c}, 10^{d}		
3,7-Dimethyluric acid	0.8	1^{a}, T^{b}			1	4^{a}, 4.5^{b}, 2^{c}, 0.5^{d}		
Dimethylallantoin		T^{b}		$<1^{b}$		T^{b}		
1-Methylxanthine	19		1	$<1^{b}$			17	11^{b}

		$6.3^a, 5^b, 22^c, 1.5^d$						
7-Methylxanthine	7.5	$3^a, 2^b, 16^c$			36	$12.5^a, 4^b, 49^c\text{-}4.5^d$	5.5	2^b
3-Methylxanthine	3	$2^a, 1^b, 4^c, 31^d$	14	3.5^b	21.5	$5^a, 6.5^b, 11.5^c, 27^d$		
1-Methyluric acid	26.5	$8.5^a, 6.5^b, 19^c, 12.5^d$	20	20^b			19	21^b
7-Methyluric acid	–	$1^a, 0.8^b, 3.5^d$			8.5	$8^a, 2^b, 3^c, 6^d$	4.5	T^b
3-Methyluric acid	0.1	$2^a, 0.3^b, 4.5^c$	1	$<1^b$	0.6	$0.5^a, T^b, 0.8^c, 1^d$		
3-β-D-Paraxanthine glucuronide	–	$20^a, 0^b$						$9–25^a$
α-[7-(1,3-Dimethylxanthinyl)] methyl methyl sulfoxide	–	$T^{a–c}$						
α-[7-(1,3-Dimethylxanthinyl)] methyl methyl sulfide	–	T^a						
α-[7-(1,3-Dimethylxanthinyl)] methyl methyl sulfone	–	T^a						
N-Methylurea	–	T^b				T^b		
N,*N*′-Dimethylurea	–	T^b						

The results are expressed as the percentage of metabolites excreted in urine (at 48 h in human). From Arnaud and Welsch (1980a, b), Callahan et al. (1982), Shively and Tarka (1983), Tarka et al. (1983), Miller et al. (1984), Arnaud (1984, 1985), Beach et al. (1985), Birket et al. (1985), Arnaud et al. (1989), Rodopoulos et al. (1996), and Rodopoulos and Norman (1997).

T traces

[a] Mouse urine (24–36 h)

[b] Rat urine (24 h)

[c] Rabbit urine (36 h)

[d] Dog urine (48 h)

[*] Value of theophylline + paraxanthine

intestinal segments studied (Murray et al. 1993). The rhythmicity in plasma levels found in theophylline disposition was not due to diurnal variation in the passive transport of the mucosa, but may be caused by differences in food intake between morning and evening, in the transit time or gastric emptying, or in the amount or composition of the gastric or intestinal fluid (Tukker and Meulendijk 1991). Exsorption of theophylline from blood to the gastrointestinal tract corresponded to 12–15%, while the extent of the drug excreted into the bile varied from 0.17 to 0.30% (Arimori and Nakano 1988). Rectal absorption of theophylline is slow but complete.

Also in humans, theophylline is rapidly and completely absorbed (Ogilvie 1978; Yesair et al. 1984). It appeared to be almost completely absorbed before it reached the jejunum and the jejunal concentrations were lower than 10% of the maximal duodenal concentrations (Brouwers et al. 2005). The absolute bioavailability of theophylline was investigated by comparing the AUC after intravenous and oral administration of theophylline. The fraction of the dose absorbed averaged 0.99 ± 0.02, thus showing a bioavailability close to 100% (Hendeles et al. 1977) as well as in neonates and young infants (Moore et al. 1989). Food decreased significantly the absorption rate of theophylline, prolonged $T_{\max}$, and decreased $C_{\max}$, but the AUC was slightly but not significantly smaller, indicating that theophylline bioavailability was not modified (Jonkman et al. 1985). Oral administration of activated charcoal is a well-established therapy for treatment of theophylline intoxication (Cooling 1993).

3.2 Distribution

Plasma theophylline concentrations in guinea pigs could be quantitatively described by a two-compartment model with nonlinear elimination kinetics and individual volume distribution of theophylline at each dose (Sato et al. 2007). Protein binding in blood was 48.8 ± 6.2% in the rats (Ingvast-Larsson et al. 1992). The theophylline dose required to achieve the narrow therapeutic concentrations (10–20 mg/mL) varies among subjects, largely because of differences in metabolism. Another important pharmacokinetic parameter is protein binding. Theophylline binds mainly to albumin and the protein binding of approximately 50% was shown to be nonlinear (Fleetham et al. 1981; Trnavská 1990) ,with little variations in healthy subjects but important changes for physiological (Shaw et al. 1982) and disease (Lesko et al. 1981; Siegel et al. 1990; Korrapati et al. 1995) states. Measured saliva levels allow predictions of the unbound serum theophylline levels. The therapeutic range for saliva, which corresponds to the accepted total serum concentration range of 10–20 μg/mL, is approximately 5.55–11.3 μg/mL (Blanchard et al. 1992).

Recovery of theophylline is much less than that of caffeine in brain, liver, muscle, and adipose tissue (Ståhle 1991), reflecting the lower lipid solubility of theophylline. Lower theophylline concentrations were found in the brain (91 μM) than in other tissues (120 μM) and this rate of penetration into the brain extracellular space was higher for caffeine than for theophylline (Ståhle et al. 1991). With microdialysis methods applied in anesthetized rats, striatum-to-blood ratios at the steady state of approximately 0.5 were shown (Sjöberg et al. 1992). These results were confirmed and compared with fetal brain AUC values of theophylline; those found in the brains of adults were lower compared with those found in plasma after a dose of 25 mg/kg, suggesting that theophylline might be selectively excluded from the adult brain (Wilkinson and Pollard 1993). Theophylline crosses the placenta and distributes in the organs of the rat fetus and the pregnant animal, except for the brain, where the exposure of the fetal brain was twice that of the adult brain (Arnaud et al. 1982a). Placental clearance of theophylline averaged 0.62 mL/min in the rabbit but it was difficult to extrapolate these results as human and rabbit placentas are structurally dissimilar (Omarini et al. 1991).

3.3 Excretion

There were no significant differences in the elimination and metabolism of [8-^{14}C] theophylline when given orally or intravenously to rats. Fecal excretion amounted to 5 ± 3% of the dose after 7 h and increased to 18 ± 2% after 24 h. About 25% of the dose was secreted in the bowels (Arnaud and Welsch 1980b; Arnaud et al. 1982a, b). After 1 day, 70 ± 7% of the dose was excreted in urine and 6 ± 1% in CO_2 and less than 1% remained in the body (Arnaud and Welsch 1980b). In pregnant rats, labeled caffeine was found in the fetus and only traces were detected in the urine. At the 18th day of pregnancy, unchanged theophylline corresponded to 70 ± 10% of urine activity, suggesting impaired metabolism when compared with 35 ± 3% in nonpregnant rats (Arnaud and Bracco 1981). Theophylline in humans is completely absorbed and fecal excretion has not been reported.

In premature infants with postconception ages of 28–42 weeks, the urinary percentages of unchanged theophylline decreased from 61 to 43%, respectively, suggesting an increased theophylline metabolism into 1,3DMU with age (Tserng et al. 1983). In 10–12-year-old asthmatic children, the percentage of unchanged theophylline excreted in the urine was 11.6 ± 1.75%. Metabolites found in urine in addition to theophylline were 3MX, 1,3DMU, and 1MU (Monks et al. 1979; Wijnands et al. 1990). Theophylline was shown to be extensively reabsorbed in the renal tubule and its renal clearance was highly urine flow dependent and urinary excretion varied with urine output (Tang-Liu et al. 1983).

3.4 Pharmacokinetics

In rats, the plasma concentration decayed according to a first-order process with an apparent half-life of about 4 h, but after 4–8 h the slope of the curves declined, resulting in elimination half-lives of about 70 min, a value similar that for lower doses. The AUC increased disproportionately with dose, indicating capacity-limited elimination, but there was no capacity-limited elimination of 1,3DMU and 1MU with the dose. These results showed that linear pharmacokinetics of theophylline in rats can be applied only to doses not exceeding 10 mg/kg (Teunissen et al. 1985). The plasma theophylline concentrations in rat declined in a monoexponential manner, while those of 1MU and 1,3DMU declined in a biexponential manner upon their injection. The total body clearances of the metabolites were fourfold to sixfold larger and their distribution volumes were 40–50% smaller than that of theophylline (Kuh and Shim 1994). The pharmacokinetics of theophylline were investigated in Cyp1(+/+) wild-type mice, Cyp1a1 (−/−) and Cyp1a2(−/−) knockout mice, and humanized hCYP1A1_1A2 mice lacking either the mouse Cyp1a1 or the mouse Cyp1a2 gene. The half-life of elimination from plasma was more than 4 times longer in Cyp1a2(−/−) mice than in Cyp1(+/+) mice. In humanized hCYP1A1_1A2 mice lacking the mouse Cyp1a2 gene, the half-life of elimination from plasma was 2–3 times longer than that in Cyp1(+/+) mice (Derkenne et al. 2005). A pharmacokinetics study conducted in dogs showed the bioequivalence of the two injectable forms containing theophylline and aminophylline (ethylenediamine salt of theophylline) and thus the lack of influence of ethylenediamine on the pharmacokinetics of theophylline (Kawai et al. 2000).

The demethylation of theophylline at high concentrations shows biphasic kinetics in the production of individual metabolites with human microsomes (Campbell et al. 1987a). In children with chronic asthma receiving two dosage levels of theophylline, the steady-state serum concentrations increased to a greater degree than predicted with a significantly lower clearance at the higher dose ($P < 0.02$). These results showed the nonlinear nature of the relationship between dose and theophylline serum concentration in these children with asthma (Weinberger and Ginchansky 1977). Theophylline elimination from blood in a 10-month-old female acutely intoxicated had a half-life of 10.0 h, an elimination that was anomalously long for a child of this age (Jarboe et al. 1986). Theophylline pharmacokinetics in asthmatic patients of 8–18 years of age showed that $C_{\max}$ of 8.4 ± 1.7 mg/L occurred 2.2 h after oral ingestion, with a mean serum half-time for theophylline of 5.8 ± 1.7 h (Becker et al. 1984). The volume of distribution of theophylline depends primarily on age; it is twofold greater in newborns than in adults (Tröger and Meyer 1995). These results were confirmed in healthy male volunteers with total plasma clearances of theophylline of 0.93 mL/min/kg, unbound plasma clearances of 1.61 mL/min/kg, half-lives of 6.2 h, a volume of distribution at steady state of 0.44 L/kg, and an unbound volume of distribution of 0.77 L/kg (Lelo et al. 1986a). Studies have established relationships between renal clearance and urine flow rate for caffeine and theophylline (Trang et al. 1985). Theophylline frequently exhibits nonlinear pharmacokinetics with a relatively large intrapatient variability in clearance over time (Pan et al. 2000). A study confirmed the intrasubject variability reported for theophylline clearance in healthy male volunteers but no significant dose dependency was observed for doses of 1 and 6 mg/kg (Fleetham et al. 1981).

3.5 Metabolism

Several CYP isoenzymes, including CYP1A2, CYP2E1, and CYP3A4, are involved in the hepatic metabolism of theophylline (Pan et al. 2000). Theophylline has (as caffeine) been used as a marker of CYP1A2 activity (Obase et al. 2003). In human adults, approximately 90% of theophylline is metabolized in the liver by CYPs, while unchanged theophylline is excreted via the kidneys (Tröger and Meyer 1995). CYP1A is responsible for theophylline N-demethylation to 3MX and 1MX (Sarkar and Jackson 1994). The positive relationship between clearance of 1MU and of 3MX in both smokers and nonsmokers ($P < 0.001$) suggests that the two N-demethylation pathways for theophylline metabolism are under common regulatory control and involve a CYP distinct from that mediating 8-hydroxylation of theophylline to 1,3DMU (Grygiel and Birkett 1981). Theophylline is metabolized by 8-hydroxylation to 1,3DMU, which accounts for about half of the clearance of the drug in humans (Ogilvie 1978), and by N-demethylation to 3MX and 1MX. Although theophylline 8-hydroxylation is catalyzed by several CYP subfamilies (Zhang and Kaminsky 1995; Gu et al. 1992; Sarkar et al. 1992), CYP1A2 is reported to play a major role only at lower substrate concentrations (Zhang and Kaminsky 1995). A 30-fold individual difference was observed for the 1MU plus 3MX to theophylline ratio in patients receiving theophylline therapy, and in healthy volunteers a 70-fold difference was found for the 1MX plus 1MU plus AFMU to 1,7DMU ratio. The CYP1A2 activities were not significantly influenced by CYP1A2*1C or CYP1A2*1F polymorphism, suggesting that these CYP genotypes are not major factors for the variability of CYP1A2 activity. The CYP1A2*1K haplotype seems to show a very low frequency in this Japanese population (Takata et al. 2006). Pretreatment in rats with an inhibitor or inducer of CYPs such as troleandomycin, 3-methylcholanthrene, orphenadrine or dexamethasone suggested that theophylline was metabolized via CYP1A1/CYP1A2, CYP2B1/CYP2B2, and CYP3A1/CYP3A2, and that 1,3DMU is primarily formed via CYP1A1/CYP1A2, and possibly CYP3A1/CYP3A2 (Yang et al. 2008).

Theophylline was metabolized in cultured hepatocytes and in liver slices of young and adult rats into 1MU, 1MX, 1,3DMU and/or 3MX, caffeine, a uracil derivative, and two unknown polar compounds. Although the same metabolites were identified in young and adult rats, the development pattern was not uniform and formation of caffeine from theophylline was not dependent on a lack of activity of other pathways. Preincubation with caffeine or theobromine inhibited theophylline metabolism (Gorodischer et al. 1986b). In human liver microsomes, the formation of 3MX, 1MX, and 1,3DMU from theophylline was reported and the two demethylation pathways seemed to be performed by the same enzyme (Robson et al. 1988). In addition, α-naphthoflavone inhibited theophylline demethylations, whereas 8-hydroxylations were generally less inhibited (Campbell et al. 1987a). In microsomes prepared from different human livers, the formation of 3MX and 1MX correlated best with amounts of the immunoreactive protein HLd (P-IA2) ($P < 0.05$), whereas formation of 1,3DMU correlated with the microsomal content of HLp (P-IIIA3) and HLj (P-IIE1). In immunoinhibition experiments, incubations conducted with a polyclonal antirat P-c/d antibody, the formation of all three theophylline metabolites was significantly inhibited ($P < 0.05$). However, addition of isoform-specific antirat-CYP-d antibodies to the microsomal mixture significantly and selectively inhibited 1-N-demethylation, with little inhibition of 3-N-demethylation or 8-hydroxylation (Sarkar et al. 1992). 1MX seemed to be mediated by CYP1A1/CYP1A2 and 3MX specifically by CYP1A2 (Sarkar and Jackson 1994). CYPs expressed in human B-lymphoblastoid cell lines showed that at high theophylline concentration (10 mM) four CYPs (CYP1A1, CYP1A2, CYP2D6, CYP2E1) catalyzed the metabolism of theophylline, but the highest affinity was for the CYP1A subfamily. CYP2E1, responsible for a relatively high intrinsic clearance by 8-hydroxylation, may be the low-affinity high-capacity isoform involved in theophylline metabolism. The affinity of theophylline for CYP1A1 was comparable with that of its homologue CYP1A2 and when induced, the participation of CYP1A1 in theophylline metabolism may be important. CYP2D6 played only a minor role and CYP3A4 was not active in the in vitro metabolism of theophylline. These results confirm the major role of CYP1A2 in theophylline metabolism and explain why the elimination kinetics of theophylline in vivo are nonlinear (Ha et al. 1995). In microsomes, at low theophylline concentrations the metabolism of theophylline to 1,3DMU was catalyzed primarily by CYP1A2, while at high substrate concentrations CYP2E1 was primarily responsible for 1,3DMU formation. At theophylline concentrations achieved in vivo, its metabolism must thus be catalyzed primarily by CYP1A2 (Zhang and Kaminsky 1995). In human, rabbit, and rat liver microsomes 1,3DMU accounted, respectively, for 59, 77, and 94% of the total metabolites formed. In both human and rabbit liver microsomes the N-demethylation of theophylline to 1MX accounted for 20% of the total metabolites formed. In human microsomes N-demethylation of theophylline to 3MX accounted for 21% of theophylline metabolism, but it was a minor pathway in rabbit and rat microsomes (McManus et al. 1988).

3.6 Sources of Variation in Theophylline Pharmacokinetics and Metabolism

Age. Differences in the half-life and total clearance were found among the age groups with a linear correlation between age and the clearance of theophylline (Kearns et al. 1986). The average mean residence time of theophylline was significantly longer in 20-month-old rats than in 2- and 14-month-old rats. A greater elimination rate constant was observed in 14-month-old rats and the apparent volume of distribution decreased from 0.71 to 0.57 L/kg in the 2- and 20-month-old rats, respectively (Jung and Nanavaty 1990). The ability of the rat fetus to methylate theophylline into caffeine was demonstrated when [8-^{14}C] theophylline was administered to pregnant rats (Arnaud et al. 1982a, b). However, the biotransformation of theophylline to caffeine reported for human neonates and the rat fetus was not observed in neonatal piglets (Kearns et al. 1986).

In premature neonates, weighing less than 1,500 g at birth, and under 32 weeks of gestational age, theophylline clearance was lower (12 mL/h/kg) and the volume of distribution (0.8–0.9 L/kg) was higher than previously reported for less premature neonates, term babies, and older children (Lee et al. 1996). The weight-normalized value of the volume of distribution in premature neonates during the first week of life was 0.63 L/kg (du Preez et al. 1999). In contrast to older children and adults, in whom theophylline disposition follows zero-order kinetics at high concentrations, a monoexponential function best described theophylline elimination in the premature newborn, with half-lives ranging from 24.7 to 36.5 h and estimated clearance ranging from 0.02 to 0.05 L/kg/h (Lowry et al. 2001). In premature neonates, only unchanged theophylline and caffeine were found in urine, indicating the absence of oxidative pathways for theophylline metabolism. In both adults and children, there was high positive correlation between urinary excretion of 3MX and 1MU. Both 3MX and 1MU correlated negatively with urinary excretion of 1,3DMU (Grygiel and Birkett 1980). The metabolism of theophylline in premature infants showed that the urinary percentages of unchanged theophylline decreased from 61% at a post-conception age of 28–32 weeks to 43% at 38–42 weeks. This increased metabolism of theophylline is explained by the production of 1,3DMU (20–34%). It was hypothesized that methylation of theophylline to caffeine is equally active in adults and premature infants and the absence of caffeine in adults is due to the maturing caffeine-metabolizing enzymes (Tserng et al. 1983).

Postnatal age was the most powerful predictor for theophylline half-life in the neonatal period, while gestational age, duration of treatment, and weight did not correlate significantly with any pharmacokinetic parameters (Dothey et al. 1989). Theophylline clearance reached adult values at 55 weeks of postconceptional age and the disappearance of serum caffeine concentrations and the maturation of theophylline clearance were primarily related ($P < 0.001$) to development of the demethylation pathway to 3MX. Postconceptional age was the major factor ($P < 0.001$) explaining the interpatient variability in theophylline clearance (Kraus et al. 1993). The total clearance of theophylline was 87–100 mL/h/kg in children and 57 mL/h/kg in adults, with important interindividual differences in the biological half-life (1.42–7.85 h) and a higher elimination rate constant (0.49 ± 0.30/h) in the children (Ellis et al. 1976; Gardner and Jusko 1982; Kolski et al. 1987; Berdel et al. 1987). There was a linear decrease in clearance with increasing age (1.3–30.0 years) regardless of the sex (Gardner and Jusko 1982).

In healthy volunteers and patients with asthma, 20–87-years old and receiving theophylline, although clearance did not fall with increasing age during younger adult life, there was a fall during late adult life, becoming apparent in the seventh, eighth, and ninth decades of age with a reduction in the basal rate of theophylline metabolism (Crowley et al. 1988; Jackson et al. 1989) and plasma clearance of theophylline was 30% lower in elderly male subjects than in young male subjects (Loi et al. 1997). A considerably higher interindividual variability in the disposition of theophylline was observed in frail elderly women (Groen et al. 1993).

Gender and hormones. There were statistically significant differences ($P < 0.01$) in the theophylline kinetic parameters, such as the elimination half-life, 8.70 ± 0.60 h during proestrus, 4.61 ± 0.16 h during estrus, and 5.01 ± 0.85 h during diestrus, and the AUC were 214.61 ± 3.58, 128.64 ± 9.64, and 165.57 ± 23.86 μg h/mL, respectively (Bruguerolle 1987). In pregnant rats, theophylline was eliminated at a slower rate than in both lactating rats and virgin controls, resulting in a longer half-life and lower clearance, while the volumes of distribution in pregnant, lactating, and control rats were not different (Brandstetter et al. 1986) and the impaired theophylline metabolism in late pregnancy exhibited increased excretion of unchanged theophylline with decreased formation (−68%) of 1,3DMU and (−30%) of 1MU (Arnaud et al. 1982a, b).

The elimination of theophylline does not differ between men and women (Jusko et al. 1979; Powell et al. 1977). Several other studies looking at the effect of gender on theophylline clearance in children (8 years) reported that gender had no effect on theophylline clearance (Ellis et al. 1976; Yano et al. 1993), but male children aged 4–20 years were shown to have significantly higher theophylline clearances (31 and 22%, respectively) than female children in other studies (Gardner and Jusko 1982; Driscoll et al. 1989; Igarashi and Iwakawa 2009). In healthy men and premenopausal women, statistically significant gender-related effects were seen for the theophylline half-life and clearance (Jennings et al. 1993). The disposition of theophylline throughout pregnancy and in the postpartum period showed that theophylline clearance was slightly affected during the first two trimesters (2.61–2.85 L/h), while a statistically significant reduction was observed late in pregnancy (2.05 L/h). The postpartum clearance values suggest an ongoing suppression relative to prepregnancy levels. A significant higher half-life of 13.00 ± 2.31 h was observed during the third trimester when compared to 9.53 ± 3.53 h in the postpartum period. The absolute volume of distribution increased with gestation (Gardner et al. 1987). Chronic oral contraceptive users exhibited significantly lower total plasma theophylline clearance (−30%) and the half-life was also significantly prolonged from 7.3 to 9.8 h, while the volume of distribution was unchanged (Tornatore et al. 1982; Teichmann 1990). In contrast, acute oral contraceptive exposure failed to induce significant changes (Gardner et al. 1983).

Physical exercise. The volumes of theophylline distribution decreased significantly after exercise in the heat, apparently due to dehydration (Schlaeffer et al. 1984; Lenz et al. 2004).

Obesity. Age was the most important determinant of theophylline clearance in pediatric patients and weight had less effect than age and did not statistically improve the model ($P > 0.005$) when combined with age (Driscoll et al. 1989). In obese and normal subjects, the apparent volume of distribution measured from the total body weight (TBW) or the ideal body weight (IBW) averaged 0.482 L/kg TBW in normal subjects and 0.382 L/kg TBW in obese subjects and 0.77 L/kg IBW in obese subjects. Clearance averaged 63.0 mL/h/kg IBW in normal subjects and 32.8 mL/h/kg TBW and 64.1 mL/h/kg IBW in obese subjects. The mean half-lives were longer in obese subjects than in normal subjects, 8.6 ± 2.0 and 6.0 ± 2.1 h, respectively (Gal et al. 1978).

Drugs. As expected from the metabolism described already, theophylline pharmacokinetics can be influenced by drugs, herbal supplements, and diet. The extensive literature on the interactions of drugs with theophylline will not described here. A review on the interaction of drugs has been published describing an increase or a decrease of theophylline clearance (Upton 1991). One aspect that needs mentioning is that theophylline is demethylated to 1MX and 1MU was produced from a rapid xanthine oxidase mediated 8-oxidation, while no 1MU was formed by 3-demethylation of 1,3DMU (Birkett et al. 1983).

Cigarette smoking appeared to induce theophylline metabolism as reflected by the mean theophylline half-life in smokers (5.4 h) versus nonsmokers (8.3 h) (Jusko 1979; Jusko et al. 1979; Powell et al. 1977). Cigarette smoking significantly altered the theophylline clearance processes (Schrenk 1998; Teichmann 1990; Jennings et al. 1993; Zevin and Benowitz 1999) and a 40% elevation in theophylline clearance was observed in women who smoked (Gardner et al. 1983).

Daily caffeine intake significantly altered the theophylline clearance processes (Gardner et al. 1983). On caffeine administration, the theophylline steady-state concentration and AUC increased by 23 and 40%, respectively, and the reduction in the apparent total body clearance and elimination rate constant of theophylline reached 29 and 31%, respectively, indicating a pronounced influence on theophylline of concomitant ingestion of caffeine in normal consumers (Jonkman et al. 1991). Abstention from methylxanthine-containing foods and beverages led to a significant decrease in the elimination half-life ($P < 0.02$) owing to increases in the elimination constants for theophylline, 3MX, and 1,3DMU (Monks et al. 1979). However, in contrast to the effect of deprivation of dietary methylxanthines, the addition of extra methylxanthines from six bottles per day of a cola beverage to the diet did not influence the disposition of theophylline (Monks et al. 1981).

Diseases. As well as for caffeine, diseases that compromise liver function, especially cirrhosis, reduce theophylline clearance in animals and man (Park et al. 1999; Nam et al. 1997; Amodio et al. 1991). There is also a small effect in diabetes mellitus rats induced by alloxan or streptozotocin (Kim et al. 2005). In patients with insulin-dependent diabetes mellitus and in sex-, age-, and weight-matched healthy nonsmokers, the pharmacokinetic parameters of theophylline, plasma clearance, elimination half-life, and volume of distribution were similar, but there was a positive correlation between hemoglobin A1c values and plasma theophylline clearance ($P < 0.05$), formation clearance of 1,3DMU ($P < 0.05$), and formation clearance of 1MU ($P < 0.05$) (Korrapati et al. 1995). Among animal and human studies, renal disease, Down syndrome, psoriasis, endotoxin-induced fever, acidosis, hypoxia, hyperlipidemia, and hypoalbuminemia were shown to alter the pharmacokinetics of theophylline.

3.7 Metabolites and Metabolic Pathway

The methylation of theophylline to caffeine was shown in rat (Gorodischer et al. 1986b) and in rat fetus (Arnaud et al. 1982a, b), in premature infants (Boutroy et al. 1979; Bory et al. 1979), and in vitro in the human fetal liver (Aranda et al. 1979).

In premature infants, plasma concentrations of caffeine increased from 1.8 mg/L at day 1 to 3.7 mg/L 7 days after initiation of theophylline therapy. Labeled caffeine, paraxanthine, and theobromine were found in plasma and urine of preterm newborns receiving [1,3-^{15}N],[2-^{13}C]theophylline for the treatment of primitive apneas, showing that theophylline was converted to caffeine by N7-methylation (Brazier et al. 1980b). Several studies confirmed this methylation pathway in the newborn (Soyka et al. 1981; Simons et al. 1981). This pathway was believed to be specific to the neonatal period, explained by the immaturity of liver enzymes. However, caffeine (0.21–0.75 mg/L) and its major metabolite, paraxanthine, were observed in plasma following oral administration of theophylline (8.1–21.5 mg/L) in a multiple-dose study in healthy subjects. In adult subjects, about 6% of the theophylline dose was converted to caffeine (Tang-Liu and Riegelman 1981; Arnaud 1984). Only 7–19% of theophylline is excreted unchanged in the urine with other metabolites, including 1,3DMU (35–55%), 1MU (13–26%), 3MX (9–18%), 1MX (0.3–4%), and 3MU (1%) (Arnaud 1984; Birkett et al. 1985; Anonymous 1991). The N3-demethylation of theophylline accounted for 34 ± 6% of the urinary metabolites, N1-demethylation of theophylline for 15 ± 3%, and C8-oxidation of theophylline for 51 ± 9%. The C8-oxidation of 1MX and 3MX corresponded to 93 ± 4 and 9 ± 4%, respectively, of the excreted monomethylxanthine and urate. In addition to theophylline, 1,3DMU and 1MU were consistently found in plasma and saliva. Theophylline accounted for 91 ± 4% of the total plasma AUC, with 1,3DMU accounting for 3.1 ± 1.4%, 3MX for 3.4 ± 1.8%, and 1MU for 2.5 ± 1.5% (Rodopoulos and Norman 1997). Urinary excretions of 1,3DMU and 1MU exhibited the highest correlations, while the poorest correlations were observed for 1MX compared with those of 1MU and 1,3DMU, suggesting that 1MU did not derive solely from 1MX and implicating 1,3DMU as an alternative precursor (Bayar and Ozer 1997). However, previous results on oral administration of 1,3DMU in healthy male volunteers showed that 1,3DMU was recovered unchanged in urine and was not demethylated to 1MU (Birkett et al. 1983). The quantitative urinary excretion of theophylline metabolites in man and in various animal species, expressed as the percentage of the administered dose, is shown in Table 1.

4 Theobromine

When compared with caffeine and theophylline, fewer studies have been performed on theobromine.

4.1 Absorption

In rats there is complete absorption of theobromine, with only 1% of the dose excreted in feces as unchanged theobromine (Arnaud and Welsch 1979a; Bonati et al. 1984) and 94–106% was recovered in urine (Shively and Tarka 1983).

A marked decrease of the absorption rate constant was observed with increased dose, but the absolute bioavailability of theobromine remained 100%. As a consequence, the peak blood level tends to appear later with larger doses (Bonati et al. 1984). Theobromine bioavailability after an oral administration in healthy, nonmedicated, nonsmoking men and after 14 days' abstention from all methylxanthine sources was 0.96 ± 0.02 (Tarka et al. 1983; Miners et al. 1982; Yesair et al. 1984). Both the rate and the extent of absorption of theobromine in chocolate were less than those of theobromine in solution and the relative bioavailability of theobromine in chocolate was 80%, suggesting food interaction with chocolate ingredients (Shively et al. 1985). Theobromine absorption after oral administration of capsules and chocolate candy was compared in volunteers who abstained from methylxanthines. A theobromine plasma $C_{\max}$ of 6.72 μg/mL was measured 3 h after ingestion of a capsule containing 370 mg theobromine and absorption of the same dose from chocolate was more rapid and produced a higher $C_{\max}$ of 8.05 μg/mL after 2 h (Mumford et al. 1996).

4.2 Distribution

One day after the oral administration of [7-Me-^{14}C]theobromine to rats, no organ accumulation of theobromine and metabolites could be seen by whole-animal autoradiography and the most labeled organ was the liver, with 0.4% of the administered radioactivity and 2% was present in the cecum and the colon coming from intestinal and bile secretion (Arnaud and Welsch 1979a). Theobromine was shown to cross the placenta in the pregnant rat (Arnaud and Gétaz 1983).

In rats, blood samples taken at various intervals, from 0.5 to 3 h, showed that the mean value of the unbound theobromine fraction was 0.88 (Bonati et al. 1984). In plasma of pregnant and nonpregnant rats, theobromine corresponds to about 99% and metabolites to less than 1% (Shively and Tarka 1983).

When [8-^{14}C]theobromine was administered to newborn rats and on the following days, it was shown that the brain/blood theobromine concentrations ratio decreased continuously from 0.96 ± 0.02 at birth to 0.60 ± 0.02 in 30-day-old rats, while the liver/blood ratio remained constant at 1.18 ± 0.05. These results have been interpreted as a postnatal blood–brain barrier for theobromine in the rat (Arnaud and Gétaz 1982). The theobromine concentration was shown to be in equilibrium between blood, brain, and liver of the fetus and blood of the pregnant rat (Arnaud and Gétaz 1983). In man, theobromine is distributed throughout the total body water (Yesair et al. 1984). Milk chocolate containing theobromine (240 mg) was ingested by nursing mothers and peak theobromine concentrations of 3.7–8.2 mg/L were found in all fluids, including plasma, saliva, and breast milk, at 2–3 h after ingestion (Resman et al. 1977). In in vitro and in vivo studies, the fraction of theobromine unbound to plasma proteins averaged 0.90 over a wide range of concentrations (Bonati et al. 1984).

Compared with the fetus, the AUC values of theobromine were lower in the brains of adults compared with plasma, confirming that theobromine might be selectively excluded from the adult brain (Wilkinson and Pollard 1993). Theobromine and caffeine milk-to-serum concentration ratios were twofold higher compared with those of paraxanthine and theophylline (McNamara et al. 1992). The mean concentration ratio of theobromine in nursing mothers was 0.82 ± 0.17 for milk/plasma and if a mother ate a 4-oz chocolate bar every 6 h and the infant nursed when the theobromine concentration in milk was at its peak, the infant could ingest about 10 mg of theobromine per day (Resman et al. 1977).

The theobromine concentrations in plasma and saliva were similar, after a 500-mg oral dose, whereas the saliva concentrations for 7MX and 3MX were found to be 63 ± 17% of the plasma concentrations for 7MX and 74 ± 13% for 3MX, respectively (Rodopoulos et al. 1996).

4.3 Excretion

In rats, urine excretion was the main excretory route and amounted to 84 ± 8% of the administered dose (Arnaud and Welsch 1979a; Bonati et al. 1984). Urinary excretion was compared in rats, mice, hamsters, and male rabbits and dogs after oral administration of [8-^{14}C]theobromine and about 60–89% of the dose was recovered in urine (Miller et al. 1984).

Theobromine was shown to be extensively reabsorbed in the renal tubule and its renal clearance was highly urine flow dependent and thus urinary excretion varied with urine output (Tang-Liu et al. 1983). After the ingestion of 1 g theobromine, 62% of the dose was recovered in 48-h urine collected in adult subjects and unchanged theobromine, 3MX, 7MX, and 7MU were identified (Cornish and Christman 1957). After a single oral dose of theobromine with a trace amount of [8-^{14}C]theobromine had been administered to healthy nonsmoking men, 50% of the radioactivity was recovered in urine after 8–12 h and the entire radioactivity administered was found after 3 days (Tarka et al. 1983). The cumulative urinary excretion of radioactivity from [8-^{14}C]theobromine in subjects who maintained 14 days of methylxanthine abstention was 86.4% (80–96%) and in the same subjects not limited in their methylxanthine consumption the excretion in urine amounted to 81.1% (81–93%) (Shively et al. 1985).

After the administration of [7-Me-^{14}C]theobromine (1–6 mg/kg) to male rats, fecal excretion amounted to 11 ± 1% of the administered dose, but only 10% was unchanged theobromine and incubation of theobromine into the gastrointestinal content showed no metabolic transformation, suggesting that the metabolites found were excreted through gastrointestinal secretion (Arnaud and Welsch 1979a). After oral administration of [8-^{14}C]theobromine to rats, similar amounts of radioactivity (2.5%) were recovered in feces as after intravenous administration (Bonati et al. 1984). In pregnant rats, the oral administration of [8-^{14}C]theobromine showed that fecal excretion amounted to 31% of the ingested dose (Arnaud and Gétaz 1983).

Fecal excretion was compared in rats, mice, hamsters, and male rabbits and dogs after oral administration of [8-^{14}C]theobromine and from 2 to 38% of the dose was recovered in feces. In male and female rats, 38.2 ± 0.8 and 16.2 ± 1.3% were excreted in feces, respectively, and the values were 8.8 ± 1.1 and 11.5 ± 1.8% for mice, 15.0 ± 6.0% and 14.3 ± 3.3% for hamsters, and 1.6 ± 0.2% male rabbits and 4.5 ± 0.1% for male dogs (Miller et al. 1984). However, bentonite was often added to the rodent diet as a pellet binder and may explain the higher fecal excretion reported in rats (Arnaud 1983). Fecal elimination of [8-^{14}C]theobromine-derived radioactivity after the oral administration of a tracer dose in subjects who maintained or did not maintain 14 days of methylxanthine abstention was 0.56–0.54 and 0.15–1.42%, respectively (Shively et al. 1985).

4.4 Pharmacokinetics

The half-life of theobromine in rats exhibited large variations from 1.9 to 6.4 h, with an average value similar to that reported for man of 6.1 ± 0.7 h (Drouillard et al. 1978). The kinetics of theobromine in rats after a dose ranging from 1 to 100 mg/kg and chronic intake showed no significant difference in the pharmacokinetic profile except for a reduction in the absorption rate constant as the dose increased. Linear pharmacokinetics was observed up to the dose of 100 mg/kg and the AUC values increased in proportion to the dose (Bonati et al. 1984). Pregnancy in rats on day 19 of gestation did not affect the pharmacokinetics of theobromine (15–100 mg/kg orally) and similar values were obtained in nonpregnant rats. No dose-dependent kinetics was observed in the theobromine plasma half-life, volume of distribution, systemic clearance, dose-normalized AUC, or $T_{\max}$ (Shively and Tarka 1984). A decrease in the elimination rate constant of theobromine was observed at the highest dose of 50 mg/kg (1–100 mg/kg/day) in rabbits, suggesting saturation (Latini et al. 1984).

The half-life of theobromine in nursing mothers after ingestion of milk chocolate containing 240 mg of theobromine averaged 7.1 ± 2.1 h, body clearance was 65 ± 20 mL/h/kg, and the apparent volume of distribution was 0.62 ± 0.13 L/kg (Resman et al. 1977). A similar mean value of the half-life was reported 1 year later from measurements in man and was 6.1 ± 0.7 h (Drouillard et al. 1978). Theobromine disposition follows first-order kinetics with a one-compartment open model and the mean theobromine half-life was 9.28 ± 0.7 h, plasma clearance was 0.87 ± 0.06 mL//min/kg, the AUC was 117 ± 7.9 mg h/L, and the volume of distribution was 0.68 ± 0.03 L/kg (Tarka et al. 1983). In healthy volunteers the total plasma clearance and renal clearance for theobromine were 46 and 67% greater than those for theophylline, respectively, but most of the difference was due to the lower protein binding of theobromine with the free fraction of 0.86 compared with 0.58 for theophylline. Clearance by 3-methyl demethylation was 3.7-fold higher for theobromine than for theophylline. There were high degrees of correlation between theophylline and theobromine plasma clearances, partial metabolic clearances, and renal clearances (Birkett et al. 1985; Lelo et al. 1986a). Correlation between renal clearance of theobromine and the urine flow rate was reported (Trang et al. 1985). A supplement of theobromine (6 mg/kg) given to healthy men did not modify significantly theobromine pharmacokinetics and a similar half-life, apparent volume of distribution, and clearance were reported (Shively et al. 1985).

4.5 Metabolism

In human liver microsomes, at least two distinct liver enzymes, isozymes of CYP, with differing substrate affinities have the potential to catalyze theobromine N-demethylations and C8-hydroxylations. At the low theobromine concentrations encountered in vivo, the high-affinity site is expected to predominate (Campbell et al. 1987a). The identification of the CYP isoforms responsible for the conversion of theobromine to its primary metabolites was studied in human liver microsomes using various specific inhibitors. Furafylline variably inhibited 7MX formation from theobromine, but had no effect on other pathways. Diethyldithiocarbamate and 4-nitrophenol, probes for CYP2E1, inhibited the formation of 3MX, 7MX, and 3,7-dimethyluric acid (3,7DMU) by approximately 55–60, 35–55, and 85%, respectively. Recombinant CYP1A2 and CYP2E1 enzymes exhibited similar values of the apparent Michaelis–Menten constant (K_m) for 7MX formation, and CYP2E1 was further shown to have the capacity to convert theobromine to both 3MX and 3,7DMU (Gates and Miners 1999). The total plasma and partial metabolic and renal clearances of theobromine determined in healthy volunteers supported the view that theobromine was metabolized by a common group of CYPs under similar regulatory control and it was proposed to use theobromine to assess the activity of these enzymes in man (Birkett et al. 1985). It was suggested that 3,7DAU and 3,7DMU are derived from a common oxidized intermediate of theobromine which is the precursor of 3,7DMU, but in the presence of glutathione (GSH) or some other cellular thiol it may be reduced to give 3,7DAU (Lelo et al. 1990). The involvement of GSH and CYPs in the conversion of theobromine to 3,7DAU and 3,7DMU has been demonstrated in rat liver microsomes, showing that the ratio of formation of 3,7DAU to 3,7DMU increased with increasing GSH concentration to a maximum of 12:1 for 2 mM. When compared with untreated animals, 3,7DAU and 3,7DMU formation was increased approximately 12- and 1.6-fold in liver microsomes of rats treated with 3-methylcholanthrene and phenobarbitone, respectively (Lelo et al. 1990).

4.6 Sources of Variation in Theobromine Pharmacokinetics and Metabolism

Gender and hormones. The kinetic parameters of pregnant and nonpregnant rats were similar at all theobromine dose levels studied (Shively and Tarka 1983). In rabbits there was also no significant difference due to either gender or pregnancy

(Latini et al. 1984). The most important theobromine metabolite excreted by mice was 3,7DAU and male mice converted theobromine to this metabolite more extensively than did female mice. There was significantly more 3,7DMU in female rats than in male rats (Miller et al. 1984).

Drugs. Allopurinol had no effect on the clearance of theobromine, suggesting that the elimination of theobromine is not dependent on xanthine oxidase (Miners et al. 1982).

Smoking. As expected, theobromine plasma clearance was 33% higher in smokers than in nonsmokers owing to induction of all metabolic pathways, but 7-demethylation was induced to a greater extent than the other pathways (Miners et al. 1985; Gates and Miners 1999).

Diet. The mean theobromine half-life, apparent volume of distribution, and clearance were unaffected by abstinence from all methylxanthines or receiving high daily doses of theobromine from chocolate for 1 week (Shively et al. 1985). However, a previous study (Drouillard et al. 1978) suggested that immediately after five daily doses of theobromine, an impairment of theobromine clearance occurred that was reversible by 4 days of dietary abstention from methylxanthines.

4.7 Metabolites and Metabolic Pathway

After the administration of [7-Me-^{14}C]theobromine (1–6 mg/kg) to male rats, the radioactivity collected in $^{14}CO_2$ corresponding to the formation of 1-methyl and 3-methyl derivatives amounted to 6 ± 1% of the administered dose. In urine unchanged theobromine (49 ± 4% of excreted metabolites), 3,7DAU (36 ± 4%), 7MX (6 ± 1%), 7MU (3.9 ± 0.5%), 3,7DMU (2.7 ± 0.2%) and trace amounts of *N*-methylurea and dimethylallantoin were found (Arnaud and Welsch 1979a). Urinary excretion of unchanged theobromine increased in the pregnant rat from 47 ± 4 to 74 ± 3%, while urinary excretion of 3,7DAU decreased from 35 ± 4% to 22 ± 2%, thus showing that pregnancy impaired theobromine metabolism (Arnaud and Gétaz 1983; Bonati et al. 1984). After an oral dose of 5 and 100 mg/kg theobromine, pregnant and nonpregnant rats showed similar qualitative metabolic patterns and the metabolites identified in the urine were theobromine (39–62%), 37DAU (20–32%), 3MX and 7MX (8–15%), 3,7DMU (5–10%), and 7MU (5–7%) (Shively and Tarka 1983). Unchanged theobromine corresponded to about 50% of urinary metabolites in rat and dog, 32.4 ± 2.2% of the administered dose in rat and 36.8 ± 5.9% in dog, about 30% of urinary metabolites for mouse and hamster, 22.1 ± 3.6% of the dose in mouse and 20.0 ± 2.8% in hamster, and less than 20% of urinary metabolites for rabbit, 13.9 ± 2.7% of the dose (Latini et al. 1984). 3,7DAU was the most important metabolite after theobromine and corresponded to 25% of urinary metabolites in rat (16.5 ± 0.9% of the dose), mouse (13.9 ± 2.1% of the dose) and hamster (14.6 ± 1.6% of the dose), while it represented only 10% of urinary metabolite in rabbit (10.0 ± 1.1% of the dose) and dog (7.5 ± 3.1% of the dose). In rabbit, 7MX (35.5 ± 3.2% of the administered dose) and 3MX (8.4 ± 0.5% of the dose) corresponded to about 50 and 11% of urine metabolites while 3,7DMU (1.5 ± 0.1%), 7MU (1.6 ± 0.2%), and 3MU (0.6 ± 0.1%) were minor metabolites. In hamster, 7MX (11.4 ± 1.0% of the administered dose) corresponded to 20% of urine metabolites and 3MX (2.3 ± 0.2%), 7MU (2.9 ± 0.4%), 3MU (0.3 ± 0.1%), and 3,7DMU (2.3 ± 0.6%) were minor metabolites. In dog, 3MX (19.9 ± 2.7% of the administered dose) corresponded to 25% of urine metabolites and 7MX (3.4 ± 0.9%), 7MU (4.4 ± 2.2%) ,and 3,7DMU (0.4 ± 0.1%) were less important. In mouse, 7MX (8.2 ± 0.4% of the administered dose) and 7MU (5.3 ± 0.2% of the dose) both corresponded to less than 10% of urine metabolites and 3MX (3.2 ± 0.2%), 3MU (0.4 ± 0.1%), and 3,7DMU (2.5 ± 0.3%) were minor metabolites. In rat, 3MX (3.9 ± 0.3% of the administered dose), 3,7DMU (2.1 ± 0.2% of the dose), and 7MX (2.5 ± 0.1% of the dose) corresponded to about 5% of urine metabolites and 7MU (1.3 ± 0.1%) was less important and 3MU could not be quantified. Demethylation of theobromine was greatest in rabbits and lowest in rats and 3-methyl demethylation predominated over 7-methyl demethylation in all species except the rat and the dog. In dog, demethylation of theobromine was most important on 7-methyl, while in rat there was no specific demethylation activity. Oxidation to uric acids was a minor metabolic pathway in all species, with the greatest activity in mice. In addition to these identified metabolites, an unidentified but apparently unique metabolite was detected in dog (Miller et al. 1984).

In healthy, nonmedicated, nonsmoking men after 14 days' abstention from all methylxanthine sources, the urine metabolites measured were theobromine (18.2 ± 2.1%), 7MX (33.6 ± 1.6%), 7MU (7 ± 0.7 %), 3MX (19.9 ± 0.9%), 3,7DAU (5.7 ± 0.9%), and 3,7DMU (1.0 ± 0.2%) (Tarka et al. 1983). After the oral administration of a dose of [8-^{14}C]theobromine, the percentage of urinary metabolites recovered consisted of 42% 7MX, 20% 3MX, 18% theobromine, 10% 7MU, and 10% 3,7DAU (Shively et al. 1985). In urine collected 48 h after administration of a 500-mg theobromine dose, unchanged theobromine accounted for 21 ± 4% of total urine excretion and 36 ± 5% for 7MX, 21 ± 4% for 3MX, 11 ± 4% for 3,7DAU, 10 ± 2% for 7MU, 1.3 ± 0.6% for 3,7DMU and 0.5 ± 0.4% for 3MU. The N3-demethylation of theobromine accounted for 58 ± 7% of the urinary metabolites, the N7-demethylation of theobromine for 27 ± 6%, the C8-oxidation of 7MX for 22 ± 4%, the C8-oxidation of 3MX for 2 ± 2%, and the formation of 3,7DAU for 13 ± 4% (Rodopoulos et al. 1996). The quantitative urinary excretion of theobromine metabolites in man and in various animal species, expressed as the percentage of the administered dose, is shown in Table 1.

5 Paraxanthine

Plant biosynthesis leads to the accumulation of caffeine, theobromine, and theophylline, while only trace amounts of paraxanthine were identified as a constituent of *Coffea arabica* (Arnaud and Enslen 1992). The absence of food containing paraxanthine has limited the number of studies investigating its metabolic fate. As 80% of caffeine ingested by humans is transformed through paraxanthine and the average serum levels of paraxanthine are two thirds those of caffeine, the contribution of paraxanthine to the pharmacological activity of caffeine needs to be considered in understanding the clinical pharmacological activity of caffeine, particularly with chronic, repetitive caffeine consumption (Benowitz et al. 1995).

5.1 Absorption and Distribution

It is believed that paraxanthine absorption from the gastrointestinal tract, like the other methylxanthines, was complete after an oral dose (Lelo et al. 1989).

The concentration of radioactivity in the blood and the liver 2 h after oral administration was the same, indicating a complete equilibrium between blood and the tissues except for the brain (Arnaud and Enslen 1992). Twenty-four hours after intravenous administration of [1-Me-^{14}C]paraxanthine to rats, there was no accumulation in the body as seen by whole-animal-body autoradiography and the cumulated dose measured in organs reached 0.2% of the dose (Arnaud and Welsch 1979b). Whole-animal-body autoradiography showed a wide distribution throughout the body in liver, heart, muscle, thymus, lungs, and the gastrointestinal tract 0.5 h after [8-^{14}C]paraxanthine had been given orally to rats. Higher concentrations were observed in the stomach, the kidney, and the bladder, showing that a fraction of the dose had still not emptied from the stomach and that another fraction had already been excreted in the urine. After 10 h, only traces of radioactivity were detected in the body (Arnaud and Enslen 1992).

Brain. The radioactivity from [8-^{14}C]paraxanthine 2 h after oral administration was lower in the brain, which is protected by a blood–brain barrier, and the brain-to-blood concentrations ratio was 0.30 for paraxanthine, while this ratio is 1 for caffeine (Arnaud and Enslen 1992). Subcutaneous injection of caffeine into rats resulted in higher concentrations of methylxanthines, particularly paraxanthine, in the striatum than in the rest of the brain and it was observed that the clearance of paraxanthine was faster in serum than in brain structures (Parra et al. 1991). However, an alternative explanation is that paraxanthine binding to abundant A_{2A} receptors in the striatum delayed elimination. Compared with the fetus, the AUC value of paraxanthine was found to be lower in the brains of adults compared with plasma after a dose of 25 mg/kg. This suggests that paraxanthine might be partly excluded from the adult brain (Wilkinson and Pollard 1993).

The distribution into milk of paraxanthine measured in lactating New Zealand white rabbits following a bolus dose of caffeine showed that the milk-to-serum paraxanthine concentration ratio was 0.358 ± 0.019. Paraxanthine and theophylline have the lowest ratios, about half those of caffeine and theobromine (McNamara et al. 1992).

5.2 Excretion

A systematic survey of individual pollutants in a sewage treatment plant receiving urban wastewater recently showed that paraxanthine was one of the main product found in concentrations over 20 ppb (Rosal et al. 2010). Paraxanthine has been identified in human urine (Salomon 1883) and then recovered unchanged in wastewater. The most frequently detected compounds in surface water on the coastline within the western Lake Erie basin were caffeine (88%) and paraxanthine (56%), which was detected with a maximum concentration of 1.8 μg/L (Wu et al. 2009). Intravenous administration of [1-Me-^{14}C]paraxanthine to rats showed that 1 day after injection 7 ± 1% was recovered in feces (Arnaud and Welsch 1979b).

Intravenous administration of [1-Me-^{14}C]paraxanthine to rats showed that the main excretory pathway was urine ,where 85 ± 3% of the administered dose was recovered (Arnaud and Welsch 1979b). In man, approximately 60% of orally administered paraxanthine may be recovered as unchanged in the urine (Arnaud and Welsch 1980a) and after caffeine administration to healthy subjects, paraxanthine was excreted in the urine in amounts sixfold higher than caffeine (Arnaud and Welsch 1980a; Callahan et al. 1982). Paraxanthine was extensively reabsorbed in the renal tubule and its renal clearance was highly urine flow dependent and thus urinary excretion varied with urine output (Tang-Liu et al. 1983).

5.3 Pharmacokinetics

The pharmacokinetics of paraxanthine given intravenously in different doses to rats showed that the fraction bound was 15% and remained constant in the plasma for a concentration range of 1–100 μg/mL. Paraxanthine elimination followed first-order kinetics for a dose up to 10 mg/kg and the blood concentrations versus time data were described by a one-compartment, open model system. The mean half-life and elimination rate constant were 1 h and 0.70/h, respectively. The average apparent volume of distribution was 1.50 L/kg and the total clearance was 0.90 L/h/kg. After larger doses (15 and 30 mg/kg), the kinetics were nonlinear and the AUC increased, but not in proportion to the dose. These findings indicated that paraxanthine in the rat is eliminated by a saturable process with an apparent K_m of about 31 μg/mL and an apparent maximum rate of metabolism of about 0.40 μg/mL/min (Bortolotti et al. 1985; Arnaud and Enslen 1992). In rabbit, paraxanthine clearance was not dose-dependent (Dorrbecker et al. 1987).

The elimination of paraxanthine after its formation has been shown not to follow linear kinetics (Tang-Liu et al. 1983). A relationship has been established between renal clearance of paraxanthine and the urine flow rate (Trang et al. 1985). The partial clearance of caffeine to paraxanthine was eightfold and 23-fold greater than that of theobromine and theophylline, respectively (Lelo et al. 1986b). The clearances of paraxanthine and caffeine were similar, 2.20 and 2.07 mL/min/kg, respectively and were twofold lower for theophylline and theobromine (Lelo et al. 1986a). After a single dose of 3–4 mg/kg paraxanthine, a mean half-life of 3.9 ± 0.7 h was reported (Lelo et al. 1986a, 1989). The volume of distribution at the steady state of paraxanthine was similar to that of caffeine and theobromine (0.63–0.72 L/kg) and was higher than that of theophylline (0.44 L/kg) (Lelo et al. 1986a).

The administration of [8-^{14}C]caffeine (40 mg/kg) to various mouse strains showed higher plasma paraxanthine concentrations in the CBA/J strain compared with the SJL/J, A/J, and SWR/J strains. This effect may be explained by differences in the capacity for paraxanthine glucuronidation. The ratio of the plasma concentration of paraxanthine to total methylxanthine was 7–12% after 0.5 h and increased after 8 h to 29% for the SJL/J and A/J strains andto 44–52% for the CBA/J and SWR/J strains. Paraxanthine was the most important methylxanthine 4 h after caffeine administration for the CBA/J and SWR/J strains but not for the SJL/J and A/J strains, where similar concentrations of paraxanthine and caffeine are observed (Arnaud et al. 1989). There were no differences in the blood pharmacokinetics of paraxanthine between CYP1A2-null and wild-type mice (Labedzki et al. 2002).

Allopurinol pretreatment had no effect on paraxanthine plasma clearance but decreased 1MU excretion and increased 1MX excretion, with the combined excretion of these metabolites remaining constant (Lelo et al. 1989). Among the high degree of interliver variation in metabolic rates, smokers showed the second highest activity among a 20-fold range in paraxanthine demethylation rates (Campbell et al. 1987a).

5.4 Metabolism

The main metabolite of paraxanthine was 1MX both in human and in wild-type mice liver microsomes. In contrast, in CYP1A2-null murine liver microsomes, the main paraxanthine metabolite was 7MX (Labedzki et al. 2002). It was suggested that the formation of paraxanthine may be a better indicator of in vivo CYP1A2 activity than caffeine levels (Bapiro et al. 2005). The high value of the CYP1A2 index defined as urinary AFMU plus 1MX plus 1MU to 1,7DMU could be explained by a low urinary concentration of 1,7DMU and was suggested to be affected by the whole deleted allele of CYP2A6 (CYP2A6*4) in healthy Japanese volunteers. It was shown that CYP2A6 and CYP1A2 exhibited high catalytic activities for the paraxanthine 8-hydroxylation, which was significantly associated with coumarin 7-hydroxylase activities ($P < 0.01$) in liver microsomes. Tranylcypromine, an inhibitor of CYP2A6, reduced the paraxanthine 8-hydroxylase activities of human liver microsomes. Paraxanthine 8-hydroxylase activities were also found to be low in liver microsomes from individuals possessing deletion of or mutations in the CYP2A6 gene, suggesting that CYP2A6 is a main paraxanthine

8-hydroxylase and this activity is reduced by the genetic polymorphisms of the CYP2A6 gene (Kimura et al. 2005). Children had a higher urine metabolite ratio of paraxanthine 7-demethylation to 8-hydroxylation ($P < 0.001$) than adults (Campbell et al. 1987b).

5.5 *Metabolites and Metabolic Pathways*

Paraxanthine is the main pathway (75–80%) of the first step of caffeine metabolism in man (Arnaud and Welsch 1980a), while it represents a maximum of 40% in rats, 65% in mice, 55% in Chinese hamsters, and less than 10% in monkeys. Rabbits seems the closest model to man, but with a relative inefficiency for further metabolizing paraxanthine (Dorrbecker et al. 1987). The mean fractional conversion of caffeine to paraxanthine was 79.6 ± 21%, while it was only 10.8 ± 2.4% for theobromine and 3.7 ± 1.3% for theophylline. Demethylation pathways accounted for 83.9 ± 5.4% for paraxanthine and only 12.1 ± 4.1% for theobromine and 4.0 ± 1.4% for theophylline (Lelo et al. 1986b). The combined formation of the 7-demethylated products (1MX, 1MU) and AFMU was found to account for 67% of paraxanthine clearance. Formation of 7MX and 1,7DMU and renal excretion of unchanged paraxanthine corresponded to 6, 8, and 9% of paraxanthine clearance, respectively. Data from the effect of allopurinol and cimetidine are consistent with 1MX and AFMU being derived from a common intermediate (Lelo et al. 1989).

Unchanged paraxanthine was the most important urine metabolite in rats, 52 ± 3%, and 1,7DMU with the uracil derivative 1,7DAU both corresponded to 15 ± 2% of the dose excreted. Paraxanthine 1-methyl demethylation with the formation of 7MX and 7MU is a minor pathway (2.3 ± 0.2%), while 7-methyl demethylation was the most important with urine excretion of 1MX (11 ± 1%) and 1MU (21 ± 3%) (Arnaud and Welsch 1979b). Important species differences were shown for paraxanthine metabolism (Arnaud 1985) and a glucuroconjugate of paraxanthine was identified only in mice (Arnaud et al. 1986b). Paraxanthine and paraxanthine glucuronide urine excretion in mice showed an inverse relationship in CBA/J (17 ± 5 and 9.5 ± 3%, respectively), SJL/J (10.5 ± 3.5 and 21.0 ± 2%), A/J (7 ± 1 and 25 ± 1.5%) and SWR/J (14.5 ± 4 and 19 ± 6%) strains (Arnaud et al. 1989).

The analysis of the metabolites in urine collected at 24-h showed with unchanged paraxanthine (10 ± 4% of the administered dose) the presence of 1MX (15 ± 4%), 1MU (17 ± 2%), 1,7DMU (15 ± 3%), 1,7DAU (4 ± 2%), 7MX (5 ± 3%), AFMU (18 ± 6%), and 7MU (4 ± 3%) (Arnaud and Welsch 1980a; Callahan et al. 1982). The quantitative urinary excretion of paraxanthine metabolites in man and in various aniumal species, expressed as the percentage of the administered dose, is shown in Table 1.

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Inhibition of Cyclic Nucleotide Phosphodiesterases by Methylxanthines and Related Compounds

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Abstract Naturally occurring methylxanthines were the first inhibitors of cyclic nucleotide (cN) phosphodiesterases (PDEs) to be discovered. To improve potency and specificity for inhibition of various PDEs in research and for treatment of diseases, thousands of compounds with related structures have now been synthesized. All known PDE inhibitors contain one or more rings that mimic the purine in the cN substrate and directly compete with cN for access to the catalytic site; this review focuses on inhibitors that contain a nucleus that is closely related to the xanthine ring of theophylline and caffeine and the purine ring of cNs. The specificity and potency of these compounds for blocking PDE action have been improved by appending groups at positions on the rings as well as by modification of the number and distribution of nitrogens and carbons in those rings. Several of these inhibitors are highly selective for particular PDEs; potent and largely selective PDE5 inhibitors are used clinically for treatment of erectile dysfunction [sildenafil (Viagra™), tadalafil (Cialis™) and vardenafil (Levitra™)] and pulmonary hypertension [sildenafil (Revatio™) and tadalafil (Adenocirca)]. Related compounds target other PDEs and show therapeutic promise for a number of maladies.

Keywords Phosphodiesterases · Cyclic AMP · Cyclic GMP · Theophylline · Xanthine · Caffeine · Phosphodiesterase inhibitors · Caffeine in beverages and foods

1 Identification of Biologically Active Alkylxanthines

1.1 History of Methylxanthines as Biological Stimulants and Inhibitors of Cyclic Nucleotide Phosphodiesterases

In 1958–1960 Earl Sutherland, winner of the Nobel Prize in Physiology or Medicine in 1971 for discovery of cyclic AMP (cAMP; Fig. 1), and his colleague Ted Rall identified caffeine (1,3,7-trimethylxanthine) (Fig. 1), a plant-derived alkaloid, as an inhibitor of cAMP breakdown by crude preparations of cyclic nucleotide (cN) phosphodiesterases (PDEs) (Sutherland and Rall 1958); this was the first PDE inhibitor to be identified and the first enzymatic effect of caffeine to be defined.

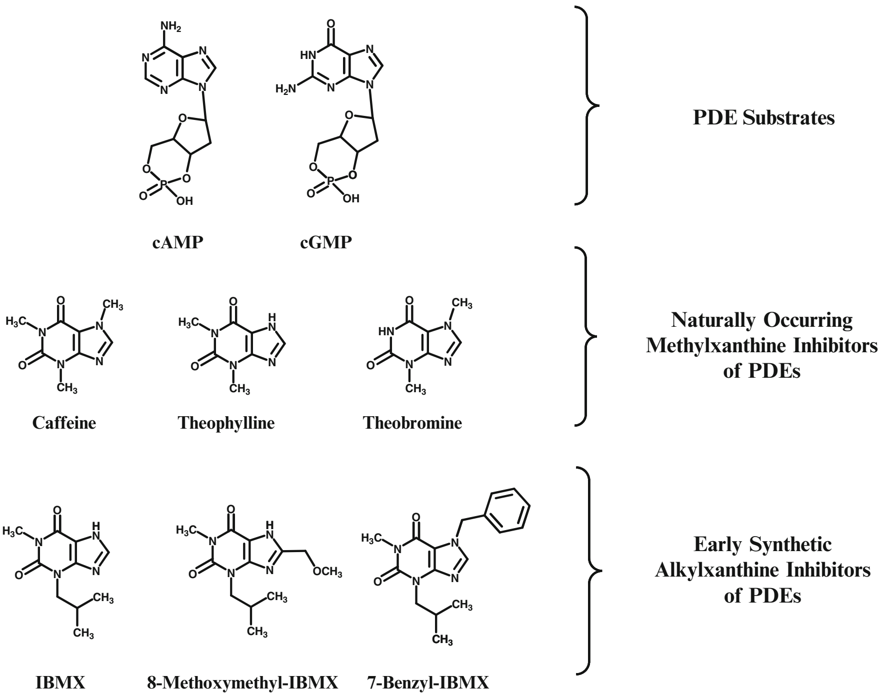


Fig. 1 Molecular structures of cyclic nucleotides (cNs) and those of naturally occurring methylxanthine inhibitors of phosphodiesterases (PDEs) and representative early synthetic alkylxanthine PDE inhibitors

In their classical studies on the phosphorylation and activation of purified phosphorylase or phosphorylase in dilute liver homogenates, Wosilait and Sutherland (1956) discovered that dephosphorylation of phosphophosphorylase by an "inactivating enzyme" (later known as the LP-phosphatase activity) was increased in the presence of caffeine or theobromine; binding of the purine to an allosteric site on phosphorylase caused a conformational change that facilitated dephosphorylation. Consequently, caffeine was included in their protocols in order to promote dephosphorylation of phosphorylase, thereby lowering the blank that was due to the active phosphophosphorylase in tissue extracts (Berthet et al. 1957). However, in studies of epinephrine or glucagon action using liver homogenates, they found surprisingly that this effect of caffeine was observed only with low concentrations of caffeine and was quite modest; at higher caffeine concentrations, the amount of phosphophosphorylase actually increased. Moreover, they found that the effect of caffeine was greatest when low concentrations of glucagon or epinephrine were employed and that inclusion of caffeine in these experiments increased sensitivity to the hormones (Berthet et al. 1957). Having already established that the effect of glucagon or epinephrine was due to a heat-stable factor (cAMP), they concluded that there must be an enzyme (now known to be PDE) that destroyed the factor and that this enzyme was inhibited by the caffeine in the reaction mixtures. Indeed, in unpublished experiments involving crude preparations from liver, brain, or heart, they found that caffeine enhanced accumulation of cAMP in these tissue extracts by blocking the PDE activity that breaks down cAMP. Thus, at low glucagon or epinephrine concentrations, caffeine acted to synergistically increase the cAMP level, thereby increasing the activities cAMP-dependent protein kinase (PKA) and phosphorylase kinase activities, resulting in increased amounts of active phospho-phosphorylase (Berthet et al. 1957). However, the exact details describing the insights that led Sutherland and Rall to the realization that caffeine might act to block PDE action and foster cAMP accumulation are not known (we are grateful to Bill Butcher for his historical insights). In 1962, Butcher and Sutherland (1962) demonstrated that theophylline (1,3-dimethylxanthine) and theobromine (Fig. 1) also inhibit PDE action; in that instance, the inhibitory potencies of caffeine and theobromine were comparable, whereas theophylline was approximately six-fold more potent (Butcher and Sutherland 1962).

The stimulatory effects of plant extracts containing caffeine and theophylline had long been appreciated, but their mode of action was not understood (Fredholm 2010). Historical accounts suggest that about 3,000 years ago Ethiopian herders observed that goats that foraged on beans of coffee plants in the daytime were restless and wakeful in the evening. They subsequently found that consumption of extracts from these beans increased their own wakefulness; that was the discovery of coffee, and the pharmacological action that they experienced was due to the stimulant effect of caffeine in the beverage. Tea, which contains caffeine and theophylline, both of which are stimulants, is thought to have been discovered in China in more distant antiquity. Caffeine along with theobromine (3,7-dimethylxanthine) is also found in cocoa beans and is an additive in many foodstuffs; paraxanthine (1,7-dimethylxanthine), theophylline, and theobromine, natural breakdown products of caffeine (Arnaud 2010), are present in the body after caffeine consumption and elicit a variety of biological effects (Fig. 1) (Guerreiro et al. 2010; Smit 2010; Müller and Jacobson 2010).

1.2 Superfamily of Mammalian PDEs

Investigators conducting early studies on the effects of caffeine to inhibit PDEs suspected that there were multiple types of PDEs in a given tissue extract, and that was soon proven to be the case (Sutherland and Rall 1958). It is now known that there are 21 genes for PDEs in the human genome (Bender and Beavo 2006). The protein products of these genes comprise the superfamily of PDEs that has been subdivided into 11 families (PDEs 1–11) (Table 1); some families are encoded by a single gene, whereas others are products of multiple genes. Further complexity results from extensive alternative splicing of the messenger RNA to produce a vast array of PDEs with different regulatory features, catalytic characteristics, tissue

Table 1 Substrate specificities and kinetic characteristics of mammalian phosphodiesterase (PDE) families

Isoenzyme	Substrate specificity	K_m (μM)		V_{max} (μmol/min/mg)		References
		cGMP	cAMP	cGMP	cAMP	
PDE1A	cAMP < cGMP	3–4	73–120	50–300	70–450	Hansen et al. (1988), Sharma et al. (1984), Snyder et al. (1999), Sonnenburg et al. (1995)
PDE1B	cAMP < cGMP	1–6	10–24	30	10	Bender et al. (2005), Sharma and Wang (1986)
PDE1C	cAMP = cGMP	1–2	0.3–1	?	?	Loughney et al. (1996), Yan et al. (1996)
PDE2A	cAMP = cGMP	10	30	123	120	Martins et al. (1982), Rosman et al. (1997)
PDE3A	cAMP > cGMP	0.02–0.2	0.2	0.3	3–6	Grant and Colman (1984), Harrison et al. (1986)
PDE3B	cAMP > cGMP	0.3	0.4	2	9	Degerman et al. (1987)
PDE4A	cAMP >> cGMP	?	3–10	?	0.6	Rena et al. (2001), Salanova et al. (1998), Wallace et al. (2005), Wang et al. (1997)
PDE4B	cAMP >> cGMP	?	2–5	?	0.1	Huston et al. (1997), Salanova et al. (1998), Wang et al. (1997)
PDE4C	cAMP >> cGMP	?	2	?	0.3	Wang et al. (1997)
PDE4D	cAMP >> cGMP	?	1–6	?	0.03–2	Salanova et al. (1998), Wang et al. (1997)
PDE5A	cGMP >> cAMP	1–6	90	1–3	1–3	Loughney et al. (1998, Thomas et al. (1990), Turko et al. (1998), Zoraghi et al. (2006)
PDE6A/B	cGMP >> cAMP	15	700	2,300	?	Gillespie and Beavo (1988), Zhang et al. (2005)
PDE6C	cGMP >> cAMP	17	610	1,400	?	Gillespie and Beavo (1988), Zhang et al. (2005)
PDE7A	cAMP >> cGMP	?	0.1–0.2	?	?	Han et al. (1997), Michaeli et al. (1993)
PDE7B	cAMP >> cGMP	?	0.03–0.1	?	?	Hetman et al. (2000), Sasaki et al. (2000, 2002)
PDE8A	cAMP >> cGMP	?	0.1	?	?	Fisher et al. (1998)
PDE8B	cAMP >> cGMP	?	0.1	?	?	Gamanuma et al. (2003)
PDE9A	cGMP >> cAMP	0.2–0.7	230	?	?	Fisher et al. (1998), Soderling et al. (1998)

(*continued*)

Table 1 (continued)

Isoenzyme	Substrate specificity	K_m (μM)		V_{max} (μmol/min/mg)		References
		cGMP	cAMP	cGMP	cAMP	
PDE10A	cAMP > cGMP	13	0.2–1	?	?	Fujishige et al. (1999), Kotera et al. (1999)
PDE11A	cAMP = cGMP	0.4–2	0.5–3	?	?	Fawcett et al. (2000), Weeks et al. (2007), Yuasa et al. (2001)

The results are compiled from listed references and reproduced with modifications and permission (Bender and Beavo 2006). The range of concentrations indicates differences among different laboratories. These are likely to be due to different assay conditions and different preparations of the respective enzymes. *Question marks* indicate no reliable data are currently available
cAMP cyclic AMP, *cGMP* cyclic GMP

distribution, and subcellular localizations. At present there are estimated to be about 100 PDEs derived from the 21 PDE genes and additional forms are being identified with some regularity (Bender and Beavo 2006; Conti and Beavo 2007).

All mammalian PDEs share a common catalytic domain that is located toward the carboxyl-terminal portion of the proteins and comprises about 270 amino acids; despite the fact that the identity of sequence among catalytic domains of PDEs varies from 24–50%, all X-ray crystal structures of these domains reveal a very similar overall structure that is composed primarily of α-helices and a similar catalytic pocket (Ke and Wang 2007). The catalytic pocket where the cNs or inhibitors bind occupies about 330 Å^3 and contains two divalent cations, which is typically a tightly bound zinc and another more loosely bound (likely a magnesium or manganese) metal that are required for hydrolysis of the cyclic phosphate bond. Some PDEs are highly specific for hydrolysis of either cAMP (PDEs 4, 7, and 8) or cyclic GMP (cGMP) (PDEs 5, 6, and 9), whereas others readily hydrolyze both nucleotides (PDEs 1–3, 10, and 11).

While all known PDE catalytic domains interact to some extent with methylxanthines and related compounds, there is a very wide range of affinities (Table 2); methylxanthine-related compounds that are profoundly potent inhibitors for certain PDEs are ineffective for inhibiting other PDEs (Beavo et al. 2006). Moreover, members of the PDE8 and PDE9 families are not significantly inhibited by methylxanthine inhibitors such as 3-isobutyl-1-methylxanthine (IBMX) that are commonly described as “nonspecific” and are used in many tissue studies in an effort to block PDE action (Lavan et al. 1989). The affinities of the various PDEs for IBMX vary by more than 100-fold, for zaprinast by more than 1,000-fold, for sildenafil by more than 7,000-fold, and for vardenafil by more than 300,000-fold (Table 2). Thus, despite the overall similarities in the catalytic pockets of these enzymes, there are elements that strongly discriminate among these compounds.

Although PDEs in general are in low abundance in cells, almost all cells contain multiple PDEs, all of which contribute important regulatory control of cNs in that

Table 2 Comparison of inhibitory potencies (IC_{50}) of various methylxanthine-related compounds for mammalian PDE families

Isoenzyme	IBMX (μM)	Zaprinast (μM)	Sildenafil (μM)	Vardenafil (μM)
PDE1	3–10	6	0.28	0.07
PDE2	6–50	NA	>30	6.2
PDE3	2–10	NA	16	>1
PDE4	5–20	NA	7.7	6.1
PDE5	2–10	0.13–0.8	0.004	0.0001–0.0004
PDE6[a]	1–5	0.03	0.005–0.01	0.0003–0.0007
PDE7	2	NA	21	>30
PDE8	>100	NA	30	>30
PDE9	>100	35	2.6	0.6
PDE10	3	11–22	~1	3.0
PDE11	25–80	11–33	2.7	0.16

The range of values in many instances reflects measurements from different laboratories using different assay conditons, substrate concentrations, etc. Values for sildenafil and vardenafil inhibition of PDEs 1–5 and 7–11 (Ballard et al. 1998; Corbin and Francis 2002; Gibson 2001)

IBMX 3-isobutyl-1-methylxanthine, *NA* not available

Values for IBMX potency of inhibition for PDEs 1–5 (Dent and Rabe 1996). Values for zaprinast inhibition of PDEs 9–11 (Gibson 2001; Nakamizo et al. 2003)

[a] Values for PDE6 (Zhang et al. 2005) and the range of concentrations reflect the potency of inhibition of rod PDE6 and cone PDE6

particular cell. These individual contributions result from the substrate selectivity and affinity for a given cN, regulatory features of the PDEs (e.g., effects of phosphorylation, allosteric cN binding, calcium/calmodulin), and subcellular localization. Representatives of particular PDE families (PDEs 1–4), are widespread and relatively abundant in mammalian tissues, whereas others (PDEs 5–11) occur in lower abundance and have a more confined distribution. Nevertheless, particular PDEs that are low in overall abundance in a particular tissue may occur in high abundance in particular regions of a cell and can thereby still significantly impact physiological and pharmacological responses. Some PDEs (PDEs 2, 5, 6, 10, and 11) contain subdomains within their regulatory domains that can bind either cGMP (PDEs 2, 5, 6, and 11) or cAMP (PDE 10), and direct regulation of enzyme function by these allosteric sites has been demonstrated for PDEs 2, 5, and 6 (Bender and Beavo 2006; Conti and Beavo 2007). These sites are evolutionarily distinct from the PDE catalytic sites and do not interact appreciably with caffeine, theophylline, or any of the other known PDE inhibitors.

1.3 Development of Potent and Selective Alkylxanthine-Related Compounds for Inhibition of cN PDEs

Until the early 1970s, naturally occurring methylxanthines constituted the majority of compounds that were available for use as PDE inhibitors in research focused on cN signaling. Caffeine and theophylline weakly inhibit most cN PDEs, with IC_{50}

values ranging from 100 to 1,000 μM (Beavo et al. 2006; Choi et al. 1988; Dent and Rabe 1996; Smellie et al. 1979), and show little selectivity among PDE families (PDEs 1–11). However, it was shown early on that very modest (less than 20%) inhibition of crude PDE activity by alkylxanthines resulted in a significant increase in lipolysis (Beavo et al. 1970) To improve potency and selectivity of these inhibitors, chemists synthesized a collection of alkylxanthines; IBMX was one of the first produced, and a number of IBMX analogs with substituents appended at N7 and C8 quickly ensued (Fig. 1). These were valuable tools for studying cN signaling and for defining characteristics of different PDEs (Fig. 1) (Garst et al. 1976; Mushlin et al. 1981; Wells and Kramer 1981). Chemists in both academia and in the pharmaceutical industry eventually synthesized thousands of compounds that were in the same general family as caffeine and theophylline, i.e., either alkylxanthines or closely related compounds (Fig. 2). (Boolell et al. 1996; Boyle et al. 2005; Buckle et al. 1994; Corbin and Francis 1999; Kaplan et al. 1995; McKenna and Muller 2006; Miyamoto et al. 1993, 1994; Sekhar et al. 1996; Silver et al. 1994; Wang et al. 2002). These efforts produced compounds with greatly improved inhibitory potency for PDEs and, in certain instances, greatly improved selectivity among PDEs; this collection continues to evolve and includes pentoxifylline (a nonselective inhibitor) (Tjon and Riemann 2001), enprofylline, isbufylline, denbufylline (PDE4 inhibitors), 8-substituted-IBMX analogs (primarily PDE1 and PDE5 inhibitors), zaprinast (a PDE1, PDE5, and PDE6 inhibitor), WIN 58237 (a PDE1/PDE5 inhibitor), *erythro*-9-(2-hydroxyl-3-nonyl)-adenine (EHNA; a PDE2 inhibitor) (Fig. 2a), and a group of potent PDE5 inhibitors including sildenafil and vardenafil [both of which are also potent for PDE6 (about threefold to tenfold lower potency than for PDE5)], tadalafil, udenafil, mirodenafil, SLX-2101, and dasantafil. (Beavo et al. 2006; Francis et al. 2009) (Fig. 2b). As interest in therapeutic use of PDE inhibitors increased, attention was also directed to modifications that improved the pharmacokinetics of these compounds, including considerations of the rate of absorption, stability in the gastrointestinal tract, metabolism, and clearance.

1.4 Mode of Action of Methylxanthine-Related Compounds in Beverages, Foods, and Medications

Despite wide use of theophylline and caffeine in beverages, foods, and medications, the exact mode and site of action of these compounds and their metabolites are still not fully understood. In the 1960s, PDE inhibition was thought to mediate the pharmacological effects of caffeine or theophylline, and evidence suggests that a number of the effects of these compounds are indeed mediated via this process (Mascali et al. 1996; Mushlin et al. 1981; Rabe et al. 1995; Sullivan et al. 1994a, b; Torphy et al. 1992). However, it is now appreciated that there are numerous modes of actions of these compounds and their metabolites. Many actions of theophylline

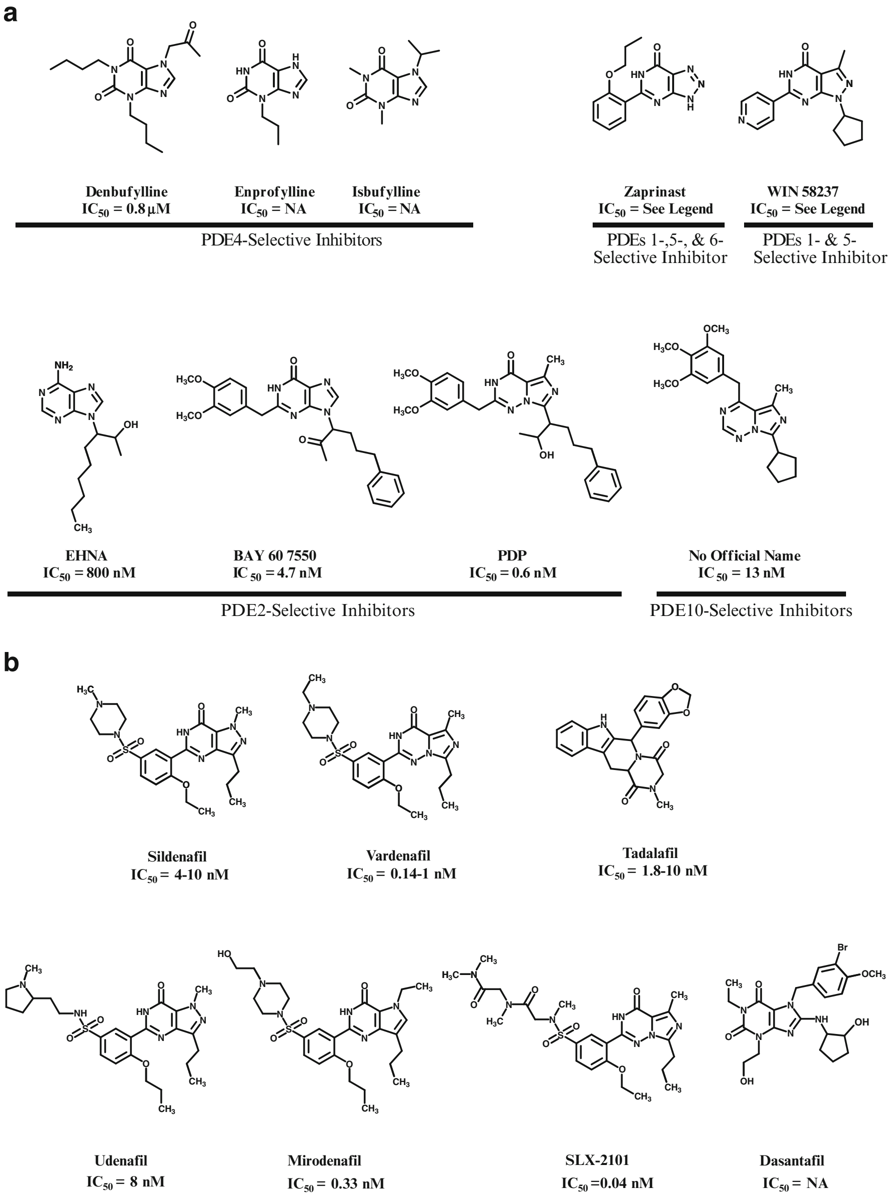


Fig. 2 Molecular structures of xanthine-related PDE inhibitors. **a** Xanthine-related inhibitors that are selective for particular PDE families; **b** Xanthine-related compounds that are highly potent and selective for PDE5. Approximate IC_{50} of zaprinast for PDE1 (6 µM) (Beavo et al. 2006), PDE5 (0.33 µM) (Zhang et al. 2005), and PDE6 (0.03 µM) (Zhang et al. 2005) and IC_{50} of WIN 58237 for PDE1 (1.5 µM) and PDE5 (0.17 µM) (Silver et al. 1994). *NA* not available

and caffeine are mediated by antagonism of adenosine effects on adenosine receptors (A_1, A_{2A}, and A_{2B}), where they have modest affinity (Sawynok and Yaksh 1993; Yang et al. 2009a, b; Müller and Jacobson 2010). Blockade of the A_1 receptor by these compounds accounts for their well-known natriuretic and diuretic effects (Rieg et al. 2005) (see Osswald and Schnermann 2010) and certain neurological effects such as wakefulness and increased alertness may be due, at least in part, to effects on one of the adenosine receptor isoforms (Boutrel and Koob 2004; Huang et al. 2005; Solinas et al. 2002; Yu et al. 2009) (see Porkka-Heiskanen 2010). Paraxanthine, the primary metabolite of caffeine, acts through the ryanodine receptor to elevate intracellular calcium levels and increases viability of neuronal cells in culture (Guerreiro et al. 2008, 2010). Therapeutic concentrations of theophylline also increase histone deacetylase activity, apparently via an indirect mechanism, thereby reducing inflammatory processes (Haskó and Cronstein 2010; Ohta and Sitkovsky 2010) (Barnes 2003a; Ito et al. 2002; Marwick et al. 2008).

1.4.1 Role of PDE Inhibition in Effects of Caffeine Derived from Beverages and Foods

Surprisingly, caffeine is found in many beverages and foods; in some instances, this caffeine is derived from the source itself, whereas in other instances, it is added. The possibility that caffeine may act in part through inhibition of PDEs is increased following consumption of caffeine-rich "energy drinks," foods, and gum alone, in combination, or in conjunction with caffeine-containing medications. Energy drinks are commonly ingested over a short period and can contain 6–14 times the amount of caffeine in a cup of coffee based on the number of milligrams per ounce (Table 3). Following consumption of beverages containing caffeine (about five or six cups of coffee daily) or therapeutic doses of caffeine, the plasma caffeine concentration is usually 10–50 μM (Benowitz 1990) although the level and persistence of caffeine and its metabolites in plasma vary widely owing to many factors. Caffeine is quickly converted to paraxanthine (84%), theobromine (12%), and theophylline (4%). Generally, caffeine inhibition of PDEs requires higher levels (100–1,000 μM) than for interaction with adenosine receptors (10–100 μM), but the potencies of its metabolites, paraxanthine and theobromine, for inhibition of most known PDEs have not been studied (Butcher and Sutherland 1962; Sattin 1971; Sattin and Rall 1970). Depending on the serving size, the caffeine content of beverages and/or food, and the variation in clearance times, it is entirely plausible that plasma and cellular caffeine levels could be within the range for pharmacological action on PDEs (Chou and Bell 2007). It is also well established that the intracellular concentration/action of a PDE inhibitor cannot be confidently predicted strictly on the basis of its extracellular concentration (Thompson 1991).

The caffeine contents of a number of common foods, certain gums, and mints are shown in Table 4. In most instances, individuals are unaware of their daily caffeine

Table 3 Compilation of caffeine content in popular beverages

Beverage	Weight (oz)	Total caffeine (mg)	Ratio (mg/oz)
Fixx Extreme	0.17	400	2,352
Ammo	1	171	171
Redline Power Rush	2.5	350	140
Mana Energy Potion	1.4	160	119
Extreme Energy 6-Hour Shot	2	220	110
Jolt Endurance Shot	2	200	100
Powershot	1	100	100
Charge! Super Shot	2	200	100
Fuel Cell	2	180	90
Stok Black Coffee Shots	0.4	40	91
Upshot	2.5	200	80
NOS Powershot	2	125	63
925 Energy Shot	2	120	60
SLAM Energy Drink	2	107	54
SPIKE shooter	8.4	300	36
Cocaine Energy Drink	8.4	280	33
Redline Princess	8	250	31
Starbucks Short Coffee	8	180	23
Wired X344	16	344	22
Dark Chocolate	1	20	20
Brewed Coffee	8	108	13
BooKoo Energy	24	360	15
Vamp	16	240	15
Rockstar Roasted	15	225	15
Dopamine Energy Drink	8.4	120	14
Arizona Caution Energy Drink	16	200	13
No Fear	16	174	11
Red Bull	8	80	10
Monster	16	160	10
Rockstar	16	160	10
Brewed tea	8	60	8
Bawls	10	67	7
Afri-Cola	12	89	7
Diet Pepsi Max	12	69	6
Mountain Dew	12	54	5
Diet Coke	12	45	4
Dr. Pepper	12	41	3
Coca Cola Classic	12	35	3
Pepsi–Cola	12	38	3

Values in this table were compiled from Energyfiend.com (Energyfiend.com 2005a)

consumption and the amount of caffeine in their beverages or foods. Use of caffeine-containing energy drinks, in their beverages or foods coffee, tea, or caffeine-rich foods in combination with medications containing caffeine or theophylline can significantly increase the risk of side effects due to higher plasma levels of these compounds. Certain “buzz beers” contain 50–60 mg of caffeine per 250 mL and combine the effects of alcohol, a depressant, and caffeine, a stimulant, both of which promote diuresis and potential dehydration (O’Brien et al. 2008).

Table 4 Compilation of caffeine content in representative foods

Food	Caffeine (mg)	Serving
Crackheads 2	600	Per box
Dark Chocolate Coated Coffee Beans	311	200 cal
Milk Chocolate Coated Coffee Beans	291	200 cal
Cocoa, Dry Powder, Unsweetened	202	200 cal
NRG Potato Chips	175	1.75-oz bag
Engobi	140	Per ounce
Alien Energy Jerky	110	Per pack
Foosh Energy Mints Powershot	100	Per mint
Buzz Bites Chocolate Chews	100	Per chew
Go Fast Energy Gum	100	Per piece
Jet Alert	100	Per tablet
Edy's Grand Espresso Chip	90	Per cup (8 oz)
Ben & Jerry's Coffee Heath Bar Crunch	84	Per cup (8 oz)
Butterfinger Buzz	80	Per Pkg^{-1}
Ben & Jerry's Fair Trade Coffee Ice Cream	70	Per cup (8 oz)
Ben & Jerry's coffee flavored ice cream	68	Per cup (8 oz)
Morning Spark Energy Instant Oatmeal	60	Per packet
Blitz Energy Gum	55	Per piece
Jelly Belly Extreme Sports Beans	50	Per 1-oz bag
Starbucks Coffee Ice Cream	50–60	Per cup (8 oz)
Haagen-Dazs coffee ice cream	48	Per cup (8 oz)
Hershey's Special Dark Chocolate Bar	31	1.45 oz
Breyer's All Natural Coffee	30	Per cup (8 oz)
Semisweet Chocolate	26	200 cal
Chocolate-Coated Graham Crackers	19	200 cal
Milky Way Midnight Bar	14	200 cal
Quaker Cocoa Blasts	11	200 cal
Chocolate Flavored Puddings	10	200 cal
Chocolate Flavored Lite Syrup	8	200 cal
Frozen Chocolate Yogurt	4–6	200 cal
Tootsie Roll	4	200 cal
Snickers Marathon Energy Bar	3	200 cal

Values in this table were compiled from Energyfiend.com (Energyfiend.com 2005b)

1.4.2 Role of PDE Inhibition by Theophylline and Caffeine in Medications

In particular, caffeine is included in many prescription and over-the-counter medicines; common over-the-counter drugs that contain caffeine include NoDoz® (200 mg/tablet), Vivarin® (200 mg/tablet), Excedrin Extra Strength® (65 mg/tablet), Anacin Maximum Strength® (32 mg/tablet), and Midol Menstrual Complete® (60 mg/tablet) (Interest 2007).

Theophylline either alone or in combination with other medications has been used clinically in the Western world for more than 60 years for treatment of bronchospasm associated with asthma or chronic obstructive pulmonary disease (COPD) (Barnes 2003a, b, 2005; Barnes and Stockley 2005; Tilley 2010), and the anti-inflammatory effects of theophylline in airways are well established (Barnes 2003a; Dent et al. 1994; Dent and Rabe 1996). Much of the credit for use of methylxanthines in treatment of asthma has been given to Herrmann and Greene (Greene et al. 1937; Herrmann et al. 1937), who studied the effectiveness of theophylline in treating this malady. However, a treatise in the *Edinburgh Medical Journal* in 1859 by a Scottish physician, H. Salter, who was both a medical expert on asthma and himself an asthmatic stated that "One of the commonest and best-reputed remedies of asthma, one that is almost sure to have been tried in any case that may come under our observation, and one that in many cases is more efficacious than any other is strong coffee" (Salter 1859). Salter "prescribed" the use of this approach by instructing patients to ingest several cups of very strong black coffee preferably in the morning on an empty stomach. The relief Salter experienced was most likely attributable to the pharmacological action of the caffeine in the coffee, but whether this effect was mediated by inhibition of PDEs is unclear.

Systematic studies of methylxanthines found that relatively high plasma concentrations of these compounds are required to achieve significant bronchodilation; in the case of theophylline, 10–20 mg/L, which translates to about 50–100 μM, is required (Barnes 2006). On the basis of IC_{50} values of theophylline for a number of PDEs, these levels could significantly diminish PDE activity and cause elevation of the levels of cNs (Butcher and Sutherland 1962; Dent and Rabe 1996). Evidence suggests that in airway smooth muscle the effects of theophylline are mediated through inhibition of PDEs 3–5 to cause increases in the levels of cAMP and cGMP and activation of signaling pathways for these cNs (Rabe et al. 1995). In industrialized countries, theophylline, either alone or in combination with other medications, is still in limited use in a subset of asthma patients, but more effective medications with fewer adverse side effects are preferred. Despite its narrow therapeutic window and problems in determining due to appropriate dosages for treatment of different patients, theophylline is still widely used in developing countries for treatment of asthma due to its low cost (Barnes 2003a, b). The effects of theophylline to blunt airway inflammation in COPD occur below 10 mg/mL plasma (Hirano et al. 2006; Kobayashi et al. 2004) and are therefore unlikely to act through PDE inhibition.

1.4.3 Role of PDE Inhibition in Effects of Derivatized Alkylxanthine Medications

Pentoxifylline (Trental™, Sanofi-Aventis), a derivatized alkylxanthine inhibitor of PDEs 1, 2, and 4 (Fig. 2a), entered the market in the 1970s for treatment of symptoms related to intermittent claudication, which is associated with chronic occlusive arterial disease of the limbs (Tjon and Riemann 2001). It reportedly improves peripheral perfusion by decreasing blood viscosity and increasing erythrocyte deformability, but its mode of action and therapeutic efficacy are debated (Aviado and Porter 1984; Regensteiner and Hiatt 2002). Sildenafil (Viagra™), a pyrazolopyrimidinone, and vardenafil (Levitra™), an imidazotriazinone, are highly selective and potent inhibitors of PDEs 5 and 6, both of which specifically hydrolyze cGMP (Cote 2006; Francis et al. 2006). The heterocyclic ring structures of these inhibitors closely mimic those of theophylline and caffeine, and both are highly successful in treatment of erectile dysfunction (Carson and Lue 2005; Corbin and Francis 1999; Francis and Corbin 2003, 2005a, b; Stief et al. 2004). Sildenafil (marketed as Revatio™) is also used for treatment of some forms of pulmonary hypertension (Galie and Branzi 2005; Ghofrani et al. 2006); each of the approved PDE5 inhibitors, as well as newly available PDE5 inhibitors (Fig. 2b), are continually being tested for use in treatment of other maladies (Al-Ameri et al. 2009; Black et al. 2008; Burnett et al. 2006; Gotshall et al. 2009; Jeong et al. 2008; Kumar et al. 2009; Levien 2006; Lubamba et al. 2008; Medeiros et al. 2008; Puzzo et al. 2008; Rutten et al. 2009; Salloum et al. 2006; Stief et al. 2008). To date, most of the known biological effects of these inhibitors are mediated through inhibition of cGMP breakdown at the PDE5 catalytic site; visual perturbations in some patients who take sildenafil or vardenafil are attributed to inhibition of PDE6 in photoreceptor cells (Francis and Corbin 2003). Both sildenafil and vardenafil have outstanding safety records, with reports of only minor and transient side effects. A number of closely related compounds (udenafil, mirodenafil, SLX-2101, dasantafil, and avanafil) have either entered or are poised to enter the market (Doh et al. 2002; Hatzimouratidis 2008).

2 Inhibition of cN PDEs by Alkylxanthine-Related Compounds

2.1 Mechanism of Action for Inhibition of PDEs by Derivatized Alkylxanthines

The general structures for cNs and the derivatized alkylxanthine inhibitors, which are competitive inhibitors, are similar (Figs. 1, 2). The heterocyclic ring of these inhibitors, which mimics the purine of cNs, comprises a six-membered pyrimidine ring conjoined with a five-membered ring containing two or more nitrogens. The affinity of a particular PDE for the various inhibitors is dictated by (1) the chemical characteristics of the particular heterocyclic ring, (2) groups appended to the ring, (3) differences in the distribution of electrons within the ring, and (4) structural restrictions of PDE catalytic sites for entry and binding of derivatized alkylxanthines with particular molecular structures. Substituents appended to the ring can impact affinity for the various PDE catalytic sites by forming new contacts with regions in and around the catalytic sites of PDEs or by interference (steric or chemical) with these regions. The distribution and number of carbons and nitrogens within the ring system can alter the electron distribution, thereby impacting the strength of the interactions (Corbin et al. 2006; Erneux et al. 1984).

2.2 Molecular Basis for High Potency of Vardenafil over Sildenafil, Substrate, and Weak Inhibitors of PDE5: An Example

Despite vardenafil and sildenafil appearing to have very similar chemical structures (Fig. 2b), the potency of vardenafil for inhibition of the PDE5 catalytic site (K_i ~ 0.1–0.4 nM) exceeds that of sildenafil (K_i ~ 4 nM) by 10–40-fold; the affinity of the PDE5 catalytic site for cGMP is 6,000–24,000 times weaker (K_m ~ 2,500 nM) than that for vardenafil. The potency of caffeine or theophylline for the PDE5 catalytic site is approximately 10-million to 40-million-fold and 600,000–2,400,000 times weaker, respectively, than that of vardenafil (Francis and Corbin 2003). The strong preference of the PDE5 catalytic site for vardenafil, a potent inhibitor, compared with that for weak inhibitors such as caffeine and theophylline is determined by unique structural features of both the inhibitor and the PDE5 catalytic site (Blount et al. 2006; Corbin et al. 2004, 2006; Sung et al. 2003; Wang et al. 2008b). The 10 to 40-fold difference in potency of vardenafil and sildenafil is largely due to features of the respective heterocyclic rings (Corbin et al. 2004, 2006), which differ in having either a carbon or a nitrogen at two positions in the five-membered ring. This difference substantially alters the electron distribution in the rings and the electronegativity of individual nitrogens (Corbin et al. 2006). Moreover, higher affinity of the PDE5 catalytic site for vardenafil requires structural features in the regulatory domain (Blount et al. 2006) since in the absence of this domain the potencies of vardenafil and sildenafil are the same. Vardenafil, like sildenafil, is also quite selective for inhibition of PDE5 compared with other PDEs (Francis and Corbin 2003) owing to the fact that their novel structures exploit unique features in PDE5 (Blount et al. 2006).

2.3 Concerns for Specificity of Derivatized Alkylxanthines as Inhibitors of PDEs

Given the abundance of cell proteins that bind or utilize purine-containing ligands (adenosine, adenine nucleotides, guanine nucleotides, etc.), the potential for interaction of alkylxanthine-related PDE inhibitors with proteins other than PDEs is always a concern. EHNA, a selective and weak inhibitor of PDE2, also inhibits adenosine deaminase (Mery et al. 1995), and zaprinast, which was historically considered to be a relatively selective inhibitor for PDEs 1 and 5, is a high-affinity agonist for GPR-35, an orphan receptor that is involved in modulating calcium homeostasis (Taniguchi et al. 2006). Some notable differences in the effects of vardenafil and sildenafil in in vivo studies have been reported (Toque et al. 2008), but whether this reflects action through non-PDE5 targets remains to be determined. Importantly, most of the alkylxanthine-based PDE inhibitors that have been studied to date do not appreciably interact with cN-binding sites on other proteins, including cN-gated channels, cN-dependent protein kinases, cAMP-modulated guanine-nucleotide exchange factors, or cN-binding allosteric sites on PDEs 2, 5, 6, 10, and 11. Use of radiolabeled inhibitors has not yet detected non-PDE targets of these compounds; however, this technique is only successful when inhibitor-binding proteins are sufficiently abundant for detection of the radioactive signal (Corbin et al. 2005).

3 Design of Potent Xanthine-Based PDE5 Inhibitors That Recapitulate Structural Features of the Entire cGMP Molecule

Early use of cN analogs such as $2'$-*O*-monobutyryl cAMP, N^2, $2'$*O*-dibutyryl cAMP, and 8-Br-cAMP as agonists for activation of PKA led to the realization that these analogs could also bind to other cN-binding proteins such as cGMP-dependent protein kinase (PKG), Epacs, cN-gated channels, and PDEs. In many instances, the cN acted as a PDE inhibitor and thereby fostered accumulation of naturally occurring cN in cells or tissue extracts. An added advantage was that these analogs could traverse the cell membrane, whereas unmodified cAMP or cGMP is typically excluded from entering the cell. The cN-binding sites on PKA, PKG, Epacs, and cN-gated channels are homologous and belong to the catabolite gene activator protein (CAP) family of cN-binding sites (Fig. 3); as a result they share many similarities in preference for particular cN analogs, although those selective for each have been identified. In contrast, the catalytic sites and allosteric cN-binding sites in PDEs are evolutionarily unrelated to the CAP family of cN-binding sites, as well as to each other (McAllister-Lucas et al. 1993; Takio et al. 1984). As a result, the analog specificities of these sites are quite different from those of the CAP-related family of cN-binding sites.

Although it was recognized early on that cN analogs acted as PDE inhibitors, the efficacy of these compounds in this capacity was often limited by their breakdown by PDEs. For these and other reasons, design of PDE inhibitors primarily mimicked only the purine of cAMP or cGMP. Increased availability of a broader spectrum of cN analogs with a range of chemical modification (Fig. 4) led to advances in understanding structural features that impact interaction of cNs and cN analogs with target proteins (kinases, cN-gated channels, Epacs, and PDEs). This, in turn, led to the development of analogs that were more potent and specific as agonists for the respective target proteins and more resistant to breakdown by PDEs, thus making them more effective PDE inhibitors. Moreover, cN analogs could be used to more rigorously define characteristics of cN-binding sites in various proteins, including PDEs (Beltman et al. 1995; Butt et al. 1995; Corbin and Doskeland 1983; Dao et al. 2006; Doskeland et al. 1983; Erneux et al. 1984, 1985; Erneux and Miot 1988; Francis et al. 1990; Rehmann et al. 2003; Thomas et al. 1992). Information that can be derived from cN analogs will be discussed in this context; cGMP analogs will be used for illustration.

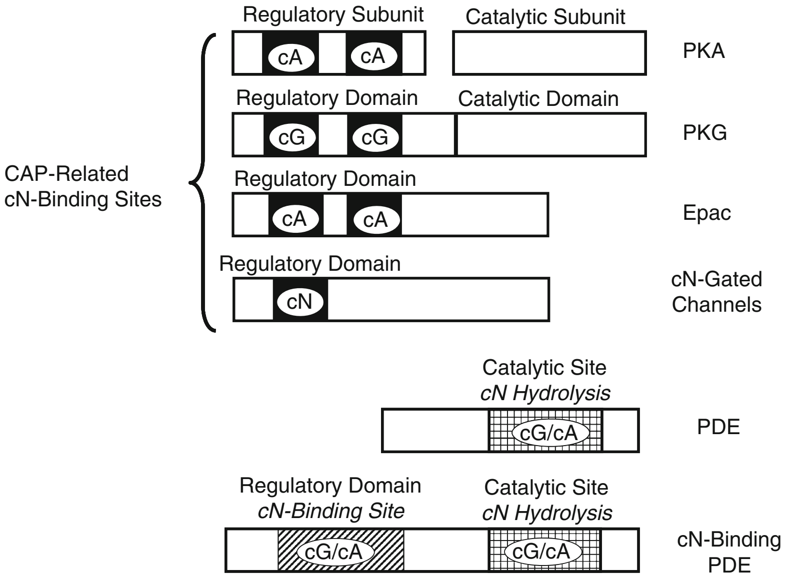


Fig. 3 cN-binding proteins in mammalian tissues. cN-binding sites in mammals fall into three categories: **a** those whose amino acid sequences are related to the bacterial catabolite gene activator protein, **b** allosteric sites on certain PDEs (PDEs 2,5,6,10, and 11), and **c** catalytic sites of PDEs. Differences in the evolutionary origin and molecular structures are indicated by different *fill patterns* in the blocks representing the sites. Sites in cyclic AMP (cAMP)-dependent protein kinase and cyclic GMP (cGMP)-dependent protein kinase are selective for cAMP and cGMP, respectively, but at high concentrations either nucleotide will bind to these sites. Epacs have very high specificity for cAMP and contain either one or two cN-binding sites. Different cN-gated channels have selectivity for either cAMP or cGMP. Allosteric sites in PDEs most commonly bind cGMP with high selectivity, but PDE10 binds cAMP. Catalytic sites of PDEs can either be highly selective for cN or hydrolyze both cNs with equal efficacy

3.1 Cyclic GMP and Cyclic AMP in Solution Exist in Equilibrium Between Two Conformations

In solution, cGMP and cAMP are in equilibrium between two conformations (*syn* and *anti*) that differ in the orientation of the ribose phosphate moiety around the glycosyl bond at N9; the example for cGMP is shown in Fig. 5a. These conformations in gaseous and aqueous phases were predicted using the computational method self-consistent reaction field. The *anti* conformation is more stable in aqueous solution according to predictions from both the Onsager model and the isodensity polarized continuum method (Salter et al. 2003). However, appending groups at various positions in the purine can introduce steric constraints that perturb the equilibrium between the conformers so that the majority of the compound is primarily in the *anti* or the *syn* conformation (Fig. 5b). As a result, the favored cGMP conformation for binding of a particular cN analog to a target protein may be

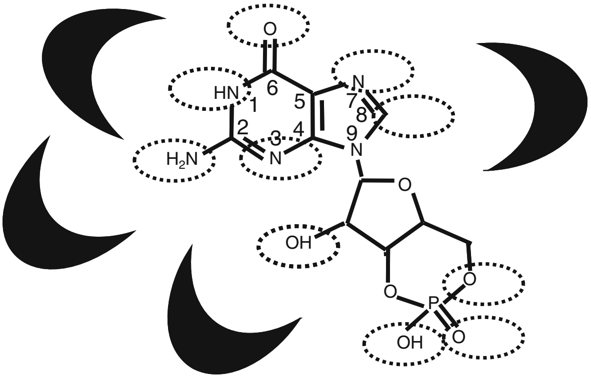


Fig. 4 Modifications of cN structure for development of analogs. cGMP is used as the example to indicate points in the structure that have been altered for development of analogs for use in biochemical studies. Positions on the purine ring are numbered. *Dotted ovals* indicate atoms that have been either removed or substituted by other atoms. *Black arcs* indicate regions that have had various types of groups appended to test for the effects of bulk and/or chemical characteristics

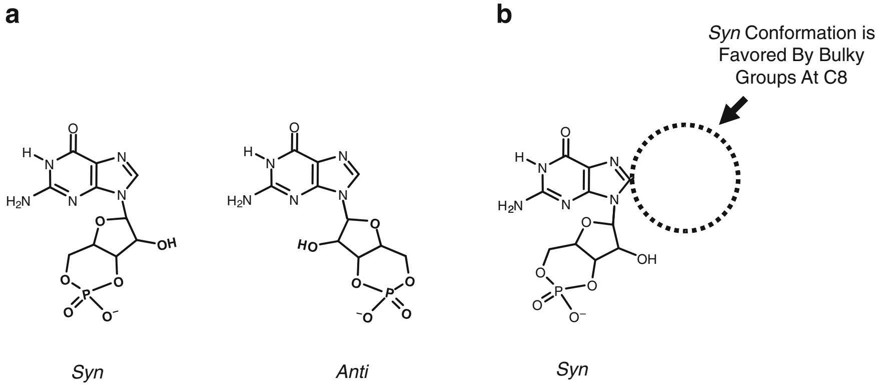


Fig. 5 cNs in solution are in equilibrium between two conformations. **a** cGMP, shown as an example, in solution is in equilibrium between the *syn* and *anti* conformations owing to free rotation of the ribose–cyclic phosphate moiety around the N9 glycosyl bond. **b** Introduction of a bulky group at C8 sterically interferes with rotation of the ribose–cyclic phosphate moiety into the *anti* conformation and therefore increases the amount of cN in the *syn* conformation

more abundant at a particular concentration of the compound; for instance, the bulk of a large group, such as Br or a phenyl appended at C8 (Fig. 5b), physically interferes with rotation of the ribose phosphate moiety around the glycosyl bond, thereby strongly favoring the *syn* conformation. Moreover, groups appended to the purine can alter other features of the molecule, including the electron distribution and the dipole moment across the ring system (Corbin et al. 2006; Erneux et al. 1984). Insights derived from predictions of the favored conformations of cN analogs and the interactions of these compounds with target proteins were used to model the cN-binding sites in proteins. Some proteins such as PKA and PKG preferentially bind cNs in the *syn* conformation (Corbin and Doskeland 1983; Sekhar et al. 1992; Wolfe et al. 1989); thus, cN analogs with large groups appended at C8, which foster the *syn* conformation, had high affinity for the cGMP-binding sites on PKG. Preference for either the *syn* or the *anti* conformer of cGMP for interaction with PDE catalytic sites varied (described later) (Beltman et al. 1995; Butt et al. 1995; Francis et al. 1990; Thomas et al. 1992). In the cN-signaling pathways, cN analogs can have dual and synergistic roles as activators of cN-dependent protein kinases and inhibitors of PDEs, whereas most alkylxanthine inhibitors of PDEs (e.g., IBMX and sildenafil) interact only with catalytic sites of PDEs but not with allosteric cN-binding sites in PKA, PKG, Epacs, cN-gated channels, or cN-binding PDEs.

3.2 Use of cN Analogs To Map cN-Binding Sites of cN-Dependent Protein Kinases

As more cN analogs with distinct modifications became available, they were used to define important points of contact with the cN-binding sites on PKA and PKG and to assess the potency and selectivity of analogs for activation of the respective phosphotransferase activities (Corbin and Doskeland 1983; Doskeland et al. 1983). These seminal studies provided a mechanistic basis for use of a catalog of cN analogs that continues to expand. This approach continues to be used today to better define the role of particular cN-binding proteins in mediating the effects of cN signaling in myriad biological processes (Beebe et al. 1984, 1985, 1988; Dremier et al. 2007; Francis et al. 1988; Petersen et al. 2008; Poppe et al. 2008; Strassmaier and Karpen 2007). To better understand the molecular basis of cN-mediated relaxation of smooth muscle, Francis et al. (1988) correlated known potencies of cGMP and cAMP analogs for activation of PKG or PKA with their potencies for relaxation of smooth muscle. The results convincingly showed that PKGIα plays a role in smooth muscle relaxation but did not support significant PKA involvement. During the course of this study, PKGIβ, which had a different pattern of cN analog selectivity, was discovered (Wolfe et al. 1989); this discovery required the design of new analogs to better define the roles of the PKGI isoenzymes.

Many new cGMP analogs were synthesized and studied for potency in activating PKGs and for blocking PDE activity (Corbin and Doskeland 1983; Sekhar et al. 1992; Wolfe et al. 1989). Analogs of cGMP with bulky groups at C8, e.g., a phenylthio group, which would strongly favor the *syn* conformer of cGMP, preferentially activated PKGIα over PKGIβ and potently relaxed precontracted pig coronary arteries. These studies strengthened the correlation between PKGIα activation and smooth muscle relaxation (Francis et al. 1988; Sekhar et al. 1992). The potency of cGMP analogs containing an 8-phenylthio group was further enhanced by appending an electron-donating substituent (e.g., hydroxyl, methoxy, amino) rather than an electron-withdrawing substituent (e.g., nitro) on the 8-phenylthio

Table 5 Comparison of selectivity of allosteric cGMP-binding sites of cGMP-dependent protein kinase (*PKG*) I isoenzymes for cGMP analogs modified at C8

	K_I' PKGIα		K_I' PKGIβ	
	Site a	Site b	Site a	Site b
8-Br-cGMP	2.5	0.6	3.5	0.59
8-*p*-OH-Ph-*S*-cGMP	6.6	1.7	5.7	2.1
8-Di-OH-Ph-*S*-cGMP	10.4	0.7	14.4	1.2
8-*p*-NH_2-Ph-*S*-cGMP	2.7	16.4	5.3	6.7
8-*p*-OMe-Ph-*S*-cGMP	0.9	2.8	2.1	1.9
8-*o*-Br-Ph-*S*-cGMP	1.6	0.9	ND	ND
8-Br-PET-cGMP	14.3	17.3	93.1	20.4

The value is the ratio of the affinity of the indicated site for a particular analog compared with that for cGMP. A high number indicates greater affinity for the analog than for cGMP
ND not determined

moiety (Sekhar et al. 1992). All of the analogs thus modified were poor activators of PKGII (Gamm et al. 1995) (unpublished data). Analogs of cGMP that were modified at N1 and C2, e.g., 1-N^2-phenyletheno-cGMP (PET-cGMP) and 8-Br-PET-cGMP, activated PKGIα and PKGIβ with nearly equal potency (Sekhar et al. 1992; Wolfe et al. 1989).

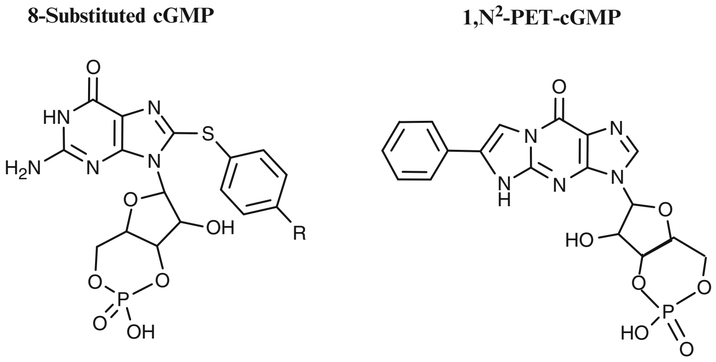
The amino acid sequences of the cGMP-binding region of PKGIα and PKGIβ are identical and each PKG monomer contains two homologous allosteric cGMP-binding sites (site a and site b) that differ in amino acid sequence and kinetic characteristics. Remarkably, the affinities of these sites for cGMP in the two PKGs and their specificities for cGMP analogs differ (Table 5), but each site is involved in activation of the phosphotransferase activity (Corbin and Doskeland 1983; Sekhar et al. 1992; Wernet et al. 1989; Wolfe et al. 1989). The K'_I values in Table 5 represent affinities of a group of cN analogs for sites a and b in PKGIα and PKGIβ relative to the affinity for cGMP; a value of 10 indicates tenfold greater affinity than that for cGMP. This information was used to design potent and specific cN activators of PKGIα and PKGIβ and further study the roles of these PKGs in smooth muscle relaxation. 8-(4-Hydroxyphenyl-*S*)-PET-cGMP, which is modified at N1, C2, and C8 of cGMP, was very potent in activating both PKGs and in relaxing smooth muscle (Sekhar et al. 1992). The results suggested that these substitutions are favorable for the cGMP-binding sites in both PKGs.

3.3 Use of cN Analogs To Map the Allosteric cGMP-Binding Sites and Catalytic Sites of PDEs

Cyclic nucleotide (cN) analogs were also used in studies to map the allosteric cGMP-binding sites and catalytic sites of several PDEs (Beltman et al. 1995; Butt et al. 1995; Francis et al. 1990; Thomas et al. 1992). The catalytic domains of PDEs are conserved and vary in amino acid sequence by 22–50% (Bender and Beavo 2006; Conti and Beavo 2007). Unlike cN-dependent protein kinases that strongly prefer cNs in the *syn* conformation, the preference of PDE catalytic sites for the *anti* versus the *syn* conformation varies (Beltman et al. 1995; Butt et al. 1995; Sekhar et al. 1996; Thomas et al. 1992). Analogs of cGMP substituted at C8 had low affinity for the PDE5 catalytic site, whereas analogs with modifications at N1 and/or C2 were well tolerated. This predicted that cGMP binds to the PDE5 catalytic site in the *anti* conformation (Fig. 5a). The allosteric cGMP-binding site of PDE5 is highly specific for cGMP and has low tolerance for most cGMP analogs or alkylxanthines (Francis et al. 1980, 1990; Thomas et al. 1992).

The large number of new cGMP analogs that were synthesized by Sekhar et al. (1996) allowed for better characterization of the sites on PDE5; in addition, the resistance of these analogs to breakdown by PDE5 was determined to better predict their usefulness as investigational tools and to understand the effects of substitutions on the hydrolytic process. Analogs of cGMP substituted at C8 such as 8-(4-aminophenylthio)-cGMP were very resistant to degradation by PDE5 compared with cGMP (Sekhar et al. 1996). Structural modeling suggested that a large group appended at C8 sterically forced the ribose cyclic phosphate into the *syn* conformation, thus displacing that moiety from the catalytic machinery of PDE5 and providing strong resistance to degradation.

Analogs modified at the 1,2-position of cGMP, e.g., PET-cGMP (Fig. 6), were less resistant than the 8-substituted analogs to hydrolysis by PDE5 (Sekhar et al. 1996). This increased susceptibility to hydrolysis could be explained by greater freedom of the ribose phosphate in PET-cGMP to assume either the *syn* or the *anti* conformation by unimpeded rotation around the N9 glycosyl bond and therefore a greater tendency to assume the *anti* conformation. This interpretation was supported by comparing the extent of hydrolysis of PET-cGMP, 8-Br-PET-cGMP, and 8-I-PET-cGMP by purified PDE5; the extent of hydrolysis was inversely correlated to the bulkiness (I > Br > H) of the C8 substitution (Sekhar et al. 1996). Space-filling models revealed that steric constraints at C8 cause the ribose phosphate group in 8-I-PET-cGMP to be largely fixed in the *syn* conformation. On the basis of results obtained with cN analogs and visualization of the conformations using



8-Substituted cGMP

1,N^2-PET-cGMP

Fig. 6 The steric effect introduced by a bulky group appended to cGMP at C8 versus one appended to N1, C2 space-filling models, it was predicted that the PDE5 catalytic site binds cGMP in the *anti* conformation (Sekhar et al. 1996; Thomas et al. 1992).

3.4 Design and Use of 8-Substituted IBMX Analogs for Potent Inhibition of PDE

3.4.1 Early Insights into the Mode of Interaction of Xanthine-Based Compounds with cN PDEs

Wells et al. (1981) studied the inhibitory potency of xanthine-based compounds substituted at C1, N3, and C8 on partially purified PDEs: calmodulin-sensitive PDE and cAMP-specific PDE. On the basis of the results, they proposed that the catalytic site of calmodulin-sensitive PDE tolerates bulky groups appended at C8 of cGMP or xanthine analogs and contains a lipophilic area with which the ribose cyclic phosphate group interacts. With some xanthine analogs, this area is partially occupied by derivatized alkyl substitutions at N3, e.g., the isobutyl of IBMX. These early studies provided crucial information about PDE catalytic sites and led to the interpretation that cNs bind to certain PDEs in the *anti* conformation. It was later shown that catalytic sites of several other PDEs prefer the *syn* conformation (Beltman et al. 1995; Butt et al. 1995).

3.4.2 Design of Potent and Novel Derivatized Alkylxanthines as PDE5 Inhibitors

With use of insights derived from studies of the interaction of cGMP analogs with the PDE5 catalytic site and the hydrolytic resistance of analogs, a design for potent PDE5 inhibitors was conceptualized and achieved long before the discovery of sildenafil (Sekhar et al. 1996). It was proposed that potent IBMX analogs would be achieved by mimicking the entire cGMP molecule and that the moiety which mimicked the ribose phosphate should occupy a space simulating an *anti* conformation, which is the conformation of cGMP bound in the PDE5 catalytic site (Thomas et al. 1992). At that time, despite known differences in analog specificities, it was thought that sites of interaction of cGMP on PKG and PDE5 could be homologous. Therefore,on the basis of results obtained from cN studies with PKG, it was proposed that a hydrophobic group, e.g., a phenyl, with electron-donating substitutions would foster affinity of analogs for PDE5 and thereby increase inhibitor potency. Prior to this, most PDE5 inhibitors contained a mimic of guanine but not of the ribose cyclic phosphate.

Using this strategy, Sekhar et al. (1996) synthesized a collection of IBMX analogs with a phenylthio substitution at C8, which were 10–30-fold more potent inhibitors for PDE5 than any known inhibitor. The cartoon in Fig. 7a depicts cGMP bound to the PDE5 catalytic site in the *anti* conformation with the ribose phosphate

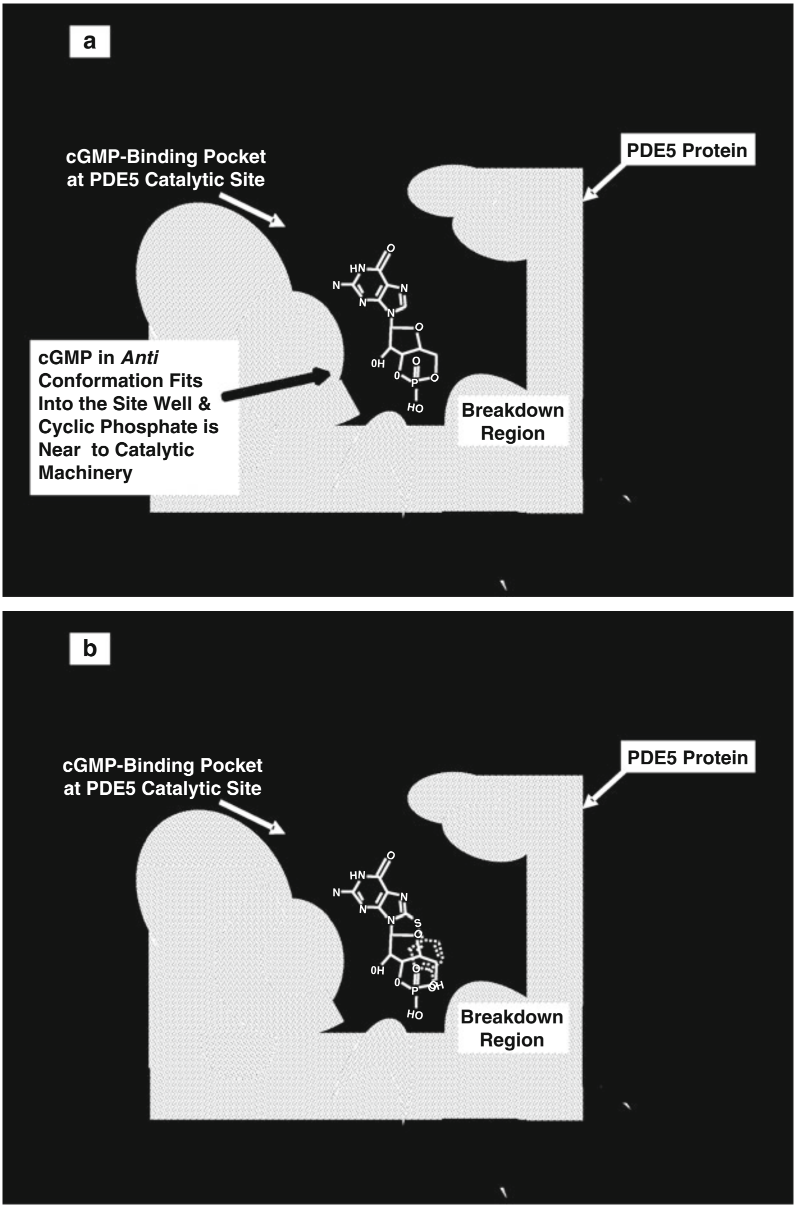


Fig. 7 The cN alignment in the PDE5 catalytic site. **a** cGMP is shown in the *anti* conformation in the PDE5 catalytic site; this orientation places the cyclic phosphate ring near the breakdown region containing the divalent cations that provide for hydrolysis. **b** The *dotted structure* depicts the fact that the 4-hydroxyphenylthio group appended at C8 in 3-isobutyl-1-methylxanthine (IBMX) occupies the space normally occupied by the cyclic phosphate moiety of cGMP, i.e. the two moieties are shown to be overlapping in this depiction

moiety located near the hydrolytic region of the site. To mimic cGMP in this *anti* conformation, related compounds were modeled so that the C8-substitution could occupy the space of the ribose cyclic phosphate group of cGMP when bound to the PDE5 catalytic site (Fig. 7b). This design avoided steric hindrance posed by substitutions at N9. IBMX analogs synthesized based on this reasoning produced highly potent PDE5 inhibitors that also had improved selectivity for PDE5 over other PDEs (Sekhar et al. 1996). The most potent compound [8-(norbornylmethyl)-IBMX] was about 7,000-fold more potent for PDE5 ($IC_{50} = 0.0015\ \mu M$) than IBMX, the parent compound ($IC_{50} = 10\ \mu M$); the potency of this compound for PDE1 ($IC_{50} = 0.03\ \mu M$) was more than 200-fold greater than that of the parent IBMX ($IC_{50} = 7\ \mu M$). This simple modification of the nonspecific and weak inhibitor IBMX dramatically improved both the potency and specificity of this compound for PDE1 or PDE5 compared with PDEs 2–4. The IC_{50} values reported for these compounds for PDE5 underestimated the true potency because for technical reasons assays were conducted with saturating cGMP substrate. The K_i of 8-(norbornylmethyl)-IBMX calculated from this IC_{50} value using the Cheng–Prusoff equation – $K_i = IC_{50}/(1 + [S]/K_m)$ – is 0.17 nM, which is similar to that of vardenafil (0.1–0.4 nM), the most potent known inhibitor for PDE5 (Blount et al. 2004; Cheng and Prusoff 1973).

Information gained from investigations of the characteristics of the cN-binding sites of PKA and PKG was a critical antecedent to subsequent studies of the sites on PDEs (Corbin and Doskeland 1983; Doskeland et al. 1983; Sekhar et al. 1992; Wolfe et al. 1989). Synthesis and studies of IBMX analogs with restricted orientation of substitutions clearly demonstrated the preference of an *anti* conformation at the catalytic site of PDE5, a conclusion that had already been drawn on the basis of cN analog studies (Thomas et al. 1992). The *cis* isomer of 8-(4-methoxystyryl)-IBMX ($IC_{50} = 0.016\ \mu M$) was 23-fold more potent than its *trans* isomer ($IC_{50} > 10\ \mu M$). These results complemented the original design strategy and emphasized the importance of studies of substrate analogs in mapping sites that interact with cNs, and in designing effective inhibitors.

4 Insights into Interaction of PDEs with Alkylxanthine-Related Compounds

To advance understanding of the interaction of cNs and alkylxanthine-related inhibitors with the catalytic sites of PDEs, a number of X-ray crystal structures of isolated C domains of PDEs in complex with either the product of hydrolysis (5′-AMP or 5′-GMP) or inhibitors have been determined. To date, there is no structure for a co-crystal of a PDE with wild-type sequence containing either cAMP or cGMP in the catalytic site since the cN (even “nonhydrolyzable” analogs) is invariably hydrolyzed during the determinations. Despite that, the structures of inactive PDE4D and PDE10A in complex with cAMP and cGMP, respectively, the structures of other PDEs in complex with inhibitors and catalytic products, site-directed mutagenesis studies, and cN analog studies have provided considerable insight into the interaction of cN with PDE catalytic sites (Corbin et al. 2006; Huai et al. 2004a; Ke and Wang 2007; Turko et al. 1998, 1999; Wang et al. 2007a, 2007b, 2008a; Zoraghi et al. 2006, 2007).

4.1 Interactions of IBMX and Other Xanthine-Related PDE Inhibitors with PDE Catalytic Sites as Determined by X-ray Crystallography

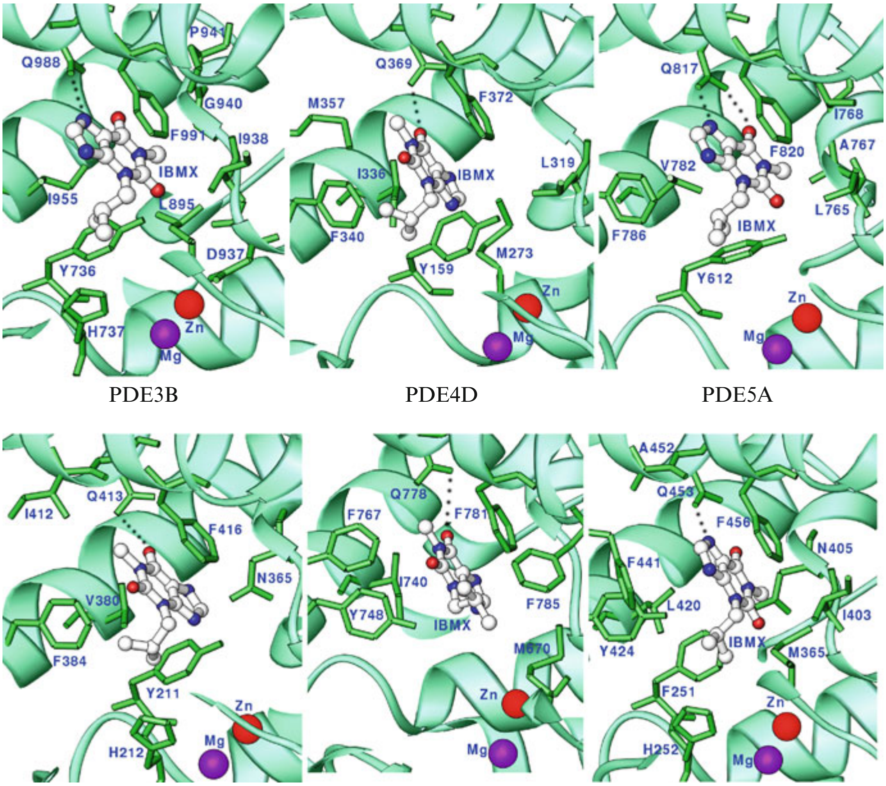
4.1.1 Identification of Direct Contacts Between PDE Catalytic Sites and Derivatized Alkylxanthine Inhibitors

Six crystal structures of isolated C domains of human PDEs in complex with IBMX (which occupies the catalytic site) have been reported; these include PDE3B (Protein Data Bank entry code 1SOJ), PDE4D (1ZKN), PDE5A (1RKP), PDE7A (1ZKL), PDE8A (3ECN), and PDE9A (2HD1) (Huai et al. 2004a, b; Ke and Wang 2007; Scapin et al. 2004; Wang et al. 2005, 2006, 2008a). In addition, X-ray crystal structures of PDE5 in complex with the derivatized alkylxanthine inhibitors sildenafil, vardenafil, and tadalafil have also been reported (Sung et al. 2003; Wang et al. 2006, 2008b).

The planar xanthine-like ring of these inhibitors mimics the purine of cN and is bound in a hydrophobic pocket of the catalytic site. In these crystal structures, the heterocyclic ring is bound by a “hydrophobic clamp” that is formed by the side chains of two amino acids [an alkyl-like group (e.g., valine or leucine) and a phenyl group of a conserved phenylalanine] (Fig. 8) (Ke and Wang 2006; Sung et al. 2003; Xu et al. 2000; Zhang 2006). IBMX binding to PDE catalytic sites shows two common features: (1) hydrophobic stacking with a conserved phenylalanine, and (2) hydrogen bonding with an invariant glutamine. For IBMX, the xanthine ring stacks against the hydrophobic side chain of Phe991 in PDE3B, Phe372 in PDE4D2, Phe820 in PDE5A1, Phe416 in PDE7A1, Phe781 in PDE8A1, and Phe456 in PDE9A2 (Fig. 8). The five-membered ring in IBMX typically forms a π–π electron-stacking interaction with the phenylalanine side chain.

Hydrophobic stacking with the phenylalanine and hydrogen bonding with the glutamine appear to be basic elements for binding of PDE inhibitors since they have been shown to be common for binding of most known PDE inhibitors (Ke and Wang 2007).

While these contacts contribute importantly to high-affinity binding in certain instances, e.g., binding of IBMX, sildenafil, vardenafil, and tadalafil to PDE5A (Zoraghi et al. 2007), they alone are not sufficient for high-affinity interaction. This is evident from the very weak interaction of IBMX with PDE8A (Wang et al. 2008a) as well as the contributions of other amino acids to high affinity of PDE5 for substrates and inhibitors (Zoraghi et al., 2007). In all PDE catalytic sites studied, IBMX forms van der Waals contacts with residues other than the phenylalanine and



PDE3B PDE4D PDE5A

PDE7A PDE8A PDE9A

Fig. 8 IBMX binding to the active sites of PDEs. The *dotted lines* represent hydrogen bonds. The PDE binuclear metal site where breakdown of the cyclic phosphate moiety of cN occurs is shown containing zinc (*large red ball*), which is bound to PDEs with high affinity and has clearly been identified in the respective X-ray crystal structures, and magnesium (*large purple ball*), which is presumed to occupy the second metal site

glutamine (Table 6); among these, some residues that are involved in IBMX binding, e.g., Ile336 of PDE4D2 or their homologs in other PDEs, are conserved within families, while different amino acids are found uniquely in particular PDE families. Despite the availability of numerous X-ray crystal structures of PDEs in co-complex with alkylxanthine-related inhibitors, there is still much that is not understood about factors that provide for high potency and selectivity for the various PDEs (Blount et al. 2006; Ke and Wang 2006; Zhang 2006).

4.1.2 Difference in Orientation of IBMX in the Active Sites of PDEs

In spite of the shared pattern of interactions, IBMX binding shows significant differences among PDEs in both its orientation and position within the respective catalytic sites and specific interactions that are formed. The most significant

Table 6 PDE catalytic domain amino acids that interact with IBMX

PDE														
PDE3B	Y736	H737	L895	D937	I938	G940	P941	I955				Q988	F991	
PDE4D2	Y159		M273		L319			I336	F340	M357		Q369	F372	
PDE5A1	**Y612**					*L765*	A767	I768	*V782*	*F786*		***Q817***	***F820***	
PDE7A1	Y211	H212				N365		V380	F384		I412	Q413	F416	
PDE8A1			M670					I744	Y748	F767		Q778	F781	F785
PDE9A2	F251	H252	M365		I403	N405		L420	Y424	F441	A452	Q453	F456	

Summary of amino acids in homologous positions in the catalytic sites of PDEs that make contact with IBMX. Site-directed mutagenesis of Tyr612, Gln817, or Phe820 (*bolded and underlined*) in PDE5 holoenzyme caused a 40–90-fold loss of affinity for IBMX. Point mutations of Leu765, Val782, or Phe786 (*italicized and underlined*) caused a tenfold or lower change in affinity for IBMX difference is the orientation of IBMX in these structures. The xanthine ring of IBMX is aligned in the same direction in the structures of PDEs 4D, 7A, and 8A, but in PDEs 3B, 5A, and 9A, the alignment is the opposite (Fig. 8). This difference in orientation results in a different pattern of hydrogen bonding. The N7 atom of IBMX forms a hydrogen bond with the Oε from the side chain of the invariant glutamine of PDEs 3B, 5A, and 9A; the carbonyl oxygen at C6 forms a hydrogen bond with the Nε of the glutamine side chain in the structures of PDEs 4D, 5A, 7A, and 8A (Fig. 8) (Huai et al. 2004a; Ke and Wang 2007). In addition, IBMX shows positional changes of several angstroms among the atoms. On the basis of the results of different positions and orientations, individual PDE families show unique interactions such as the interaction between N3 of IBMX and OH of Tyr424 in the PDE9A structure (Huai et al. 2004b).

Differences in the interactions of IBMX in these structures may be due to the fact that IBMX has a much smaller molecular volume than do the PDE catalytic site pockets (about 330 $Å^3$) (Xu et al. 2000). As a result, IBMX can assume different orientations and exploit potential interactions, while retaining hydrophobic stacking with the conserved phenylalanine and hydrogen-bond formation with the glutamine. These differences in the IBMX binding should be useful for design of PDE-family selective inhibitors.

4.1.3 Extended Effects of Derivatized Alkylxanthines on the Structure of the PDE5 Catalytic Site

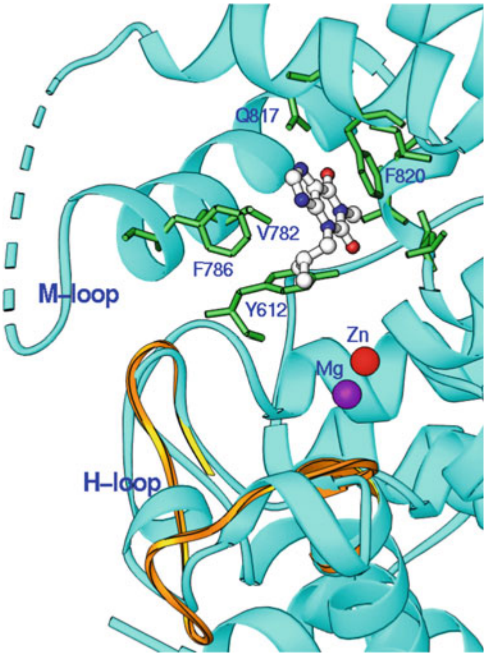
Movement of the H-Loop of PDE5

In most PDE families, the conformations of the C domain appear to be rigid. The structure of the PDE5 C domain appears to be unique among PDE families (Ke and Wang 2007) since the H-loop in the C domain can assume at least five different conformations (Chen et al. 2008; Wang et al. 2006). While some inhibitors such as sildenafil and icarisid II directly interact with the H-loop and cause 10–20-Å shifts, IBMX does not directly contact the H-loop and causes up to 7-Å movement of the H-loop (Fig. 9). The H-loop of the PDE5A–IBMX complex contains two short α-helices that are comparable within most other PDE families. However, in unliganded PDE5 C domain, the H-loop is more like a random coil. The explanation for the H-loop movement is not straightforward. Apparently, IBMX binding to the pocket of the active site imposes an allosteric effect on H-loop movement (Wang et al. 2006), but the impact of the binding of other inhibitors could be due to both direct interaction and an allosteric effect.

Inhibitor-Induced Structural Changes in the PDE5 Catalytic Site

Another characteristic of binding of alkylxanthine-related compounds to PDE5 involves an inhibitor-induced change in affinity for the inhibitor. The molecular

Fig. 9 Comparison of the position of PDE5 H-loop in the unliganded PDE5A versus its position in the IBMX-bound form. Ribbon structure of the PDE5 catalytic domain in the unliganded state and with IBMX bound is shown. The location of IBMX in the catalytic site is shown by the stick model and amino acids surrounding IBMX are indicated. The image emphasizes the different positions and structural features of the PDE5 H-loop in the unliganded state (*blue*) and the IBMX-bound form (*golden ribbon*)



basis for these kinetic effects has not been resolved. Binding of certain inhibitors (sildenafil, vardenafil, and tadalafil) to either PDE5 holoenzyme or isolated C domain exhibited two rates, a high-affinity component and a low-affinity component (Blount et al. 2007). However, prolonged incubation of PDE5 holoenzyme with these inhibitors converted the low-affinity component to the high-affinity component, indicating that there is a time-dependent conformational change that optimizes contacts between the compound and PDE5 catalytic site. This shift in affinity required a portion of the regulatory domain of PDE5, i.e., the GAF B subdomain, and did not occur in the isolated C domain. Since heterogeneity of the binding pattern was not evident in the X-ray crystallographic structure of the isolated C domain, there is no straightforward explanation of this phenomenon. However, it is worth noting that binding of rolipram, which is not an alkylxanthine derivative, to PDE4 occurs with low- and high-affinity states that apparently reflect binding activity of a single site on PDE4, i.e., the catalytic site (McKenna and Muller 2006). Whether the PDE4 catalytic site converts between the two kinetic states is unknown. However, these types of kinetic differences in the catalytic sites of PDEs may be more common than appreciated and add another degree of complexity to understanding ligand interactions with PDE catalytic sites.

4.2 Use of Site-Directed Mutagenesis To Quantify the Impact of Amino Acid Contacts with Substrates and Xanthine-Related PDE Inhibitors

Results of site-directed mutagenesis in PDE5 holoenzyme have biochemically quantified and verified the importance of the contact(s) observed in the X-ray crystallographic studies of the isolated C domain (Zoraghi et al. 2007); contacts observed in crystal structures of other PDEs have not yet been studied in this manner. In PDE5 holoenzyme, conversion of the conserved phenylalanine (Phe820) to alanine caused a dramatic loss (60–450-fold) of affinity for substrate as well as inhibitors such as vardenafil, sildenafil, tadalafil, and IBMX. Mutation of the side chain of the invariant glutamine (Gln869) also caused a major loss (60–500-fold) of affinity for substrate as well as inhibitors (Ke and Wang 2006, 2007; Zoraghi et al. 2007); mutation of this same glutamine in PDE11 also caused a major loss of affinity for substrates (cGMP and cAMP) as well as tadalafil, a reasonably potent inhibitor (Weeks et al. 2009). Substitution of alanine for the invariant tyrosine in PDE5 (Tyr612), which also makes contacts with certain inhibitors in PDE5 catalytic site, also caused a major loss of affinity (14–120-fold) for substrate and inhibitors. Replacement of this tyrosine by phenylalanine increased the affinity for some inhibitors, consistent with a positive contribution of the tyrosine side chain to the hydrophobicity of the catalytic pocket (Corbin et al. 2006; Huai et al. 2004a; Sung et al. 2003). Most of the mutations of other amino acids that had been shown by X-ray crystallography to be in contact with inhibitors had more modest effects. Thorough mutagenesis studies have not been conducted in most other PDE families.

4.3 Use of cN Analogs to Probe Structural Elements; Comparison of Findings Using X-ray Crystallography

Studies with cN analogs to characterize the catalytic sites of PDEs have provided an important adjunct to interpretations arrived at through the use of X-ray crystallographic structures and site-directed mutagenesis. Studies with cN analogs as well as those using site-directed mutagenesis are particularly important because both approaches can employ PDE holoenzymes to assess the importance of various contacts between the PDE and the substrate and/or inhibitors. Studies with cN analogs have shown major differences in the catalytic sites of PDEs, so it is difficult to make general comments. Studies with cN analogs have shown that (1) some PDEs prefer cN in the *anti* conformation, while others prefer the *syn* conformation, and these results have subsequently been validated by X-ray crystallographic studies (Wang et al. 2007a, b), (2) contact with the 2′-hydroxyl is not particularly important for any PDE tested, (3) interaction with N1 in either cAMP or cGMP is important in many, but not all PDEs, (4) the importance of interaction with N7 varies, and (5) interaction with the substituent at C6 is in some cases important, but is not universal (Beltman et al. 1995; Butt et al. 1995; Francis et al. 1990; Thomas et al. 1992; Weeks et al., unpublished results).

5 Concluding Remarks

Shortly after the discovery of cAMP, the methylxanthines and related compounds became critical tools for research in cN signaling pathways. They inspired synthesis of thousands of compounds that might act as more specific and potent PDE inhibitors. Many of these compounds have become highly useful in clinical treatment of a number of disease processes, and potential treatment of many other diseases is being investigated. However, despite great advances in understanding the mode of interaction of these compounds with PDEs, the pharmacological action of these agents are not completely understood.

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Methylxanthines and Ryanodine Receptor Channels

Serge Guerreiro, Marc Marien, and Patrick P. Michel

Contents

Abstract Methylxanthines of either natural or synthetic origin have a number of interesting pharmacological features. Proposed mechanisms of methylxanthine-induced pharmacological effects include competitive antagonism of G-coupled adenosine A_1 and A_{2A} receptors and inhibition of phosphodiesterases. A number

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of studies have indicated that methylxanthines also exert effects through alternative mechanisms, in particular via activation of sarcoplasmic reticulum or endoplasmic reticulum ryanodine receptor (RyR) channels. More specifically, RyR channel activation by methylxanthines was reported (1) to stimulate the process of excitation coupling in muscle cells, (2) to augment the excitability of neurons and thus their capacity to release neurotransmitters, and also (3) to improve their survival. Here, we address the mechanisms by which methylxanthines control RyR activation and we consider the pharmacological consequences of this activation, in muscle and neuronal cells.

Keywords Calcium · Methylxanthines · Muscle cell contraction · Neuroprotection · Neurotransmitter release · Ryanodine receptors

Abbreviations

Ca_{cyt}^{2+}	Cytoplasmic Ca^{2+}
ER	Endoplasmic reticulum
RyR	Ryanodine receptor
SR	Sarcoplasmic reticulum

Methylxanthines are purine derivatives that have in common a xanthine core molecule with methyl groups attached in various combinations to nitrogens, at positions 1, 3, 7, or 9. Methylxanthines are generally from natural origin but a number of synthetic congeners have also been designed. The most common methylxanthines are caffeine (1,3,7-trimethylxanthine) and its hypomethylated analogues theophylline (1,3-dimethylxanthine), theobromine (3,7-dimethylxanthine), and paraxanthine (1,7-dimethylxanthine). Caffeine, theophylline, and theobromine are produced by different plant species, whereas paraxanthine occurs essentially as a caffeine metabolite in biological fluids (Magkos and Kavouras 2005)[1].

Proposed mechanisms of methylxanthine-induced pharmacological actions include competitive antagonism of G-coupled adenosine A_1 and A_{2A} receptors and also inhibition of phosphodiesterases (Jacobson and Gao 2006; Daly 2007)[2]. A number of studies have shown, however, that methylxanthines can exert effects through alternative mechanisms, in particular via the activation of sarcoplasmic reticulum (SR) or endoplasmic reticulum (ER) ryanodine receptor (RyR) channels. RyR channel activation by methylxanthines was reported originally to stimulate

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[1] Botanical sources and bioconversion pathways of methylxanthines are described in detail in Ashihara et al. (2010) and Arnaud (2010), respectively.

[2] See Francis et al. (2010) for details on the pharmacological effects of methylxanthines that are mediated by adenosine receptor blockade or phosphodiesterase inhibition.

excitation coupling in muscle cells, a process that connects myocyte membrane depolarization and muscle contraction (Fill and Copello 2002; Zalk et al. 2007). Other studies have shown that methylxanthines can augment the excitability of neurons and thus their capacity to release neurotransmitters (Pessah et al. 1987) and may improve their survival as well (Guerreiro et al. 2008). Note that methylxanthines were also found to activate RyRs in other cell types, including pancreatic β cells (Bruton et al. 2003) and T cells (Ritter et al. 2001), but work on the subject is relatively limited.

The aim of this chapter is to provide an overview of the mechanisms by which methylxanthines trigger RyR channel activation in muscle and neuronal cells and to report on the pharmacological consequences of this activation. As a first step, it appears pertinent, however, to begin with a brief description of the mechanisms involved in the release of calcium (Ca^{2+}) via RyR channels.

1 RyR Channels: Regulation and Function

RyR channels are intracellular Ca^{2+} channels that share some structural and functional similarities with inositol 1,4,5-trisphosphate receptors (Fill and Copello 2002). They are large tetrameric channel proteins sharing a rather peculiar four-leaf-clover-like structure when observed by electron microscopy (Fill and Copello 2002; Zalk et al. 2007). The large cytoplasmic domain of RyRs serves as a scaffold for proteins that bind to the channel and modulate its function. The RyR family comprises three major receptor subtypes. The three channel subtypes are encoded by three distinct genes that share approximately 70% sequence homology. The predominant isoform in skeletal muscle is RyR_1, and in cardiac muscle it is RyR_2. In neurons, RyR_1, RyR_2, and RyR_3 are all present. All three RyRs can be activated by Ca^{2+} from the cytosolic side. In addition, RyR channels are modulated by numerous factors, including a number of physiological agents (e.g., ATP, adenosine, cyclic ADP ribose, and Mg^{2+}) and various cellular processes (e.g., redox state and energy charge phosphorylation) (Sitsapesan et al. 1995; Zalk et al. 1997).

RyR channels directly control intracellular Ca^{2+} release in skeletal and cardiac muscle cells, activating muscle contraction during excitation–contraction coupling. In neurons RyR channels can modulate neurotransmitter release. Two mechanisms account for RyR-mediated Ca^{2+} release (Verkhratsky 2005; Zalk et al. 2007). Ca^{2+} release through RyRs can result from a direct physical interaction between plasmalemmal voltage-gated Ca^{2+} channels located on the transverse tubule membrane of striated muscle cells and RyR_1 channels on the SR (Fill and Copello 2002; Magkos and Kavouras 2005). Alternatively, in cardiac cells and also in neurons, Ca^{2+} release is triggered through a process known as Ca^{2+}-induced Ca^{2+} release (Fabiato 1983). In that case, the activation of voltage-gated channels triggers a small elevation in the level of cytoplasmic Ca^{2+} ($Ca_{cyt}{}^{2+}$), which in turns serves to activate Ca^{2+} release through RyR activation. Ligand-gated Ca^{2+}-induced Ca^{2+} release makes the kinetics of intracellular Ca^{2+} release slower when compared with protein-mediated activation of RyR_1 in skeletal muscle (Näbauer et al. 1989).

The RyR channel binds specifically the plant alkaloid ryanodine, which is the reason for its name. Ryanodine binds the channel with high affinity in a Ca^{2+}-dependent manner (Sutko et al. 1997). The two ryanodine-binding sites, a high-affinity and a low-affinity binding site, which have been described at the C-terminus of the receptor may account for the fact that low micromolar concentrations of ryanodine cause the stimulation of RyR channels, while higher concentrations have the opposite effect and can even provoke their complete closure (Buck et al. 1992). Whereas ryanodine operates as preferential ligand, methylxanthines are also potent activators of RyR channels. The mechanisms by which methylxanthines cause the activation of RyR and the pharmacological consequences of this activation are described hereafter.

2 Methylxanthines and RyR Channels in Muscle Cells

2.1 Effects of Caffeine on Muscle Cell Contractility

The first evidence that caffeine could increase the contractility of isolated skeletal muscles dates back to experiments performed by Veley and Waller (1910). Subsequent studies using muscle preparations demonstrated that contractures induced by caffeine can occur in the absence of external Ca^{2+} and without any change in resting membrane action potential (Conway and Sakai 1960; reviewed in Magkos and Kavouras 2005). These early studies showed that caffeine's effects were dose-dependent, and that threshold effective concentrations for activation were on the order of 1–2 mM, with a peak activation occurring at 5–10 mM.

It again took a number of years before several groups could demonstrate that caffeine operates by releasing Ca^{2+} from ER stores (Endo 1977; Fabiato 1983; Kirino and Shimizu 1982). The site of caffeine's action was ascribed to RyR channels using preparations in which the channel proteins from striatal or cardiac muscles were incorporated into an artificial planar lipid bilayer (Pessah et al. 1987; Hymel et al. 1988; Meissner et al. 1988; Xu and Meissner 1988). This explains why cultured myotubes isolated from a dyspedic (IB5) mouse lacking RyR_1 channels were insensitive to caffeine, while the response to caffeine could be restored in this preparation by transfection with a complementary DNA encoding RyR_1 (Fessenden et al. 2006) (Fig. 1). A number of studies demonstrated that the activation site for caffeine was within the pore region of RyRs channels, i.e., in the C-terminal portion of the protein (Du and MacLennan 1999). In particular, several mutations in the C-terminal portion of RyR_1 have been reported to modulate the response of RyR_1 channels to caffeine in central core disease, an autosomal dominant, slow progressive or nonprogressive congenital myopathy (Du et al. 2004). Furthermore,

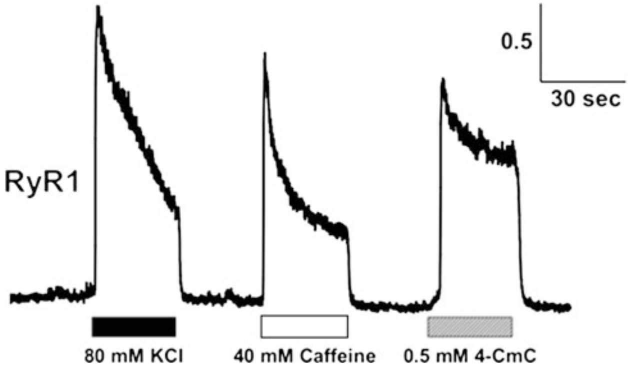

Fig. 1 Caffeine-induced elevation of intracellular calcium levels in cultured 1B5 myotubes transfected with the ryanodine receptor isoform RyR_1. Caffeine stimulates the release of sarcoplasmic reticulum calcium by directly activating RyR_1 channels. An elevated K^+ level was initially applied to the preparation to test for depolarization-induced calcium release, e.g., calcium release occurring via RyR_1, as the result of the activation of plasmalemmal voltage-gated calcium channels. Note that untransfected 1B5 myotubes cells are insensitive to both caffeine and depolarization induced by high K^+ concentration as they derive from RyR null/dyspedic mice. The phenol-based compound 4-chloro-*m*-cresol, which is unrelated structurally to methylxanthines, can also operate as a potent activator of RyRs. The figure depicts changes in Fluo-4 fluorescence (F) normalized to the average resting fluorescence (F_0) of the cell. Calibration bar = 0.5 F/F_0 arbitrary units versus 30 s. (Data from Fessenden et al. 2006 adapted with permission)

site-directed mutagenesis of the same region in RyR_2 channels generated mutants that were insensitive to caffeine (Wang et al. 2004).

Rousseau et al. (1988) demonstrated that caffeine increases the channel mean open time and open probability of both skeletal (RyR_1) and cardiac (RyR_2) SR Ca^{2+} release channels but not their conductance. The response to caffeine was like that of ryanodine, in that it was suppressed by Mg^{2+} and ruthenium red (Zucchi and Ronca-Testoni 1997). The regulatory effect of caffeine on excitation–contraction coupling appeared to be bimodal with (1) a Ca^{2+}-dependent activation with threshold concentrations greater than 100 μM caffeine making RyRs (RyR_1 and RyR_2) more sensitive to endogenous activators (e.g., Ca^{2+}, ATP, ADP, AMP, adenosine) (Pessah et al. 1987; Magkos and Kavouras 2005) and (2) a Ca^{2+}-independent activation observed at high (millimolar) caffeine concentrations (Sitsapesan and Williams 1990; Herrmann-Frank et al. 1999). When applied to SR vesicles, caffeine caused a significant increase in the efflux rate of Ca^{2+} when Ca^{2+} in the release medium was in the range of nanomolar concentrations. However, when the external Ca^{2+} concentration was set at micromolar concentrations or above, caffeine had no stimulatory effect on Ca^{2+} mobilization (Meissner and Henderson 1987; Moutin and Dupont 1988). It is worth noting that caffeine-mediated Ca^{2+} release via RyRs was more sensitive to cytosolic Ca^{2+} in cardiac preparations than in skeletal SR preparations (Meissner and Henderson 1987).

In most cases, the effect of caffeine reproduced that of adenine nucleotides. However, several lines of evidence suggested that caffeine and adenine nucleotides acted on different but possibly interacting sites: (1) caffeine and adenine nucleotides had synergistic effects on channel gating; (2) adenine and adenosine inhibited the response to ATP analogues possibly by competition, yet they stimulated the response to caffeine (McGarry and Williams 1994). The phenol-based compound 4-chloro-*m*-cresol, which is unrelated structurally to methylxanthines, also reproduced some of the effects of caffeine on RyRs (Fessenden et al. 2006). Similar to caffeine, 4-chloro-*m*-cresol activated RyR_1 by increasing the affinity of Ca^{2+} binding to its activator site. Yet, these two compounds differed in that 4-chloro-*m*-cresol was approximately 25 times more potent than caffeine in activating RyR . The activation profiles of caffeine and 4-chloro-*m*-cresol were also different: whereas 4-chloro-*m*-cresol was a much more effective activator of RyR_1 and RyR_2 compared with RyR_3 (Fessenden et al. 2006), caffeine was much more potent in activating RyR_2 and RyR_3 compared with RyR_1 (Du and MacLennan 1999).

Finally, unlike many receptors that are activated only transiently and undergo a rapid process of desensitization (Kelly et al. 2008), skeletal and cardiac RyR channels did not undergo conventional inactivation upon caffeine exposure. Indeed, multiple additions of submaximal concentrations of caffeine could each induce a partial and transient Ca^{2+} release from intracellular Ca^{2+} stores in cells expressing RyRs, a phenomenon known as “quantal” Ca^{2+} release (Dettbarn and Palade 1997; Kong et al. 2008). These observations can be explained by assuming either that different Ca^{2+} release channels have different sensitivities to caffeine or that the SR is composed of several compartments, which are discharged by different concentrations of caffeine (Verkhratsky 2005).

2.2 Effects of Other Naturally Occurring Methylxanthines

Theophylline, theobromine, and paraxanthine, which occurs essentially as a metabolite of caffeine, shared several of the properties of caffeine toward RyRs. They all stimulated the release of $^{45}Ca^{2+}$ from heavy SR vesicles and they also increased [^{3}H]ryanodine binding to skeletal muscle SR vesicles (Liu and Meissner 1997). Rousseau et al. (1988) reported that paraxanthine, theobromine, and theophylline were more effective than caffeine in stimulating Ca^{2+} release. In the same preparation, 1,3-dimethyluracil was ineffective, suggesting that the integrity of the imidazole ring (methylxanthines are formed from a pyrimidine-dione ring and an imidazole ring fused together) was required for this process. Hawke et al. (2000) also demonstrated that paraxanthine, theophylline, and theobromine were all able to transiently increase Ca_{cyt}^{2+} concentrations to subcontracture levels in resting mammalian skeletal muscles, in a concentration-dependent fashion. The ability of procaine to inhibit this effect confirmed that the SR was the primary source for the increased Ca_{cyt}^{2+} concentrations.

2.3 Effects of Synthetic Methylxanthines

Two methylxanthines which are structurally related to theophylline and paraxanthine, 3-isobutyl-1-methylxanthine and 7-isobutyl-1-methylxanthine, respectively, retained the ability of their parent compounds to stimulate Ca^{2+} release through RyR channels in SR vesicles (Rousseau et al. 1988; Wyskovsky 1994). Rousseau et al. (1988) also reported that among methylxanthines having an *N*-methyl substitution at position 9, 3,9-dimethylxanthine was as potent as caffeine in release experiments, whereas 1,9-dimethylxanthine was minimally effective. Using a preparation of sea urchin egg homogenates, Cavallaro et al. (1999) also compared the potential of a number of synthetic caffeine derivatives to potentiate the effects of cyclic ADP ribose, a metabolite of NAD^+, which is also a physiological activator of RyRs (Galione et al. 1991; Lee 1993). These compounds had their methyl substituent at positions 1 or 7 replaced with alkyl chains containing different functional groups. Two of these compounds, 1,3-dimethyl-7-(7-hydroxyoctyl)xanthine and 3-methyl-7-(7-oxooctyl)-1-propargylxanthine, were more potent than caffeine itself in potentiating cyclic ADP ribose induced Ca^{2+} release, while 1,3-dimethyl-7-(5-ethylcarboxypentyl)xanthine was more efficacious (Cavallaro et al. 1999).

3 Methylxanthines and RyRs in Neuronal Cells

3.1 Stimulation of ER Calcium Release

A number of studies have also shown that methylxanthines, including caffeine, theophylline, and 3-isobutyl-1-methylxanthine, can cause elevations of ${Ca_{cyt}}^{2+}$ concentrations in ganglionic neuronal cells via activation of RyRs (McPherson et al. 1991; Usachev et al. 1993; Usachev and Verkhratsky 1995; Walz et al. 1995; Smith and Cunnane 1996). The contribution of RyR channels to this effect was established by showing that ${Ca_{cyt}}^{2+}$ transients evoked by methylxanthines were blocked by high concentrations of ryanodine, by procaine, and dantrolene as well. As expected, the response to methylxanthines persisted in Ca^{2+}-free extracellular solutions. Similar to what was observed in muscle cells, caffeine and other methylxanthines appeared to sensitize RyR channels to ${Ca_{cyt}}^{2+}$ ions so that Ca^{2+} release could develop even at resting ${Ca_{cyt}}^{2+}$ concentrations.

Of importance, methylxanthines also caused the activation of RyRs in brain neurons, in particular hippocampal neurons, midbrain dopamine neurons, and Purkinje cells (Kano et al. 1995; Garaschuk et al. 1997; Sharma and Vijayaraghavan 2003; Patel et al. 2009). Caffeine was generally sufficient to trigger Ca^{2+} elevations at resting ${Ca_{cyt}}^{2+}$ concentrations (Sharma and Vijayaraghavan 2003; Patel et al. 2009). However, in Purkinje cells from rat cerebellar slices, application of 20 mM caffeine to the resting cells rarely resulted in Ca^{2+} release. Yet, when the Ca_{cyt}^{2+} concentration was slightly elevated by moderate depolarization (experiments were done under voltage clamp) caffeine produced robust Ca_{cyt}^{2+} concentration elevations (Kano et al. 1995). Similarly, in CA_1 hippocampal neurons, a small elevation of Ca_{cyt}^{2+} concentration prior to caffeine treatment led to a several-fold increase of the subsequent caffeine-induced Ca_{cyt}^{2+} response (Garaschuk et al. 1997).

3.2 Impact on Neurotransmitter Release

The transient increase in Ca^{2+} which triggers exocytosis after invasion of nerve terminals by the action potential is thought to arise principally through the activation of voltage-dependent Ca^{2+} channels located close to the sites of neurotransmitter release. However, the demonstration that methylxanthines can cause elevations of Ca_{cyt}^{2+} concentrations in neuronal cells indicated that RyR channels might also possibly intervene in neurotransmitter release (Galante and Marty 2003). Consistent with this view, the application of caffeine onto mossy fiber nerve terminals in hippocampal slices led to a burst of high-frequency and large-amplitude glutamatergic miniature excitatory postsynaptic currents in CA_3 pyramidal neurons (Sharma and Vijayaraghavan 2003). A similar effect was also observed when nicotine was substituted for caffeine to activate presynaptic nicotinic receptors, indicating that glutamate release occurred via a mechanism that was independent of incoming action potentials. Demonstrating an obligatory requirement for stored Ca^{2+} release via RyR channels in this process, caffeine and nicotine responses were absent after depleting intracellular Ca^{2+} stores with thapsigargin, and the effect of nicotine was abolished by a high concentration of ryanodine (Sharma and Vijayaraghavan 2003). It is worth noting that caffeine also exerted a facilitatory effect on the release of glutamate evoked by 4-aminopyridine, a potassium channel blocker that depolarizes nerve terminals in vitro, in a manner corresponding to in vivo depolarization. Yet, this effect required presynaptic adenosine A_1 receptor blockade by caffeine and was independent of RyR-gated Ca^{2+} stores (Wang 2007).

Caffeine was also reported to stimulate somatodendritic release of dopamine when applied to dopamine cell bodies of the substantia nigra pars compacta in midbrain slices (Patel et al. 2009). This effect was prevented either by the RyR blocker dantrolene or by the inhibitor of the ER Ca^{2+}-ATPase cyclopiazonic acid that causes depletion of Ca^{2+} stores, suggesting that caffeine operated via activation of RyR channels (Patel et al. 2009). As expected, the effect of caffeine was accompanied by a concomitant rise in Ca_{cyt}^{2+} concentration in dopamine neurons. This finding is also in agreement with reports showing that somatodendritic release of dopamine in the substantia nigra pars compacta shows limited dependence on extracellular Ca^{2+} concentration, at variance with what is observed for axonal release of dopamine in the striatum (Chen et al. 2006). Likewise, caffeine was also reported to stimulate somatodendritic release of serotonin in leech Retzius neurons (Trueta et al. 2004).

3.3 Impact on Neuronal Survival

The mechanisms by which methylxanthines cause RyR channel activation and calcium release have been essentially studied with respect to skeletal and cardiac muscle contraction and neurotransmitter release. However, intracellular Ca^{2+} signaling also intervenes crucially in the control of neuronal survival during development and also in the adult brain (Michel et al. 2007). Therefore, one may assume that RyR channels might play a role in neurodegenerative conditions of aging and that methylxanthines may also have an impact in these disorders. This may be particularly true in the case of Parkinson's disease: (1) the CD157 gene which encodes an ectoenzyme that catalyses the production of the RyR channel endogenous agonist cyclic ADP-ribose, is a susceptibility gene for this disorder (Satake et al. 2009); (2) results of case-control and prospective studies have indicated that consumption of caffeine in coffee, tea, and caffeinated beverages may significantly reduce the risk of developing the disease after accounting for smoking and other potentially confounding factors such as estrogen replacement therapy (Ascherio et al. 2001; Elbaz and Moisan 2008; Sääksjärvi et al. 2008).

Caffeine and its primary metabolite paraxanthine produced through liver enzymatic biotransformation were shown to exert protective effects for dopamine neurons in mouse models of Parkinson's disease, but these effects were attributed to the antagonistic action of these compounds for A_{2A} adenosine receptors (Xu et al. 2010; Xu et al. 2006; Jacobson and Gao 2006). Using a model system of spontaneous dopaminergic cell death, we have shown, however, that paraxanthine was protective by a mechanism that was unrelated to adenosine receptor blockade (Guerreiro et al. 2008). We also excluded a possible mechanism involving the action of glial-cell-line-derived neurotrophic factor (Guerreiro et al. 2008), a trophic peptide which exerts neurotrophic effects for dopamine neurons in the developing and aging brain (Love et al. 2005; Kramer et al. 2007). In fact, a number of arguments suggested that survival promotion by paraxanthine was mediated by RyR channel activation (Fig. 2): (1) ryanodine mimicked the rescuing effects of paraxanthine; (2) survival-promoting effects of paraxanthine or ryanodine were abolished by blockade of RyRs with dantrolene; (3) the rescuing effects of both paraxanthine and ryanodine caused a moderate rise in ${Ca_{cyt}}^{2+}$ concentration which was also prevented by dantrolene; (4) the Ca^{2+} rise persisted in the absence of extracellular Ca^{2+}. Unlike paraxanthine, caffeine provided only limited neuroprotection to dopamine neurons in this preparation. Of interest, the activation of RyR channels by paraxanthine also provided protection in an unrelated paradigm of dopamine cell death in which neuronal demise was triggered by the mitochondrial complex I inhibitor 1-methyl-4-phenylpyridinium (Guerreiro et al. 2008), the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a toxin which can cause irreversible parkinsonian symptoms in man when self-administrated accidentally (Langston et al. 1999). Interestingly, ryanodine was also neuroprotective in the same paradigm (Guerreiro et al. 2008). Altogether, these data suggest that some of

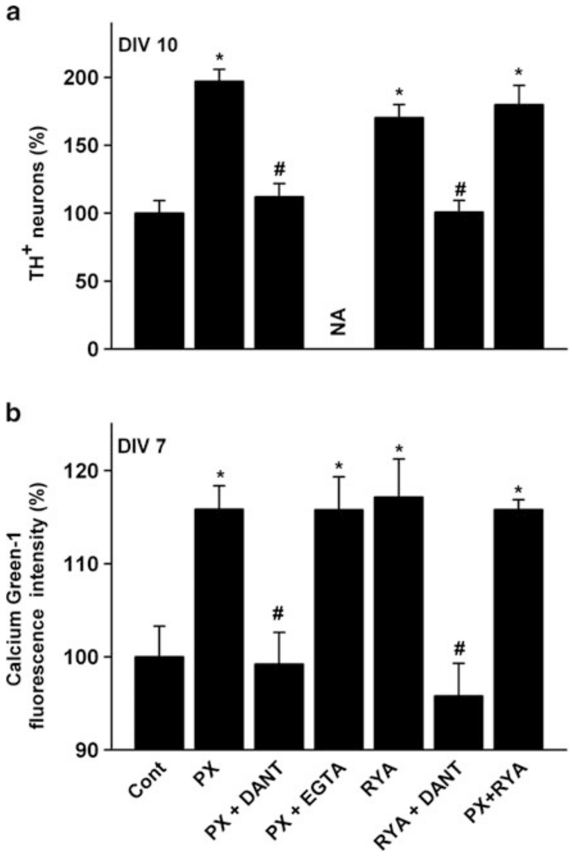

Fig. 2 Role of RyR calcium release channels in the neuroprotective effect of paraxanthine on dopamine neurons in culture. (**a**) Number of dopamine (tyrosine hydroxylase immunopositive) neurons at 10 days in vitro in midbrain cultures exposed chronically to optimal concentrations of paraxanthine (PX) or ryanodine (RYA) in the presence or absence of dantrolene (DANT), an antagonist of RyR channels. (**b**) Calcium Green-1 fluorescence levels in 7 day in vitro cultures exposed for a short time to the same treatments as in **a**. Some measurements were performed in the presence of 3 mM ethylene glycol bis(2-aminoethyl ether)tetraacetic acid to eliminate extracellular calcium from the culture medium. Note that the effects of paraxanthine and ryanodine were not additive. Paraxanthine was used at 800 μM, ryanodine at 10 μM, and dantrolene at 30 μM. $^{*}P < 0.05$, compared with corresponding control (cont) cultures; $^{\#}P < 0.05$, significant inhibition of the effect of the test compound (One-way ANOVA followed by Student Newman Keuls post-hoc test). *NA* not applicable, since widespread degeneration occurs when low-calcium conditions are maintained for prolonged periods of time in our culture model. (Data reproduced from Guerreiro et al. 2008 with permission)

the protective effects of caffeine reported earlier in epidemiology studies on Parkinson's disease might be due in part to paraxanthine acting through the activation of RyR channels. This hypothesis needs, however, to be further substantiated by in vivo studies.

Additional studies established that the N7- and N1-demethylated metabolites of paraxanthine, 1-methylxanthine, and 7-methylxanthine, were either less potent than paraxanthine itself or totally inactive, respectively. Incidentally, these results and the observation that 3-methylxanthine and xanthine itself failed to protect dopamine neurons, also indicated that N-substitution at position 1 was required for survival promotion by methylxanthine derivatives and that this effect was largely modulated by the presence of other N-substituents. Of interest, the extent of neuroprotection afforded by methylxanthines, including caffeine, was strictly correlated to their efficacy to elevate Ca_{cyt}^{2+} levels via RyR activation. It is worth noting that the Ca^{2+} level elevation elicited by optimal concentrations of paraxanthine (about 15% above control levels) was relatively modest and much lower than that produced by concentrations of glutamate causing excitotoxic stress (Guerreiro et al. 2008). Overall, these results are consistent with the idea that paraxanthine helped to maintain Ca_{cyt}^{2+} within levels that are optimal for the survival of dopamine neurons (Douhou et al. 2001; Salthun-Lassalle et al. 2004). The effects of methylxanthines on RyR channels reported in this paper are summarized in Table 1.

4 Are the Effects of Methylxanthines In Vitro Physiologically Relevant In Vivo?

In summary, the effects of methylxanthines on RyR channels are fairly well documented. In particular, methylxanthines are useful probes to explore the effects of RyR channel activation in striated and cardiac muscle cells and in neuronal cells. The possibility that RyR modulation may also play a role in the physiological or therapeutical response to methylxanthines remains, however, speculative. Indeed, the concentrations of methylxanthines required to elicit RyR receptor activation in vitro, which are generally in the range 0.3–20 mM, are several-fold higher than corresponding plasma and brain concentrations (Hawke et al. 2000; Guerreiro et al. 2008). Nevertheless, a variety of endogenous modulators, such fatty acyl-CoA esters, cyclic ADP ribose, and ATP have been reported to sensitize RyRs to exogenous agonists, suggesting that the effects of methylxanthines on these receptors may manifest themselves in vivo at lower concentrations than those determined from in vitro studies (Magkos and Kavouras 2005). Consistent with this view, paraxanthine was reported to increase Ca_{cyt}^{2+} concentrations in intact skeletal muscle preparations at 10 μM, a concentration that is physiologically relevant after systemic administration of its parent compound caffeine (Ferré et al. 1990), whereas concentrations 50-fold higher were required to produce the same effect on single isolated muscle cells (Hawke et al. 2000). In addition, twitch potentiation has also been documented in isolated rat soleus muscle after exposure to 10–100 μM caffeine (Connett et al. 1983), and in frog semitendinous muscles in the presence of 50 μM of the theophylline dimer aminophylline (Block et al. 1992).

Table 1 Comparative effects of methylxanthines on ryanodine receptor (*RyR*) channels

Common names of methylxanthines	*N*-Methyl substituents	Other substituents	RyR activation	References[a]
From plants				
Caffeine	1,3,7	none	+++	Rousseau et al. (1988), Fill and Copello (2002), Magkos and Kavouras (2005), Zalk et al. (2007)
Theophylline	1,3	none	++++	Rousseau et al. (1988)
Theobromine	3,7	none	++++	Rousseau et al. (1988)
1-Methylxanthine	1	none	++	Guerreiro et al. (2008)
3-Methylxanthine	3	none	0	Guerreiro et al. (2008)
7-Methylxanthine	7	none	0	Guerreiro et al. (2008)
Produced essentially by bioconversion				
Paraxanthine	1,7	none	++++	Hawke et al. (2000), Guerreiro et al. (2008), Rousseau et al. (1988)
Produced by synthesis				
3-Isobutyl-1-methylxanthine	1	3-Isobutyl	+++	Rousseau et al. (1988), Usachev and Verkhratsky (1995)
–	1	7-Isobutyl	+++	Rousseau et al. (1988), Islam et al. (1998), Wyskovsky (1994)
–	3,9	none	+++	Rousseau et al. (1988)
	1,9	none	0	Rousseau et al. (1988)
–	1,3	7-(5 Ethylcarboxypentyl)	++++	Cavallaro et al. (1999)
–	1,3	7-(7-Hydroxyoctyl)	++++	Cavallaro et al. (1999)
–	3	7-(7-Oxooctyl)-1-propargylxanthine	++++	Cavallaro et al. (1999)

Paraxanthine, the main metabolite of caffeine in man (Magkos and Kavouras 2005), is only produced in low amounts in plants. 1-Methyxanthine is also a secondary metabolite of paraxanthine and a metabolite of theophylline (Birkett et al. 1985; Magkos and Kavouras 2005). 3-Methylxanthine is a metabolite of caffeine, theophylline, and theobromine (Birkett et al. 1985; Magkos and Kavouras 2005). 7-Methylxanthine is the main metabolite of theobromine (Birkett et al. 1985)

[a] Limited to those references cited in the present article.

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Xanthines as Adenosine Receptor Antagonists

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Abstract The natural plant alkaloids caffeine and theophylline were the first adenosine receptor (AR) antagonists described in the literature. They exhibit micromolar affinities and are non-selective. A large number of derivatives and analogues were subsequently synthesized and evaluated as AR antagonists. Very potent antagonists have thus been developed with selectivity for each of the four AR subtypes.

Keywords Adenosine receptors · A_1 receptor antagonists · A_{2A} receptor antagonists · A_{2B} receptor antagonists · A_3 receptor antagonists · Caffeine · Deazaxanthines · Molecular probes · Paraxanthine · Theobromine · Theophylline · Tricyclic xanthine derivatives · Xanthines

1 Caffeine and Theophylline: Historical Aspects and Early Structural Modification

1.1 Naturally Occurring Xanthines

The earliest adenosine receptor (AR) antagonists identified were the naturally occurring alkylxanthines, most notably among these being caffeine (1,3,7-trimethylxanthine, **1**) and theophylline (1,3-dimethylxanthine, **2**) (see Fig. 1) (Daly 1982; Fredholm et al. 1999; Stefanovich 1989). Another simple natural xanthine, theobromine (**3**), was shown to have only weak activity as an AR antagonist (Müller et al. 1993). The major caffeine metabolites in humans, paraxanthine (**4**) and 1-methylxanthine (**5**) (Krämer and Testa 2008), the latter also being the major metabolite of theophylline, are as potent as caffeine and theophylline and may contribute to their activity (Daly et al. 1986a; Müller et al. 1993). These simple alkylxanthines are of micromolar affinity, at best, at the ARs, and variation of affinity between species has been documented (see Table 1). This affinity range was later shown to apply generally to all human AR subtypes, A_1, A_{2A}, A_{2B}, and A_3, but only to three of the AR subtypes of the rat (A_1, A_{2A}, and A_{2B}). At the rat A_3 AR, the

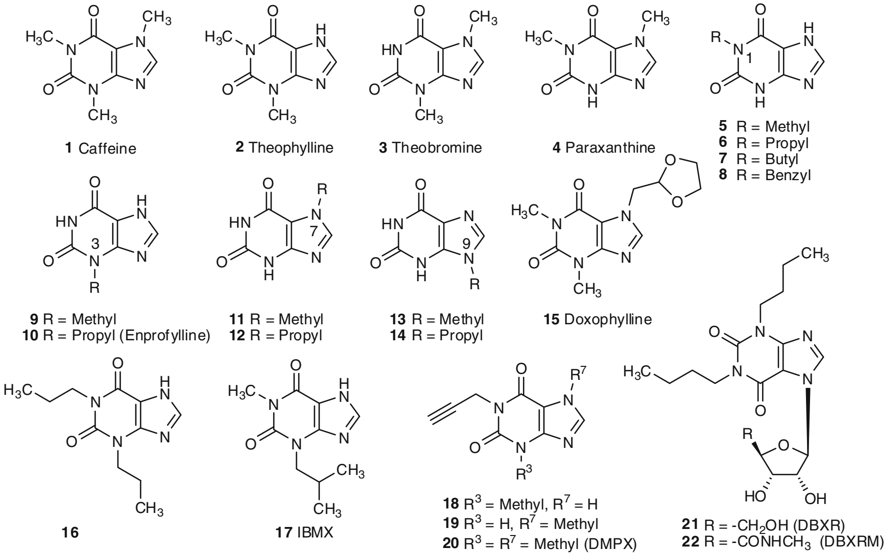


Fig. 1 8-Unsubstituted xanthine derivatives

simple alkylxanthines were shown to have much higher K_i values of approximately 10^{-4} M or higher (Fredholm et al. 1994; van Galen et al. 1994).

1.2 Early Modification of 8-Unsubstituted Xanthine Derivatives

The first xanthine analogues with enhanced affinity at the ARs were modified from caffeine and theophylline (Daly 2000, 2007; Müller and Scior 1993; Fredholm and Jacobson 2009). In the early 1980s, one particular type of modification of the xanthine structure proved to be especially useful in enhancing affinity: the elongation of the 1,3-dimethyl groups to propyl or larger alkyl groups (Bruns 1981; Ukena et al. 1986b). For example, substitution of the 1,3-dimethyl groups with 1,3-dipropyl groups in **16** increased affinity at the rat A_1 AR by approximately 20-fold (Table 1). Substitutions at the 1-, 3-, or 7-positions, particularly small hydrophobic groups, were generally much better tolerated in AR binding than substitution at the 9-position (Daly 1982; Müller and Scior 1993). Evaluation of a series of monosubstituted xanthines (**5**–**14**) showed that substitution at N1 was most important for all AR subtypes (Müller et al. 1993). While a 1-propyl residue was best for A_{2B} and A_3 ARs, a 1-benzyl residue was optimal for the A_1 and A_{2A}AR subtypes (see Table 1). 1-Propylxanthine (**6**) shows the highest affinity of the small, simple xanthine derivatives for the human A_{2B}AR (K_i 360 nM), along with some selectivity (at least sevenfold vs. the other subtypes) (Kim

Table 1 Adenosine receptor affinities of 8-unsubstituted xanthine derivatives

	Name	K_i (nM)[a]			
		A_1	A_{2A}	A_{2B}	A_3
Natural xanthine derivatives					
1	Caffeine (1,3,7-trimethylxanthine)	10,700 (h)[d] 44,900 (h)[e] 41,000 (r)[f] 44,000 (r)[g] 47,000 (gp)[h] 44,000 (c)[h]	23,400 (h)[e] 9,560 (h)[d] 45,000 (r)[g] 32,500 (r)[i] 48,000 (r)[d]	33,800 (h)[j] 10,400 (h)[k] 20,500 (h)[l] 30,000 (r)[m] 13,000 (m)[m]	13,300 (h)[d] >100,000 (r)[n]
2	Theophylline (1,3-dimethylxanthine)	6,770 (h)[o] 14,000 (r)[p] 8,740 (r)[d] 7,060 (gp)[q] 4,710 (rb)[q] 9,050 (s)[q] 6,330 (c)[q]	1,710 (h)[o] 6,700 (h)[d] 22,000 (r)[p] 25,300 (r)[d]	9,070 (h)[k] 74,000 (h)[l] 15,100 (r)[k] 5,630 (m)[r] 11,000 (gp)[s] 17,700 (rb)[r] 38,700 (d)[r]	22,300 (h)[d] 86,400 (h)[o] >100,000 (r)[n] 85,000 (r)[t] >100,000 (d)[u]
3	Theobromine (3,7-dimethylxanthine)	105,000 (r)[p] 83,400 (r)[v]	>250,000 (r)[p] 187,000 (r)[v]	130,000 (h)[v]	>100,000 (r)[n]
4	Paraxanthine (1,7-dimethylxanthine)	21,000 (r)[p]	32,000 (r)[p]	4,500 (h)[w]	>100,000 (r)[n]
Monosubstituted xanthine derivatives					
5	1-Methylxanthine	36,000 (r)[p] 11,400 (r)[v]	47,000 (r)[p] 36,200 (r)[v]	6,600 (h)[v]	>100,000 (r)[n]
6	1-Propylxanthine	13,000 (r)[p]	33,000 (r)[p]	360 (h)[k] 1,880 (r)[k]	2,370 (h)[k]
7	1-Butylxanthine	9,000 (r)[p]	61,000 (r)[p]	421 (h)[k]	4,610 (h)[k]
8	1-Benzylxanthine	2,800 (r)[p]	22,000 (r)[p]	ND	ND
9	3-Methylxanthine	>100,000 (r)[p] 35,000 (r)[x]	59,000 (r)[p]	87,000 (h)[w]	>100,000 (r)[n]
10	Enprofylline (3-propylxanthine)	156,000 (h)[y] 42,000 (h)[d] 32,000 (r)[p] 29,100 (r)[v] >100,000 (d)[u]	32,000 (h)[y] 81,300 (h)[d] 137,000 (r)[p] 103,000 (r)[v]	7,000 (h)[y] 4,730 (h)[k] 19,800 (h)[z] 26,000 (r)[aa] 5,630 (m)[r] 5,840 (rb)[r] 6,960 (d)[r]	92,600 (h)[d] 65,000 (h)[y] 93,000 (r)[d] >100,000 (d)[u]
11	7-Methylxanthine	33,000 (r)[p]	59,000 (r)[p]	97,000 (h)[w]	>100,000 (r)[n]
12	7-Propylxanthine	18,000 (r)[p]	>200,000 (r)[p]	ND	ND
13	9-Methylxanthine	>250,000 (r)[p]	>250,000 (r)[p]	>1,000,000 (h)[w]	>100,000 (r)[n]
14	9-Propylxanthine	>250,000 (r)[p]	>250,000 (r)[p]	ND	ND
Di- and trisubstituted xanthine derivatives					
15	Doxofylline	~100,000[ab]	~100,000[ab]	ND	ND
16	1,3-Dipropylxanthine	700 (r)[p] 450 (r)[v] 1,310 (gp)[h] 340 (c)[h]	6,600 (r)[p] 5,160 (r)[v]	1,110 (h)[k] 680 (h)[w]	1,940 (h)[d]
17	3-Isobutyl-1-methylxanthine	7,000 (r)[p] 2,460 (r)[v] 8,600 (gp)[h] 4,400 (c)[h]	16,000 (r)[p] 13,800 (r)[v]	3,500 (h)[v]	ND
18	3-Methyl-1-propargylxanthine	820 (r)[p] 5,830 (r)[k]	4,800 (r)[p] 33,600 (r)[k]	511 (h)[k] 2,150 (r)[k]	10,900 (h)[k]
19	7-Methyl-1-propargylxanthine	22,000 (r)[p]	16,000 (r)[p]	ND	ND
20	3,7-Dimethyl-1-propargylxanthine	45,000 (r)[g] 11,000 (r)[h]	16,000 (r)[g] 5,600 (r)[i]	4,130 (h)[k]	>10,000 (r)[ac]

(*continued*)

Table 1 (continued)

	Name	K_i (nM)[a]			
		A_1	A_{2A}	A_{2B}	A_3
		25,800 (gp)[h] 16,400 (c)[h]			
21	DBXR	4,190 (r)[n]	19,500 (r)[n]	ND	6,030 (r)[n,b]
22	DBXRM	37,300 (r)[ad]	>100,000 (r)[27]	ND	229 (r)[ad,c]

DBXRM *N*-methyl-1,3-dibutylxanthine-7-β-D-ribofuronamide

[a] *h* human, *c* cow, *d* dog, *gp* guinea pig, *m* mouse, *r* rat, *rb* rabbit, a few A_{2B} data are from functional (cyclic AMP) studies; *ND* no data available

[b] Partial agonist

[c] Full agonist

[d] Jacobson et al. (1999)

[e] Abo-Salem et al. (2004)

[f] Grahner et al. (1994)

[g] Daly et al. (1991)

[h] Ukena et al. (1986b)

[i] Müller et al. (2000)

[j] Borrmann et al. (2009)

[k] Kim et al. (2002)

[l] Bertarelli et al. (2006)

[m] Brackett and Daly (1994)

[n] van Galen et al. (1994)

[o] Klotz et al. (1998)

[p] Müller et al. (1993b)

[q] Klotz et al. (1991)

[r] Auchampach et al. (2009)

[s] Fozard et al. (2003)

[t] Jacobson et al. (1995)

[u] Auchampach et al. (1997)

[v] Bruns et al. (1986)

[w] Bruns (1981)

[x] Shamim et al. (1989)

[y] Robeva et al. (1996)

[z] Ji et al. (2001)

[aa] Alexander et al. (1996)

[ab] Cirillo et al. (1988)

[ac] Müller and Ferré (2007)

[ad] Kim et al. (1994b)

et al. 2002), while 3-propylxanthine (enprofylline, **10**) is less potent (K_i human A_{2B} AR 4,730 nM), but even more selective (at least 14-fold).

3,7-Dimethyl-1-propargylxanthine (DMPX, **20**) was the first A_2-AR-selective antagonist described in the literature (Ukena et al. 1986b; Seale et al. 1988). It is similarly potent at A_{2A} and A_{2B} ARs, but the degree of selectivity versus A_1 ARs is low (Jacobson et al. 1992a). A comparison with the 7-unmethylated derivative 3-methyl-1-propargylxanthine (**18**) indicated that a 7-methyl group led to a large decrease in A_1 AR affinity and thus increased selectivity for A_{2A} or A_{2B} AR (Müller et al. 1993; Kim et al. 2002). The theophylline derivative doxofylline (**15**), bearing a 1,3-dioxolan-2-ylmethyl residue in the 7-position, is virtually inactive at A_1 and

A_{2A}ARs and is believed to exert its antiasthmatic activity via inhibition of phosphodiesterases (Cirillo et al. 1988; Shukla et al. 2009).

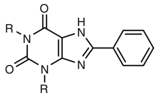
The branched analogue 3-isobutyl-1-methylxanthine (IBMX, **17**) shows potency as a phosphodiesterase inhibitor in the same concentration range as is required to block ARs (Ukena et al. 1993).

1,3-Dibutylxanthine-7-ribosides (**21**, **22**) were found to bind effectively at A_3 ARs, indicating that the ribose group – as in adenosine – can act as a secondary anchor or recognition moiety in the receptor binding site (van Galen et al. 1994; Kim et al. 1994b; Park et al. 1998). This series of xanthine-7-ribosides also provided an early indication of modes of ligand binding at ARs, i.e. the overlay of receptor-bound positions of xanthine and adenine moieties. The 5′-uronamide modification of the CH_2OH group of the ribose moiety greatly enhanced A_3AR affinity as was shown for adenosine agonists, such that *N*-methyl-1,3-dibutylxanthine-7-β-D-ribofuronamide (DBXRM, **22**) was 143-fold selective (Kim et al. 1994b). DBXRM was found to be a full agonist at the A_3 AR, unlike other xanthine derivatives. It is proposed that the ribose moiety contains the essential structure and required flexibility to effect the conformational change of the receptor needed for activation (Gao et al. 2002).

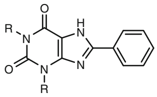
1.3 Progression to Xanthines with Subtype Selectivity

In addition to substitution of the 1,3-dimethyl groups with larger 1,3-dialkyl groups, a means of increasing affinity at the rat A_1 AR was found to be the introduction of 8-aryl substituents (Fig. 2, Table 2) (Bruns 1981; Daly et al. 1986b; Jacobson et al. 1988, 1992a; Müller and Stein 1996). For example, placement of a phenyl group at the 8-position, generally increased A_1 AR affinity by at least an order of magnitude (Table 2). The first analogue having both 1,3-dialkyl and 8-phenyl modifications to be studied in detail was 1,3-diethyl-8-phenylxanthine (DPX, **25**), which displayed a K_i value of 44 nM at the rat A_1 AR and was beginning to show selectivity for that subtype (Bruns et al. 1987a). [^{3}H]DPX was demonstrated as the first AR antagonist radioligand, but its hydrophobicity limited its use (Bruns et al. 1980). Homologation to the 1,3-propyl groups in the 8-aryl analogue (**26**) provided a desired boost in affinity; however, the unintended consequence of rapidly diminishing aqueous solubility made this series unable to be used in typical pharmacology studies (Ukena et al. 1986b; Bruns and Fergus 1989). The 8-*p*-hydroxyphenyl-substituted derivative (**33**) (NPC-205) was slightly more potent than **26** (Shamim et al. 1988). The low aqueous solubility, which is both a function of the lipophilic groups present on the xanthine and the tendency of 8-arylxanthine derivatives to form highly stable crystal lattices, resulted in low bioavailability of **26** and similar compounds (Müller and Stein 1996; Frédérick et al. 2005).

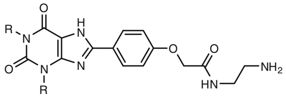
Several approaches were taken to increase the water solubility. A sulfonate group was introduced at the *para* position of the 8-phenyl ring, which greatly increased the solubility (Daly et al. 1985; Shamim et al. 1989). However, this



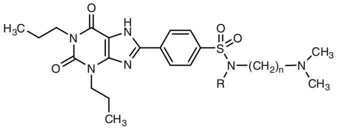
23 R = H
24 R = Methyl (8-PT)
25 R = Ethyl (DPX)
26 R = Propyl



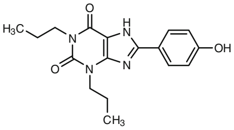
27 R = Methyl (8-SPT)
28 R = Propyl (DPSPX)



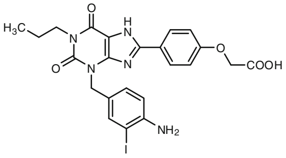
29 R = Ethyl
30 R = Propyl (XAC)



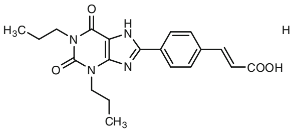
31 R = H, n = 3 (PD113,297)
32 R = Methyl, n = 2 (PD115,199)



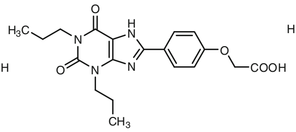
33 NPC-205



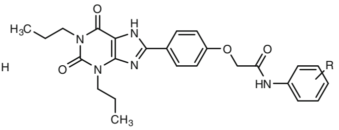
34 I-ABOPX



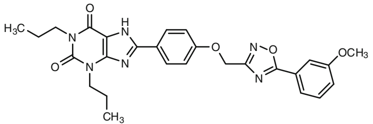
35 BWA1433



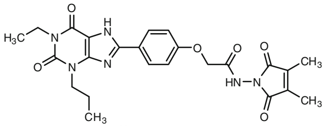
36 XCC



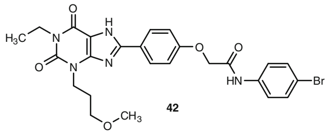
37 R = *p*-CN (MRS1754)
38 R = *p*-COCH3 (MRS1706)
39 R = *p*-I (MRS1765)



40 CVT-5440



41 MRS1595



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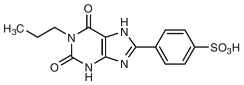
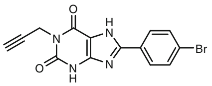
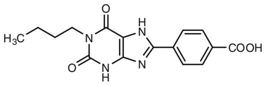


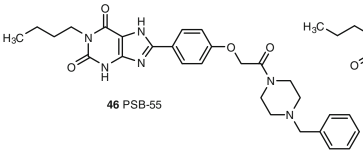
43 PSB-1115



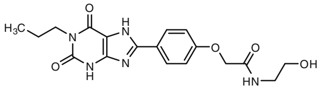
44 PSB-50



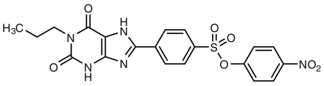
45 PSB-53



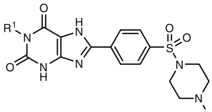
46 PSB-55



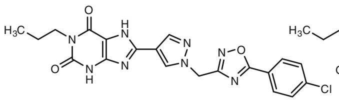
47 PSB-298



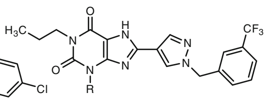
48



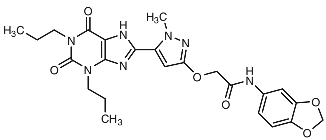
49 R^1 = Propyl, R^2 = Benzyl (PSB-601)
50 R^1 = Ethyl, R^2 = *p*-CF_3-Benzyl (PSB-09120)
51 R^1 = Propyl, R^2 = *p*-Cl-Benzyl (PSB-0788)
52 R^1 = Propyl, R^2 = *p*-Cl-Phenyl (PSB-603)



53 CVT-6694



54 R = H (CVT-7124)
55 R = Ethyl (CVT-6883)



56 MRE-2029-F20

Fig. 2 (Continued)

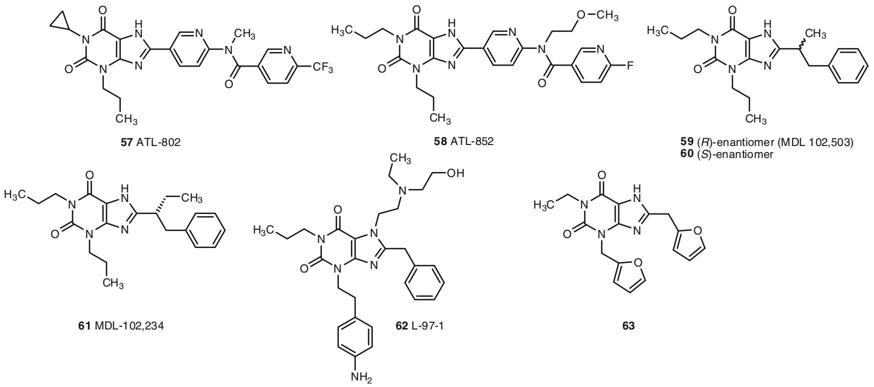


Fig. 2 8-Phenyl- and 8-phenylalkyl-substituted xanthines and heteroaromatically substituted derivatives

modification tended to decrease both the affinity and the selectivity in comparison with the uncharged 8-phenyl analogues. Thus, SPT (**27**) and DPSPX (**28**), the latter of which is somewhat more potent, were both useful in pharmacological experiments where a blockade of all AR subtypes is required. It has to be kept in mind that these compounds do not block rat A_3 ARs but are active in other species, such as human and sheep (Table 2). SPT (**27**) was shown not to penetrate into the brain owing to its high polarity (Baumgold et al. 1992).

2 A_1 Adenosine Receptor Antagonists

2.1 8-Aryl- and 8-Arylalkyl-Substituted Xanthines

An alternative approach to the introduction of charged groups for increasing water solubility resulted in the synthesis of the 8-aryl derivatives in which the charged group was separated from the phenyl ring by a spacer group. Various substitutions of an 8-phenyl ring indicated that an electron-donating group provided a favourable AR affinity (Jacobson et al. 1985b; Shamim et al. 1988). Thus, a methoxy substituent was replaced by a carboxymethyloxy group, resulting in the carboxylic congener XCC (**36**) and the amine congener 8-[4-[[[[(2-aminoethyl)amino]carbonyl] methyl]oxy]phenyl]-1,3-dipropylxanthine (XAC, **30**) (Fig. 2, Table 2) (Jacobson et al. 1985b, 1999). By placement of the charged group at a distance from the 8-aryl ring, it was possible to maintain and even enhance the high affinity seen with neutral, but poorly soluble analogues. Thus, XAC was found to have a K_i value of 1.2 nM at the rat A_1 AR and approximately 50-fold selectivity in comparison with the rat A_2 AR. The initial measure of A_2 AR affinity used in Jacobson et al. (1985b)

Table 2 Adenosine receptor affinities of 8-phenyl- and 8-phenylalkyl-substituted xanthines and heteroaromatically substituted derivatives

	Name	K_i (nM)[a]			
		A_1	A_{2A}	A_{2B}	A_3
First-generation 8-phenylxanthine derivatives					
23	8-Phenylxanthine	2,500 (r)[c]	21,000 (r)[c]	810 (h)[d]	ND
24	8-Phenyltheophylline	1,340 (h)[e] 115 (h)[f] 86 (r)[g] 76 (r)[h] 1,540 (gp)[h] 7.6 (c)[h]	454 (h)[e] 850 (r)[g]	415 (h)[i] 436 (m)[j] 249 (rb)[j] 371 (d)[j]	1,250 (h)[e] >100,000 (r)[k]
25	1,3-Diethyl-8-phenylxanthine	44 (r)[l]	860 (r)[l] 190 (h)[m]	62.0 (h)[i]	ND
26	1,3-Dipropyl-8-phenylxanthine	10 (r)[h] 0.22 (c)[h] 20.9 (gp)[h]	180 (r)[n] 2100 (h)[o]	18.9 (h)[i]	ND
27	SPT	1,000 (h)[e] 4,500 (r)[g] 1,000 (r)[h] 10,100 (gp)[h] 6,460 (d)[p] 300 (c)[h]	7,050 (h)[e] 14,000 (r)[g]	1,330 (h)[i] 1,590 (r)[q] 4,990 (m)[j] 2,190 (gp)[q] 2,370 (rb)[j] 7,240 (d)[j] 224 (d)[q]	5,890 (h)[r] 11,000 (h)[s] >>10,000 (r)[r] 25,300 (d)[p]
28	DPSPX	210 (r)[g] 140 (r)[k]	1,400 (r)[g] 790 (r)[k]	568 (m)[j] 200 (rb)[j] 721 (d)[j]	183 (s)[t] >100,000 (r)[k] 22,500 (rb)[u]
29		12 (r)[v]	83 (r)[v]	ND	ND
30	XAC	6.8 (h)[w] 29.1 (h)[x] 1.2 (r)[y] 0.49 (r)[u] 5.49 (gp)[u] 0.45 (rb)[u] 0.09 (s)[u] 0.03 (c)[u] 159 (d)[u]	18 (h)[w] 1.00 (h)[x] 63 (r)[y]	7.8 (h)[y] 16.0 (h)[i] 42.7 (r)[q] 4.51 (m)[j] 17.8 (gp)[q] 4.47 (rb)[j] 29.8 (d)[j] 3.55 (d)[q]	91.9 (h)[x] 26 (h)[w] 71 (h)[p] 29,000 (r)[p] 106 (rb)[p] 180 (s)[t,z] 138 (d)[p]
31	PD113,297	5.59 (r)[n]	70.0 (r)[n]	ND	ND
32	PD115,199	14 (r)[aa] 4.05 (r)[v]	16 (r)[aa] 3.86 (rb)[ab]	160 (m)[ac]	ND
33	NPC-205	3.5 (r)[ad]	48 (h)[ad]	50 (gp)[ae]	ND
34	I-ABOPX (BW-A522)	70 (h)[au] 37 (r)[af] 601 (d)[p]	95 (h)[au] 700 (r)[af]	30 (h)[au]	18 (h)[z] 1,170 (r)[ag] 1,500 (r)[p] 179 (rb)[p] 37.5 (d)[p]
35	BWA1433	20 (r)[af] 132 (d)[p]	nd	15.6 (h)[au]	54 (h)[z] 15,000 (r)[p] 384 (rb)[p] 1,880 (d)[p]

(*continued*)

Table 2 (continued)

	Name	K_i (nM)[a]			
		A$_1$	A$_{2A}$	A$_{2B}$	A$_3$
36	XCC	175 (h)[w] 58 (r)[y]	2200 (h)[w] 595 (h)[w]	13.6 (h)[y] 40 (h)[i] 2,200 (r)[y]	3,910 (h)[w] 75,700 (r)[w]
A$_{2B}$-selective 8-phenylxanthine derivatives and heteroaromatically substituted derivatives					
37	MRS1754	403 (h)[w] 16.8 (r)[w]	503 (h)[w] 612 (r)[w]	1.97 (h)[w] 12.8 (r)[w] 16.6 (r)[q] 3.39 (m)[j] 9.12 (gp)[q] 1.79 (rb)[j] 12.8 (d)[j] 12.3 (d)[q]	570 (h)[w]
38	MRS1706	157 (h)[w] 38 (r)[w]	112 (h)[w] 548 (r)[w]	1.4 (h)[w]	230 (h)[w]
39	MRS1765	152 (h)[w] 15.7 (r)[w]	293 (h)[w] 1640 (r)[w]	2.13 (h)[w]	1270 (h)[w]
40	CVT-5440	>10,000 (h)[ah]	>10,000 (h)[ah]	50 (h)[ah]	>10,000 (h)[ah]
41	MRS1595	3,030 (h)[w] 11.1 (r)[w]	1,970 (h)[w] 126 (r)[w]	26.6 (h)[w]	670 (h)[w]
42		100 (r)[ai]	97.7 (h)[ai]	2.88 (h)[ai]	1,290 (h)[ai]
43	PSB-1115	>10,000 (h)[d] 2,200 (r)[c]	24,000 (r)[c]	53.4 (h)[d]	>10,000 (h)[d]
44	PSB-50	60 (r)[d]	199 (r)[d]	6.8 (h)[d]	477 (h)[d]
45	PSB-53	1,181 (h)[d] 481 (r)[d]	~10,000 (h)[d] 3,800 (r)[d]	24 (h)[d]	4,622 (h)[d]
46	PSB-55	122 (h)[d] 37 (r)[d]	~10,000 (r)[d] 550 (r)[d]	1.3 (h)[d]	475 (h)[d]
47	PSB-298	68 (h)[d] 35 (r)[d]	2,139 (r)[d]	1.2 (h)[d] 60 (h)[aj]	422 (h)[d]
48		3.6 (r)[ak]	74 (r)[ak]	5.4 (h)[ak]	≥10,000 (h)[ak]
49	PSB-601	2,067 (h)[al] 260 (r)[al]	484 (h)[al] 93.7 (r)[al]	3.6 (h)[al]	>1,000 (h)[al]
50	PSB-09120	>10,000 (h)[am] >1,000 (r)[am]	22.7 (h)[am] 122 (r)[am]	0.157 (h)[am]	>10,000 (h)[am]
51	PSB-0788	2,240 (h)[am] 386 (r)[am]	333 (h)[am] 1,730 (r)[am]	0.393 (h)[am]	>1,000 (h)[am]
52	PSB-603	>10,000 (h)[am] >10,000 (r)[am]	>10,000 (h)[am] >10,000 (r)[am]	0.553 (h)[am] K_D 0.403 (h)[am] K_D 0.351 (m)[am]	>10,000 (h)[am]
53	CVT-6694	>6,000 (h)[an]	>5,000 (h)[an]	7 (h)[an]	>9,000 (h)[an]
54	CVT-7124	>6,000 (h)[an]	>5,000 (h)[an]	6 (h)[an]	>9,000 (h)[an]
55	CVT-6883	1,940 (h)[ao]	3,280 (h)[ao]	22 (h)[ao]	1,070 (h)[ao]
56	MRE-2029-F20	200 (h)[ap]	>1,000 (h)[ap]	5.5 (h)[ap]	>1,000 (h)[ap]
57	ATL 802	369 (h)[aq] 9,583 (m)[aq]	654 (h)[aq] 8,393 (m)[aq]	2.36 (h)[aq] 8.58 (m)[aq]	>1,000 (h)[aq] >10,000 (m)[aq]
58	ATL 852	ND	ND	28.5 (h)[b]	ND
8-Phenylalkyl-substituted xanthines					
59	MDL 102,503	6.9 (r)[ar]	157 (r)[ar]	ND	ND
60		60.7 (r)[ar]	848 (r)[ar]	ND	ND

(*continued*)

Table 2 (continued)

	Name	K_i (nM)[a]			
		A_1	A_{2A}	A_{2B}	A_3
61	MDL 102,234	23.2 (r)[ar]	3,510 (r)[ar]	ND	ND
62	L-97-1	580 (h)[as]	>100,000 (h)[as]	>100,000 (h)[as]	ND
63		102 (r)[at]	83.2 (h)[at]	7.41 (h)[at]	10,000 (h)[at]

XAC 8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-l,3-dipropylxanthine

[a] *h* human, *c* cow, *d* dog, *gp* guinea pig, *m* mouse, *r* rat, *rb* rabbit, a few A_{2B} data are from functional (cyclic AMP) studies, *ND* no data available

[b] Personal communication (J. Linden), see also Cagnina et al. (2009)

[c] Müller et al. (1993b)

[d] Hayallah et al. (2002)

[e] Kim et al. (1999)

[f] Ferkany et al. (1986)

[g] Daly (1991)

[h] Ukena et al. (1986b)

[i] Kim et al. (2002)

[j] Auchampach et al. (2009)

[k] van Galen et al. (1994)

[l] Bruns et al. (1987a)

[m] Ukena et al. (1986a)

[n] Bruns et al. (1986)

[o] Shamim et al. (1989)

[p] Auchampach et al. (1997)

[q] Fozard et al. (2003)

[r] Abo-Salem et al. (2004)

[s] Martin et al. (1996)

[t] Linden et al. (1993)

[u] Klotz et al. (1991)

[v] Jacobson et al. (1988)

[w] Kim et al. (2000)

[x] Klotz et al. (1998)

[y] Jacobson et al. (1999)

[z] Salvatore et al. (1993)

[aa] Bruns et al. (1987b)

[ab] Ji et al. (1991)

[ac] Brackett and Daly (1994)

[ad] Shamim et al. (1988)

[ae] Daly et al. (1986b)

[af] Linden (1994)

[ag] Kim et al. (1994a)

[ah] Zablocki et al. (2005)

[ai] Nieto et al. (2009)

[aj] Bertarelli et al. (2006)

[ak] Yan and Müller (2004)

[al] Yan et al. (2006)

[am] Borrmann et al. (2009)

[an] Kalla et al. (2008)

[ao] Elzein et al. (2008)

[ap] Baraldi et al. (2004)

[aq] Cagnina et al. (2009)

[ar] Peet et al. (1993)

[as] Obiefuna et al. (2005)

[at] Balo et al. (2009)

[au] Linden et al. (1999)

was the inhibition of cyclic AMP accumulation in rat brain slices, which corresponds more closely to the A_{2B} AR, rather than the A_{2A} AR. However, subsequent tests at the rat A_{2A} AR confirmed that there was still a margin of selectivity of XAC in binding to the A_1 AR in rat (Ukena et al, 1986c). The substitution of the 1,3-dipropyl groups of XAC with 1,3-diethyl increased the affinity at the A_{2A} AR while decreasing it at the A_1 AR (Jacobson et al. 1987a). The aqueous solubility of XAC was found to be 25 μM, which was an improvement over the uncharged 8-aryl derivatives. Therefore, XAC was suitable for use in pharmacological experiments as a general AR antagonist and was the first such antagonist to display moderate A_1 AR selectivity, at least in rat.

Given the promise of a relatively water soluble and somewhat selective xanthine derivative, this amine-functionalized derivative of 1,3-dipropyl-8-phenylxanthine was specifically radiolabelled on the 1,3-dipropyl groups by catalytic reduction of a 1,3-diallyl precursor. The resulting [^{3}H]XAC was useful as a radiotracer in binding experiments at rat cerebral cortical A_1 ARs, with a K_D value of approximately 1 nM, and was thus the first generally useful antagonist radioligand for study of this receptor (Jacobson et al. 1986a). [^{3}H]XCC (**36**) was also introduced as a high-affinity radioligand for the A_1 AR (Jarvis et al. 1987).

Another rationale for the design of XAC with a primary amino group was the "functionalized congener approach" to drug design (Jacobson et al. 1986b; Jacobson 2009). By this approach, a chemically functionalized chain is incorporated at an insensitive site on the xanthine pharmacophore and can be extended to enable a conjugation strategy. Such high-affinity conjugates are useful for AR characterization and can be coupled to radioactive or spectroscopic reporter groups without them losing their ability to bind to the receptor (Jacobson et al. 1987b). XAC was also used as an immobilized high-affinity ligand for the purpose of affinity chromatography leading to the isolation of both A_1 and A_{2A} ARs and their purification to homogeneity (Olah et al. 1989; Weiss and Grisshammer 2002). While in XAC the polar, basic residue was connected to the 8-phenyl ring via ether and amide linkages, in another series, sulfonamide-linked derivatives were investigated (**31**, **32**, Fig. 2). Compound **31** and its congeners were potent A_1 AR antagonists, but showed only a moderate degree of selectivity (Table 2) (Bruns et al. 1986, 1987a).

Besides 8-phenylxanthine derivatives, 8-benzyl-substituted xanthines (**62**) and 8-phenylethyl-substituted xanthines (**59**–**61**) have also been investigated and optimized with respect to A_1 AR affinity (Peet et al. 1993). Among 8-(arylalkyl)-xanthine derivatives, 3-[2-(4-aminophenyl)ethyl]-8-benzyl-7-{2-ethyl(2-hydroxyethyl)amino]ethyl}-1-propyl-3,7-dihydropurine-2,6-dione (L-97-1, **62**) is a weaker binder than typical 8-arylxanthine probes, but is a water-soluble A_1 AR antagonist bearing a basic substituent at N7 that has been proposed for the treatment of asthma (Obiefuna et al. 2005). 1,3-Dipropyl-8-phenylethylxanthine derivatives with a methyl (**59**, **60**) or ethyl (**61**) substituent at the α-carbon atom adjacent to the xanthine C8 position showed particularly high affinity and selectivity for the A_1 AR, with a configurational preference for the *R* enantiomer over the *S* enantiomer (Peet et al. 1993).

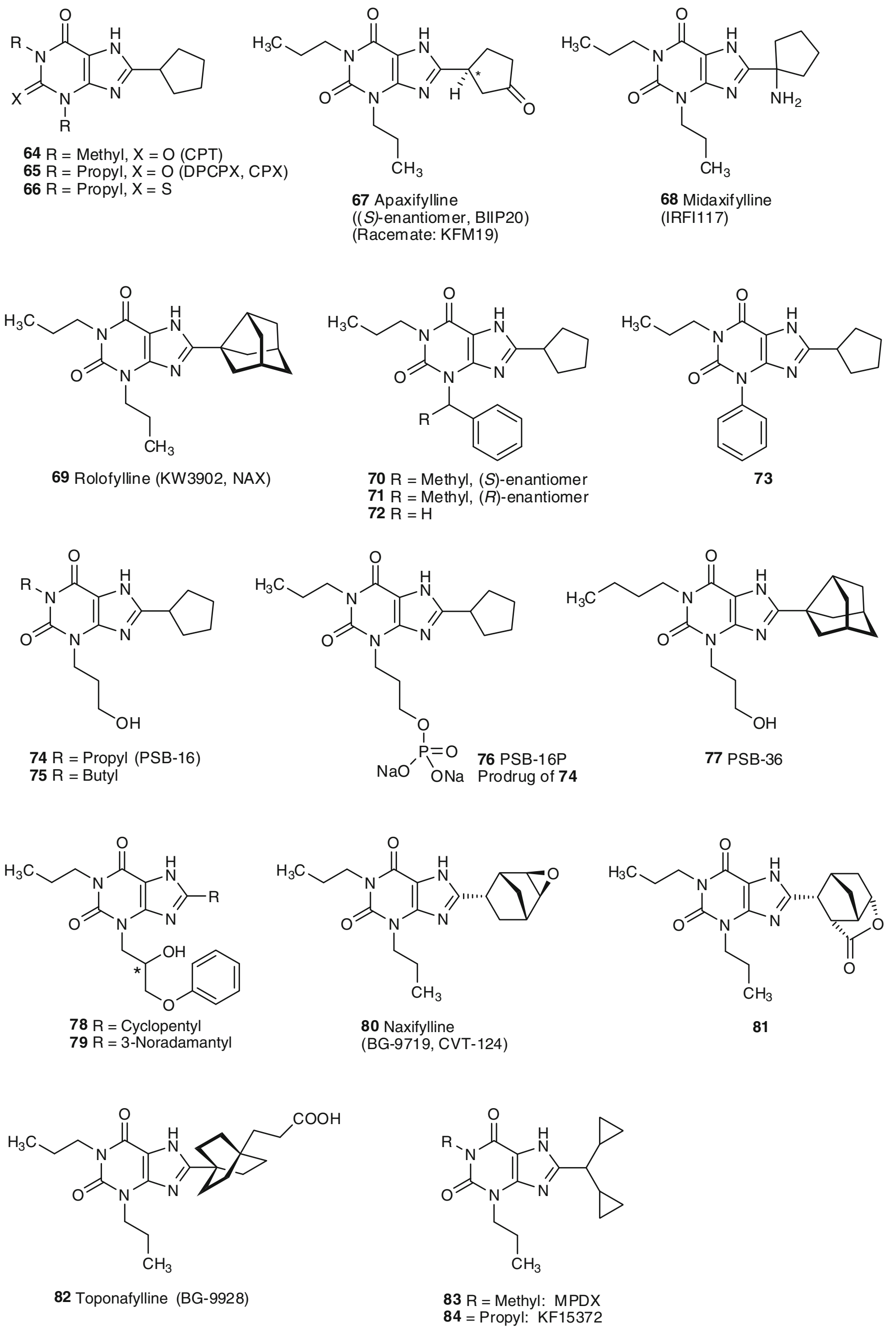
2.2 8-Cycloalkylxanthines

The introduction of 8-cycloalkyl groups instead of 8-aryl groups proved to be beneficial for affinity at the A_1 AR, and also allowed sufficient aqueous solubility for broad pharmacological application (Fig. 3, Table 3). The 8-cycloalkylxanthine derivative that is most widely used as a pharmacological tool is 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (**65**, also known as CPX), which is approximately 500-fold selective for the rat A_1 AR in comparison with the A_{2A} AR (Bruns et al. 1987a). Among the human ARs, the A_1 AR selectivity is less than in the rat (Table 3). The corresponding cyclohexyl analogue has a similar pharmacological profile (Shamim et al. 1989). Curiously, DPCPX was in clinical trials for the treatment of cystic fibrosis through a non-AR related mechanism. It was found to act on the cystic-fibrosis-related chloride transporter to enhance the level of chloride in cell systems, an action that is unrelated to its AR antagonism (Cohen et al. 1997; Sorbera et al. 2000).

Bulkier cycloalkyl substituents in the xanthine 8-position, such as 3-noradamantyl [e.g. rolofylline (KW3902, **69**) and **77**], (substituted) norbornyl [naxifylline (BG-9719, CVT124, **80**), and the lactone **81**], dicyclopropylmethyl (MPDX, **83**, and KF15372, **84**), and bicyclo[2.2.2]octyl [toponafylline (BG-9928, **82**)] yielded very potent and selective A_1 AR antagonists (Fig. 3, Table 3).

The 2-thio analogue of DPCPX (**65**), 2-thio-DPCPX (**66**), was as potent and selective as **65**, showing that in the 2-position a hydrogen-bond acceptor (such as a keto group) was not required (Jacobson et al. 1989b). Replacement of the 3-propyl residue in DPCPX by a phenyl (**73**), a benzyl (**72**), or a chiral methylbenzyl residue (**70**, **71**) was well tolerated (Weyler et al. 2006). However, the affinity for the human A_3 AR was increased by the lipophilic, aromatic residues, and the compounds lost A_1 AR selectivity in comparison with the A_3 AR affinity. The introduction of polar hydroxy groups at the 3-position was well tolerated by the A_1 AR, but not by the A_3 AR, leading to very potent and highly selective A_1 AR antagonists (**74**, **75**, **77**–**79**) (Weyler et al. 2006; Massip et al. 2006). In fact, 1-butyl-3-(3-hydroxypropyl)8-(3-noradamantyl)xanthine (PSB-36, **77**) is one of the most potent A_1 AR antagonists described to date, showing K_i values of 0.124 nM (rat) and 0.7 nM (human). The hydroxylated DPCPX derivative **74** (PSB-16) was converted to its phosphoric acid ester disodium salt, yielding a highly water soluble A_1 AR antagonist prodrug suitable for parenteral application without the need for detergents and organic solvents (Weyler et al. 2006).

Several other polar derivatives and analogues of DPCPX were developed in order to further improve water solubility and bioavailability. Apaxifylline (**67**), with a keto group at C3 of the cyclopentyl ring, was clinically evaluated as a memory-enhancing drug for the treatment of dementia (Schingnitz et al. 1991). An amino-substituted DPCPX derivative, midaxifylline (**68**), has also been investigated (Ceccarelli et al. 1995). A promising second-generation compound currently undergoing clinical trials for the treatment of chronic heart failure is toponafylline (**82**), which contains a carboxylate function (Doggrell, 2005). Rolofylline (**69**), an A_1 AR antagonist of the first generation, had shown promising results in a pilot phase III study in patients with acute heart failure, but in a recently published larger



64 R = Methyl, X = O (CPT)
65 R = Propyl, X = O (DPCPX, CPX)
66 R = Propyl, X = S

67 Apaxifylline
((*S*)-enantiomer, BIIP20)
(Racemate: KFM19)

68 Midaxifylline
(IRFI117)

69 Rolofylline (KW3902, NAX)

70 R = Methyl, (*S*)-enantiomer
71 R = Methyl, (*R*)-enantiomer
72 R = H

73

74 R = Propyl (PSB-16)
75 R = Butyl

76 PSB-16P
Prodrug of **74**

77 PSB-36

78 R = Cyclopentyl
79 R = 3-Noradamantyl

80 Naxifylline
(BG-9719, CVT-124)

81

82 Toponafylline (BG-9928)

83 R = Methyl: MPDX
84 = Propyl: KF15372

Fig. 3 8-Cycloalkylxanthines

Table 3 Adenosine receptor affinities of 8-cycloalkylxanthines

	Name	K_i (nM)[a]			
		A_1	A_{2A}	A_{2B}	A_3
64	8-Cyclopentyltheophylline	24 (r)[c] 6.3 (r)[e] 26.1 (gp)[e] 6.4 (rb)[e] 2.9 (s)[e] 1.4 (c)[e]	1,400 (r)[c] 3,170 (r)[ac]	710 (h)[d] 902 (h)[ac]	~100,000 (h)[c] >10,000 (r)[ac]
65	DPCPX	3.0 (h)[f] 0.50 (r)[f] 1.0 (r)[g] 0.18 (r)[e] 1.06 (gp)[e] 3.9 (gp)[h] 0.21 (rb)[e] 0.10 (s)[e] 0.05 (c)[e] 0.29 (c)[h] 11.4 (d)[i]	129 (h)[j] 60 (h)[k] 157 (r)[l] 500 (r)[g]	51 (h)[f] 63.8 (h)[g] 186 (r)[g] 200 (r)[m] 86.2 (m)[n] 145 (gp)[m] 96.0 (rb)[n] 147 (d)[n] 132 (d)[m]	795 (h)[o] 243 (h)[f] 509 (h)[i] 3,960 (h)[j] >10,000 (r)[k] 43,000 (r)[i] 708 (rb)[i] 115 (d)[i]
66	2-Thio-DPCPX	0.655 (r)[p]	314 (r)[p]	2800 (h)[q]	nd
67	Apaxifylline [*S*-(-)-configured enantiomer] [KFM19 (racemate), BIIP-20 (*S*-(-)]	10.5 (mk)[r,b] 3 (r)[s]	1,512 (mk)[r,b] 2,640 (r)[s]	nd	nd
68	Midaxifylline [8-(1-aminocyclopentyl)-1,3-dipropylxanthine, IRFI117]	26[t]	54,600[t]	nd	nd
69	Rolofylline [KW3902, NAX, 1,3-dipropyl-8-(3-noradamantyl)xanthine]	0.72 (h)[u] 8.0 (h)[v] 0.19 (r)[v] 12.6 (r)[u]	108 (h)[u] 673 (h)[v] 380 (r)[w] 510 (r)[u]	296 (h)[v]	4,390 (h)[v]
70	1-Propyl-3-(*S*)-1-methylbenzyl-8-cyclopentylxanthine	10.1 (r)[k]	3,500 (r)[k]	8,000 (h)[k]	85 (h)[k] >10,000 (r)[k]
71	1-Propyl-3-(*R*)-1-methylbenzyl-8-cyclopentylxanthine	23.8 (r)[k]	2,400 (r)[k]	2,960 (h)[k]	370 (h)[k]
72	1-Propyl-3-benzyl-8-cyclopentylxanthine	24.3 (h)[k] 8.70 (r)[k]	511 (r)[k]	8,000 (h)[k]	54.6 (h)[k]
73	1-Propyl-3-phenyl-8-cyclopentylxanthine	7.1 (h)[k] 1.01 (r)[k]	1,200 (h)[k] 492 (r)[k]	625 (h)[k]	395 (h)[k]
74	1-Propyl-3-(3-hydroxypropyl)-8-cyclopentylxanthine (PSB-16)	5.74 (h)[k] 0.57 (r)[k]	664 (r)[k]	194 (h)[k]	3,100 (h)[k]
75	1-Butyl-3-(3-hydroxypropyl)-8-cyclopentylxanthine	0.45 (r)[k]	582 (r)[k]	ND	1,190 (h)[k]
77	1-Butyl-3-(3-hydroxypropyl)-8-(3-noradamantyl) xanthine (PSB-36)	0.7 (h)[k] 0.124 (r)[k]	980 (h)[k] 552 (r)[k]	187 (h)[k]	2,300 (h)[k] 6,500 (r)[k]
78		49 (h)[x] 55 (r)[x]	>10,000 (h)[x] >10,000 (r)[x]	ND	3,550 (h)[x]

(*continued*)

Table 3 (continued)

	Name	K_i (nM)[a]			
		A_1	A_{2A}	A_{2B}	A_3
79		29 (h)[x] 21 (r)[x]	>10,000 (h)[x] >10,000 (r)[x]	ND	>10,000 (h)[x]
80	Naxifylline (BG-9719, CVT-124)	0.45 (h)[u] 12 (h)[w] 0.67 (r)[u]	1,100 (h)[u] 1,660 (h)[w] 1,250 (r)[u]	611 (h)[w] 1,010 (m)[n] 470 (rb)[n] 742 (d)[n]	4,810 (h)[w]
81		18 (h)[w] 3.0 (r)[w]	657 (h)[w] 264 (r)[w]	802 (h)[w]	>1,000 (h)[w]
82	Toponafylline (BG-9928)	7.4 (h)[v] 3.9 (mk)[y] 1.3 (r)[v] 29 (d)[y]	6,410 (h)[v] 943 (mk)[y] 2,440 (r)[v] 4307 (d)[y]	90 (h)[v]	>10,000 (h)[v]
83	MPDX (1-methyl analogue of KF 15372)	4.2 (r)[z]	>100 (r)[z]	ND	ND
84	KF 15372	0.99 (r)[aa] 3.0 (r)[ab] 3.0 (gp)[aa]	430 (r)[aa]	ND	ND

DPCPX 1,3-dipropyl-8-cyclopentylxanthine

[a] *h* human, *c* cow, *d* dog, *gp* guinea pig, *m* mouse, *mk* monkey, *r* rat, *rb* rabbit, a few A_{2B} data are from functional (cyclic AMP) studies, *ND* no data available

[b] Data for the racemate (KFM-19)

[c] van Galen et al. (1994)

[d] Bruns et al. (1986)

[e] Klotz et al. (1991)

[f] Bulicz et al. (2006)

[g] Kim et al. (2002)

[h] Ukena et al. (1986b)

[i] Auchampach et al. (1997)

[j] Klotz et al. (1998)

[k] Weyler et al. (2006)

[l] Müller et al. (2000)

[m] Fozard et al. (2003)

[n] Auchampach et al. (2009)

[o] Hayallah et al. (2002)

[p] Jacobson et al. (1989b)

[q] Jacobson et al. (1999)

[r] Schingnitz et al. (1991)

[s] Müller (1997)

[t] Ceccarelli et al. (1995)

[u] Pfister et al. (1997)

[v] Kiesman et al. (2006b)

[w] Kiesman et al. (2006a)

[x] Massip et al. (2006)

[y] Doggrell (2005)

[z] Noguchi et al. (1997)

[aa] Suzuki et al. (1992a)

[ab] Shimada et al. (1992)

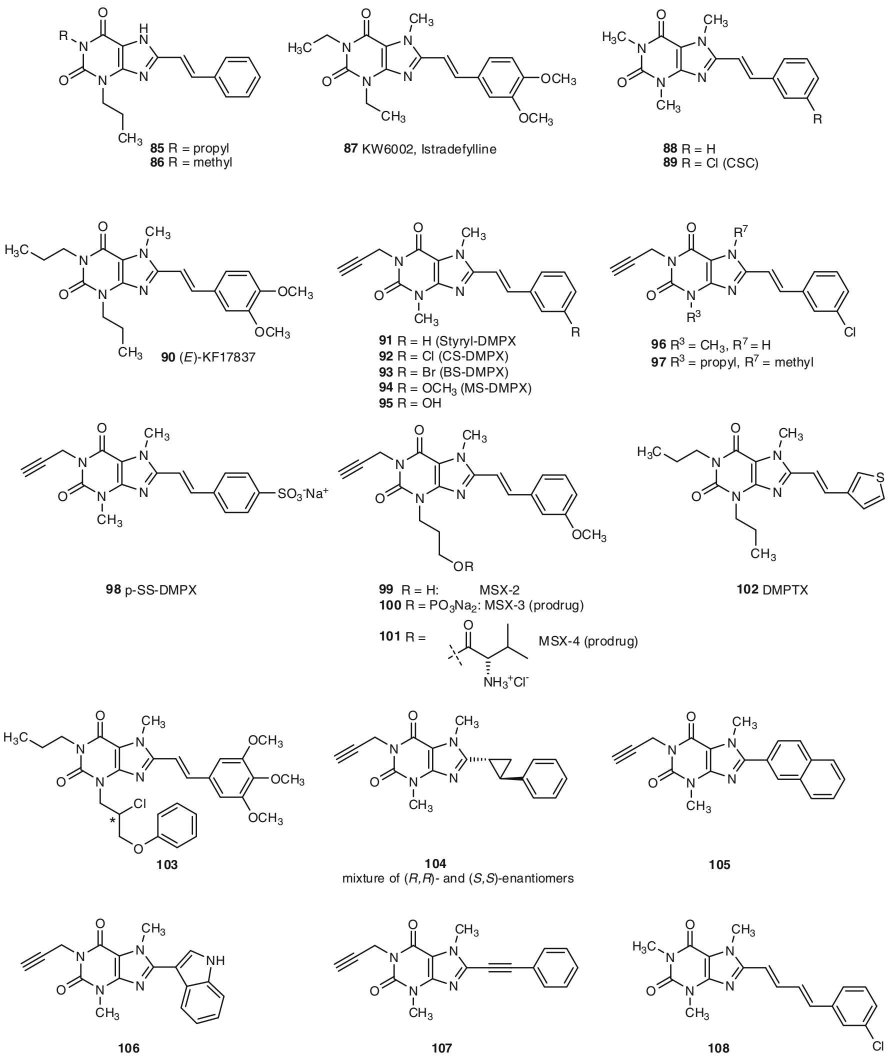


Fig. 4 8-Styrylxanthines and configurationally stable analogues

phase III study (PROTECT) it did not exhibit significant improvement over a placebo (Slawsky and Givertz 2009). Further potential applications for A_1 AR antagonists include hypertension and renal diseases owing to their diuretic and kidney-protective effects. The feasibility of designing kidney-selective prodrugs of an A_1 AR antagonist has been demonstrated (Barone et al. 1989). Yet other applications are cardiac arrhythmia, asthma and other respiratory disorders, and the prevention of organ damage, e.g. resulting from transplantation (Jacobson et al. 1992a; Müller and Stein 1996; Müller 1997; Jacobson and Gao 2006; Moro et al. 2006; Givertz 2009).

Table 4 Adenosine receptor affinities of 8-styrylxanthines and configurationally stable analogues

	Name	K_i (nM)[a]			
		A_1	A_{2A}	A_{2B}	A_3
Styrylxanthines[b]					
85	1,3-Dipropyl-8-stryrylxanthine	22.2 (r)[f]	85.1 (r)[f]	ND	ND
86	1-Methyl-3-propyl-8-styrylxanthine	31.1 (r)[f]	46.5 (r)[f]	ND	ND
87	Istradefylline (KW-6002) (K_i MAO-B 28,000 nM)[g]	841 (h)[c] 230 (r)[c]	12 (h)[h] 91.2 (h)[c] 2.2 (r)[i] 4.46 (r)[j]	>10,000 (h)[c]	4,470 (h)[c]
88	8-Styrylcaffeine (K_i MAO-B 2,864 nM)[k]	3,890 (r)[l]	94 (r)[l]	ND	ND
89	*m*-Chlorostyrylcaffeine (K_i MAO-B 80.6 nM)[j]	28,000 (r)[l]	54 (r)[l]	8,200[m]	>10,000 (r)[n]
90	KF17837	390 (r)[o]	7.9 (r)[o] (*E*/*Z*) 1.0 (r)[o] (*E*)	1,500 (h)[o]	ND
91	Styryl-DMPX	1,100 (r)[p]	27 (r)[p]	ND	ND
92	*m*-Chlorostyryl-DMPX	1,300 (r)[p]	13 (r)[p]	ND	ND
93	*m*-Bromostyryl-DMPX	1,200 (r)[p]	8.2 (r)[p] 10 (r)[q]	>10,000 (h)[r]	>10,000 (h)[r]
94	*m*-Methoxystyryl-DMPX	1,280 (r)[r]	12 (r)[r]	ND	ND
95	*m*-Hydroxystyryl-DMPX	940 (r)[r]	21 (r)[r]	ND	ND
96	7-Unsubstituted analogue of m-Chlorostyryl-DMPX	250 (r)[p]	410 (r)[p]	ND	ND
97	3-Propyl analogue of m-Chlorostyryl-DMPX	102 (r)[p]	5.1 (r)[p]	ND	ND
98	*p*-Sulfostyryl-DMPX	4,900 (r)[q]	240 (r)[q]	ND	ND
99	MSX-2	900 (r)[s] 2,500 (h)[s]	8.04 (r)[r,s] 5.38 (h)[s,d] 14.5 (h)[s,e]	>10,000 (h)[s] 2,900 (h)[t]	>10,000 (h)[s]
102	DMPTX	561 (r)[u]	19 (r)[u]	ND	ND
103		44 (r)[v]	>10,000 (r)[v]	ND	ND
Analogues of styrylxanthines					
104	Phenylcyclopropyl-DMPX (*trans, rac*)	4,600 (r)[w]	1,700 (r)[w]	ND	ND
105	β-Naphthyl-DMPX	980 (r)[w]	380 (r)[w]	ND	ND
106	3-Indolyl-DMPX	1,000 (r)[w]	300 (r)[w]	ND	ND
107	Phenylethynyl-DMPX	>3,000 (r)[w]	314 (h)[c] 300 (r)[w]	ND	5,000 (h)[c]
108	Phenylbutadienylxanthine (K_i MAO-B 42.1 nM)[j]	ND	104 (r)[w]	ND	ND

MSX-2 3-(3-hydroxypropyl)-7-methyl-8-(*m*-methoxystyryl)-1-propargylxanthine, *DMPX* 3,7-dimethyl-1-propargylxanthine, *DMPTX* [8-(3-thienylethenyl)-1,3-dipropylxanthine], *MAO*-B monoaminoxidase type B

[a] *h* human, *c* cow, *d* dog, *gp* guinea pig, *m*mouse, *mk* monkey, *r* rat, *rb* rabbit, a few A_{2B} data are from functional (cyclic AMP) studies, *ND* no data available

[b] Most data probably represent data from mixture of *E*/*Z* isomers since in dilute solutions light-induced isomerization occurs very fast and is difficult to avoid under standard testing conditions

[c] Müller, C.E., Hockemeyer, J., Diekmann, M. unpublished data

[d] Recombinant receptors expressed in Chinese hamster ovary cells

[e] Native receptors (postmortem human brain cortex)

2.3 Species Differences

Among the human ARs, the A_1 AR affinity and selectivity (vs. A_{2A} and A_{2B} ARs) of 8-cycloalkyl and 8-aryl derivatives are typically less than in the rat (see Tables 2, 3). Several groups have studied the species dependence of the AR affinity of xanthine derivatives (Ukena et al. 1986b; Klotz et al. 1991; Müller et al. 1993; Müller 1997; Kull et al. 1999; Fozard et al. 2003; Auchampach et al. 2009). An early conclusion was that the affinity of typical 8-substituted analogues (both aryl and cycloalkyl) was greatest at the bovine A_1 AR, intermediate at the rat A_1 AR, and lowest at the porcine A_1 AR. Later, it was found that the human A_1 AR most closely resembled the porcine A_1 AR, in that respect. At the A_{2A} AR and the A_{2B} AR, the opposite is true, although the differences are moderate: 8-substituted xanthines, such as XAC and DPCPX, are more potent at the human receptor than at the rat orthologue. The largest species differences are observed for the A_3 AR: 8-phenylxanthines and 8-cyclopentylxanthines are typically much more potent at the human than at the rat A_3 AR (Linden 1994; Ji et al. 1994; Jacobson 1998; Müller 2001, 2003).

2.4 Deazaxanthines and Azaxanthines

Analogues of xanthine derivatives, such as caffeine, theophylline, and 1,3-dialkyl-8-phenylxanthine, have been synthesized which are lacking either the N7 ("7-deazaxanthines") or the N9 ("9-deazaxanthines") nitrogen atom in the imidazole partial structure (compounds **109–116**, Fig. 5, Table 5). It was found that the nitrogen atom at the 9-position was not required for high receptor affinity, the

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[f] Erickson et al. (1991)
[g] Petzer et al. (2003)
[h] Kase (2003)
[i] Shimada et al. (1997)
[j] Pretorius et al. (2008)
[k] Vlok et al. (2006)
[l] Jacobson et al. (1993a)
[m] Daly and Jacobson (1995)
[n] van Galen et al. (1994)
[o] Nonaka et al. (1994a)
[p] Müller et al. (1997a)
[q] Müller et al. (1998b)
[r] Müller et al. (2000)
[s] Sauer et al. (2000)
[t] Solinas et al. (2005)
[u] Del Giudice et al. (1996)
[v] Massip et al. (2006)
[w] Müller et al. (1997a)

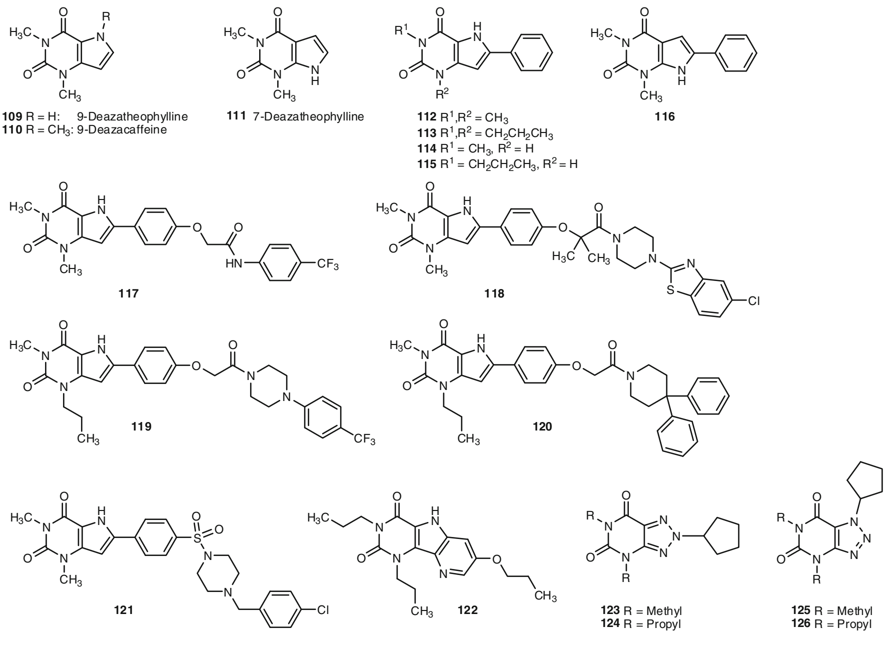

Fig. 5 Deazaxanthines and azaxanthines

9-deazaxanthines being even slightly more potent at the A_1 AR in comparison with the corresponding xanthine derivatives (Grahner et al. 1994). In contrast, 7-deazaxanthines were much less potent, proving that the xanthines will bind as 7*H* rather than 9*H* tautomers to the receptors (Grahner et al. 1994). The addition of another nitrogen atom to the 8-position of xanthines was less successful: 8-azaxanthines (**123**–**126**, Fig. 5, Table 5) showed only moderate affinity for the receptors (Franchetti et al. 1994) which can be explained by the lack of the N7 hydrogen atom that is required as a hydrogen-bond donor for high-affinity binding.

2.5 Tricyclic Xanthine Derivatives

Several different types of tricyclic xanthine derivatives have been prepared and investigated (Fig. 6, Table 6). Cycloalkyl-substituted dihydroimidazo[2,1-*i*]purinones (**127**, **128**) showed high A_1 AR affinity and selectivity combined with improved water solubility owing to the presence of a basic nitrogen atom that can be protonated (Suzuki et al. 1992b; Vu et al. 2006). A new class of heterotricyclic xanthine derivatives in which the 3-alkyl substituent is tethered to the N9 atom – pyrimido[1,2,3-*cd*]purinediones (**151**–**153**) – was synthesized and investigated (Fig. 6) (Weyler et al. 2006). Interestingly, the cyclopentyl-substituted derivative **151**, an analogue of DPCPX, was only weakly active, probably due to the lack of

Table 5 Adenosine receptor affinitities of deazaxanthines and azaxanthines

	Name	K_i (nM)[a]			
		A_1	A_{2A}	A_{2B}	A_3
Deazaxanthines					
109	9-Deazatheophylline	5,400 (r)[b]	12,000 (r)[b]	ND	ND
110	9-Deazacaffeine	32,000 (r)[b]	72,000 (r)[b]	ND	ND
111	7-Deazatheophylline	43,000 (r)[b]	>250,000 (r)[b]	ND	ND
112	1,3-Dimethyl-8-phenyl-9-deazaxanthine	47 (r)[b]	510 (r)[b]	ND	ND
113	1,3-Dipropyl-8-phenyl-9-deazaxanthine	13 (r)[b]	450 (r)[b]	ND	ND
114	1-Methyl-8-phenyl-9-deazaxanthine	97 (r)[b]	2,000 (r)[b]	520 (h)[c]	2,098 (h)[c]
115	1-Propyl-8-phenyl-9-deazaxanthine	45 (h)[c] 39 (r)[b]	>10,000 (h)[c] 1,200 (r)[b]	42 (h)[c]	380 (h)[c]
116	1,3-Dimethyl-8-phenyl-7-deazaxanthine	3,100[b]	12,000[b]	ND	ND
117		14.8 (h)[d]	64.6 (h)[d]	3.02 (h)[d]	>1,000 (h)[d]
118		>1,000 (h)[e]	10,000 (h)[e]	11.0 (h)[e]	>1,000 (h)[e]
119		89.1 (h)[e]	324 (h)[e]	2.04 (h)[e]	2,240 (h)[e]
120		676 (h)[e]	3,550 (h)[e]	5.26 (h)[e]	>1,000 (h)[e]
121		183 (h)[f]	ND	1 (h)[f]	12,260 (h)[f]
122	Tricyclic 9-deazaxanthine	346 (h)[g]	164 (h)[g]	ND	3.82 (h)[g]
8-Azaxanthines					
123	1,3-Dimethyl-8-cyclopentyl-8-azaxanthine	110,000 (c)[h]	58,000 (c)[h]	ND	ND
124	1,3-Dipropyl-8-cyclopentyl-8-azaxanthine	1,300 (c)[h]	13,000 (c)[h]	ND	ND
125	1,3-Dimethyl-7-cyclopentyl-8-azaxanthine	11,000 (c)[h]	292,000 (c)[h]	ND	ND
126	1,3-Dipropyl-7-cyclopentyl-8-azaxanthine	340 (c)[h]	10,000 (c)[h]	ND	ND

[a] *h* human, *c* cow, *r* rat, a few A_{2B} data may be from functional (cyclic AMP) studies, *ND* no data available

[b] Grahner et al. (1994)

[c] Hayallah et al. (2002)

[d] Carotti et al. (2006)

[e] Stefanachi et al. (2008)

[f] Esteve et al. (2006)

[g] Ishiyama et al. (2009)

[h] Franchetti et al. (1994)

the N7 hydrogen atom. In contrast, the 3-noradamantyl-substituted analogues (**152**, **153**) showed relatively high A_1 AR affinity. While propyl derivative **152** (PSB-63) was very selective compared with the other AR subtypes, butyl derivative **153** was also quite potent at the human A_3 AR (Table 6). Another novel tricyclic analogue of DPCPX, the oxazolo[3,2-*a*]purinone derivative **154**, showed only weak affinity for ARs (Table 6) (Müller 1994). In a series of tricyclic pyrimido[2,1-*f*]purinediones the *N*,*N*-dipropyl-substituted derivative **139** (Fig. 6), bearing a *m*-chlorobenzyl

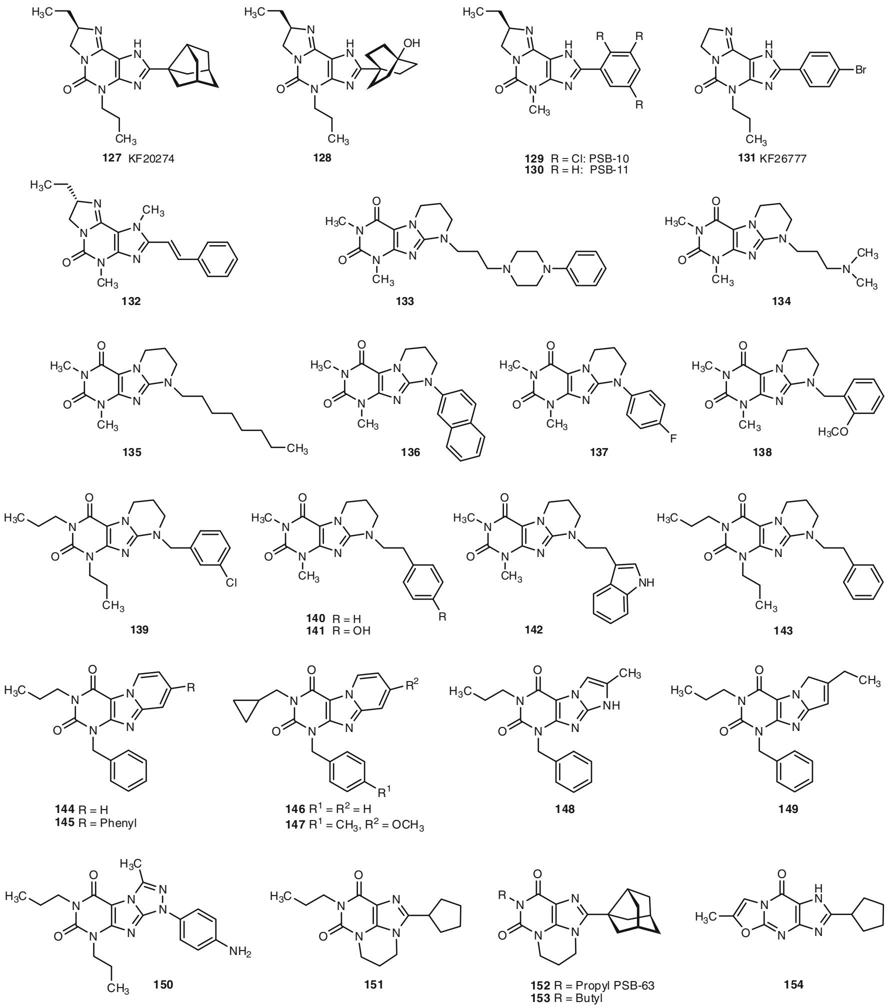

Fig. 6 Tricyclic xanthine derivatives

residue attached to the additional ring, was a relatively potent A_1 AR antagonist with some selectivity (Table 6) (Drabczynska et al. 2007a).

3 A_{2A} Adenosine Receptor Antagonists

A_{2A}-AR-selective antagonists of both xanthine and nonxanthine classes have been developed and some have entered clinical trials for Parkinson's disease, based on the opposing action of adenosine and dopamine in the striatal pathways in the brain

Table 6 Adenosine receptor affinities of tricyclic xanthine derivatives

	Name	K_i (nM)[a]			
		A_1	A_{2A}	A_{2B}	A_3
Imidazo[2,1-*i*]purin-5-ones					
127	KF20274	2.7 (r)[b]	290 (r)[b]	ND	ND
128		22 (h)[c] 6 (r)[c]	4,400 (h)[c] 2,700 (r)[c]	580 (h)[c]	>10,000 (h)[c]
129	PSB-10	1,700 (h)[d] 805 (r)[e]	2,700 (h)[d] 6,040 (r)[e]	ND	0.441 (h)[e]
130	PSB-11	1,640 (h)[d] 440 (r)[d]	1,280 (h)[d] 2,100 (r)[d]	2,100 (m)[e]	2.34 (h)[d] K_D 4.9 (h)[f]
131	KF26777	1,800 (h)[g]	470 (h)[g]	620 (h)[g]	0.20 (h)[g]
132		14,900 (r)[e]	424 (r)[e]	3,700 (m)[e]	30,600 (h)[e]
Pyrimido[2,1-*f*]purinediones					
133		15,000 (r)[h]	16,000 (r)[h]	ND	ND
134		20,000 (r)[h]	>250,000 (r)[h]	ND	ND
135		>25,000 (r)[i]	998 (r)[i]	5,200 (h)[i]	12,300 (h)[i]
136		26,800 (h)[j] ≥25,000 (r)[j]	2,870 (h)[j] 219 (r)[j]	~10,000 (h)[j]	>10,000 (h)[j]
137		16,700 (h)[j] >25,000 (r)[j]	1,880 (h)[j] 147 (r)[j]	~10,000 (h)[j]	>10,000 (h)[j]
138		>25,000 (r)[k]	11,300 (h)[k] 699 (r)[k]	ND	ND
139		89 (r)[k]	478 (r)[k]	ND	1,290 (h)[k]
140		>10,000 (h)[l] >25,000 (r)[l]	2,890 (h)[l] 320 (r)[l]	~10,000 (h)[l]	>10,000 (h)[l]
141		>25,000 (h)[l] ~25,000 (r)[l]	630 (h)[l] 230 (r)[l]	7,200 (h)[l]	>10,000 (h)[l]
142		>25,000 (h)[l] ~25,000 (r)[l]	4,560 (h)[l] 330 (r)[l]	~10,000 (h)[l]	>10,000 (h)[l]
143		620(r)[l]	860(r)[l]	590 (h)[l]	3,660 (h)[l]
Pyrido[2,1-*f*]purinediones					
144		50 (h)[m]	119 (h)[m]	ND	4.0 (h)[m]
145		>10,000 (h)[m]	>10,000 (h)[m]	ND	35 (h)[m]
146		>1,000 (h)[n]	242 (h)[n]	ND	4.2 (h)[n]
147		>1,000 (h)[n]	>1,000 (h)[n]	>1,000 (h)[n]	2.24 (h)[n]
Imidazopurinediones, pyrrolopurinediones, and triazolopurinediones					
148		>1,000 (h)[o]	>1,000 (h)[o]	>1,000 (h)[o]	0.8 (h)[o]
149		>1,000 (h)[o]	>1,000 (h)[o]	>1,000 (h)[o]	3.5 (h)[o]
150		>10,000 (h)[p]	2,050 (h)[p]	>100,000 (h)[p]	1,330 (h)[p]
4,5-Dihydro-6*H*,8*H*-pyrimido[1,2,3-*cd*]purine-8,10-diones					
151		1,440 (r)[q]	12,400 (r)[q]	42,600 (h)[q]	ND
152	PSB-63	16.9 (r)[q] 90.6 (h)[q]	22,000 (r)[q] 34,500 (h)[q]	3,190 (h)[q]	>10,000 (h)[q]
153		40.6 (r)[q] 13.8 (h)[q]	23,400 (r)[q] ~25,000 (h)[q]	22,300 (h)[q]	188 (h)[q]
Oxazolo[3,2-*a*]purinone					
154		770 (r)[r]	20,600 (r)[r]	ND	ND

[a] *h* human, *m* mouse, *r* rat, a few A_{2B} data may be from functional (cyclic AMP) studies, *ND* no data available

[b] Suzuki et al. (1992b)

(Richardson et al. 1997; Schapira et al 2006; Schwarzschild et al. 2006; Müller and Ferré 2007; Baraldi et al. 2008). The cellular mechanisms of the motor and neuroprotective effects of A_{2A} AR antagonists have been explored (Yu et al. 2008). Recently, ameliorating effects of A_{2A} AR antagonists including xanthine derivatives on animal models of Alzheimer's disease and cognitive dysfunction have been reported (Dall'Igna et al. 2007; Cunha et al. 2008; Takahashi et al. 2008). Since the early 1990s, there has been a major medicinal chemical effort to increase the A_{2A} AR selectivity of simple xanthines by structural modification.

Prior to the synthesis of truly A_{2A}-AR-selective antagonists, certain high-affinity xanthines were used in a non-selective fashion as probes of the A_{2A} AR. For example, [^{3}H]XAC (**30**) was useful as a radiotracer in binding experiments at the A_{2A} AR in human platelets and was therefore the first antagonist radioligand with high affinity at the A_{2A} AR (Ukena et al. 1986a). PD115,199 (**32**) was prepared in tritiated form and shown to bind with high affinity to the rat A_{2A} AR (Bruns et al. 1987b).

The first "selective" A_{2A} AR antagonist described in the literature was the caffeine analogue DMPX (**20**, Fig. 1, Table 1) (Ukena et al. 1986b). Like caffeine, the compound possesses low A_{2A} AR affinity and moderate selectivity compared with A_1 ARs. Nevertheless, this compound has been widely used in in vivo studies because of its good water solubility and bioavailability (Seale et al. 1988; Thorsell et al. 2007). Later it was found that DMPX is as potent at the A_{2B} AR as at the A_{2A} AR. The species dependence of affinity at the A_{2A} AR of 1,3,7- and 1,3,8-trisubstituted xanthines has been reported (Stone et al. 1988).

An early example of a caffeine analogue that displayed selectivity for the A_{2A} AR was 8-trifluoromethylcaffeine, but the affinity was still low, with a K_i value in binding at the rat A_{2A} AR of 29 μM (Jacobson et al. 1993b). This effect of the 8-trifluoromethyl group was not observed in the corresponding (inactive) theophylline derivative. An 8-(*trans*-2-carboxyvinyl) derivative of caffeine also proved to be similarly selective for the A_{2A} AR.

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[c] Vu et al. (2006)
[d] Ozola et al. (2003)
[e] Müller et al. (2002a)
[f] Müller et al. (2002b)
[g] Saki et al. (2002)
[h] Geis et al. (1995)
[i] Drabczynska et al. (2004)
[j] Drabczynska et al. (2006)
[k] Drabczynska et al. (2007a)
[l] Drabczynska et al. (2007b)
[m] Priego et al. (2002)
[n] Priego et al. (2008)
[o] Baraldi et al. (2005)
[p] Pastorin et al. (2005)
[q] Weyler et al. (2006)
[r] Müller (1994)

3.1 8-Styrylxanthines

The observation that N7-methylation in 8-substituted xanthine derivatives was better tolerated by the A_{2A} AR than the A_1 AR (Shamim et al. 1989) and that the 8-substituent had to be coplanar to achieve high A_{2A} AR affinity (Erickson et al. 1991) led to the first highly potent and selective A_{2A} AR antagonists: the 1,3,7-alkyl-substituted 8-styrylxanthine derivatives **87–95** and **99** (Fig. 4, Table 4).

A small alkyl group at N1 (methyl, ethyl, propyl, propargyl) proved to be optimal for high A_1 AR affinity and selectivity, while methylation is required at the 7-position (Jacobson et al. 1993a; Nonaka et al. 1994a; Shimada et al. 1997; Müller et al. 2000; Kase 2003). The 8-styryl residue has to be *E*-configurated, and *m*-chloro or *m*-methoxy substitution improved affinity and selectivity. The *meta* position of the 8-styryl ring can be substituted with elongated chains with retention of A_{2A} AR selectivity and enhancement of water solubility (Jacobson et al. 1993a). The phenyl ring in the 8-styryl residue can be substituted by heterocyclic rings, such as a 3-thienyl ring (**102**) (Del Giudice et al. 1996).

The most common substituents at N3 in A_{2A}-AR-selective xanthine derivatives are small alkyl residues, such as methyl, propyl, and 3-hydroxypropyl (reviewed in Müller 2000; Cacciari et al. 2003; Vu 2005; Yuzlenko and Kiec-Kononowicz 2006; Müller and Ferré 2007; Cristalli et al. 2007, 2009). Recently, the development of a new synthetic approach allowed the preparation of a series of xanthine derivatives with more variations in the 3-position (Massip et al. 2006). It was found that the A_{2A} AR tolerated bulky, functionalized substituents at the 3-position. For instance, *N*3-phenoxypropyl-substituted 8-(methoxystyryl)xanthine derivatives (e.g. **103**) are potent and selective A_{2A} AR antagonists (Massip et al. 2006).

Some of the best A_{2A} AR antagonists were istradefylline (KW6002, **87**), *m*-chlorostyrylcaffeine ((*E*)-8-*m*-Chlorostyrylcaffeine, **89**), *m*-bromostyryl-DMPX (**93**), and 3-(3-hydroxypropyl)-7-methyl-8-(*m*-methoxystyryl)-1-propargylxanthine (MSX-2, **99**). (*E*)-8-(3-Chlorostyryl)caffeine (**89**) is not only a potent A_{2A} AR antagonist (K_i rat A_{2A} AR 54 nM), but in addition, it has been reported to be a potent inhibitor of monoaminoxidase type B (MAO-B) (baboon MAO-B, K_i 80.6 nM), an enzyme which metabolizes dopamine (van den Berg et al. 2007; Petzer et al. 2009). This activity may contribute to the potency of CSC in in vivo studies, e.g. in animal models of Parkinson's disease. All other styrylxanthine derivatives investigated so far, including 8-styrylcaffeine (**88**) and istradefylline (**87**), are considerably less potent as MAO-B inhibitors than CSC. Recently, a chain-extended homologue of CSC, 8-(*m*-chlorophenylbutadienyl)caffeine (**108**), has been described as showing similar dual activity as an A_{2A} AR antagonist and an MAO-B inhibitor (Pretorius et al. 2008).

Istradefylline (KW-6002, **87**) has been intensively studied in in vitro and in a number of animal models. Until recently (Fernandez et al. 2010), it was in phase IIIb clinical trials for Parkinson's disease. In phase II clinical trials istradefylline reduced motoric dysfunction without producing dyskinesias (reviewed by Knutsen and Weiss 2001). A ^{11}C-labelled version of istradefylline has been prepared and used for positron emission tomography (PET) studies in healthy human brain (Hirani et al. 2001).

A major drawback of styrylxanthine derivatives, however, is their high lipophilicity and low water solubility. Introduction of a polar sulfonate group into 8-styryl-DMPX, resulting in compound **98**, led to an almost tenfold reduction in A_{2A} affinity, but increased water solubility (Müller et al. 1998). A more successful approach has been the preparation of water-soluble prodrugs, particularly of the 3-(3-hydroxypropyl)-substituted 1-propargyl-8-styrylxanthine derivative MSX-2 (**99**) (Müller 2009). MSX-3 (**100**) is a water-soluble phosphate prodrug of MSX-2, which is cleaved in vivo by ubiquitous phosphatases to release the A_{2A} AR antagonist MSX-2 (Sauer et al. 2000). MSX-3 has proven useful for animal studies and is widely used for studying the in vivo effects of A_{2A} AR antagonists (Hauber et al. 1998, 2001; Strömberg et al. 2000; Ferré et al. 2001, 2008; Nagel et al. 2003; Blum et al. 2003; Schindler et al. 2004, 2005; Antoniou et al. 2005; Karcz-Kubicha et al. 2003a, b; Filip et al. 2006; Fuxe et al. 2007; Ishiwari et al. 2007; Farrar et al. 2007; Carriba et al. 2007; Salamone et al. 2008a, b; Marcellino et al. 2008; Mott et al. 2009; Worden et al. 2009). Owing to its very high water solubility at the physiological pH of 7.4 (9 mg/mL), it can be directly injected into specific brain areas, but is also an effective A_{2A} AR antagonist after systemic application. Recently, an amino acid ester prodrug of MSX-2, MSX-4 (**101**), was synthesized, and was found to be very soluble in water, highly stable in artificial gastric fluid, but readily cleaved by esterases and may be a suitable prodrug for peroral administration (Vollmann et al. 2008).

Care has to be taken when using the *E*-configurated styrylxanthines since they easily undergo light-induced isomerization in dilute solutions yielding mixtures of *E* and *Z* isomers, the *Z* isomers being only weakly active or inactive (Nonaka et al. 1993; Müller et al. 1998). This isomerization does not occur in concentrated solution, e.g. during synthesis of the compounds, or when the compounds are applied as solid dosage forms. However, styrylxanthines can also undergo light-induced dimerization ([2 + 2] cycloaddition reaction) in the solid state, and therefore have to be rigorously stored under the exclusion of light (Hockemeyer et al. 2004).

3.2 Configurationally Stable Analogues of 8-Styrylxanthines

To overcome the problem of photoisomerization, the styryl moiety has been replaced with different, more stable bioisosteric groups (e.g. replacement of the double bond for a cyclopropyl ring in **104**, a 2-naphthyl residue in **105**, a triple bond in **107** (Müller et al. 1997c), or a tricyclic constrained structure (**133**–**143**) (Kiec-Kononowicz et al. 2001; Drabczynska et al. 2003, 2004, 2006, 2007b; Fhid et al. 2003). In most cases, a significant loss of affinity was observed by such modifications. The most promising compounds were the pyrimido[2,1-*f*] purinedione derivative (**141**) (K_i human A_{2A} AR 630 nM, rat A_{2A} AR 230 nM) and 8-phenylethynyl-DMPX (**107**, K_i human A_{2A} AR 314 nM, rat A_{2A} AR 300 nM), both endowed with high selectivity. The latter class of compounds has been optimized for increased A_{2A} AR affinity and the highly potent and selective A_{2A} AR antagonists obtained were described in a recent patent (Müller et al. 2008). Furthermore, a substitution of the ethenyl group with a diazo structure has been performed. The compounds obtained retained selectivity but showed only moderate affinity (Müller et al. 1997b).

3.3 A_{2A}-Adenosine-Receptor-Selective Radiolabelled Xanthine Derivatives

The tritiated derivative of the 8-styrylxanthine KF17837S [the equilibrium mixture of (*E*)-KF17837 and (*Z*)-KF17837 isomers] was shown to bind to rat striatal membranes in a saturable and reversible way, with K_D values of low nanomolar concentration (Nonaka et al. 1994b). Another A_{2A} AR antagonist radioligand was prepared, [^{3}H]MSX-2. This molecule showed high affinity ($K_D = 8.0$ nM) for rat and human A_{2A} ARs, with saturable and reversible binding, and also a A_{2A}-selectivity of at least 2 orders of magnitude compared with all other AR subtypes (Müller et al. 2000).

3.4 Heterocyclic Compounds Related to Xanthines

A tricyclic styryl-substituted imidazo[2,1-*i*]purin-5-one derivative (**132**, Fig. 6, Table 6) showed enhanced water solubility but reduced A_{2A} AR affinity and moderate selectivity (Müller et al. 2002a).

4 A_{2B} Adenosine Receptor Antagonists

4.1 Aryl-Substituted 1,3-Dialkylxanthines

From the initial studies of Daly and coworkers using cyclic AMP studies in the brain slice, it was recognized that 1,3,7- and 1,3,8-trisubstituted xanthines have considerable affinity at the A_{2B} AR. Also, the simple xanthine enprofylline (**10**) was discovered to have slight selectivity for the A_{2B} AR, which was proposed to be responsible for its antiasthmatic action in the clinic (Stefanovich 1989; Daly 2000, 2007). Screening efforts by Bruns (1981) followed by more detailed studies by Müller, Daly, and Jacobson showed that 1-monosubstituted xanthine derivatives, such as 1-propylxanthine (**6**) and 1-butylxanthine (**7**), were about tenfold more potent than enprofylline at A_{2B} AR and equally selective (Müller et al. 1993; Kim et al. 2002).

In fact, the unintended interaction at the A_{2B}AR of widely used xanthine antagonists of the ARs has proven to be a complication in pharmacology studies.

Many known xanthines were screened at the A_{2B}AR to identify leads for the design of novel A_{2B} AR antagonists. The first successful efforts to enhance the activity of 1,3,8-trisubstituted xanthines at the A_{2B}AR by Jacobson and colleagues resulted in one compound of intermediate selectivity at the human, but not rat A_{2B} AR, MRS1595 (**41**), which is a hydrazide derivative of XCC (Fig. 2, Table 2) (Kim et al. 2000). Then, further probing of the structure–activity relationship culminated in the introduction of MRS1754 (**37**), which was the first selective A_{2B} AR antagonist with nanomolar affinity at the human receptor (Kim et al. 2000). The degree of selectivity for the human A_{2B} AR was more than 120-fold, but selectivity for the rat A_{2B} AR was considerably less (Fig. 2, Table 2). Thus, it remained a challenge to design a rat A_{2B}-AR-selective xanthine antagonist. Another drawback in the series of anilide derivatives of XCC is the low aqueous solubility, which is partly remedied in related antagonists such as MRS1706 (**38**). Nevertheless, [^{3}H] MRS1754 has found application as a useful radioligand of the A_{2B} AR (Ji et al. 2001). Structurally related 8-phenylxanthine derivatives include CVT-5440 (**40**), in which additional aromatic rings were attached by an ether linkage, and **42**, with a modified 3-substituent (3-methoxypropyl), and were developed as potent and selective A_{2B} AR antagonists (Kim et al. 2000; Nieto et al. 2009). Newer derivatives in this series, which have two pyridine rings linked by an amide group, include the highly selective A_{2B} AR antagonists ATL-802 (**57**) and ATL-852 (**58**). [^{3}H] ATL-852 has been reported as a high-affinity radioligand at this receptor (Cagnina et al. 2009).

8-Pyrazolyl-substituted xanthines that have been developed as selective human A_{2B} AR antagonists include MRE-2029-F20 (**56**), which was also reported as a high-affinity radioligand (Baraldi et al. 2004). A different series of isomeric 8-pyrazolylxanthines yielded the highly potent A_{2B} AR antagonists CVT-6694, CVT-7124, and CVT-6883 (**53**–**55**) (Kalla et al. 2008; Elzein et al. 2008; Kalla and Zablocki 2009). High selectivity at human receptors has been found for all of these pyrazolylxanthines, but no data for rodent receptors have been reported. CVT-6883 (**55**) is a promising candidate for the treatment of diabetes or asthma and has entered phase I clinical trials. Pain treatment is another potential area under consideration for A_{2B} AR antagonists (Abo-Salem et al. 2004; Akkari et al. 2006; Bilkei-Gorzo et al. 2008; Michael et al. 2010).

4.2 1,8-Disubstituted Xanthines

The observation that 1-monosubstituted and 1,8-disubstituted xanthine derivatives showed high affinity and increased selectivity for the A_{2B} AR led to the development of a series of 1-alkyl-8-phenylxanthine derivatives (**43**–**52**) (Hayallah et al. 2002; Yan and Müller 2004; Yan et al. 2006; Borrmann et al. 2009). These compounds also appeared to show reduced affinity at the rat A_1 AR and therefore increased A_{2B} AR selectivity in rat. 1-Propyl-8-*p*-sulfophenylxanthine (PSB-1115, **43**) was developed as a water-soluble A_{2B} AR antagonist, useful as a pharmacological tool for in vivo studies (Müller et al. 1993; Kirfel et al. 1997; Abo-Salem et al. 2004; Bilkei-Gorzo et al. 2008). 1-Butyl-8-(*p*-carboxyphenyl)xanthine (PSB-53, **45**) showed similar affinity and selectivity. 1-Propargyl-8-*p*-bromophenylxanthine (PSB-50, **44**) was more potent, but somewhat less selective and much less water soluble. The 8-phenylxanthine derivatives PSB-55 (**46**), a benzylpiperazine derivative, and PSB-298 (**47**), a hydroxyethylamide, were synthesized to obtain more polar compounds with high A_{2B}AR affinity. PSB-298 was obtained in tritiated form and was found to have a low degree of non-specific binding (Bertarelli et al. 2006). However, its affinity and selectivity were not satisfactory.

Starting from the sulfonate PSB-1115 (**43**), sulfonic acid esters (e.g. **48**) and sulfonamides (e.g. **49**–**52**) were obtained (Yan and Müller 2004; Yan et al. 2006). Compound **48** can be envisaged as a lipophilic prodrug of the highly polar sulfonate **43**, which may show peroral bioavailability and release of **43** after absorption (Yan and Müller 2004). However, **48** has high A_{2B}AR affinity itself and can therefore be classified as a limited prodrug, although without selectivity versus the A_1 AR (Fig. 2, Table 2). The most potent and selective A_{2B}AR antagonists described to date are the sulfonamide derivatives **50**–**52**, whose development was based on PSB-601 (**49**), an already very potent and selective A_{2B}AR antagonist (K_i 3.6 nM). Compounds **50**–**52** show subnanomolar affinity for A_{2B} ARs and very high selectivity in humans and in rodents. [^{3}H]PSB-603 was prepared as a selective, high-affinity A_{2B}AR antagonist radioligand with K_D values of 0.403 nM at human and 0.351 nM at mouse A_{2B}AR (Borrmann et al. 2009).

4.3 9-Deazaxanthines

Several series of 9-deazaxanthine derivatives (**115**, **117**–**121**) were developed as A_{2B}AR antagonists, and were structurally related to the 8-phenylxanthine derivatives described earlier (Fig. 5, Table 5). A number of compounds with low nanomolar affinity were obtained, and some were selective for the human A_{2B}AR (Carotti et al. 2006; Esteve et al. 2006; Stefanachi et al. 2008).

4.4 8-Furylmethyl-Substituted Xanthines

8-(2-Furyl)methyl-substituted xanthined derivatives, e.g. **63**, have been developed as A_{2B} AR antagonists (Fig. 2, Table 2). Some of them showed high A_{2B} AR affinity but only moderate selectivity (Balo et al. 2009).

5 A_3 Adenosine Receptor Antagonists

In the search for A_3AR antagonists, alkylxanthines were initially rejected as a suitable lead in favour of non-xanthine chemically diverse heterocycles, because of the exceptionally low affinity of alkylxanthines at the rat A_3AR. For example, the classic adenosine antagonists caffeine and theophylline have K_i values of more than 100 μM at the rat A_3AR (Table 1). Initial structure–activity relationship studies at the rat A_3 AR were conducted using multiply substituted xanthines, many of which retained selectivity for the A_3AR (van Galen et al. 1994). Only slight A_3AR selectivity was observed for analogues containing 8-alkyl and 2-thio substitutions (Kim et al. 1994b). However, when other orthologues of the A_3AR were cloned and studied pharmacologically, such as sheep, and human A_3AR, many xanthines were found to display good affinity for those A_3ARs (Linden 1994). Thus, attention returned to the xanthines as a source for A_3AR antagonist leads.

One of the earliest approaches to enhancing the affinity of xanthines at the A_3 AR was to attach a ribose group at the 7-position (**21**, **22**). The 5′-uronamide derivative DBXRM (**22**) is 140-fold selective for the A_3AR, but the presence of the uronamide function increases the efficacy such that it is an agonist at this receptor (van Galen et al. 1994; Kim et al. 1994b; Bridson et al. 1998).

5.1 8-Aryl-Substituted Xanthine Derivatives

8-Phenylxanthine derivatives bearing a carboxylate group attached to the phenyl ring via an ethylene (**35**) or an oxymethylene (**34**) spacer were initially found to be potent antagonists at the human A_3 AR, but both compounds are also very potent A_1 and $A_{2B}AR$ antagonists (Fig. 2, Table 2). $[^{125}I]$I-ABOPX (BW-A522, **34**) has been used as a radioligand for labelling the A_1 AR as well as human A_3 and human A_{2B} ARs (Patel et al. 1988; Salvatore et al. 1993; Linden et al. 1999). The corresponding *p*-azido derivative was previously used for photoaffinity labelling of the A_1AR. The xanthine derivative **34** is characterized by a *p*-amino-*m*-iodobenzyl residue at N3 of the xanthine core (Fig. 2) and is therefore quite lipophilic. As observed for other xanthine antagonists, **34** is much less potent (65-fold) at rat than at human A_3 ARs.

5.2 Tricyclic Xanthine and Deazaxanthine Derivatives

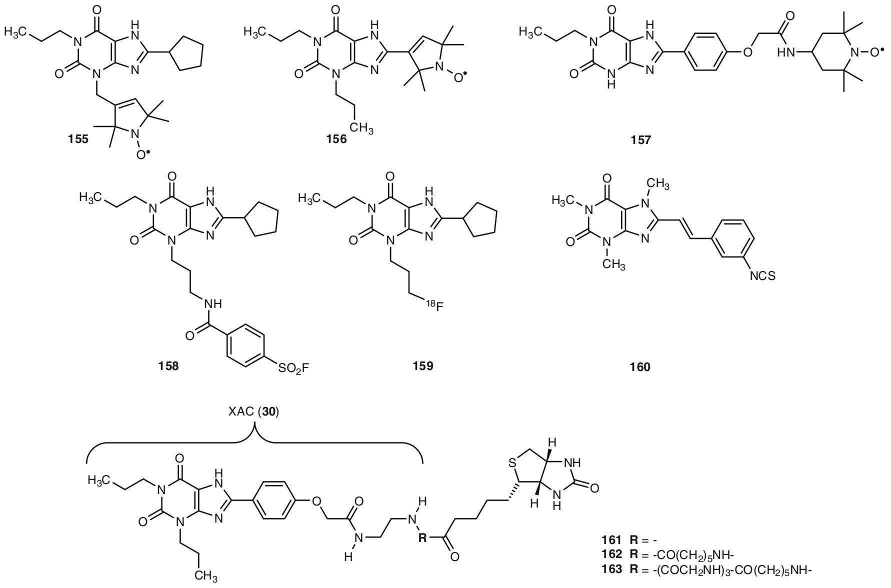
Cyclized derivatives of xanthines, such as (8*R*)-8-ethyl-4-methyl-2-phenyl-4,5,7,8-tetrahydro-1*H*-imidazo[2.1-*i*]purin-5-one (PSB-11, **130**), its trichlorophenyl-substituted derivative PSB-10 (**129**), and the 8-unsubstituted 8-bromophenyl derivative **131** are very potent A_3-AR-selective antagonists (Fig. 6, Table 6) (Müller et al. 2002a; Saki et al. 2002; Ozola et al. 2003). PSB-11 (K_D 4.9 nM) was prepared as a radioligand by catalytic hydrogenation from the polychlorinated precursor PSB-10 (Müller et al. 2002b; Burbiel et al. 2003). Owing to its increased polarity and solubility in comparison with xanthines, [^{3}H]PSB-11 shows only low nonspecific binding. Further tricyclic xanthine derivatives, in which an additional ring was attached to the imidazole rather than the pyrimidine ring of the xanthine core structure (pyridopurinediones, imidazopurinediones, pyrrolopurinediones, and triazolopurinediones **144–150**), were developed as A_3-AR-selective antagonists (Priego et al. 2002; Baraldi et al. 2005; Pastorin et al. 2005; Priego et al. 2008). An aromatic residue (mostly benzyl) attached to N3 (xanthine numbering) increases A_3AR affinity in xanthine derivatives, including the tricyclic ones.

Besides tricyclic xanthine derivatives, tricyclic deazaxanthines have also been obtained (Ishiyama et al. 2009). Compound **122** (Fig. 5, Table 5) was one of the most potent and selective compounds in this series.

6 Xanthine Derivatives Used as Molecular Probes

6.1 Irreversible Ligand Probes

The amine congener XAC (**30**) was coupled to a variety of bifunctional cross-linking reagents to form products containing a single chemically reactive group (Fig. 7, Table 7). For example, 1,3-phenylene and 1,4-phenylene diisothiocyanates



161 R = -
162 R = $-CO(CH_2)_5NH-$
163 R = $-(COCH_2NH)_3-CO(CH_2)_5NH-$

Fig. 7 (Continued)

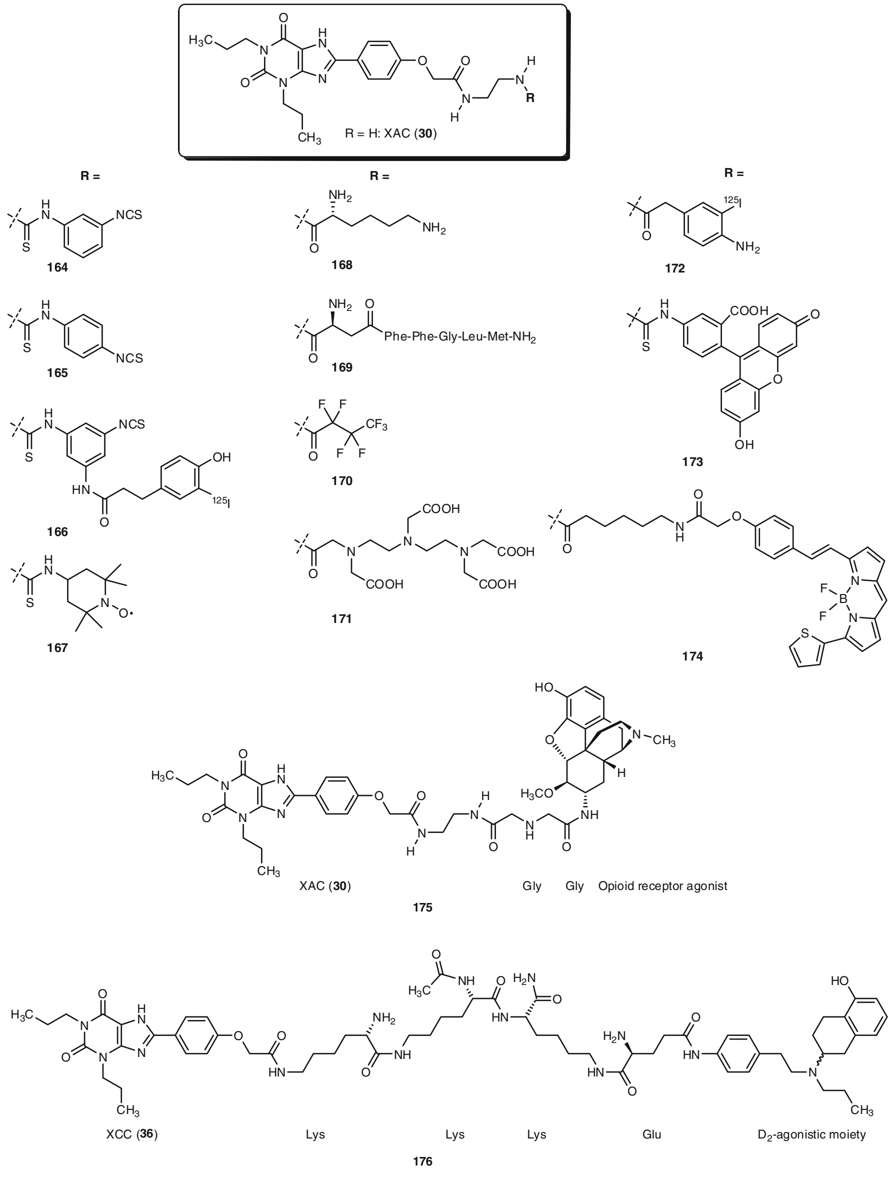


Fig. 7 Functionalized xanthines as molecular probes

were conjugated to XAC to form the A_1-AR-selective antagonists *m*-phenylenediisothiocyanate-XAC (**164**) and *p*-DITC-XAC (**165**), which were demonstrated to bind irreversibly at submicromolar concentrations to the A_1AR to act as covalent affinity labels. Similarly, prosthetic reagents designed for radiolabelling and photoaffinity labelling could be coupled to XAC and similar amine congeners

Table 7 Adenosine receptor affinities of functionalized xanthines as molecular probes

	Name	K_i (nM)[a]			
		A_1	A_{2A}	A_{2B}	A_3
Spin-labeled probes					
155		5.47 (r)[b]	8,780 (r)[b]	>1,000 (h)[b]	1,700 (h)[b]
156		8.23 (r)[b]	3,800 (r)[b]	3,100 (h)[b]	~10,000 (h)[b]
157		15.7 (r)[b]	1,270 (r)[b]	48 (h)[b]	350 (h)[b]
167	TEMPO-XAC	4.9 (r)[c] 0.30 (c)[c]	ND	ND	ND
Irreversible ligands					
158	FSCPX[d,e]	10 (r)[d]	ND	ND	ND
160	ISC	42,600 (r)[f] 51,400 (gp)[f] 89,500 (rb)[f] 63,400 (c)[f]	146 (r)[f] 160 (gp)[f] 413 (rb)[f] 516 (c)[f]	ND	ND
164	*m*-DITC-XAC	2.39 (r)[g] 52 (r)[h]	ND	ND	ND
165	*p*-DITC-XAC	6.60 (r)[g] 27 (r)[h]	ND	ND	ND
Radioligands					
159	$[^{18}F]$CPFPX	1.26 (h)[i] 0.63 (r)[i] 1.37(p)[i] 0.18 (c)[i]	940 (h)[i] 812 (r)[i]	ND	ND
166		40 (IC_{50}) (c)[i]	ND	ND	ND
172	$[^{125}I]$PAPA-XAC	0.1 (c)[k]	ND	ND	ND
Biotin conjugates					
161		54 (r)[l,m]	ND	ND	ND
162		50 (r)[l,m]	ND	ND	ND
163		60 (r)[m]	ND	ND	ND
Various conjugates					
168	D-Lys-XAC	1.74 (IC_{50}) (r)[n]	159 (IC_{50}) (r)[n]	ND	ND
169		35 (r)[m]	ND	ND	ND
170		8.1 (r)[c] 0.8 (c)[c]	ND	ND	ND
171	DTPA-XAC	59.5 (r)[c] 3.25 (c)[c]	ND	ND	ND
Fluorescent ligands					
173	FITC-XAC	125 (r)[c] 9.3 (c)[c]	ND	ND	ND
174	XAC-BY630	151 (h)[o]	ND	ND	ND
Bivalent ligand conjugates					
175[l,p]		31 (r)	ND	ND	ND
176	A_{2A} antagonist/D_2 agonist for A_{2A}/D_2 receptor heteromers (K_i D_2 (s) = 1.0 nM)[q]	ND	55 (s)[q]	ND	ND

TEMPO 2,2,6,6-tetramethylpiperidine-1-oxyl, *FSCPX* 8-cyclopentyl-3-*N*-[3-((3-(4-fluorosulphonyl)benzoyl)-oxy)-propyl]-1-*N*-propyl-xanthine, *ISC* 8-(3-isothiocyanatostyryl)caffeine, *DITC* phenylenediisothiocyanate, *PAPA* p-phenylacetyl, *DTPA* diethylenediaminepentaacetic acid, *FITC* fluorescein isothiocyanate

(Stiles and Jacobson 1988; Jacobson et al. 1989a). The conjugate with the *p*-aminophenylacetyl moiety was readily iodinated to form the radioligand **172**, which could then be converted to the photoactivatable *p*-azide (Stiles and Jacobson 1987). This azide then served to cross-link a radiolabelled AR antagonist suitable for the A_1AR protein, which could be visualized by gel electrophoresis. The sulfonyl fluoride group present in **158** was also a means of cross-linking a xanthine derivative to the A_1AR (Scammels et al. 1994; van Muijlwijk-Koezen et al. 2001).

6.2 *Spectroscopic Probes: Spin-Labelled and Fluorescent Probes*

Other types of reporter groups could be similarly incorporated into xanthine-functionalized congeners with retention of moderate AR affinity, for example chelating groups capable of complexing radioactive metal ions; spin labels for electron spin resonance spectroscopy, e.g. **167**; a perfluorinated acyl prosthetic group, as in **170**, intended for use in fluorine-NMR spectroscopy; and fluorescent dyes, e.g. **173** and **174** (Jacobson et al. 1987b). The fluorescent conjugate (**174**) of XAC and BODIPY [6-(((4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-yl)styryloxy)acetic acid], with an intermediate ε-aminocaproyl spacer, has proven useful in fluorescence correlation spectroscopy to characterize ligand complexes of the A_1 AR (Briddon et al. 2004).

Spin-labelled probes that retained high A_1 AR affinity were obtained by inserting the spin label into the molecule as part of the pharmacophore. The most potent and A_1-AR-selective compounds were the DPCPX analogues **155** (replacement of the 3-substituent by a spin label) and **156** (substitution of the cyclopentyl ring by a structurally related spin label). Both compounds showed affinity for the A_1 AR in the low nanomolar range combined with high selectivity compared with the other receptor subtypes (Ilas et al. 2005). The 1-propyl-8-phenyl derivative (**157**), in

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[a] *h* human, *c* cow, *d* dog, *gp* guinea pig, *p* pig, *r* rat, *rb* rabbit, *s* sheep
[b] Ilas et al. (2005)
[c] Jacobson et al. (1987b)
[d] Scammels et al. (1994)
[e] van Muijlwijk-Koezen et al. (2001)
[f] Ji et al. (1993)
[g] Jacobson et al. (1989a)
[h] Stiles and Jacobson (1988)
[i] Holschbach et al. (2002)
[j] Jacobson et al. (1992b)
[k] Stiles and Jacobson (1987)
[l] Jacobson (2009)
[m] Jacobson et al. (1987c)
[n] Jacobson et al. (1986b)
[o] Briddon et al. (2004)
[p] Jacobson, K.A., Kirk, K.L., Daly, J.W., Lipkowski, A.W., Rice, K.C., and Jacobson, A.A., unpublished
[q] Soriano et al. (2009)

which the spin label was integrated into the 8-substituent, showed good affinity for A_1 as well as A_{2B} ARs (Fig. 7, Table 7).

6.3 *Specialized Radioligand Probes Based on Conjugation*

Trifunctional probes derived from XAC were synthesized for the purpose of cross-linking to both a reporter group and the receptor (Boring et al. 1991). By this means, the xanthine would deliver a radioactive or spectroscopic prosthetic group to the receptor, to which it would react irreversibly by virtue of an electrophilic group such as an isothiocyante. This approach was illustrated with a series of analogues of *m*-DITC-XAC containing a third substituent in the phenyl isothiocyanate ring. For example, in **166** the third substituent contained a 3-(4-hydroxyphenyl)propionate moiety for radioiodination (Jacobson et al 1992b). This antagonist derivative effectively radiolabelled the bovine A_1AR in a covalent manner. Similar trifunctional xanthine probes for covalent labelling of ARs that furthermore contained a cleavable disulfide linkage within the chain linked to the xanthine moiety were reported (Jacobson et al. 1995). The intended strategy was to be able to remove the label after isolation of the modified receptor in order to regenerate the binding ability of the receptor.

6.4 *Xanthine Radioligand Probes for Positron Emission Tomography*

There is a need for the development of imaging agents based on high-affinity ligands for ARs. For example, ligands for in vivo PET imaging of A_1, A_{2A}, and A_3 ARs have been developed. The high-affinity A_1 AR antagonist DPCPX gave rise to the high-affinity analogue in which a terminal hydrogen of the 3-propyl group has been substituted with radiofluorine: 8-cyclopentyl-1-propyl-3-(3-[^{18}F]fluoropropyl)-xanthine ([^{18}F]CPFPX, **159**), similar in structure to DPCPX. This tracer is being developed for PET imaging of the A_1AR in the brain (Holschbach et al. 2002; Bauer and Ishiwata 2009).

PET ligands for the A_{2A} AR in the 8-styrylxanthine series that are structurally related to KW6002 have been developed: for example [7-methyl-^{11}C]-(*E*)-8-(3,4,5-trimethoxystyryl)-1,3,7-trimethylxanthine([^{11}C]TMSX) (Ishiwata et al. 2000a). This compound was alternatively named [^{11}C]KF18446 ([7-methyl-^{11}C]-(*E*)-8-(3,4,5-trimethoxystyryl)-1,3,7-trimethylxanthine; Ishiwata et al. 2000b, 2002, 2003a, 2003b). Ex vivo autoradiography for this molecule showed a high striatal uptake and a high uptake ratio of the striatum in comparison with other brain regions; [^{11}C]KF18446 was therefore proposed as a suitable radioligand for mapping the A_{2A}AR of the brain by PET (Mishina et al. 2007). In 2001 the synthesis and the testing of the 8-styrylxanthine derivative [^{11}C]KW-6002 as a PET ligand was reported. This molecule showed high retention in the striatum but it bound also to extrastriatal regions, so its potential as a PET ligand appeared to require further investigation (Hirani et al. 2001; Brooks et al. 2008).

In an earlier study, ^{11}C-labelled (*E*)-KF17837 was synthesized and tested, and it was proposed as a potential PET radioligand for mapping the A_{2A} AR in the heart and the brain (Ishiwata et al. 1996, 1997). Further studies on radiolabelled xanthine derivatives as A_{2A} AR radioligands were carried out by preparing and testing a ^{11}C-labelled selective antagonist, (*E*)-8-(3-chlorostyryl)-1,3-dimethyl-7-[^{11}C]methylxanthine [^{11}C](*E*)-8-*m*-chlorostyrylcaffeine). This molecule was shown to accumulate in the striatum, and PET studies on rabbits showed a fast brain uptake of [^{11}C] CSC, reaching a maximum in less than 2 min (Marian et al. 1999). A few years later, iodinated and brominated styrylxanthine derivatives labelled with ^{11}C were tested as in vivo probes (Ishiwata et al. 2000c). [7-Methyl-^{11}C]-(*E*)-3,7-dimethyl-8-(3-iodostyryl)-1-propargylxanthine ([^{11}C]IS-DMPX) and [7-methyl-^{11}C]-(*E*)-8-(3-bromostyryl)-3,7-dimethyl-1-propargylxanthine ([^{11}C]BS-DMPX) showed K_i affinities of 8.9 and 7.7 nM respectively, and high A_{2A}/A_1 AR selectivity values. Unfortunately, biological studies proved that the two ligands were only slightly concentrated in the striatum, and that they were not suitable as in vivo ligands because of low selectivity for the striatal A_{2A} ARs and a high non-specific binding (Ishiwata et al. 2000c).

6.5 Conjugated Ligand Probes and Bivalent Ligands

Three biotin conjugates (**161**–**163**) of 1,3-dipropyl-8-phenylxanthine (Fig. 7) were reported as being able to bind competitively to the rat A_1 AR, but in the case of **161** and **162** only in the absence of avidin. This was in contrast to similar conjugates of functionalized nucleoside agonists, which more readily bound simultaneously to both avidin and the A_1AR. The results were interpreted in terms of the possible reorientation of the ligands at the receptor binding site (Jacobson et al. 1985a; Jacobson 1990).

Two different pharmacophores, one being a xanthine AR antagonist, have been tethered with the intention to create a dual selectivity in a single functional unit. For example, XAC was coupled covalently through an L-Lys linker to a segment derived from the neurotransmitter peptide substance P to form a binary drug (**169**) (Jacobson et al. 1987c). The L-Lys linker served to increase aqueous solubility and to preserve A_1 AR by virtue of a free amino group in the spacer chain. Conjugate **169** bound to the rat A_1 AR with a K_i value of 35 nM and to the neurokinin type 1 receptor with a K_i value of 300 nM. Similarly, XAC was coupled to functionalized agonist ligands for opioid receptors, e.g. **175**, and for D_2 dopamine receptors, e.g. **176** (Fig. 7, Table 7) (Jacobson 2009; Soriano et al. 2009). Each of these conjugates bound effectively to both relevant receptors.

7 Conclusions

The pharmacological activity of the natural xanthines currently used in therapy, namely theophylline (as an antiasthmatic) and caffeine (as a CNS stimulant, for the treatment of apnoea in newborn babies and as an analgesic in combination therapy e.g. for the treatment of headaches) is mainly mediated by a (non-selective) inhibition of AR subtypes. AR-subtype-selective xanthine derivatives with high potency have been developed and evaluated in animal models and clinical trials.

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Theobromine and the Pharmacology of Cocoa

Hendrik Jan Smit

Contents

Abstract The effects of theobromine in man are underresearched, possibly owing to the assumption that it is behaviourally inert. Toxicology research in animals may appear to provide alarming results, but these cannot be extrapolated to humans for a number of reasons. Domestic animals and animals used for racing competitions need to be guarded from chocolate and cocoa-containing foods, including foods

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containing cocoa husks. Research ought to include caffeine as a comparative agent, and underlying mechanisms need to be further explored. Of all constituents proposed to play a role in our liking for chocolate, caffeine is the most convincing, though a role for theobromine cannot be ruled out. Most other substances are unlikely to exude a psychopharmacological effect owing to extremely low concentrations or the inability to reach the blood–brain barrier, whilst chocolate craving and addiction need to be explained by means of a culturally determined ambivalence towards chocolate.

Keywords Chocolate · Cocoa · Comparative · Craving · Liking · Myths · Pharmacology · Psychology · Theobromine · Toxicology

1 Background

Chocolate is an excellent example of a dichotomous food commodity. The current scientific and popular media focus on health issues has produced two conflicting health labels for chocolate – antioxidant benefits versus increased risk of weight gain. This is a change from the 1990s, when the focus was on a search for psychoactive constituents of chocolate that would explain not only its appeal, but also its craving-inducing, even its alleged addictive qualities. First phenylethylamine (PEA; Hamilton 1992) and later anandamide (Tytgat et al. 2000) were at the centre of this debate (see Sects. 4.1, 5, respectively).

The only pharmacologically active substance that has generally been ignored in this respect is theobromine, at least in part because of an early and persistent notion that it does not stimulate the central nervous system (CNS) (e.g. "does not show any central activity worth mentioning" – Czok 1974; "ineffective by itself" – Sprugel et al. 1977; "virtually inactive" – Rall 1980, p. 593; "behaviourally inactive" – Snyder et al. 1981; "possesses little pharmacological activity and is almost devoid of effects on the CNS and cardiovascular system" – Gates and Miners 1999; "does not affect the nervous system" – Bonvehi and Coll 2000). This may explain why relatively few studies or reviews on the effects of theobromine have been published, especially in comparison with caffeine. However, some recent findings have created a renewed interest in theobromine. Indeed, although at first glance there appear to be very few relevant publications on the effects of theobromine, the reader will notice that a surprisingly large number of studies and other communications surfaced as work on this chapter progressed.

The main aim of this chapter is to assess the role theobromine plays in the pharmacological activity of chocolate – the main supplier of theobromine to the human diet. In addition, other (potentially) pharmacologically active chocolate constituents will be discussed.

2 Theobromine

2.1 Characteristics

As a purified chemical, theobromine is a white powder, and is mainly produced from cocoa husks as a by-product of chocolate manufacture (The Merck Index 2006), although it can also be synthesised from (3-methyl-)uric acid (The Merck Index 2006; Thorpe 1893, p. 697). It is only very slightly soluble in water (1 g/2,000 ml) and alcohol (1 g/2,220 ml 95%), and only slightly more soluble in boiling water (1 g/150 ml), though it dissolves in dilutions of alkali hydroxides and in mineral acids (The Merck Index 2006; European Pharmacopoeia 2005; IARC 1991).

Theobromine is considered a diuretic, a smooth muscle relaxant, a myocardial stimulant and a vasodilator (Dorland's Illustrated Medical Dictionary 2007). Unlike caffeine, it is a very mild CNS stimulant (Mumford et al. 1994), and it has both antioxidant and pro-oxidant characteristics (Azam et al. 2003).

2.2 Natural Occurrence

Of all structurally related purine alkaloids (methylxanthines), theobromine is the predominant member present in chocolate (Apgar and Tarka 1998). Therefore, chocolate and other cocoa products are the main sources of theobromine in our Western diet. However, it can also be found in small quantities in tea (*Camilla sinensis*; Hicks et al. 1996), guarana (*Paullinia cupana*; Weckerle et al. 2003), mate (*Ilex paraguariensis*; Cardozo et al. 2007) and cola nut (Souci et al. 1981; Burdock et al. 2009), whilst its presence in coffee is negligible at a mere 10% of that in tea (see Table 1 for a general overview of theobromine content in foods).

Note that different tea varieties contain different typical levels of methylxanthines (Hicks et al. 1996). A relatively recently discovered tea variety, *Camellia ptilophylla*, is naturally free of caffeine, but contains high levels of theobromine instead – around 15–18 times the level in of green tea (Yang et al. 2007; He et al. 2009), hence its familiar name "cocoa tea". Likewise, cocoa bean varieties differ in their theobromine content, with Forastero varieties generally containing the highest amounts (Brunetto et al. 2007; Timbie et al. 1978), although some results do not agree with this (Hammerstone et al. 1994). See also Ashihara et al. (2010; Sect. 2.3), and Sect. 2.3.1 below.

2.3 Synthesis, Catabolism and Pharmacokinetics

The brief overview below cannot pretend to represent the complexities of this topic, although I have attempted to cover the most relevant and informative aspects. For a more detailed and in-depth approach, please refer to Ashihara et al. (2010) and Arnaud (2010).

Table 1 Theobromine content of various products. (After Smit and Rogers 2001)

Product	Portion size[a]	Concentration (mg per portion)
Chocolate, dark	50 g	378 (237–519)[b]; 221[c]
Chocolate, milk	50 g	95 (65–160)[b]; 94[c]
Cocoa powder	10 g	189 (146–266)[b]; 203[c]; 260[d] 206 (178–240)[e] 263 (219–284)[f]
Tea (regular, bag)	230 ml	3.1 (1.4–4.4)[b, g]
Coffee (filter/percolated)	7.6 g/200 ml	0.3 (0.3–0.3)[b]
Coffee (instant)	1.6 g/200 ml	0.2 (0.1–0.5)[b]
Cola drinks	Can (330 ml)	ND[h]

ND not detected

[a] MAFF (1988)

[b] MAFF (1998); figures recalculated using comments in Annex C of this reference where appropriate

[c] Craig and Nguyen (1984)

[d] Risner (2008)

[e] Bonvehí and Coll (2000)

[f] De Vries et al. (1981)

[g] This is in accord with values of first brew in Hicks et al. (1996).

[h] Dried kola nut contains 0.05–0.10% theobromine (Souci et al. 1981; see also Duke 1992 in Burdock et al. 2009)

2.3.1 Theobromine Synthesis and Catabolism in *Theobroma cacao*

In the cocoa plant, theobromine accumulates in young leaves, and the concentrations decline as the leaves mature (Koyama et al. 2003). In the cocoa pod, theobromine is synthesised in both the pericarp (fleshy, outer layer) and the cotyledons (seed embryos) of young cocoa fruits, though during the ripening phase, pericarp theobromine concentrations decline sharply, whilst cotyledon (cocoa bean) theobromine concentrations increase. This suggests that the major site of theobromine synthesis is the cocoa bean itself, whilst not excluding a minor role for theobromine migration between pericarp and cocoa bean (Zheng et al. 2004). Whilst theobromine is synthesised from AMP via xanthosine, it is metabolised by demethylation via xanthine, both in the cocoa bean (Zheng et al. 2004; see Ashihara et al. 2008 for a review) as well as in the cocoa leaf (Koyama et al. 2003).

Methylxanthine (including theobromine) concentrations in the cocoa bean are broadly variety-dependent, although publications do not always agree: Brunetto et al. (2007) found cocoa bean theobromine levels varying between 0.7 and 2%, with the highest levels found in the Forastero varieties, whilst the theobromine-to-caffeine ratios varied between 2 and 12, with Criollo, Trinitario and Forastero varieties shown in order of increasing theobromine-to-caffeine ratio. Likewise, Timbie et al. (1978) found cocoa bean theobromine levels of 1.2–3.9%, with the highest average levels found in Forastero, the lowest level in Criollo (which had the highest caffeine content) and increasing theobromine-to-caffeine ratios from Criollo (1.1) through to Forastero (75.1; recalculated from data provided). Hammerstone et al. (1994), however, provided entirely different figures for the same varieties.

Their highest average theobromine content was found in the Criollo varieties (2.3%), with Trinitario, Criollo and Forastero showing increasing theobromine-to-caffeine ratios. Although the analytical procedures are very similar between the publications, minor variations in these procedures may account for some of the differences found. However, the ripening stage at which fruit is picked (Timbie et al. 1978) and possibly also other factors such as growing conditions in terms of soil quality/composition and weather may all affect the methylxanthine content.

Whilst the cocoa beans are being processed (fermentation, roasting, etc.), the theobromine content changes mainly during the fermentation stage. During this stage methylxanthines migrate from the bean into the shell, causing a decrease in cocoa bean theobromine content of around 25% (Timbie et al 1978). Additionally, it is not unreasonable to assume that the microorganisms involved in the fermentation process could further reduce the theobromine content, as is the case with tea (Wang et al. 2008).

2.3.2 Theobromine Uptake, Metabolism and Pharmacokinetics in Man

Following oral administration in man, theobromine absorption from the digestive tract is slow, especially compared with caffeine, with an estimated peak plasma time of 2.5 h (compared with 0.5 h for caffeine) (Mumford et al. 1996). Moreover, theobromine absorption is not complete, at least in some people (less than 90%; Cornish and Christman 1957). Interestingly, the theobromine peak plasma time after chocolate consumption is somewhat faster at 2 h after consumption (Mumford et al. 1996). Although this seems counterintuitive because of plausible increases in the release time from the chocolate food matrix and binding to phenolic compounds (Czok 1974), Mumford et al. (1996) suggested the shorter theobromine peak plasma time following chocolate administration may be caused by stimulating bile production, shown in other studies to improve drug absorption. Note, however, that the same study reported slower caffeine uptake from both chocolate and cola. Despite the explanation provided for the latter (delayed gastric emptying), the plasma concentration curves for both foods are strikingly similar, and suggest a possible sucrose-mediated suppression of the excitatory effects of caffeine (Chauchard et al. 1945). Clearly, more research is needed to uncover the factors relevant to methylxanthine absorption from food.

In humans, methylxanthines are metabolised by demethylation (removal of methyl side groups) by the enzyme cytochrome P450 (CYP). Hence, theobromine (3,7-dimethylxanthine) is broken down to 3-methylxanthine and 7-methylxanthine by CYP. 7-Methylxanthine is then further metabolised into 7-methyluric acid by xanthine oxidase (this is not the case for 3-methylxanthine), whilst metabolism of theobromine into 3,7-dimethyluric acid and 3,7-diaminouracil is less well understood, although this is at least in part CYP-mediated (Gates and Miners 1999).

Note that theobromine does not metabolise into other dimethylxanthines (i.e. theophylline or paraxanthine), nor does it "upgrade" to the trimethylxanthine caffeine (Mumford et al. 1996), although the latter does happen in young leaves of the *Theobroma cacao* plant (Koyama et al. 2003). However, humans are exposed to theobromine though demethylation of caffeine, in addition to the ingestion of theobromine.

The clearance rate for acutely administered theobromine is around 1.2 ml/min/kg, around half of that of caffeine (Lelo et al. 1986), whereas after 4 days of chronic administration, Miners et al. (1982) found a clearance rate of 0.75 ml/min/kg. Likewise, Drouillard et al. (1978) found acute theobromine clearance rates of 0.94 ml/min/kg (1.47 after a 2-week methylxanthine abstinence), reduced to 0.81 after 5 days of chronic administration (figures calculated from published data), suggesting that the chronic exposure-related reduction in theobromine clearance is reversed after dietary theobromine abstinence (Drouillard et al. 1978). Note that interindividual differences in theobromine clearance rates may be substantial, as is the case for caffeine (Lelo et al. 1986 measured a 1.2 ± 0.4 ml/min/kg theobromine clearance rate; Balogh et al. 1992 measured 79% interindividual variance in caffeine clearance rates). Moreover, tobacco smokers have a substantially increased theobromine clearance compared with non-smokers (Miners et al. 1985).

2.4 Mechanism of Action

Although various effects of caffeine have in the past been attributed to the release of intracellular calcium and inhibition of cyclic nucleotide phosphodiesterases, ordinary human consumption of dietary methylxanthines would be insufficient to reach the levels needed for these processes to be activated (Fredholm et al. 1999). The main mechanism of action for methylxanthines has long been established as an inhibition of adenosine receptors (Snyder et al 1981; see Fredholm et al. 1999 for an extensive review). A range of secondary effects of adenosine antagonism may explain the variety of effects of methylxanthines on the human system in more detail. The interaction of adenosine A_{2A} receptors with dopamine D_2 receptors (Fredholm et al. 1999) is one such example. Interestingly, theobromine shows a much lower affinity for adenosine receptors than caffeine (Daly et al. 1983; Fredholm and Lindström 1999), which may explain why it is generally regarded as behaviourally inert. However, caffeine and theobromine show differential affinities for different adenosine receptor subtypes. Daly et al. (1983) found that theobromine is 2–3 times less active than caffeine as an adenosine A_1 receptor antagonist, but at least 10 times less active than caffeine as an A_2 receptor antagonist. Fredholm and Lindström (1999) gave similar values, but with a clear difference in caffeine-to-theobromine affinity ratios for stratium compared with cortex A_1 receptor antagonism (theobromine was found to be 4.7 and 11.8 times less active than caffeine, respectively). Nevertheless, the authors suggested caffeine and theobromine are non-selective receptor antagonists.

Interestingly, the much higher presence of theobromine in chocolate compared with that of caffeine (theobromine-to-caffeine ratio average 10; milk chocolate

11.3; dark chocolate 14.0; cocoa powder 9.0; recalculated from Tables 1 and 2 in Smit and Rogers 2001), clearly do not make up for the lower average adenosine receptor affinity of caffeine compared with that of theobromine (again, of around a factor 10 in Fredholm and Lindström 1999, akin to the difference in locomotor stimulation threshold between caffeine and theobromine reported by Snyder et al. 1981). Moreover, because A_1 receptors determine the effects of caffeine on fluid intake (Rieg et al. 2007), whilst the A_{2A} receptors play a role in the desire for caffeine (El Yacoubi et al. 2005), the differential affinities for different receptor types provide a possible explanation for the observation that caffeine and theobromine exert different effects. Note that additionally, the caffeine dimethylxanthine metabolites paraxanthine and theophylline have adenosine receptor affinities even stronger than caffeine (Daly et al. 1983; Fredholm and Lindström 1999), thereby explaining part of the effects of caffeine, whilst theobromine, also a dimethylxanthine, does not have such metabolites. Moreover, the reduced and delayed uptake of theobromine compared with that of caffeine may further diminish the in vivo effect of theobromine as an adenosine receptor antagonist in terms of its central and peripheral effects.

2.5 Effects in Animals

The effects of theobromine in animals as reported in the scientific literature can broadly be categorised into three groups: (1) toxicology studies; (2) case studies or reports of theobromine poisoning; (3) pharmacology studies; and (4) behavioural studies. Additionally, concern regarding the use of theobromine as a doping agent in equine and related sports has also penetrated the scientific literature.

Dietary theobromine intake in animals originates from two sources: (1) domestic chocolate and chocolate- or cocoa-containing foods as consumed by humans; (2) animal feed containing cocoa shell. The use of cocoa shell in animal has seen a drastic increase since the discovery that (1) it contains high levels of vitamin D, (2) its addition to the cattle's winter diet raised the vitamin D level to that which it typically is during the summer months, and (3) milk fat content was also raised when using this feed (Knapp and Coward 1934; Kon and Henry 1935; Golding and Burr 1937 in Dowden 1938). It is likely that experience from the use of this feed taught the equine sports that it was beneficial to animal performance, though this is not clear from the literature. Note that McDonald et al. (2002; p. 596) mentioned another feed derived from the cocoa bean, that is "extracted cocoa bean meal", which also contains theobromine and which the authors therefore also did not recommend being fed to racing horses. Moreover, the European Food Safety Authority has mentioned cocoa bean meal, cocoa husk meal, cocoa germs, cocoa bean shells and discarded chocolate confectionery as sources for animal feed in Europe (EFSA 2008).

2.5.1 Toxicology Studies

Toxicology studies mainly concern teratology and male reproductive toxicology, presumably following a study by Friedman et al. (1979), which reported testicular atrophy in nearly all rats fed caffeine or theobromine at a dietary concentration of 0.5% for over 14 weeks, although the detrimental effects in the caffeine condition were greater. However, Gans (1984) reported the reverse, that is, testicular atrophy and spermatogenic cell destruction following feeding with theobromine were much greater than they were following feeding with caffeine. Though the latter study used a dietary concentration of 0.8% theobromine with an exposure time of 7 weeks, subsequent studies switched to daily doses of 25–500 mg/kg body weight (Wang et al. 1992), and have shown similar effects for a shorter test duration, even after 2 weeks (Funabashi et al. 2000). Lower toxicity has been shown for cocoa powder containing the same amount of theobromine (Wang and Waller 1994). Additionally, Tarka et al. (1981) showed that when rats were fed chow containing 0.6 and 0.8% theobromine for 7 weeks, testicular weight decreased significantly compared with feeding with 0 and 0.2% theobromine. Moreover, they showed this effect was irreversible as measured during the subsequent 7 weeks. Although the underlying mechanism is unclear, its effects are seen also in terms of degeneration and necrosis in spermatogenic cells (Gans 1982; Wang and Waller 1994). Similarly to theobromine, cocoa powder at 5% of the diet showed testicular atrophy and decreased spermatogenesis (Tarka et al. 1991). The effects of theobromine on the male reproduction system described above have been validated in several other publications (Weinberger et al. 1978; Soffietti et al. 1989; Tarka et al. 1979). Note that similar atrophy effects have been observed for the thymus gland in rats (Tarka et al. 1979), appearing sooner than testicular damage (Gans 1982), and with theobromine producing higher decreases in thymus weight than caffeine (Gans 1984), though these effects were not found in dogs (Gans et al. 1980). Because this gland “plays an important role in cellular immunity by generating circulating T lymphocytes” (Nishino et al. 2006), the effects of theobromine reported on this gland may suggest an increase in overall immune response suppression.

The toxic effects of theobromine also include growth reduction and weight loss, possibly achieved through loss of appetite and food intake (Tarka et al. 1979).

Theobromine doses as low as 6 mg/day in the diet of mother mice reduces embryo weight and embryo tissue angiogenic activity (i.e. the rate at which new blood vessels are formed in growing tissue), and reduces neonatal relative limb size and spleen weight, suggesting that this is caused by a theobromine-induced reduction in the formation of new blood vessels in embryos (Chorostowska-Wynimko et al. 2004). The same research group showed a similar effect of chocolate (Skopinski et al. 2004) but attributed this to its epigallocatechin content owing to the correlations found between effect size and epigallocatechin content. Although this appears strange as the theobromine concentrations would have produced the same conclusion for theobromine and confirmed the results of their other publication of the same year, yet another study confirmed the link between dietary cocoa flavanol dose and embryonic (and tumour) angiogenesis (Wasiutynski et al. 2005).

Further studies will need to point out differential roles or mechanisms for these effects of theobromine and cocoa polyphenols, respectively, and evidence for similar effects in man ought to be sought. Nevertheless, Tarka et al. (1986a, b) pointed out that at much lower doses (25–200 mg/kg body weight/day), only their highest doses showed teratogenic effects (a delay in osteogenesis in rats; maternal toxicity/mortality, fetal malformations and osteogenic delays in rats), whilst the theobromine intake in these doses in rats and rabbits would be equivalent to an unrealistic human consumption of 7.5–10 lb (3.4–4.5 kg) milk chocolate/day, possibly explaining why no human teratogenic effects of theobromine have been reported. Alternatively, a 5% cocoa powder as used by Tarka et al. (1991) would not be impossible to implement in the human diet, though the effects of this on the male (and the female) reproduction system are unknown.

Interestingly, and in line with the findings reported above, angiogenesis in tumour growth is also inhibited by theobromine, an example of how a toxic effect can have a positive outcome (eBarcz et al. 1998; Gil et al. 1993). This effect is mediated through inhibition of adenosine receptors (Barcz et al. 2000) present in the carcinoma itself (Ryzhov et al. 2008) and their role in carcinoma hypoxia (Ryzhov et al. 2007), which would explain why similar effects are found with caffeine (Merighi et al. 2007). Conversely, theobromine intake has been associated with the prevalence of prostate (Slattery and West 1993) and testicular (Giannandrea 2009) cancer, although these associations were inconsistent over several decades Giannandrea (2009), and have not been tested further. Nevertheless, theobromine can reduce copper, thereby generating oxygen radicals (Shamsi and Hadi 1995 in Schmid et al. 2007). Moreover, because caffeine can impair DNA double strand repair (Sarkaria et al. 1999), it is possible this may also apply to theobromine, lending theobromine, as is the case for caffeine (Azam et al. 2003), both pro- and anticarcinogenic properties. Investigating the effects of cocoa powder, Tarka et al. (1991) found no evidence of a carcinogenic effect. However, the phenolic content of cocoa is likely to counteract any carcinogenic activity of other cocoa constituents (Lee et al. 2006; Jourdain et al. 2006).

Note that the toxic effects of theobromine may depend on other dietary constituents (e.g. protein content) and species-specific tolerance levels. Therefore, the marked differences in theobromine's toxic effects observed between animal species may make extrapolations to the human system very complex (Tarka et al. 1979), if not impossible.

2.5.2 Case Studies of Animal Poisoning

Many cases of animal poisoning reportedly result from the consumption of chocolate. Dogs, unlike cats, find chocolate a most palatable food, and are therefore most vulnerable to chocolate poisoning, especially when kept indoors. Strachan and Bennett (1994) reported acute cardiac arrest in a dog on the morning after the consumption of cocoa powder on the evening before, with an estimated theobromine exposure of 80 mg/kg body weight. Stidworthy et al. (1997), however, reported similar symptoms in two dogs who died within 1 h after an estimated consumption of 20–30 g dark chocolate each (using Table 1 and the reported average animal weight of 24 kg, this equates to an estimated theobromine exposure of 8 mg/kg), whilst two similar animals fed the same appeared unaffected. Interestingly, Gans et al. (1980) showed that acute doses of 200 mg/kg and less were not lethal. Other cases of dog poisoning have been reported following the consumption of garden mulch made of chocolate beans and shells, although these animals were successfully treated and recovered within 5 days (Hovda and Kingston 1994). The symptoms are varied, but include vomiting, restlessness, diarrhoea, haematuria (blood in urine), tachycardia (rapid heart beat) and hyperpnoea (deep breaths due to hypoxia) (Hovda and Kingston 1994), shivering and convulsions (Strachan and Bennett 1994), and panting, restlessness and muscle tremors (Gans et al. 1980).

However, deaths following the consumption of chocolate have also been found in wildlife. Reportedly, parrots (Gartrell and Reid 2007), foxes and badgers (Jansson et al. 2001), and undoubtedly more animal species, have been the victim of the consumption of chocolate left unattended.

Even the consumption of cocoa products as an ingredient in cattle feed or other animal feed (i.e. cocoa meal, cocoa husks or chocolate waste from the food or catering industry) can lead to livestock poisoning, even death (e.g. poultry – Black and Barron 1943; calves – Curtis and Griffiths 1972; ducks – Gunning (1950); fowl, ducks and horses – see Blakemore and Shearer 1943 for a review of several early cases).

The toxicity of chocolate to animals has inspired research into coyote pest control in the USA, resulting in an optimal mortality caffeine-to-theobromine ratio of 1:5 (Johnston 2005), not dissimilar to that of chocolate and other cocoa products, reconfirming the danger of this food in domestic animals. Note, however, that the latter publication reiterated the importance of the *combination* of caffeine and theobromine in the effects found, suggesting a focus on theobromine alone as the active toxicant it is not justified when toxic effects or death are caused by the consumption of chocolate.

2.5.3 Equine Sports and Theobromine Doping

In equine sports, caffeine and theobromine are considered doping agents owing to their stimulant effects. Hence, horse urine should not contain any caffeine (exposure detection level set at 0.1 μg/ml), whilst theobromine levels should not exceed 2 μg/ml (IFHA 2007). Although this appears to be a fairly generous level for a doping substance, this can be easily exceeded by feeding a horse 20 chocolate-coated peanuts per day (equivalent to 1.5 such peanuts per day for a human being on a weight basis), and could therefore be interpreted as extremely conservative (Budhraja et al. 2007). Logically, the use of by-products from the cocoa industry in horse feed also increases urine theobromine levels (Haywood et al. 1990), again increasing the risk of doping detection. Upon theobromine exposure, Delbeke and Debackere (1991) recommend a 2-day washout period to ensure urinary theobromine levels are below the legal threshold, although for other methylxanthine-containing foods, e.g., guarana, this may be insufficient: Salvadori et al. (1994) identified theobromine in horse urine up to 318 h (13 days) after guarana administration. Moreover, like many other drugs, toxins and trace elements and/or their metabolites, theobromine can also be detected in equine hair as a means for assessing drug history (Dunnett and Lees 2003). Whilst methylxanthine doping is also an issue in greyhound racing (Wells et al. 1988; Loeffler et al. 2000) it would be interesting to see if the relevant sports organisations will follow the example of the World Anti-Doping Agency of moving caffeine from the "Prohibited List" to the "Monitoring Program" for detecting patterns of misuse rather than imposing a ban. The reasons for this change include (1) the presence of a great interperson variability in caffeine metabolism, (2) the notion that above the traditionally used 12 μg/ml threshold level, caffeine has a detrimental effect on performance, but also (3) that lowering the detection threshold increases the risk of being penalised for consuming caffeine through everyday food and drink (WADA 2008). It is likely that some, if not all, of these arguments are applicable to dogs and horses, where chocolate treats and potential contamination of feed with cacao may impose more problems than the benefits for both racing organisations and competitors.

Much like horse racing in Western countries, camel racing is as important a sport in, for example, the United Arab Emirates, where methylxanthines are assessed in camel urine using a zero-tolerance approach in doping control (Wasfi et al. 2000).

2.5.4 Pharmacology Studies

Unlike toxicology studies, only a few studies have investigated theobromine metabolism in animals, one of which recorded this in detail in rats (Bonati et al. 1984), and did not find a clear difference between acute and chronic administration on the pharmacokinetics, though the absorption rates declined with increased theobromine doses. Shively and Tarka (1983) found that theobromine metabolism was slower in rats than in humans, whilst in rats it was not affected by pregnancy status. Moreover, in a study comparing five mammalian species (rats, mice, hamsters, rabbits and dogs), Miller et al. (1984) found that theobromine was most extensively metabolised in male mice and rabbits, and that theobromine metabolism shows only quantitative differences between species and sexes.

2.5.5 Behavioural Studies

Kuribara and Tadokoro (1992) reported that the mean 3-h post-treatment ambulatory activity in mice was increased after oral doses of both 10 mg/kg theobromine and 1 g/kg cocoa powder, whilst response rates were increased in the shuttle avoidance task at 3 mg/kg theobromine. However, the performance in the avoidance tasks was disrupted at 100 mg/kg theobromine or higher (Kuribara and

Tadokoro 1992). Similar results were reported by the same group in a different paper (Kuribara et al. 1992), where only the 10 mg/kg theobromine dose increased the avoidance rate in mice, and where at the 1,000 mg/kg dose, half of the mice died within a few hours. Because the measurements were taken over a 3-h period, this may explain why Snyder et al. (1981) found no effect on locomotor activity in mice at 5–100 μmol/kg (1–18 mg/kg) during their 1-h post-treatment observation. He et al. (2009), however, found no effects of 30 mg/kg theobromine or 200 mg/kg cocoa tea (*Camellia ptilophylla*; see Sect. 2.2) on ambulatory behaviour in mice during a 2h post-treatment observation period. Instead, they reported that only in combination with caffeine (as chemicals or as green tea) was a synergistic effect found compared with caffeine alone. Although the caffeine dose (10 mg/kg) at which the synergistic effects with theobromine were shown would have been unusually high in humans and surely not relevant to chocolate consumption, this study provides important evidence for furthering our understanding of the behavioural effects of the methylxanthines in chocolate. Conversely, Heim et al. (1971) and Sprugel et al. (1977) found that locomotive activity, oxygen consumption and brain cyclic GMP and cyclic AMP levels in white mice were affected by caffeine, but that this effect was prevented by theobromine, whilst theobromine itself did not affect these measures (Heim et al. 1971; Sprugel et al. 1977). Only 2–3 h after treatment did effects of theobromine alone occur (Heim et al. 1971). Moreover, after caffeine versus saline discriminative stimulus training in male Sprague-Dawley rats, several methylxanthines, but not theobromine, generalised to the caffeine cue at most doses tested (10–75 mg/kg for theobromine; Carney et al. 1985). These findings suggest that, at least in mice, the theobromine concentrations in chocolate may have a behavioural consequence, that this consequence is of an interactive nature with other methylxanthines, and that behavioural effects of theobromine may be delayed compared with those of caffeine.

Only a few other animal species have been the subject of investigations regarding behavioural effects of theobromine. After previously having identified some purines and other potentially behaviourally active substances from hornet queens, Ishay and Paniry (1979) investigated the effects of the main methylxanthines on hornet behaviour. They found that unlike the effects of purine and hypoxanthine, the effects of caffeine, theobromine and theophylline included nervousness, shaky movement and unsteady gait, reduced physical contact and positive geotropism, with no marked differences between the methylxanthines.

2.6 *Effects in Man*

Although theobromine is the most predominant methylxanthine present in chocolate, research into the effects of theobromine in man is relatively scarce compared with that into the effects of caffeine, and compared with research in animals. This section aims to present the research on theobromine in man to date.

2.6.1 Psychopharmacological Effects

Several inappropriately substantiated popular claims about the psychopharmacological activity of chocolate constituents (e.g. PEA, see later) resulted in the investigation of the ecological potential of a range of such substances (Smit and Rogers 2001). It was concluded that caffeine and theobromine were the only likely substances to play a role in the psychopharmacological activity of chocolate. This idea was confirmed when the same authors (Smit et al. 2004) showed that the combination of caffeine (19 mg) and theobromine (250 mg) contained in a 2-oz bar (approximately 50 g) of dark chocolate has significant effects on energetic arousal, reaction time and information processing. Subsequent work reported that the same combination of methylxanthines increased the liking for the flavour of a 'novel' drink when combined with the (encapsulated) active substances compared with an encapsulated 'placebo' (Smit and Blackburn 2005). These results show a role for chocolate methylxanthines in our liking for chocolate. Additionally, they provide a very clear explanation for why we prefer milk chocolate over white chocolate, and why dark chocolate is an easily acquired taste. However, a study comparing the individual effects of caffeine and theobromine with the effect of their combination (as used in Smit et al. 2004) using identical, ecologically valid amounts has not been performed to date. Such a study would clarify whether the effects found are either solely or partly attributable to caffeine, and whether caffeine and theobromine provide an additive or synergistic effect.

Only a very few early publications have reported individual and combined effects of caffeine and theobromine. Dorfman and Jarvik (1970) gave volunteers 300 mg caffeine and/or 300 mg theobromine before the volunteers retired for the evening. Those in the caffeine and caffeine + theobromine condition showed a longer sleep latency and lower sleep quality than those in the theobromine condition. Additional data confirmed that sleep latency increases were related to caffeine dose and not to theobromine. Finally, they did not find any interactive effects of the two methylxanthines.

In a study of a more exploratory nature, Mumford et al. (1994) provided some valuable insights into the comparative effects of caffeine and theobromine on mood and cognition by investigating their subjective effects. Despite the small sample size ($N = 7$), and the use of relatively high doses of methylxanthines [the doses used were the lowest discriminable caffeine dose in the least sensitive volunteer (178 mg) and the highest tolerated dose of theobromine by the most sensitive volunteers (560 mg)], this study presented some very interesting and important findings. First of all, it shows how theobromine possesses caffeine-like qualities by means of the subjective effect descriptions of the most theobromine sensitive participant: "Energy", "Motivation to work", "Alert", "Sleepy" (decreased), whilst these effects were emphasised by an additional effect on the measure "Magnitude of drug effect". Second, the discrimination threshold phase of the study showed a wide range of reliable discrimination thresholds amongst the volunteers, although this was not further investigated. This study only provided limited information with regard to the role of the individual methylxanthines in the psychopharmacological effects of chocolate, although clearly a role for the effects of theobromine cannot be ruled out, and may depend on the individual's sensitivity to these effects.

Further evidence for caffeine-like effects of theobromine, albeit anecdotal, was provided by Ott (1985; pp. 79–80), who replaced his dietary caffeine intake with a daily dose of 600 mg theobromine (200 mg in the morning, afternoon and evening) for 7 days. Upon acute theobromine deprivation, the author described how he "developed a tension headache, muscle tension in his shoulders and neck, and became extremely lethargic" within 16 h. These symptoms were reversed within 60 min of the consumption of another 200-mg dose of theobromine, suggesting that the symptoms were that of theobromine withdrawal. Because this one-man experiment was not performed according to double-blind conventions, Ott advocated that the scientific community carry out a proper study looking into these effects.

In summary, theobromine produces only very minor subjective effects compared with caffeine. In sensitive individuals these effects may be more marked, but can also be detrimental in the form of headaches (Mumford et al. 1994), as can caffeine. However, anecdotal evidence suggests that theobromine behaves like caffeine by means of its capability of producing withdrawal and providing subsequent withdrawal-reversal effects. Unfortunately, no data on the effects of theobromine on mood and cognition in humans other than those presented above have been reported, confirming that this area is seriously underresearched. Although the psychopharmacological effects of theobromine may be smaller than those of caffeine, they have been reported. Taking into account habitual caffeine and theobromine intake, and discriminable and/or tolerable doses, these may help to provide a more sensitive method for uncovering clearer effects of theobromine on mood and mental performance.

2.6.2 Physiological Effects

Cardiovascular

Theobromine is generally regarded both as a bronchodilator and as a vasodilator (Reynolds 1993) and may therefore have an effect on the heart. Indeed, Czok (1974) claimed theobromine provides an effect of medium strength on the heart in general, an effect less strong than the related theophylline, but stronger than caffeine. However, no more precise explanation than that was provided, nor were any citations listed. Effects of theobromine on the heart were confirmed by anecdotal evidence reported in Ott (1985, p. 82), where the author described experiencing cardiac-stimulating effects of an oral dose of 200 mg theobromine within 15 min of administration. Interestingly, theobromine has also been prescribed for relief from pain caused by angina pectoris in some patients, presumably by means of its vasodilating effects (Dock 1926). Although Baron et al. (1999) did not find any cardiac or haemodynamic effects of theobromine, it is possible that the cocoa polyphenols in their chocolate may have obscured any theobromine-related effects.

Note that other studies have also investigated haemodynamic effects of chocolate, but attributed these effects to cocoa polyphenols (Taubert et al. 2003; Grassi et al. 2005) whilst not taking into account the potentially confounding effects of theobromine, although Kelly (2005) argued for this to be addressed.

Geraets et al. (2006) found strong inhibitory effects of theobromine on the activity of the nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1), which is implied in acute and chronic inflammatory diseases such as stroke, ischaemia–perfusion and diabetes, and implied in chronic obstructive pulmonary disease. For this reason, they emphasised that methylxanthines (including theobromine) with higher PARP-1 inhibition rates are potentially helpful dietary agents in the treatment of vascular dysfunction and inflammation.

On balance, very few studies have been published investigating cardiovascular effects of theobromine, though some limited evidence suggests that theobromine exerts cardiovascular effects by means of vasodilation and cardiac stimulation. Because the effects of caffeine on cardiovascular functions are expressed though noradrenalin release from sympathetic nerves acting on α_2-adrenergic receptors, with a possible, but much less important role for adenosine (A_1) receptor antagonism (Fredholm et al. 1999), similar effects of theobromine can be expected, although possibly of lower magnitude. Finally, further research is needed to validate the hypothesis that theobromine can be used for the prevention and treatment of vascular dysfunction and inflammation.

Respiratory

Theobromine improves bronchodilation in asthma patients (Simons et al. 1985), although this effect is stronger with theophylline and caffeine (Becker et al. 1984). However, note that the order of bronchodilation efficacy for these three methylxanthines is different in Apgar and Tarka 1999, who listed theobromine as stronger than caffeine for this effect. Presumably owing to its superior diffusion in bronchial tissue (van Zyl et al. 2008), theophylline (1,3-dimethylxanthine) is still used as a medication for asthma patients but can have serious side effects (Barnes and Pauwels 1994; El-Bitar and Boustany 2009), whilst caffeine and theobromine are not in use as such. Nevertheless, caffeine does improve lung function (Bara and Barley 2001), as is also supported by epidemiological evidence (Pagano et al. 1988), suggesting that asthma and bronchitis patients may be self-dosing on caffeine to relieve symptoms, even if this is subliminally achieved by means of positive reinforcement. Because theobromine *also* improves lung function (10 mg/kg; Simons et al. 1985), there may be a similar role for the consumption of chocolate in the relief of asthmatic symptoms. Indeed, I have anecdotal evidence (from a personal acquaintance whose partner is suffering from asthma) of a clear association between periods of heightened asthmatic symptoms and a marked increase in consumption of both chocolate and cola drinks. Interestingly, it was not the patient herself, but was her partner who became aware of this association. Note that whilst the tobacco industry claims to add cocoa powder to cigarettes as a flavouring agent, it may also conveniently serve to enhance the uptake of nicotine (and thereby increase the addictive property of tobacco) through the bronchodilating properties of theobromine (Rambali et al. 2002), as well as possibly suppressing smoke-induced cough reflex (see below).

A complementary beneficial effect of theobromine on the airways relates to theobromine's more recently identified cough reflex suppressant ("antitussive") properties through suppression of vagus nerve activity. This effect was shown in response to both inhalation of a citric acid aerosol in guinea pigs and to inhalation of a capsaicin aerosol in humans. Interestingly, although there was no clear difference between theobromine and codeine in suppressing citric acid induced cough in guinea pigs, the suppressant effects of theobromine on cough induced by a capsaicin aerosol in human volunteers were greater than those of codeine. Moreover, and unlike codeine, theobromine was free from side effects (Usmani et al. 2005), an important notion in the context of a strong need for antitussives without side-effects (Chung and Chang 2002). This could lend theobromine a direct medical application in the reduction of cough, as cough is a common symptom in cancer (Walsh et al. 2000), and usually responds well to one or more medications (Table 1 in Estfan and Walsh 2008). This application could be extended to chocolate, where a corresponding portion of dark chocolate could reduce the cost of conventional medicine where proven effective. Hence, dark chocolate is currently being investigated as an alternative to medicine for its potential to reduce cough in cancer patients for whom cough is a troubling symptom (Halfdanarson and Jatoi 2007).

Though seemingly related, not in the least by the effect theobromine has on either, the regulation of smooth muscle relaxation and that of cough suppression are different. Smooth muscle relaxation is regulated by β_2-adrenergic receptors (whereby β_2-adrenergic receptor agonists acutely improve bronchodilation, although chronic exposure can have detrimental effects on the control of asthma; Lipworth and Williamson 2009), and by adenosine receptors (Brown et al. 2008). Although methylxanthines have bronchodilating effects and act as adenosine receptor antagonists, this may not be the main mode of action for theobromine as a bronchodilator or smooth muscle relaxant. Indeed, Lunell et al. (1983) reported strong bronchodilator effects of a xanthine derivative without any CNS effects. However, both A_1 and A_{2B} receptors have been implied in the pathogenesis of asthma, and although roles for A_{2A} and A_3 receptors are likely, they are still unclear (Brown et al. 2008).

The cough reflex is triggered by three different kinds of sensory nerve receptors in the respiratory tract, whose signals are relayed via the vagus nerve and the brainstem to the "cough centre" or "central cough generator", where the physical cough response is coordinated (Chung and Pavord 2008). Although current antitussives are mainly opiates and opiate derivatives acting on the central cough pathway, their side effects call for the development of other substances that achieve the same goal through different mechanisms. Therefore, new and proposed antitussives acting centrally may target sigma or GABA receptors, or act through other mechanisms yet to be identified (Reynolds et al. 2004). Alternatively, they may act peripherally by directly targeting neuronal pathways, for example, ion channels, nerve fibres and relevant receptor sites (Chung and Chang 2002; Reynolds et al. 2004). Moreover, guinea pig sensory nerve activity and human sensory nerve activity in the airways are inhibited by activating cannabinoid CB_2 receptors (Patel et al. 2003; Belvisi et al. 2008). Although Usmani et al. (2005) showed that theobromine also inhibits guinea pig vagus nerve activity, its modus operandi has not been established. Nevertheless, the authors suggested that theobromine is likely to exert its effect through suppression of phosphodiesterase activity and by inhibiting bronchoconstricting adenosine A_1 receptors, though alternative modes of action (e.g. activation of Ca^{2+}-activated K^+ channels) cannot be ruled out (Usmani et al. 2005).

Concluding, both the antitussive and the bronchodilating effects of theobromine are at least in part related to the adenosine receptor antagonistic properties of theobromine as part of the methylxanthine family, and it could be that different effects are expressed through different adenosine receptor subtypes and though other receptors, such as β_2-adrenergic receptors. The exact pathways for the bronchodilating and antitussive effects of theobromine are unclear, and whilst other pathways may be involved, further investigation is clearly needed to clarify this topic.

Renal

Because adenosine plays an important role in regulating blood flow, it also plays an important role in renal haemodynamics, affecting renal blood flow and glomerular filtration rates. The renal vascular system, however, unlike the main vascular system, is regulated by adenosine A_1 receptors in addition to A_2 receptors (see Hansen and Schnermann 2003; Vallon et al. 2006 for reviews of the role of adenosine in the kidney). The finding that A_1 receptors also determine the effects of caffeine on fluid intake (Rieg et al. 2007) may be related to this. Because theobromine has a lower overall adenosine receptor affinity than caffeine and theophylline, though all three methylxanthines are non-selective adenosine antagonists (Fredholm and Lindström 1999), a small, but possibly insignificant, diuretic effect of theobromine would be predicted not to be functionally different from the other methylxanthines. Indeed, despite a previous and unjustified claim that theobromine has a stronger effect on the kidney than caffeine (Czok 1974), Dorfman and Jarvik (1970) and Massey and Whiting (1993) reported that unlike caffeine, oral administration of 300 mg theobromine did not increase urinary calcium or sodium excretion, although Dorfman and Jarvik (1970) found no change in the overnight urine volume following oral administration of 300 mg caffeine or 300 mg theobromine compared with ‘no drug’.

Dental

The consumption of chocolate, as a sugar-containing confectionery, is inevitably associated with dental caries (i.e. chocolate is seen as a cariogenic food). However, both theobromine (added to the diet in hamsters – Strålfors 1967; applied to human teeth in vitro – Sadeghpour 2007), and cocoa (reviewed in Naylor 1984) reportedly inhibit dental caries. Sadeghpour (2007) found that regular exposure to theobromine increased the enamel surface microhardness compared with sodium fluoride, and helped surface recrystallisation. Kashket et al. (1985) found that defatted cocoa inhibits dental plaque formation, as did cocoa extracts (Srikanth et al. 2008) and cocoa polyphenol extracts (Percival et al. 2006). Whilst the preparations used in the work reported in the latter publication may or may not have been free of methylxanthines, the authors did not make any reference to this, and also other publications have reported effects of polyphenol-containing drinks on plaque formation without referring to its methylxanthine content (Hannig et al. 2009). Whilst there may also be a role for caffeine in combating dental caries (Strålfors 1967), it is likely that methylxanthines and polyphenols may have an effect on dental caries by means of separate mechanisms, suggesting that a combined application may be more beneficial, although more research is necessary to confirm this. The strong inhibition of the metabolic activity of anaerobic bacteria by fluoride in wastewater treatment (Ochoa-Herrera et al. 2009) may well prove to be another decisive factor for the promotion of theobromine- and polyphenol-containing toothpaste in the near future.

2.7 Therapeutic Applications

Theobromine is currently not in use as a medicinal drug. However, *Stedman's Medical Dictionary* (Stedman's Medical Dictionary 1976) describes theobromine as "used as a diuretic, myocardial stimulant, dilator of coronary arteries, and smooth muscle relaxant" and according to Landau (1986), theobromine was used to treat arteriosclerosis and some peripheral vascular diseases, whilst Reynolds (1993) added angina pectoris and hypertension to this list. Rall (1980), however, mentions that it has almost disappeared from the medical scene owing to its low effectiveness in its pharmacological actions compared with caffeine and theophylline, and whilst Tarka (1982) wrote that there was no therapeutic use for theobromine, Simons et al. (1985) mentioned its use in antiasthma medication.

Recent research, however, has identified theobromine as a PARP-1 inhibiting (Geraets et al. 2006), dental enamel strengthening (Sadeghpour 2007) and antitussive (Usmani et al. 2005) agent (see earlier), suggesting there is still a future for theobromine as a medicine, preventative or curative.

3 Caffeine

Unlike theobromine, the effects of caffeine have been extensively investigated. Absorption of caffeine is rapid and complete following oral administration, though in the presence of sugar, absorption is slower but still complete (Yesair et al. 1984), and the maximum blood plasma concentrations (peak plasma time) are reached within 1 h (James 1991). Indeed, after oral administration of 72 mg caffeine, Mumford et al. (1994) found an onset of subjective effects at 21 min (10–45 min) followed by a caffeine peak plasma time at 30 min after treatment. By means of its adenosine receptor antagonistic properties, caffeine stimulates the CNS and increases blood pressure, respiration, lipolysis, renin and catecholamine release, urine output, and intestinal peristalsis (Landau 1986; James 1991).

Consumption of excessive amounts (more than 1 g/day or more than ten cups of strong coffee per day) can result in tachycardia, dyspepsia (disturbed digestion, decreased appetite, oppressive feeling in the stomach and unpleasant taste), irritability and insomnia, also referred to collectively as “caffeinism” (Landau 1986). Other publications have described symptoms following the intake of high doses of caffeine as “signs and symptoms indistinguishable from those of anxiety neurosis”, and nervousness, irritability, tremulousness, occasional muscle twitching, insomnia and sensory disturbances (Tarka 1982) and “a variety of unpleasant subjective states including anxiety, dysphoria and depression” (Mumford and Holtzman 1991).

As a psychostimulant, caffeine increases feelings of energy (more alert, less tired, etc.) and improves other aspects of mood, and enhances psychomotor and cognitive performance when taken in amounts consumed in coffee and tea (Rogers and Dernoncourt 1998; also reviewed in James 1991). Because caffeine reverses overnight caffeine-withdrawal symptoms, which include headache and lethargy (reviewed in Smit and Rogers 2007), it is a powerful (“negative”) reinforcer in learned behaviour as indicated, for example, by its ability to increase flavour preference (Rogers et al. 1995; Yeomans et al. 1998). It is this ability which is thought to lie at the heart of the fact that coffee and tea are the world’s most popular and widely consumed drinks despite their innate bitterness. Because doses as low as 12.5 mg caffeine have shown behavioural effects (Smit and Rogers 2000), and because such amounts are present in easily consumable portions of chocolate (despite their much higher presence in tea and coffee; see Smit and Rogers 2001), one can only assume that caffeine in chocolate has pharmacological activity, and that caffeine reinforcement could contribute to our liking for chocolate.

4 Biogenic Amines

Cocoa and cocoa products contain biogenic amines (e.g. PEA, tyramine, tryptamine and serotonin) and their precursors (phenylalanine, tyrosine and tryptophan) in fairly high concentrations, which increase during fermentation of the cocoa beans, and decrease during roasting and alkalisation (Ziegleder et al. 1992). In general, these concentrations are irrelevant in healthy people, since biogenic amines are metabolised by the monoamine oxidase (MAO) enzymes in the mucosa of the small intestine, and in the liver and kidneys (Askar and Morad 1980). Because of the endogenous abundance of MAO enzymes, “even the intraduodenal injection of amines in the absence of enzyme inhibition would be unlikely to lead to their absorption and appearance in systemic blood unless the amount was sufficiently large to swamp the deaminating mechanisms" (Marley and Blackwell 1970). The effects of biogenic amines are therefore only expressed in people with an MAO deficiency, as has been suggested for migraine sufferers (Marley and Blackwell 1970), and in patients receiving medication containing MAO inhibitors (Askar and Morad 1980). These effects, however, can include headaches, increased blood pressure and even a life-threatening "amino shock" (Askar and Morad 1980). Realistically, these adverse effects would presumably lead to the avoidance of chocolate rather than provide an explanation for cravings for chocolate, yet their endogenous biological function may have provided an alleged basis for any wrongfully presumed positive effects. The biogenic amines considered in the following sections have been discussed in the scientific and popular media in this respect.

4.1 Phenylethylamine

2-Phenylethylamine, or ß-phenylethylamine (PEA), is the basic molecule or structure for all compounds that make up the PEA family. This includes the stimulant and hallucinogenic substances amphetamine and mescaline, and the endogenous neurotransmitters dopamine, adrenalin and noradrenalin (Passmore and Robson 1970). Although it has been assumed that chocolate contains large amounts of PEA (e.g. 6 mg/100 g according to the British Food Manufacturing Industries Research Association, cited in Sandler et al. 1974), more recent works suggest much smaller amounts (Koehler and Eitenmiller 1978; Ingles et al. 1985; Hurst and Toomey 1981, with a maximum observed concentration of 0.66 mg/100 g for one particular (milk) chocolate sample – Hurst and Toomey 1981).

Endogenously, PEA occurs in minute quantities (single nanograms per gram of nervous tissue) in the mammalian brain, where it is synthesised by decarboxylation of phenylalanine, almost certainly in dopaminergic neurones. It appears to coexist in the brain with dopamine, and is proposed to be a modulator of catecholamine neurotransmission, though it is rapidly metabolised by MAO type B (Paterson et al. 1990).

Although low levels of endogenous PEA have been linked to depression and high levels have been linked with mania, the evidence for this is mixed and inconclusive (Davis and Boulton 1994). Even so, Liebowitz and Klein (1979) identified an affective disorder involving atypical depression and attention-seeking behaviour ("hysteroid dysphoria") and linked this to an abnormal regulation of PEA. Whilst the authors did not refer to any published evidence, they claimed that "depressed, hysteroid dysphorics often binge on chocolate, which is loaded with phenylethylamine", and that the production of PEA is "stimulated by positive life events". Moreover, PEA has been linked to the euphoric feelings that are part of courtship and sexual activity, mainly on the basis of animal experiments where PEA was injected into the brain (Kohl and Francoeur 1995, after Liebowitz 1983; see also Crenshaw 1996). This, in combination with the notion that PEA is the basic structure of all amphetamines, has led the popular media to link PEA with romance, love and sex, branding PEA a "love drug", making chocolate a "sex substitute".

Obviously, oral consumption and cerebral injection are entirely different modes of administration, and the idea that people eat chocolate to feel "sexier" or more "sensual" because eating chocolate raises endogenous PEA is simply a myth. However, overlooking this distinction may have been used as a convenient tool for the popular media to promote chocolate as a "sex substitute", a message further reinforced when a calculation error resulted in suspiciously high PEA concentrations in chocolate (Hamilton 1992; a value of 660 mg/100 g chocolate miscalculated by a factor of 1,000 from either Table 9 in Hurst and Toomey 1981 or from Table 3 in Hurst et al. 1982, same data). Note that PEA is still freely used to commercially promote the sales of PEA as a nutraceutical, e.g. http://www.americannutrition.com/store/Nootropics.html, accessed 6 August 2009.

On the bais of the evidence available, it is very doubtful that oral intake of PEA causes any beneficial psychopharmacological effects. Indeed, when assessing the effects of a large variety of synthesised amphetamines, administered (usually orally) in various doses, Shulgin and Shulgin (1991) were surprised to find that only PEA did not induce any subjective effects, either orally (200–1,600 mg) or intravenously (25–50 mg). Clearly, PEA needs side groups to function as an active amphetamine, and these findings further substantiate the "PEA myth" of chocolate.

4.2 Tyramine

Tyramine is present in a variety of foods, but its levels in chocolate are relatively low and are akin to those of PEA (Koehler and Eitenmiller 1978; Ingles et al. 1985; Hurst and Toomey 1981). Like PEA, tyramine has also been implicated in migraine attacks, and in the "cheese reaction" (*tyros* is Greek for "cheese"; Passmore and Robson 1970): prescribed in the late 1950s and the 1960s for depression and hypertension, MOA inhibitors made patients sensitive to the toxic effects of tyramine, found in some cheeses in relatively high amounts – up to 62.5 mg/100 g was measured by Ingles et al. (1985) and ten Brink et al. (1990). Symptoms of the "cheese reaction" included hypertensive crisis and severe headache, sometimes even leading to intracranial bleeding or cardiac failure (Joosten 1988). However, there appears to be no published evidence suggesting any beneficial effects of tyramine on mood and behaviour.

4.3 Serotonin and Tryptophan

As a neurotransmitter in the CNS and the peripheral nervous system, serotonin (5-hydroxytryptophan) plays an important role in the regulation of mood and behaviour (Young 1993). Although it has been identified in a range of foods, bananas, pineapples and chocolate contain somewhat higher than average concentrations (2.5, 4.2 and 2.7 mg/100 g – averages calculated from Smith 1981; Marley and Blackwell 1970; Hurst and Toomey 1981), although the highest concentrations of serotonin have been found in walnuts (55 mg/100g; Smith 1981). Note that as for all biogenic amines, also serotonin is metabolised rapidly after oral intake, and consumption of foods containing serotonin will not directly affect brain levels of serotonin. This fits with the observation that cravings for walnuts are not common, certainly when compared with the prevalence of cravings for chocolate.

As a *precursor* of serotonin, the amino acid tryptophan is *not* prone to deamination. However, large pharmacological doses of tryptophan (much larger than our normal dietary intake of 1–1.5 g/day) can be an effective antidepressant (Young et al. 1986), which is consistent with the idea that a deficit in serotonergic activity is important in the vulnerability to depression (Maes and Meltzer 1995). Likewise, tryptophan has shown improvements in depressive symptoms in seasonal affective disorder (McGrath et al. 1990) and premenstrual syndrome (Steinberg et al. 1986), and people prone to depression show deteriorated mood following the administration of tryptophan-depleted mixtures of amino acids (Young et al. 1986; Benkelfat et al. 1994). Although these studies suggest a clear role for the serotonergic system in the cause of depression, altered brain levels of tryptophan and therefore serotonin are not expected to occur when tryptophan is consumed through the regular diet owing to competition for uptake into the brain with other large neutral amino acids (Young 1993; Rogers 1995). It is therefore extremely unlikely that any mood changes that may arise from the consumption of chocolate are caused by its tryptophan content.

5 Anandamide

Anandamide (arachidonylethanolamide), an endogenous ligand for the cannabinoid receptor that binds competitively to brain cannabinoid receptors, has been identified in minute concentrations (0.05 μg/g) in chocolate, where this compound is contained in the cocoa solids, as its presence was not confirmed in white chocolate (di Tomaso et al. 1996). Unsubstantiated, the authors suggest that anandamides present in food might “heighten sensitivity and produce euphoria” and in doing so, intensify the orosensory effects of chocolate. However, the bioavailability of anandamide is no more than 5% (Di Marzo et al. 1998). Note also that Δ^9-tetrahydrocannabinol, the main psychoactive compound in cannabis, showed a noticeable “high” in human volunteers at doses as low as 18.77 μg/kg body weight (equivalent to 1.3 mg for a 70-kg person) (Perez-Reyes et al. 1973). It then follows that, even if one were to make the generous assumption that anandamide is as bioavailable, stable and potent (magnitude of drug effect) as Δ^9-tetrahydrocannabinol, a blood plasma concentration of 18.77 μg/kg body weight can only be achieved by consuming 25 kg chocolate in a single sitting – a most uncomfortable, if not impossible task with potentially lethal consequences. This therefore also contradicts the suggestion of di Tomaso et al. that their findings “point to an unexpected link between non-drug craving and the endogenous cannabinoid system”. The fact that a cannabis user tried to convince the court of having consumed “a massive amount of chocolate" in defence against the accusation of using and supplying cannabis (this involved a positive routine urine test; Tytgat et al. 2000) only confirms how the discovery of di Tomaso et al. resulted in yet another myth about our liking and cravings for chocolate.

6 Salsolinol and Tetrahydro-ß-carbolines

Salsolinol (SAL) and tetrahydro-ß-carbolines (THBCs) are neuroactive alkaloids generated endogenously following the consumption of alcohol through a reaction between the primary alcohol metabolite acetaldehyde and dopamine to create SAL,[1] or between acetaldehyde and indoleamines (e.g. serotonin, tryptamine, tryptophan) to create THBCs (Quertemont et al. 2005). Both SAL (Haber et al. 2002) and THBCs (Myers 1989) have been implied as an important factor in alcoholism, and investigated as such (Quertemont et al. 2005).

Additionally, SAL and THBCs have been identified in chocolate: SAL has been found in milk and dark chocolate and cocoa at 5, 20 and 25 mg/kg respectively; Melzig et al. 2000), whilst THBCs were identified in comparable though slightly lower amounts (1.4, 5.5 and 3.3 mg/kg respectively; Herraiz et al. 1993). In part driven by their implication in alcoholism, SAL (Melzig et al. 2000) and THBCs (Herraiz 2000) have independently been named as potentially involved in cravings for chocolate.

Again, also here, a role for SAL and THBCs in the cause of chocolate cravings would require that their consumption results in raised blood plasma levels of these compounds. Unfortunately, the literature is not clear whether this occurs or not. Even if they could be freely absorbed, THBCs are also mild MAO inhibitors (see Sect. 4 for MAO inhibition), potentially amplifying the effects of biogenic amines in chocolate and thereby contributing to migraines following the consumption of THBCs (Baker et al. 1987 in Herraiz 2000; Herraiz and Chaparro 2006) or SAL (Heikkilla et al. 1971 in Melzig et al. 2000) in chocolate. Furthermore, although SAL reportedly shows positive effects on heart rate and muscle contractions (Sokolova et al. 1990 and Chavez-Lara et al. 1989, respectively, in Melzig et al. 2000), again the route of administration is not clear. Finally, a particular THBC has been found in the tubers of the South American maca plant (*Lepidium meyenii* Walp.). Whilst this plant has been ascribed various therapeutic benefits, and is used by athletes as an alternative to anabolic steroids (Brack Egg 1999 in Piacente et al. 2002), it is perfectly possible that these effects are related to one or more of the other maca constituents, especially glucosinolates.

On the basis of the evidence available, it is unlikely that chocolate cravings can be induced by SAL or THBCs. Like PEA, tyramine and anandamide, SAL and

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[1] SAL is the most widely researched example of a tetrahydroisoquinoline; tetrahydroisoquinolines are formed from acetaldehyde and catecholamines (Quertemont et al. 2005*)*

THBCs may have to be added to the ever-growing list of myths surrounding this topic as there is no direct evidence indicating biological activity through oral intake of either substance.

7 Magnesium

Chocolate has been mentioned as a relevant source of dietary magnesium (Gibson 1990). Indeed, according to some publications, it has one of the highest magnesium levels of all foods listed (Seelig 1989; Rozin et al. 1991). Moreover, magnesium therapy has been claimed to reduce premenstrual tension (Abraham 1980) and to reduce chocolate cravings in women on hormone replacements (Roach 1989; A. Weil, personal communication). Although this appears to indicate an explanation for "why women crave for chocolate at that particular time of the month", the following findings need to be taken into consideration before any such claims can be made.

First of all, dark chocolate contains 90–100 mg magnesium/100 g, whilst milk chocolate magnesium levels are slightly lower, at 43–50 mg magnesium/100 g (FSA 2002; Souci et al. 1986). Although chocolate may have the potential to contribute to the dietary intake of magnesium, and to even counteract magnesium deficiency, other foods contain similar or even much larger amounts of magnesium, for example Brazil nuts (410 mg/100 g), roasted and salted cashew nuts (250 mg/100 g), peanuts (210 mg/kg) and All-Bran cereal (240 mg/100 g) (FSA 2002). Interestingly, cravings for these foods do not appear to be very common in sufferers from premenstrual tension, as is confirmed by the observation that chocolate is the main target in female food cravings (Hill and Heaton-Brown 1994; Rodin et al. 1991), followed by ice-cream (Rodin et al. 1991). Indeed, "the food cravings reported... were hunger-reducing, mood-improving experiences, directed at wanting to consume highly pleasant tasting food" (Hill and Heaton-Brown 1994).

Therefore, despite speculated associations between changes in mood, food preferences and the menstrual cycle (Bancroft et al. 1988; Wurtman and Wurtman 1989), there is no reliable evidence to suggest that magnesium-deficient people show an increased craving or liking for chocolate.

8 Conclusions and Considerations

Most of the pharmacologically active substances present in chocolate that have been highlighted by both scientists and the popular media do not exude an effect in man owing to extremely low concentrations, the inability to cross or even reach the blood–brain barrier, or other inabilities that may at times have been conveniently ignored in order to justify a message that appeals to the general public. Of all constituents proposed to play a role in why we like chocolate over and above its innate appeal as a sweet and creamy tasting food (Drewnowski and Greenwood 1983), caffeine appears to provide the clearest evidence, based on effects found with ecologically relevant doses (Smit and Rogers 2001). Although theobromine is a promising “candidate”, synergistic or detrimental interactions as found in animal research (Heim et al. 1971; He et al. 2009) cannot be ruled out and need to be investigated with doses relevant to generally consumed amounts, as well as the possibility that some people are much more sensitive to the effects of theobromine than others (Mumford et al. 1994; Ott 1985). Research ought to include caffeine as a comparative agent, and underlying mechanisms need to be further explored, especially in the case of theobromine as an antitussive (Usmani et al. 2005) or as a dental enamel protective (Sadeghpour 2007) agent. In animals, theobromine is much better researched, despite this being fairly limited to toxicology studies. Additionally, a range of case studies of animal poisoning point out the dangers of chocolate and other cocoa-containing products to a wide range of animal species. Finally, doping control and therefore potential disqualifications are an issue in animal racing sports. However, the effects found in animals, whether they be of a toxicological, behavioural or other nature, cannot necessarily be translated to the human system (Tarka et al. 1979; Miller et al. 1984).

Clearly, the focus of interest regarding the effects of theobromine has charged over time, and new developments are promising an interesting future for a much underresearched substance. Hence, theobromine is in need of further investigation, and the following points need to be addressed:

1. A theobromine-to-caffeine affinity ratio for adenosine receptors of possibly 1:10 is not compensated for the estimated 10:1 theobromine-to-caffeine prevalence ratio in chocolate in terms of its mood, mental performance, or subjective effects.
2. It appears some individuals may be sensitive to the subjective effects of theobromine, though this needs validating, and a theoretical basis for this needs to be established.
3. Although some interactive effects of caffeine and theobromine have been observed, and although the effects of low doses of caffeine and of relevant caffeine–theobromine combinations on mood and performance have been found, effects of the individual components in relation to their combination have not yet been reported.
4. Although antitussive and enamel-strengthening effects of theobromine may have been found, similar effects of cocoa or chocolate ingestion, or comparative effects with caffeine and maybe other methylxanthines need to be investigated, in part to gain better insight into the possible mechanisms involved.

Note that although the methylxanthines in chocolate appear to represent its pharmacological activity (Smit et al. 2004), the list of minor chocolate constituents presented here is not exhaustive, nor does it address potential interactive effects between compounds that are not explained by their individual effects (Perez-Reyes et al. 1973). Moreover, although pharmacological activity *can* play a role in the liking for a food (see Smit and Blackburn 2005 for the combination of caffeine and theobromine as an example of their role in the liking for chocolate), this does not translate into *cravings* for such a food. Indeed, Michener and Rozin (1994) showed that only the sensory experience of a food, and not the pharmacologically active constituents, could fulfil such cravings. Note also that cravings for chocolate are usually directed at milk chocolate, containing lower quantities of the active constituents, and that people rarely describe strong urges for the consumption of coffee and tea, even when caffeine intake is reduced because of changes in daily routine (Rogers and Dernoncourt 1998). Finally, unlike chocolate, caffeine intake is rarely resisted ("dietary restraint"). Taking these general observations and experimental findings into account, we must seek the most plausible explanation for the existence of cravings for chocolate and even chocolate "addiction" in a culturally determined ambivalence towards chocolate (Cartwright and Stritzke 2008; Smit and Rogers 2001; Rogers and Smit 2000), and not in a role for any chocolate constituents.

Summarising, despite the assumption of being a behaviourally inert substance, theobromine has shown a range of interesting effects, both in man and in other animal species. Novel findings may have caused a renewed interest in this caffeine-related compound, and much is yet to be clarified. Also, with regard to my personal interest in the psychopharmacological effects of chocolate, I can only conclude that the last word on theobromine has not yet been heard.

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Propentofylline: Glial Modulation, Neuroprotection, and Alleviation of Chronic Pain

Sarah Sweitzer and Joyce De Leo

Contents

Abstract Propentofylline is a unique methylxanthine with clear cyclic AMP, phosphodiesterase, and adenosine actions, including enhanced synaptic adenosine signaling. Both *in vitro* and *in vivo* studies have demonstrated profound neuroprotective, antiproliferative, and anti-inflammatory effects of propentofylline. Propentofylline has shown efficacy in preclinical models of stroke, opioid tolerance, and acute and chronic pain. Clinically, propentofylline has shown efficacy in degenerative and vascular dementia, and as a potential adjuvant treatment for schizophrenia and multiple sclerosis. Possible mechanisms of action include a direct glial modulation to decrease a reactive phenotype, decrease glial production and release of damaging proinflammatory factors, and enhancement of

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This chapter is dedicated to Peter Schubert for introducing propentofylline to J. De Leo and for 23 years of scientific and personal mentorship and friendship.

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astrocyte-mediated glutamate clearance. This chapter reviews the literature that supports a myriad of protective actions of this small molecule and implicates propentofylline as a potential therapeutic for the treatment of chronic pain. From these studies, we propose a CNS multipartite synaptic action of propentofylline that includes modulation of pre- and postsynaptic neurons, astrocytes, and microglia in the treatment of chronic pain syndromes, including, but not limited to, neuropathic pain.

Keywords Astrocytes · Cytokines · Glia · Microglia · Neuropathic pain · Opioids · Tolerance

1 Introduction

Propentofylline (PPF) is an atypical synthetic methylxanthine [1-(5′-oxohexyl)-3-methyl-7-propylxanthine]. In this chapter, we will review the 30-year history of this compound from small-molecule development to clinical trials, describing its actions both in animals and in patients. PPF is closely related to pentoxifylline, which has been used for decades for intermittent claudication. PPF, previously known as HWA 285 (see Fig. 1), has a profile distinct from the profiles of other typical methylxanthines. Although it inhibits cyclic AMP (cAMP) and cyclic GMP phosphodiesterases, it is also a weak antagonist of the adenosine A1 receptor and blocks adenosine transport. However, the elucidation of its cellular and molecular mechanisms of action is still under way. A recent PubMed search that spanned 1981–2009 resulted in 215 publications that demonstrate PPF's diversity of actions, including, but are not limited to, vasodilatory, anti-inflammatory, antiproliferative, neuroprotective, platelet aggregation inhibitor, glial modulator, and glutamate inhibitor. These diverse actions translate into a plethora of potential clinical uses, including for ischemia, dementia, spinal cord injury, multiple sclerosis, transplantation, and chronic pain. We will take the reader on a journey highlighting the history of PPF focusing on its neuroprotection and inhibition of pain states in the quest for both a mechanism of action and a clinical translation of PPF for the treatment of chronic pain.

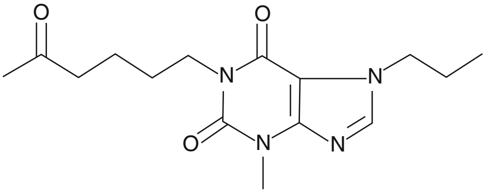

Fig. 1 Chemical structure of propentofylline (PPF)

1.1 PPF in Neuroprotection

PPF is a potent inhibitor of cAMP and phosphodiesterases (Nagata et al. 1985). In the mid-1980s, PPF was found to increase cerebral blood flow without a concomitant increase in cerebral glucose utilization (Grome and Stefanovich 1985). Distinct from many classic methylxanthines, PPF also enhances the actions of adenosine partly as an inhibitor of adenosine uptake (Fredholm and Lindstrom 1986). It was postulated that PPF may reduce neuronal activity and increase the vasodilatory effects of adenosine on blood vessels. Thus, it was questioned whether such a drug would have clinical utility (Fredholm and Lindstrom 1986).

In 1987, we demonstrated a neuronal protective action of PPF in a gerbil model of cerebral ischemia (DeLeo et al. 1987). In addition to a decrease of Nissl staining in the CA1 region of the hippocampus, systemic PPF also inhibited the marked glial fibrillary acidic protein (GFAP) astrocytic response and selective calcium accumulation in the transition zone between the hippocampal CA1 and CA3 areas (see Fig. 2). The most remarkable finding of that study was that treatment with PPF exerted a protective action against postischemic damage. In an effort to investigate mechanisms of neuronal protection, the efficacy of PPF was compared with that of pentobarbital. In contrast to PPF, pentobarbital afforded no protection when administered 1 h after bilateral carotid occlusion, suggesting divergent drug mechanisms of PPF and pentobarbital (DeLeo et al. 1988b). Electrophysiological experiments demonstrated no evidence of a direct depressant action of PPF on neuronal firing (P. Schubert, unpublished data). In a later study, we determined that PPF protects hippocampal neurons against ischemic damage in the presence of the adenosine antagonist theophylline, supporting a non-adenosine-mediated action (DeLeo et al. 1988a). Interestingly, the mechanism of action of this neuroprotection has not been clearly delineated and only recently has a novel mechanism been uncovered (see Sect. 2.1).

1.2 PPF as a Glial Modulator in Ischemia

Of importance to future research directions utilizing PPF is the novel finding in 1987 that PPF decreased GFAP immunoreactivity in the CA1 region of the hippocampus following transient cerebral ischemia (Fig. 3). This was the first indication of a direct glial modulating action which would not be fully realized until almost a decade later. It was proposed by Schubert and others that pathological microglial activation contributes to progressive neuronal damage in neurodegenerative diseases by the release of potentially toxic agents and by triggering reactive astrocytic changes (Schubert et al. 1997). With use of primary neonatal cultured microglia, it was demonstrated that PPF enhanced cAMP-dependent intracellular signaling (Si et al. 1998). PPF dose-dependently inhibited lipopolysaccharide-induced release of both tumor necrosis factor α (TNFα) and interleukin (IL)-1β

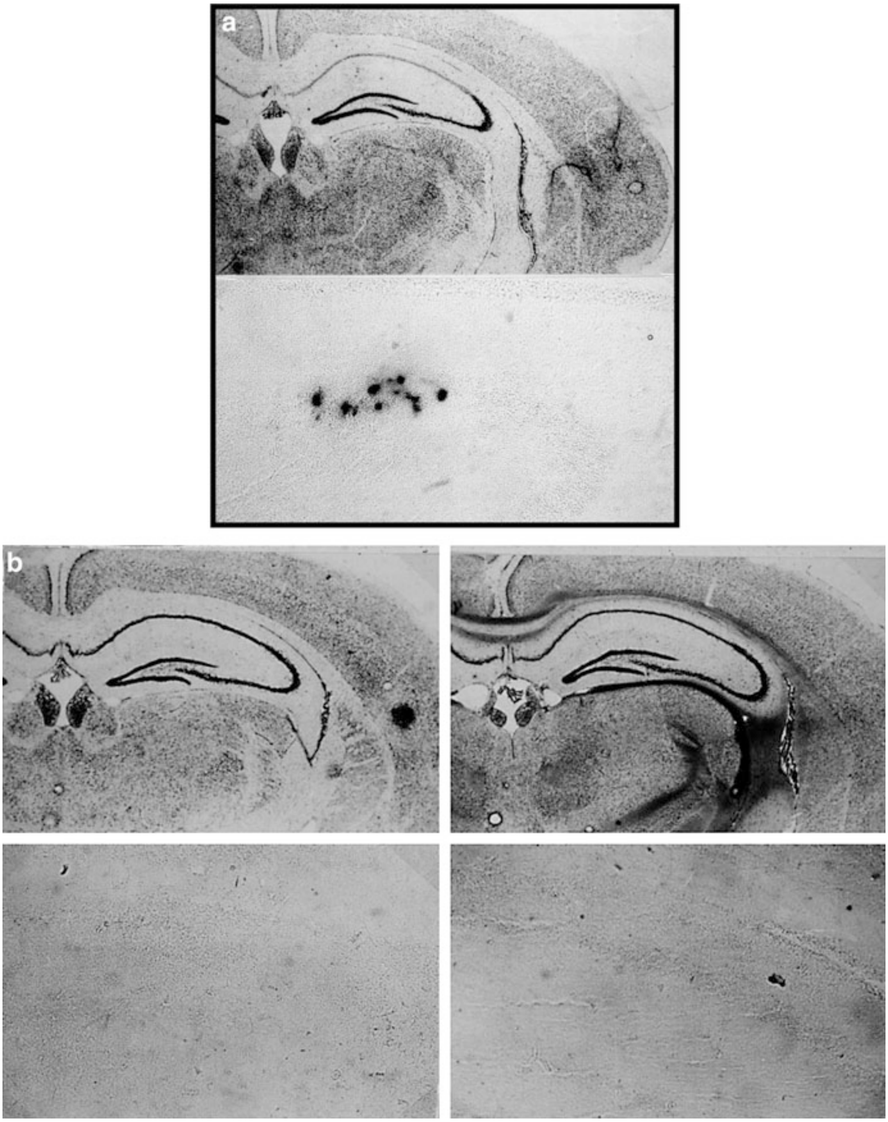

Fig. 2 Two days following 5-min bilateral carotid occlusion. **a** Selective decrease of Nissl staining in the CA1 region of the hippocampus and calcium accumulation in the CA1/CA2 transition zone. **b** Effect of PPF treatment (10 mg kg^{-1}, intraperitoneal) 15 min prior to cerebral ischemia on Nissl staining and calcium accumulation. *Left*: 5-min period of bilateral carotid occlusion. *Right*: 12-min period of bilateral carotid occlusion

(Si et al. 1998). Microglial proliferation was also dose-dependently inhibited by PPF, demonstrating a direct action of PPF on microglial function (Si et al. 1996).

These results suggest that PPF, probably via increases in cAMP intracellular signaling, alters the profile of newly adopted glial/immune properties in a way that

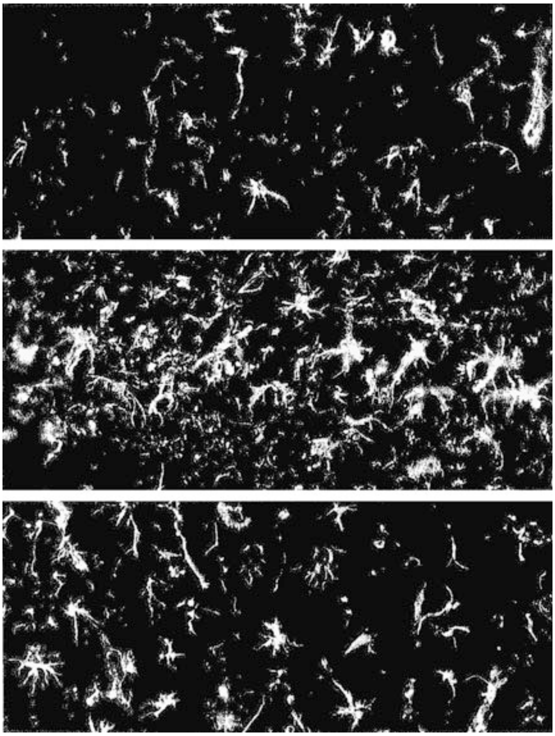

Fig. 3 Glial fibrillary acidic protein (GFAP) immunoreactivity in the CA1 region of the hippocampus. *Top*: control animal. *Middle*: 2 days following 5 min of cerebral ischemia. *Bottom*: 2 days following 5 min of cerebral ischemia after treatment with PPF (10 mg kg^{-1}, intraperitoneal)

inhibits potentially neurotoxic functions while maintaining beneficial functions. This differential regulation of microglial activation may explain the neuroprotective mechanism exerted by PPF. Schubert further posited that glial functional changes are controlled by an altered balance of the second messengers Ca^{2+} and cAMP which can be inhibited by the endogenous cell modulator adenosine via enhanced cAMP-dependent signaling (Schubert et al. 1998; Schubert and Rudolphi, 1998). The homeostatic adenosine effects on glial cells by PPF may prove to be a mediator of neuroprotection. Furthermore, the glial modulatory effect of PPF was observed in the spinal cord following cerebral ischemia, demonstrating PPF's effects at a site distant from the original site of injury, and in a site long considered to be the "gate" for pain transmission from the periphery to the CNS (Wu et al. 1999). These concepts laid the groundwork for investigations into the role of glial modulators, namely, PPF, in reducing CNS sensitization, the electrophysiological correlate to chronic pain via a direct glial–neuronal interaction.

1.3 PPF as an Antiallodynic Agent

Pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage"

(International Association for the Study of Pain 1986). Acute pain serves a very important physiological function to protect the body from tissue damage and to prevent further tissue damage following injury. In contrast, pain of greater than 3 months in duration is termed “chronic pain.” Chronic pain can develop in the absence of identifiable injury or as a result of infection (herpes zoster, HIV), physical injury (disc herniation, spinal cord injury, surgical incision, complex regional pain syndrome), chemical injury (alcohol, chemotherapy), autoimmune disease (multiple sclerosis), inflammatory disease (rheumatoid arthritis, osteoarthritis), metabolic disorders (diabetes), and cancer. One common form of chronic pain is neuropathic pain that arises from an injury or dysfunction in the peripheral nervous system or the CNS. Neuropathic pain has a prevalence of 3–8% in the US population and is characterized by paresthesia (abnormal skin sensations such as burning and shooting pain), dysesthesia (uncomfortable sensation from light touch), allodynia (pain in response to a normally nonpainful stimuli), and hyperalgesia (increased sensitivity to painful stimuli). Neuropathic pain is often refractory to current drug therapies. Over the last decade evidence has grown that reactive microglia and astrocytes modulate neuronal responsiveness to painful sensory stimuli. Seminal studies using PPF in preclinical models of neuropathic pain have not only highlighted the importance of reactive microglia and astrocytes in the transition from acute to chronic pain, but also the potential clinical utility for PPF in the treatment of chronic neuropathic pain.

Early studies suggesting the involvement of reactive glia in pain and nociception used the glial metabolic inhibitor fluorocitrate to attenuate acute formalin-induced nociception (Watkins et al. 1997) and zymosan-induced inflammatory hyperalgesia (Meller et al. 1994) or the NMDA antagonist MK-801 to attenuate chronic sciatic nerve constriction induced astrocyte activation (Garrison et al. 1991, 1994). A subsequent study demonstrated that the degree of glial changes in the dorsal horn of the spinal cord (a site where primary nociceptive afferents synapse on ascending spinothalamic tract neurons) correlated with the duration and severity of mechanical allodynia. This study showed mild and transient allodynia and glial reactivity following an acute noxious stimulus, moderate allodynia and glial reactivity following an intermediate duration inflammatory insult, and robust and long-lasting allodynia and glial reactivity following a spinal nerve transection (Sweitzer et al. 1999).

With the growing evidence for phenotypic changes in glial protein expression in the dorsal horn of the spinal cord in a number of peripheral nerve injury models of neuropathic pain (Colburn et al. 1997, 1999; Hashizume et al. 2000), we undertook a study of PPF in a preclinical model of chronic neuropathic pain (Sweitzer et al. 2001b). Daily systemic or intrathecal administration of PPF initiated prior to nerve injury prevented the development of mechanical allodynia (Fig. 4). Impressively, daily administration of PPF produced an approximately 50% reduction in astrocytic and microglial reactive changes (Fig. 5). Similarly, daily systemic administration of PPF reduced astrocytic and microglial reactive changes and attenuated mechanical allodynia in a vincrinstine model of chemotherapy-induced neuropathic pain (Sweitzer et al. 2006). In the L5 spinal nerve transection model,

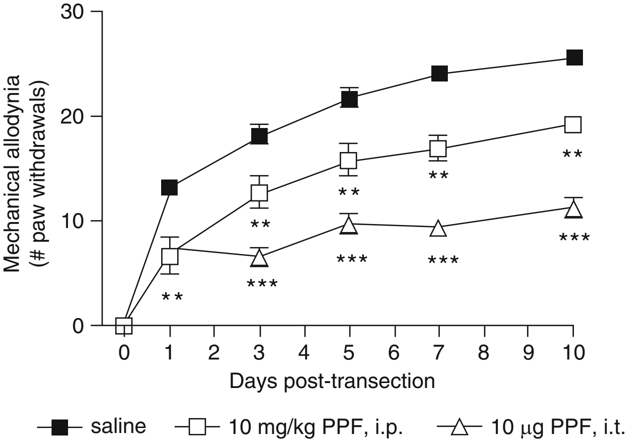

Fig. 4 PPF attenuates mechanical allodynia in a preclinical model of neuropathic pain. Daily intraperitoneal or intrathecal administration of PPF beginning on the day of nerve transection reduced mechanical allodynia over the 10 days of the study. $^{**}p<0.01$, $^{***}p<0.005$ compared with saline-vehicle-treated controls

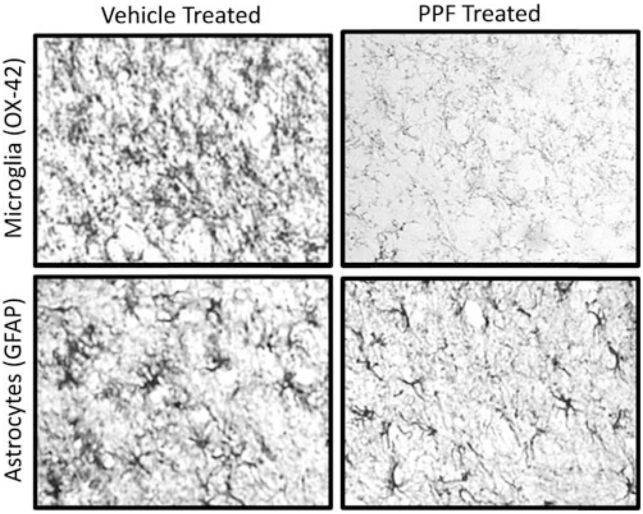

Fig. 5 Daily PPF treatment attenuated microglial (OX-42) and astrocytic (GFAP) reactivity in the dorsal horn of the spinal cord on day 10 after nerve transection as compared with saline vehicle controls

systemic administration of PPF starting on day 4 after injury prevented the further development of allodynia (Sweitzer et al. 2001a). In a crossover study, discontinuation of PPF at day 7 after injury resulted in the rapid return of allodynia, while initiation of therapy on day 7 produced only a small drop in established allodynia (Sweitzer et al. 2001a).

In a follow-up study, minocycline, a microglial specific inhibitor in *in vitro* studies, attenuated the development of mechanical allodynia and thermal hyperalgesia, but was unable to attenuate existing allodynia and hyperalgesia. While both pre- and posttreatment with minocycline reduced microglial reactivity, only pretreatment with minocycline reduced astrocytic reactivity (Raghavendra et al. 2003a). This suggests that preventative treatment with PPF (Sweitzer et al. 2001b) was much more effective by inhibiting both microglial and astrocytic changes that may promote a proinflammatory phenotype. A subsequent study showed a second phase of microglial involvement in neuropathic pain-associated behaviors that could be attenuated with daily systemic treatment with PPF for 14 days beginning 2 weeks after nerve transection (Tawfik et al. 2007). These studies highlight the temporal importance of astrocytes and microglia in chronic neuropathic pain. The question of how glial inhibition attenuates behavioral hypersensitivity remained unknown.

A subsequent study with daily intrathecal administration of PPF confirmed a decrease in the levels of CDllb, a marker of microglial reactivity, and GFAP, a marker of astrocytic reactivity at both the messenger RNA (mRNA) level and the protein level (Raghavendra et al. 2003b). As mentioned above, PPF had been shown to reduce the levels of proinflammatory cytokines in microglial cultures (Si et al. 1996), and thus cytokine expression was examined in the spinal cord of neuropathic animals treated with PPF. PPF decreased mRNA and protein expression for the proinflammatory cytokines TNFα, IL-1β, and IL-6 in the spinal cord. It had previously been shown that inhibition of these three proinflammatory cytokines attenuated allodynia (Arruda et al. 2000; Sweitzer et al. 2001a). Furthermore, PPF pretreatment was shown to attenuate IL-1β-induced C-fiber mediated windup in dorsal horn neurons, an electrophysiological measure of CNS sensitization (Arriagada et al. 2007). Together these findings suggest that PPF inhibits glial reactivity, decreases synthesis and release of proinflammatory cytokines, and prevents cytokine-induced sensitization of central nociceptive neurons and attenuation of neuropathic pain-associated behaviors.

More recent studies support this mechanism involving a multipartite synapse involving microglia, astrocytes, presynaptic neurons, and postsynaptic neurons. Acute bolus administration of PPF has been shown to reverse thermal and mechanical hyperalgesia and decreases phosphorylation of p38 and p42/44 mitogen-activated protein kinases in the spinal cord in the chronic constriction model of neuropathic pain (Garry et al. 2005). Similarly, direct spinal application of PPF attenuates neuronal hyperexcitability and pain that originates below the level of the spinal cord lesion (Gwak and Hulsebosch 2009). At presynaptic neuron terminals, PPF may prevent the downregulation of glutamic acid decarboxylase 65, which is the rate-limiting enzyme in the production of the inhibitory neurotransmitter GABA, thus increasing endogenous inhibitory tone (Gwak et al. 2008).

While the focus of the majority of pain research using PPF has examined activity at the level of the spinal cord, there is some evidence for PPF activity outside the spinal cord. A potential local antinociceptive action of PPF on phase II formalin-induced nociception was reported following PPF administration in the hind paw either before or after formalin injury (Dorazil-Dudzik et al. 2004). A supraspinal site of action of PPF is suggested with the report of a prolonged antihyperalgesic/allodynic effect of a single administration of PPF in the cingulate cortex 24 h prior to nerve injury (Kuzumaki et al. 2007). It remains to be seen whether post-treatment is similarly effective in this paradigm. Follow-up studies are eagerly awaited to identify the mechanism(s) of action by which PPF produces antinociception in neuropathic pain following systemic, local, spinal, and brain-site administration. Within the spinal cord, attenuation of central sensitization by decreasing proinflammatory cytokine production/release and a reactive glial phenotye, and increasing endogenous inhibitory tone are potential mechanisms for PPF efficacy in neuropathic pain. However, there may be additional mechanisms involved within the peripheral skin microenvironment and brain.

2 Attenuation of Morphine Tolerance and Hyperalgesia in Acute and Chronic Pain

The previous section detailed the mounting evidence for an antinociceptive action of PPF in acute and chronic pain. There is also growing evidence that PPF may have a second, equally important beneficial effect in both acute and chronic pain treatment by preventing opioid tolerance. Owing to robust efficacy in a variety of acute and chronic severe pains, opioids have been the mainstay for the treatment of pain. Unfortunately, the use of opioids in the treatment of chronic pain remains limited by the need for high doses, which are associated with undesirable side effects, and the development of tolerance, which necessitates dose escalation and further adverse side effects. Daily intrathecal administration of PPF prevents morphine tolerance in a preclinical model of neuropathic pain (Raghavendra et al. 2003b) or in normal animals (Raghavendra et al. 2004). Similarly, intrathecal coadministration of PPF is able to prevent stereo-selective cross-tolerance between dextromorphine and levomorphine (Wu et al. 2005) and morphine tolerance induced enhancement of δ-opioid receptor analgesia (Holdridge et al. 2007). Systemic PPF administration has been shown to delay the onset of morphine (Narita et al. 2006; Shumilla et al., 2005) or entorphine (Narita et al. 2006) tolerance. Similar to preclinical models of neuropathic pain, PPF attenuated spinal GFAP and CD11b expression (Holdridge et al. 2007; Raghavendra et al. 2004) and proinflammatory cytokine expression in animals repeatedly exposed to morphine (Fig. 6). These findings provide further evidence that neuropathic pain and opioid tolerance share similar mechanisms, including the importance of glia and the potential for PPF to be both antinociceptive and opioid-sparing in the treatment of neuropathic pain.

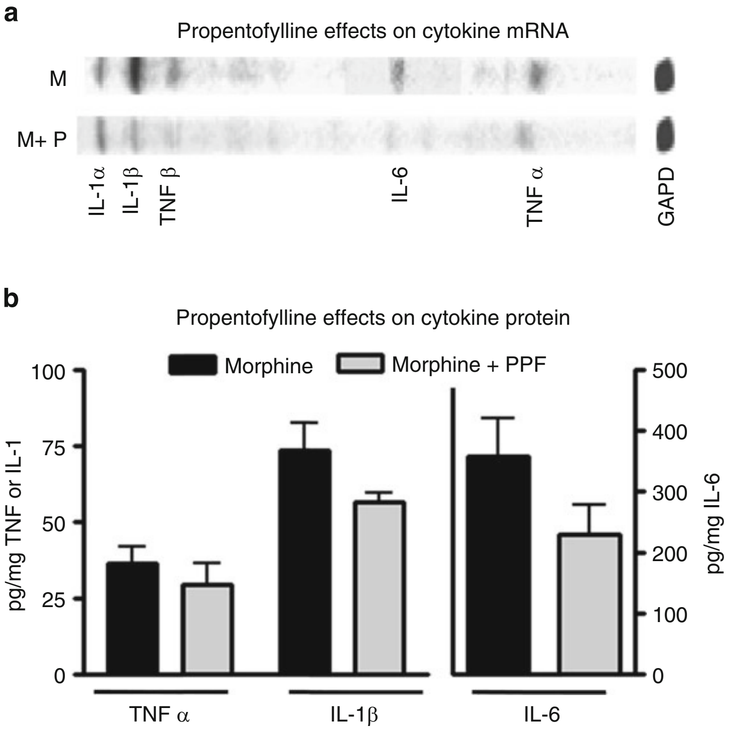

Fig. 6 Daily intrathecal PPF administration decreased morphine-induced upregulation of proinflammatory cytokines in the dorsal horn of the spinal cord. (A) With use of RNAse protection assay, administration of morphine with PPF (M+P) decreased messenger RNA levels for interleukin (IL)-1β, tumor necrosis factor α (TNFα), and IL-6 as compared with morphine alone (M). (B) Administration of morphine with PPF decreased protein expression of IL-1β, TNFα, and IL-6 as compared with morphine alone (M)

3 PPF increases GLT-1 Expression *In Vitro* and *In Vivo*: Novel Mechanisms of Action

In a series of more recent *in vitro* and *in vivo* studies, we sought to further elucidate PPF's mechanism of action to attenuate nerve-injury-induced behavioral hypersensitivity. One of the key unifying neurotransmitters in the pathogenesis of neurodegeneration and neuronal sensitization is glutamate. PPF has previously been shown to reduce glutamate release in gerbil hippocampus following transient forebrain ischemia (Miyashita et al. 1992). In relation to chronic pain, peripheral nerve injury results in a decrease in glutamate uptake (Binns et al. 2005). Mature, differentiated astrocytes are known to express the GLT-1 (EAAT2) transporter, which is responsible for over 90% of synaptic glutamate clearance (Tanaka et al. 1997). Reactive astrocytes display decreased levels of glutamate transporters such as GLT-1 and as a result, synaptic glutamate clearance is impaired. In addition, these reactive astrocytes become immunocompetent and release algesic mediators which can sensitize neurons in the spinal cord. It was previously demonstrated that the level of GLT-1 is reduced in the dorsal horn of the spinal cord in rodent models of neuropathic pain (Cata et al. 2006; Sung et al. 2003; Weng et al. 2005). We evaluated the effect of PPF on cultured cortical astrocytes. Primary astrocyte cultures, which represent an activated phenotype with a polygonal morphology and low GLT-1 expression, were treated for 3 or 7 days with 10, 100, or 1,000 μM PPF or db-cAMP, a known inducer of GLT-1 expression. PPF dose-dependently induced astrocytes to display a mature phenotype, with elongated processes and a stellate shape. In addition, PPF dose-dependently increased GLT-1 immunoreactivity. Real-time reverse transcription PCR and western blot analysis clearly demonstrated that PPF caused a potent dose-dependent induction of GLT-1 mRNA and protein in astrocytes. Importantly, the observed increase in the level of glutamate transporters was found to have a functional effect. PPF significantly enhanced glutamate uptake in astrocytes at both 100 and 1,000 μM concentrations, which was sensitive to dihydrokainate inhibition, suggesting a robust GLT-1-mediated effect. In addition, PPF decreased both MCP-1 (CCL2) and MIP-2 (CXCL2) release from astrocytes, while db-cAMP significantly enhanced this chemokine expression. These findings suggest that PPF is capable of differentiating astrocytes to a homeostatic, mature phenotype, competent for glutamate clearance and distinct from that induced by db-cAMP.

In a parallel series of experiments, we determined whether PPF-induced glial modulation alters the levels of spinal glutamate transporters, GLT-1 and GLAST *in vivo*, which may contribute to reduced behavioral hypersensitivity after nerve injury. Rats received PPF (10 μg) or saline via lumbar puncture starting 1 h prior to L5 spinal nerve transection and then daily for 4 or 12 days. In addition to a marked suppression of mechanical allodynia, PPF increased mRNA levels for GLT-1, but not GLAST after injury. In support of previous data, western blot analysis demonstrated a decrease in the level of GLT-1 at day 12 after nerve injury, which was reversed with PPF treatment. In order to specifically examine the expression of spinal glutamate transporters, a novel line of double transgenic eGFP-GLT-1/DsRed-GLAST reporter mice were used (Tawfik et al. 2008). PPF restored transporter levels on the injured side as evidenced by an equal number of GLT-1 and GLAST puncta in both dorsal horns. As demonstrated in previous studies, PPF induced a concomitant reversal of L5 spinal nerve transection-induced expression of GFAP, a marker of astrocytic activation. The ability of PPF to alter the levels of glial glutamate transporters highlights the importance of controlling aberrant glial activation in neuropathic pain and suggests one possible mechanism for the antiallodynic action of this drug.

4 Previous Clinical Data Summary

During the late 1990s, there were extensive clinical trials testing PPF in patients with mild to moderate Alzheimer's disease and vascular dementia. The phase II and phase III placebo-controlled trials were conducted by Aventis and Hoechst

Marion Roussel Pharmaceuticals in Europe, Canada, Japan, and the USA. PPF was also marketed in Japan from 1988 to 1998 for the symptoms of cerebrovascular disease. Therefore, PPF has been administered to thousands of patients over more than a decade. The efficacy in the Alzheimer's disease and vascular dementia trials varied among studies, with decreased efficacy attributed to drug–food interactions resulting in poor PPF absorption and, thus, decreased therapeutic plasma levels. It is difficult to determine the exact cause of reduced efficacy retrospectively given the long period since these trials. In addition, the primary end points used to assess efficacy were very different from the parameters that would be used today to assess treatment in Alzheimer's disease and vascular dementia. Even considering these caveats, the data demonstrate modest but positive effects on cognition with PPF compared with placebos and effects equivalent to those of other drugs clinically available for Alzheimer's disease. Considering the cost of these large trials, it is understandable but unfortunate that further testing has not been performed. Importantly, the overall profile of the safety data in thousands of elderly patients with underlying health problems supports future clinical trials.

5 Conclusions

This chapter highlighted key experimental findings demonstrating diverse actions and mechanisms of the atypical methylxanthine, PPF, in a variety of preclinical and clinical situations. Our focus was on its actions to reduce behavioral hypersensitivity in acute and chronic pain animal models and to attenuate opioid tolerance. These preclinical data from both our laboratory as well as many other investigators have culminated in an ongoing multicenter, multicountry (USA, Germany, Russia, and India) clinical trial for the treatment of postherpetic neuralgia. The importance of this trial is underscored by the first attempt to utilize a CNS glial modulator for the treatment of a chronic pain syndrome. As shown in Fig. 7, we describe a multipartite synapse where PPF may have distinct actions at each component of the synapse. There are extensive data demonstrating PPF's direct actions on microglia, including the inhibition of proliferation, migration, and a reactive phenotype, including suppression of proinflammatory factors. In addition, we and others have shown that PPF also decreases the levels of astrocytic cytosolic markers of reactivity, produces astrocyte differentiation, and increases GLT-1 expression after injury. Direct effects on neurons have also been described herein, which may lead to an inhibitory action partly due to enhancing GABAergic transmission. Further studies are needed to determine the specific effects of PPF on glial–neuronal signaling that would result in decreased CNS sensitization.

As mentioned previously, the potent neuroprotective effects we observed in a gerbil model of global ischemia were not reversed by an adenosine antagonist. In addition, PPF's actions in producing neuroprotection, glial modulation, and antiallodynia are significantly greater than the actions reported with other

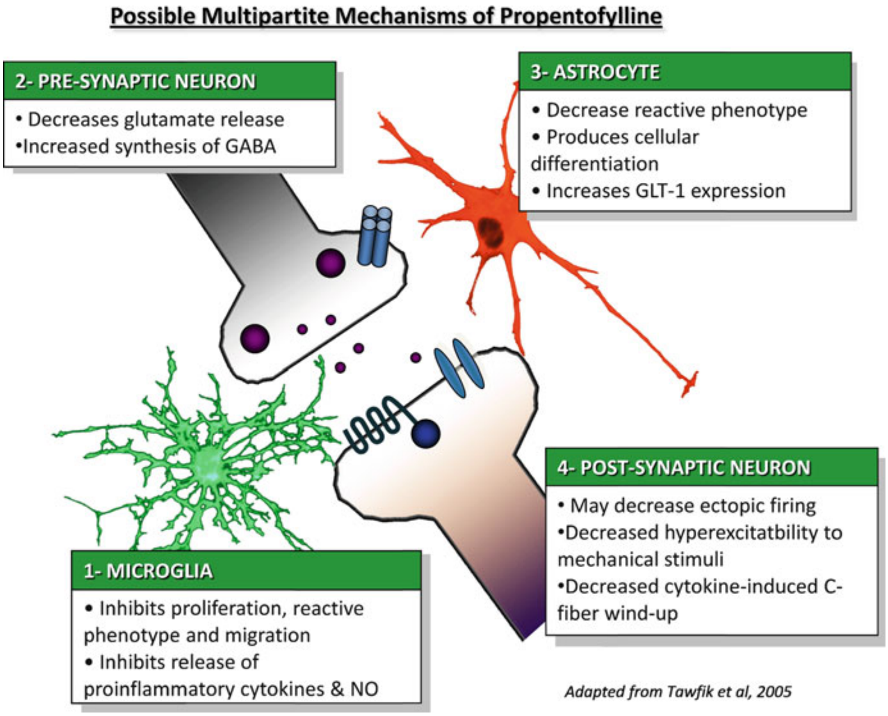

Fig. 7 Conceptual diagram of proposed PPF mechanisms of action on neurons, astrocytes, and microglia

methylxanthines or phosphodiesterase inhibitors. Similarly, the astrocytic GLT-1 enhancement we demonstrated with PPF was not seen with the typical methylxanthine, caffeine (Tawfik et al. 2006). Therefore, it seems unlikely we can attribute the mechanisms of action of PPF to a direct potentiation of adenosine or via phosphodiesterase inhibition. PPF does appear to have a pharmacodynamic profile distinct from that of other methylxanthines in many aspects, which may afford exciting drug development opportunities. As a potent glial modulator, cytokine release and functional migration assays in glial cultures are used as screening tools to enhance potency and improve pharmacokinetics of future compounds.

In conclusion, this small molecule has withstood the test of time by demonstrating both efficacy and safety in a plethora of preclinical and clinical studies. Fortuitously, PPF has more recently stimulated the fields of neuroimmunology and glial biology. As a powerful tool, it has elucidated novel molecular and cellular mechanisms of injury-induced CNS glial responses. These studies may one day lead to the utilization of glial modulators as novel pharmacological agents for the treatment of many neurodegenerative and chronic pain disorders.

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Methylxanthines, Seizures, and Excitotoxicity

Detlev Boison

Contents

Abstract Clinical evidence, in particular the wide use of theophylline as a bronchodilator, suggests that methylxanthines can cause seizures in patients without known underlying epilepsy. Theophylline is also known to be an added risk factor for seizure exacerbation in patients with epilepsy. The proconvulsant activity of methylxanthines can best be explained by their antagonizing the brain's own anticonvulsant adenosine. Recent evidence suggests that adenosine dysfunction is a pathological hallmark of epilepsy contributing to seizure generation and seizure spread. Conversely, adenosine augmentation therapies are effective in seizure suppression and prevention, whereas adenosine receptor antagonists such as

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methylxanthines generally exacerbate seizures. The impact of the methylxanthines caffeine and theophylline on seizures and excitotoxicity depends on timing, dose, and acute versus chronic use. New findings suggest a role of free radicals in theophylline-induced seizures, and adenosine-independent mechanisms for seizure generation have been proposed.

Keywords Adenosine · Adenosine kinase · Caffeine · Epilepsy · Toxicity

1 Introduction

Seizures, ranging from altered states of consciousness to clonic and/or tonic convulsions, are commonly encountered in patients who do not have epilepsy (Delanty et al. 1998). Among other potential triggers, such nonepileptic seizures can be provoked by medication or medication withdrawal. Within this context, seizures are potentially severe or fatal complications of theophylline therapy. Theophylline can trigger seizures in patients without known underlying epilepsy and is an added risk factor for seizure exacerbation in patients with epilepsy. Most of these seizures result from toxic theophylline serum concentrations and are difficult to control. Nevertheless, clinical diagnosis and management of theophylline-induced seizures are underappreciated compared with clinical diagnosis and management of other drug-induced seizures. Despite a long clinical history of theophylline-induced seizures, relatively little is known about the underlying molecular mechanisms that contribute to methylxanthine-induced seizure generation. Knowledge gained from patient data, but most notably from animal or in vitro studies aimed at elucidating the role of endogenous adenosine in seizure control, contributes to our current understanding of how methylxanthines influence the excitability of the brain.

2 Clinical Findings

Anecdotally, caffeinated beverages are "known" to lower seizure thresholds in patients with epilepsy and the avoidance of excessive caffeine has been recommended in patients with epilepsy (Kaufman and Sachdeo 2003). However, owing to the lack of well-designed, randomized, and placebo-controlled clinical trials, this concept has been challenged (Asadi-Pooya et al. 2008). Clinical findings in support of a proconvulsant role of methylxanthines are largely based on theophylline (or aminophylline, a mixture of theophylline with ethylenediamine that is 20 times more soluble than theophylline alone), which, clinically, is widely used to manage bronchospasms in reversible airway obstruction associated with stable asthma and chronic bronchitis (Barnes 2005; Van Dellen 1979). In addition, aminophylline is indicated in asystolic cardiac arrest and periarrest bradycardia refractory to atropine, whereas caffeine is used to treat diabetic cardiac autonomic neuropathy (Duby et al. 2004). Theophylline has a narrow therapeutic window, with an optimal plasma concentration of 10–20 mg l^{-1} (55–110 mmol l^{-1}). Above this concentration, side effects such as arrhythmias and convulsions may occur, especially when theophylline is given rapidly by intravenous injection (Nolan et al. 2005). Theophylline-associated seizures (TAS) are considered a neurological emergency with potentially fatal outcome (Nakada et al. 1983). These seizures – largely focal onset generalized motor seizures – tend to be the only sign of theophylline toxicity, and can occur in neurologically intact patients (Aminoff and Simon 1980; Nakada et al. 1983). Remarkably, anticonvulsant therapy is ineffective in controlling these seizures, which often progress to status epilepticus and become intractable (Nakada et al. 1983; Yoshikawa 2007). In a recent clinical study the usual first-line treatment of diazepam was found to be more likely to be ineffective in TAS cases compared with non-TAS cases (Yoshikawa 2007); the failure of diazepam to stop those seizures might be based on interactions of theophylline with benzodiazepines (see later) (Yoshikawa 2007).

Interestingly, TAS is most common in pediatric patients under 5 years of age (Korematsu et al. 2008; Yoshikawa 2007), who can be considered to be naïve to theophylline or caffeine. In a recent study of eight pediatric TAS cases without underlying epilepsy, all the patients had fever at the onset of TAS (more than 38°C), and six of them had a family history of febrile seizures and/or idiopathic epilepsy (Korematsu et al. 2008). The authors of this study concluded that in infants with an idiopathic reduced seizure threshold and fever, theophylline administration might possibly be sufficient to trigger a seizure. Apart from TAS discussed here, methylxanthine-induced seizures have also been described after the consumption of caffeinated energy drinks (Iyadurai and Chung 2007), and theophylline, caffeine, and aminophylline are used clinically to prolong seizure durations in electroconvulsive therapy for major depression (Stern et al. 1999). The potential risks associated with theophylline therapy are now well recognized. Owing to concerns of CNS stimulant effects, theophylline use in patients with insomnia was included in the 2002 Criteria for Potentially Inappropriate Medication Use in Older Adults (Fick et al. 2003).

Pharmacokinetic drug interactions of methylxanthines also need to be considered. Theophylline is largely metabolized by the hepatic enzyme CYP1A2, which is induced not only by a variety of antibiotics (Gillum et al. 1993) but also by the commonly used enzyme-inducing antiepileptic drugs phenobarbital, phenytoin, carbamazepine, and primidone, and might require an increase in the therapeutic dose of theophylline (Patsalos et al. 2002; Spina et al. 1996). In view of the potential seizure-inducing effects of theophylline, the use of theophylline in patients with epilepsy is now limited despite the fact that second-generation antiepileptic drugs do not interfere with the pharmacokinetics of theophylline (Patsalos et al. 2002). Of note, caffeine comedication in combination with phenobarbital during the first trimester of pregnancy leads to a significant increase in the number of congenital malformations in offspring (Samren et al. 1999).

3 Experimental Findings

The proconvulsant potential of methylxanthines has been corroborated in countless animal studies that go back more than 35 years (Roussinov et al. 1974). Early studies suggested slight differences in the convulsant role of methylxanthines: Intraperitoneal administration of caffeine produced immediate excitation and seizures followed by an encephalopathy, whereas progression from encephalopathy to seizures was observed following aminophylline administration (Chu 1981). The proconvulsant and convulsant effects of methylxanthines generally depend on the dose and mode of application: Aminophylline at 100 mg kg^{-1} is known to increase the susceptibility of rats to pilocarpine- or pentylenetetrazole-induced seizures (Chakrabarti et al. 1997; Turski et al. 1989), whereas higher doses of aminophylline (250 mg kg^{-1}) lead to seizures and death in rats (Chakrabarti et al. 1997). These detrimental effects of high doses of aminophylline could be avoided by using equivalent doses of theophylline in preparations of acepifylline (theophylline ethanoate of piperazine) (Chakrabarti et al. 1997). Aminophylline-induced seizures directly depended on cerebrospinal fluid concentrations of theophylline and were not influenced by metabolites of theophylline (Ramzan and Levy 1986). In several experimental combinations it was shown that methylxanthines reduce or abolish the anticonvulsant activity of several antiepileptic drugs (Kulkarni et al. 1991). In contrast, the anticonvulsant effectiveness of felbamate was only affected at higher doses of aminophylline and caffeine (Gasior et al. 1998), and aminophylline did not alter the ability of gabapentin to protect mice against seizures induced by electroconvulsive shock (Luszczki et al. 2007). The concept that methylxanthines can exacerbate seizures in epilepsy has recently been challenged by Loscher (2009), arguing that CNS stimulants exert (pro)convulsant activity only at supratherapeutic doses.

Whereas methylxanthine-induced seizures are refractory to diazepam in patients, it is important to point out that levetiracetam and several other antiepileptic drugs that do not act via activation of $GABA_A$ receptors are highly effective in suppressing caffeine-induced seizures in mice (Klitgaard et al. 1998). Astemizole, a novel histamine H_1 receptor antagonist, at a dose of 2 mg kg^{-1} increased the threshold for aminophylline-induced seizures (Swiader et al. 2005), an interesting observation since these drugs are usually combined during the treatment of asthma.

Pharmacokinetic and pharmacodynamic drug interactions have also been studied in animal models. Of note are interactions of the fluoroquinolone class of antibacterials with theophylline. In one study, chronic pretreatment of rats with the fluoroquinolone pefloxacine was shown to exacerbate aminophylline-induced seizures without altering brain concentrations of theophylline (Imperatore et al. 1997). Likewise, certain environmental toxins, such as toluene, were shown to reduce the thresholds for methylxanthine-induced seizures (Chan and Chen 2003).

4 Adenosine, Seizures, and Excitotoxicity

Several potential mechanisms have been discussed that could explain the proconvulsant role of acute theophylline (Yoshikawa 2007): (1) general decrease of seizure thresholds; (2) inhibition of adenosine A_1 receptors (A_1Rs) that normally suppress seizures by blocking the release of excitatory amino acids; (3) inhibition of cerebral blood flow via adenosine antagonism (Puiroud et al. 1988); (4) inhibition of 5′-nucleotidase and decrease in endogenous adenosine production; (5) inhibition of pyridoxal kinase, an enzyme needed for the synthesis of GABA; (6) increase in cyclic GMP that is involved in maintaining the epileptic discharge; and (7) a presumed direct inhibition of the $GABA_A$ receptor (Sugimoto et al. 2001), although interactions between $GABA_A$ receptors and the adenosine system might also be involved (Bonfiglio and Dasta 1991; Phillis 1979). Overall, it appears that theophylline does not trigger seizures as such, but rather potentiates preexisting brain hyperexcitability, a mechanism consistent with the role of A_1Rs in preventing seizure spread and in mediating seizure arrest (Fedele et al. 2006; Lado and Moshe 2008; Young and Dragunow 1994). Given the dominant role of the adenosine system in seizure control within the context of theophylline toxicity, the following sections focus on the role of adenosine in epilepsy.

4.1 Adenosine Deficiency and Seizure Generation

The role of adenosine as an endogenous regulator of hippocampal excitability was first recognized by Dunwiddie (1980) almost 30 years ago. In a subsequent study it was shown that theophylline and other alkylxanthines antagonized electrophysiological responses to adenosine and adenosine-stimulated cyclic AMP formation, indicating that alkylxanthines increase hippocampal excitability by antagonizing the actions of adenosine (Dunwiddie et al. 1981). Several adenosine receptor agonists that activate the A_1R were shown to suppress seizures in a variety of models, albeit accompanied by sedative and hypothermic side effects (Dunwiddie and Worth 1982). Endogenous adenosine is a potent regulator of hippocampal activity and was recently shown to control hippocampal sharp waves in CA3 via activation of A_1Rs (Wu et al. 2009). It is now well recognized that adenosine is an endogenous anticonvulsant and regulator of brain activity (Boison 2005; Dunwiddie and Masino 2001; Fredholm et al. 2005a, b; Ribeiro et al. 2002). The anticonvulsant activity of adenosine is largely mediated by activation of A_1Rs, since A_1R knockout mice experience spontaneous seizures (Li et al. 2007a) and are highly susceptible to seizure spread (Fedele et al. 2006). Conversely, A_1R agonists are highly effective in the suppression of seizures (Benarroch 2008; Fredholm 2003; Jacobson and Gao 2006), and have been demonstrated to suppress seizures that are resistant to conventional antiepileptic drugs (Gouder et al. 2003). Decreased extracellular adenosine levels and reduced

A_1R activation as a consequence of kindling or caused by hypercapnia in a hippocampal slice preparation provide a plausible mechanisms for seizure generation (Dulla et al. 2005; Rebola et al. 2003).

In adult brain, synaptic levels of adenosine are largely regulated by an astrocyte-based adenosine cycle (Boison 2008). Under physiological conditions, synaptic adenosine is largely derived from vesicular release of ATP from astrocytes followed by extracellular cleavage into adenosine (Pascual et al. 2005), although astrocytic release of ATP via hemichannels has been demonstrated (Iglesias et al. 2009; Kang et al. 2008). In adult brain, adenosine is rapidly phosphorylated into AMP by the astrocyte-based enzyme adenosine kinase (ADK; EC 2.7.1.20) (Boison 2006, 2008). In contrast to conventional neurotransmitters, such as glutamate and glycine, there is no transporter-based regulatory mechanism to terminate the synaptic activity of adenosine. Owing to the presence of two types of equilibrative nucleoside transporters in the astrocyte membrane (Baldwin et al. 2004), intracellular ADK is able to fulfill the role of a metabolic reuptake system for adenosine (Boison 2008). On the basis of its low K_M for adenosine, ADK is the key regulator for ambient concentrations of adenosine (Boison 2006; Etherington et al. 2009; Lloyd and Fredholm 1995).

ADK has recently been identified as a molecular link between astrogliosis and neuronal hyperexcitability in epilepsy (Li et al. 2008). Astrogliosis – a pathological hallmark of the epileptic brain – is associated with upregulation of the adenosine-removing enzyme ADK (Gouder et al. 2004; Li et al. 2008). Remarkably, the development of spontaneous electrographic seizures coincides both spatially (Li et al. 2008) as well as temporally (Li et al. 2007a) with astrogliosis and upregulated ADK. Uncoupling of astrogliosis from epileptogenesis in ADK-transgenic mice (Adk-tg) (Li et al. 2009) has demonstrated that overexpression of ADK, rather than astrogliosis per se, can be the cause for seizures. In line with these findings, Adk-tg mice express spontaneous recurrent electrographic seizures (Li et al. 2007a). Conversely, therapeutic augmentation of the adenosine system is very effective in suppressing seizures (Boison 2009). Together, these findings demonstrate that adenosine deficiency and therefore deficient activation of A_1Rs can be a direct cause for seizures. This conclusion supports the notion that methylxanthines have proconvulsant activity owing to antagonizing the function of the endogenous anticonvulsant adenosine.

4.2 Adenosine Deficiency and Excitotoxicity

Adenosine, acting via A_1Rs, is not only an endogenous anticonvulsant of the brain, but also a powerful neuroprotectant (Cunha 2005; Fredholm 1997). Thus, in addition to a proconvulsant role of A_1R deficiency or increased adenosine clearance (overexpression of ADK), these conditions lead to increased vulnerability to excitotoxic injury. Consequently, A_1R knockout mice are highly susceptible to seizure-induced (Fedele et al. 2006) or traumatic (Kochanek et al. 2006) brain injury and they experience highly aggravated neuronal cell loss after status epilepticus (Li et al. 2007a).

Pharmacological studies in a model of oxygen glucose deprivation suggest that whereas A_1Rs desensitize after prolonged agonist exposure, adenosine A_{2A} receptor ($A_{2A}R$)-mediated facilitation of glutamate release by endogenous adenosine remains fully operational under long-term oxygen glucose deprivation (Sperlagh et al. 2007). Thus, the inhibition of $A_{2A}Rs$ might be a more effective approach to attenuate glutamatergic excitotoxicity than the stimulation of A_1Rs (Cunha 2005). Consequently, $A_{2A}R$ antagonists are actively investigated clinically for their neuroprotective potential (Chase et al. 2003; Hauser et al. 2003).

4.3 Adenosine-Based Therapeutic Approaches

Given the prominent role of adenosine as an endogenous anticonvulsant and neuroprotectant, adenosine augmentation therapies are highly effective in preventing seizures (Boison 2009). Pharmacologically, seizures can be suppressed by A_1R agonists (Benarroch 2008) or by ADK inhibitors (McGaraughty et al. 2005); however, systemic augmentation of the adenosine system is associated with significant side effects, including the suppression of cardiac function and depression of blood pressure, and is therefore not a therapeutic option (Dunwiddie and Masino 2001). Alternatives are focal adenosine augmentation therapies to avoid systemic side effects and to restore adenosinergic signaling within a localized area of adenosine dysfunction, which can be equated with an epileptogenic focus (Li et al. 2008). Strategies that have been explored include the implantation of adenosine-releasing silk-based polymers into the infrahippocampal fissure in kindled rats. Rats treated with these polymers were protected both from established seizures as well as from developing epilepsy (Szybala et al. 2009). Likewise, rats with focal implants of adenosine-releasing encapsulated fibroblasts or ADK-deficient stem cells were protected from kindled seizures or kindling development, respectively (Huber et al. 2001; Li et al. 2007b). Stem-cell-derived adenosine-releasing implants that were placed into the infrahippocampal fissure in mice were shown to suppress acute chemoconvulsant-induced seizures with associated injury (Ren et al. 2007), and to suppress epilepsy development and spontaneous seizure expression in a model of CA3-selective focal epileptogenesis (Li et al. 2008). Together, these data demonstrate that focal reconstitution of adenosine signaling within an area of acquired adenosine dysfunction (i.e., within an epileptogenic focus) constitutes a powerful approach to suppress seizures.

5 Methylxanthines, Seizures, and Excitotoxicity

The previous sections suggest that methylxanthines – via antagonizing adenosine's anticonvulsant and neuroprotective actions (Fredholm et al. 1999; Nehlig et al. 1992) – are proconvulsants that aggravate excitotoxicity. There are, however, additional interactions that need to be considered: the influence of methylxanthines on seizures and excitotoxicity is context- and receptor-dependent, and appears to be influenced by pathways not related to adenosine.

5.1 Acute Versus Chronic Caffeine

Whereas the proconvulsant activity of acute methylxanthines has long been recognized (see earlier), the chronic dosing of caffeine has different effects. Caffeine administered at a dose of 60–70 mg kg^{-1} per day in mice over a period of 2 weeks (resulting in plasma levels of caffeine in the range 6–14 μM, corresponding to chronic caffeine use in humans) reduced *N*-methyl-D-aspartate-, bicuculline-, and pentylenetetrazol-induced seizures in mice in the absence of changes in A_1R, $A_{2A}Rs$, or $GABA_A$ receptors (Georgiev et al. 1993; Johansson et al. 1996). The effect was due to the combined effects of theophylline, to which caffeine is metabolized in the brain, and caffeine itself, but could not be ascribed to changes in A_1Rs, $A_{2A}Rs$, or $GABA_A$ receptors (Johansson et al. 1996). In contrast, higher plasma concentrations of caffeine (100 μM) after chronic dosage for 12 days resulted in increased A_1R densities, whereas messenger RNA levels or $A_{2A}Rs$ were not affected (Johannson et al. 1993). Remarkably, chronic caffeine administration in rats (40 mg kg^{-1}, twice daily for 7 days) increased the thresholds for subsequent theophylline-induced seizures (Zhi and Levy 1990). This phenomenon of effect inversion might be an explanation why children (who are considered to be caffeine-naïve) appear to be more sensitive to TAS (see earlier). Effect inversion of chronic adenosine receptor antagonists has also been described within the context of ischemic excitotoxicity (de Mendonca et al. 2000). Whereas acute methylxanthines generally aggravate ischemic injury, the chronic use of caffeine or of the A_1R-selective antagonist DPCPX protects the brain from ischemic injury (de Mendonca et al. 2000). The phenomenon of effect inversion of acute versus chronic caffeine has been studied intensively and has been explained by antagonism of an endogenous agonist that downregulates A_1Rs without affecting gene transcription (Jacobson et al. 1996). Evidence for effect inversion by caffeine or adenosine receptor ligands has been obtained through changes in physiological outcome parameters such as susceptibility to seizures or to seizure- and ischemia-induced neuronal cell death (Jacobson et al. 1996). Despite these clear physiological changes, the molecular mechanisms behind this phenomenon appear to be more complex since upregulation of A_1Rs as a consequence of chronic caffeine was not always observed (Georgiev et al. 1993; Johansson et al. 1996). Later studies have ruled out upregulation of A_1Rs as a consequence of the long-term use of caffeine or theophylline in reasonably normal doses, indicating that upregulation of A_1Rs is triggered only by excessively high or toxic doses of methylxanthines (Svenningsson et al. 1999). Thus, selected doses and durations of exposure and withdrawal as well as $A_{2A}R$-mediated effects (see later) might play an important role.

In a recent study a single dose of acute caffeine (40 mg kg^{-1} intraperitoneally) given *after* the onset of seizures in a new mouse model of sudden unexplained death in epilepsy (SUDEP) significantly increased the survival time from 24 to 55 min (Shen et al. 2009). This protective effect of acute caffeine can best be explained by caffeine antagonizing a seizure-induced surge of adenosine, which had experimentally been exacerbated by pharmacological disruption of adenosine clearance. In this model of SUDEP, excessive seizure-induced concentrations of adenosine are thought to induce cardiac and respiratory failure by overstimulation of brainstem adenosine receptors, an effect that can be ameliorated by caffeine-induced blockade of these receptors (Shen et al. 2009).

5.2 *Caffeine: A_1R- and A_{2A}R-Mediated Actions*

Whereas the anticonvulsant role of A_1Rs is well established, newer findings suggest that A_{2A}Rs play an important role in modulating the susceptibility to seizures. Thus, A_{2A}R knockout mice are partially resistant to limbic seizures induced by chomoconvulsants or to seizures induced by ethanol withdrawal (El Yacoubi et al. 2001, 2009). Interestingly, the attenuation of clonic pentylenetetrazole-induced seizures in A_{2A}R knockout mice could be mimicked in wild-type mice exposed to chronic caffeine (0.3 g l^{-1} caffeine in drinking water) during a period of 14 days prior to the seizure tests (El Yacoubi et al. 2008). However, A_{2A}R knockout mice under chronic caffeine were less protected from clonic seizures than water-treated A_{2A}R knockout mice, a conflicting result that was not further addressed (El Yacoubi et al. 2008). Together, these findings indicate that the protective effects of chronic caffeine might best be explained by antagonizing the A_{2A}R and thus causing a state of decreased neuronal excitability; however, these studies also indicate a proconvulsant role of chronic caffeine under conditions during which A_{2A}R-dependent signaling is abolished.

5.3 *$GABA_A$ Receptor and Phosphodiesterase Inhibition*

In contrast to adenosine receptors, which are affected by caffeine plasma concentrations attainable by normal human caffeine consumption, 10–100 times higher concentrations are needed to inhibit $GABA_A$ receptors or phosphodiesterase (Fredholm et al. 1999). Therefore, a direct proconvulsant role of "physiological" doses of methylxanthines via $GABA_A$ receptors or phosphodiesterase appears to be unlikely. However, caffeine can inhibit the binding of benzodiazepines to the $GABA_A$ receptor (Marangos et al. 1979), which might contribute to a convulsant role of high or toxic doses of methylxanthines. Inhibition of benzodiazepine binding to $GABA_A$ receptors might be an explanation for the clinical findings that TAS are usually refractory to treatment with diazepam or other drugs that act via the $GABA_A$ receptor (Yoshikawa 2007).

5.4 Ryanodine-Receptor-Activated Calcium-Induced Calcium Release

Changes in Ca^{2+} homeostasis and persistent increases in the intracellular Ca^{2+} level contribute to the initiation and maintenance of acquired epilepsy (DeLorenzo et al. 2005). Ryanodine-receptor-mediated calcium-induced calcium release plays a key role in regulating intracellular calcium concentrations in epileptic conditions (Pal et al. 2001). The brain ryanodine receptor is a caffeine-sensitive calcium-release channel and mediates the caffeine-induced mobilization of Ca^{2+} from internal stores (McPherson et al. 1991; Usachev et al. 1993). The caffeine-induced release of Ca^{2+} from ryanodine-sensitive calcium stores in the neuronal endoplasmic reticulum and pathological mechanisms that potentiate this response may render neurons more vulnerable to excitotoxicity and to the expression of seizures (Chan et al. 2000; Verkhratsky 2005). Interestingly, in cultured hippocampal neurons, the newer antiepileptic drug levetiracetam led to a 61% decrease in caffeine-induced peak height of the intracellular Ca^{2+} level (Nagarkatti et al. 2008), indicating that levetiracetam might interact with adenosine-related signaling.

5.5 Free Radicals in Theophylline-Induced Seizures

A possible role of free radicals in theophylline-induced seizures was recently suggested (Gulati et al. 2005, 2007; Ray et al. 2005). In the underlying studies, aminophylline (50–250 mg kg^{-1}) dose-dependently induced convulsions and mortality in rats. Seizures and mortality were attenuated by antioxidants (melatonin, *N*-acetylcysteine) and by nitric oxide (NO) synthase inhibitors [N^{ω}-nitro-L-arginine methyl ester (L-NAME), 7-nitroindazole]. Combination of antioxidant and NO-reducing treatments augmented the anticonvulsant effects of single treatments. Further, the authors found increased concentrations of malondialdehyde and NO metabolites in brain homogenates of mice with aminophylline-induced seizures; accumulation of these metabolites could be attenuated by melatonin or L-NAME pretreatment. These studies suggest the contribution of free radicals in the mechanism of theophylline-induced ictogenesis.

5.6 Inhibition of TREK-1 Channels by Methylxanthines

TREK-1, a member of the two-pore-domain K^+ channel superfamily, plays a major role in regulating the resting membrane potential of neurons, and thus contributes to controlling neuronal excitability (Honore 2007). Using whole-cell patch-clamp recordings on human TREK-1 channel expressing Chinese hamster ovary cells, Harinath and Sikdar (2005) demonstrated reversible inhibition of the channels, and depolarization of the membrane potential, by caffeine and theophylline in a concentration-dependent manner. Inhibition by caffeine and theophylline was attenuated in channels with a mutation of a protein kinase A (PKA) consensus sequence, indicating involvement of the cyclic AMP/PKA pathway. Thus, inhibition of TREK-1-dependent membrane depolarization may contribute to seizure generation by toxic doses of caffeine or theophylline.

6 Conclusions and Outlook

Although adenosine-independent mechanisms have been proposed, the majority of evidence indicates that the proconvulsant roles of methylxanthines are based on antagonism of the brain's endogenous adenosine-based seizure control system. Whereas inhibition of A_1Rs by methylxanthines can directly contribute to ictogenesis and seizure spread, under certain conditions methylxanthines can also contribute to seizure suppression. First, this can be the case after chronic drug exposure leading to inversion and alterations in gene expression (Svenningsson et al. 1999). Second, antagonism of A_{2A}Rs by methylxanthines may have direct anticonvulsant and neuroprotective consequences.

A detailed understanding of the convulsant role of methylxanthines is of importance since many new drugs that act on adenosine receptors are in clinical trials. For example, in recent clinical trials conducted with the A_1R antagonist rolofylline, which facilitates diuresis and preserves renal function in patients with acute heart failure with renal impairment, the occurrence of seizures was described in some patients who were treated with higher doses of the drug (Cotter et al. 2008). This example demonstrates that caution is needed when evaluating the clinical use of new adenosine-related therapeutic agents; however, understanding the mechanisms involved in the adenosine-related control of seizure mechanisms will allow the safe use of novel drugs that act on new therapeutic principles. New approaches using gene-array-based strategies might unravel novel pathways and interactions that might help explain the complex role of methylxanthines in determining the brain's susceptibility to seizures and excitotoxicity (Yu et al. 2009).

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Impacts of Methylxanthines and Adenosine Receptors on Neurodegeneration: Human and Experimental Studies

Jiang-Fan Chen and Yijuang Chern

Contents

Abstract Neurodegenerative disorders are some of the most feared illnesses in modern society, with no effective treatments to slow or halt this neurodegeneration. Several decades after the earliest attempt to treat Parkinson's disease using caffeine, tremendous amounts of information regarding the potential beneficial effect

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of caffeine as well as adenosine drugs on major neurodegenerative disorders have accumulated. In the first part of this review, we provide general background on the adenosine receptor signaling systems by which caffeine and methylxanthine modulate brain activity and their role in relationship to the development and treatment of neurodegenerative disorders. The demonstration of close interaction between adenosine receptor and other G protein coupled receptors and accessory proteins might offer distinct pharmacological properties from adenosine receptor monomers. This is followed by an outline of the major mechanism underlying neuroprotection against neurodegeneration offered by caffeine and adenosine receptor agents. In the second part, we discuss the current understanding of caffeine/methylxantheine and its major target adenosine receptors in development of individual neurodegenerative disorders, including stroke, traumatic brain injury Alzheimer's disease, Parkinson's disease, Huntington's disease and multiple sclerosis. The exciting findings to date include the specific *in vivo* functions of adenosine receptors revealed by genetic mouse models, the demonstration of a broad spectrum of neuroprotection by *chronic* treatment of caffeine and adenosine receptor ligands in animal models of neurodegenerative disorders, the encouraging development of several A_{2A} receptor selective antagonists which are now in advanced clinical phase III trials for Parkinson's disease. Importantly, increasing body of the human and experimental studies reveals encouraging evidence that regular human consumption of caffeine in fact may have several beneficial effects on neurodegenerative disorders, from motor stimulation to cognitive enhancement to potential neuroprotection. Thus, with regard to neurodegenerative disorders, these potential benefits of methylxanthines, caffeine in particular, strongly argue against the common practice by clinicians to discourage regular human consumption of caffeine in aging populations.

Keywords A_1 receptor · A_{2A} receptor · Alzheimer's disease · Adenosine receptors · Huntington's disease · Caffeine · Cognitive enhancer · Multiple sclerosis · Neuroprotection · Parkinson's disease · Stroke · Traumatic brain injury

1 Introduction

Strictly on the basis of the duration of the insults, neurodegeneration may be broadly divided into acute degeneration, such as stroke and traumatic brain injury (TBI), and chronic degeneration, such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) (Mattson 2000). Neurodegenerative disorders are some of the most feared illnesses in modern society. Ischemic stroke is the second most common cause of death in the USA/Europe and the third most common in Japan (Lo et al. 2003). TBI is the leading cause of death in the USA for individuals under 37 years old (Rutland-Brown et al. 2006; Moppett 2007).

Ischemic and traumatic insults produce necrosis and apoptosis as a result of overstimulation of neurons due to excitotoxicity and calcium overload (Lo et al. 2003). Chronic neurodegenerative disorders generally occur in mid to late life with both genetic and environmental etiological factors that result in severe and progressive cognitive and motor deficits. AD and PD are the first and second most common neurodegenerative disorders: AD affects 5% of people over 65, and another 1% of this population has PD (Lang and Lozano 1998b; Olanow 2004; Perrin et al. 2009). The pathological hallmark of chronic neurodegenerative disorders is neuronal cell death within specific regions and cell subpopulations, frequently characterized by prominent proteinaceous inclusions that accumulate in the extracellular milieu or within interacellular compartments of affected neurons (Braak and Braak 1997; Hardy and Gwinn-Hardy 1998; Dauer and Przedborski 2003; Perrin et al. 2009). Unfortunately, there are no successful treatments currently available that can slow or halt this chronic neurodegeneration (Shoulson 1998; Mattson 2004; Olanow 2004; Lopez-Diego and Weiner 2008; Mestre et al. 2009). Meanwhile, the number of people with neurodegenerative disorders is rapidly increasing as average lifespan increases. For example, the number of individuals afflicted by PD is expected to double by 2030 in line with the aging population and increases in life expectancy (Dorsey et al. 2007). Thus, there are critical and immediate medical needs for new pharmacological approaches to neurodegenerative disorders.

In the absence of an effective treatment for neurodegenerative disorders, epidemiological and experimental investigations into potential risk factors (including dietary factors) that may allow individuals to decrease their risk for neurodegenerative disorders become compelling. Caffeine is doubtless the most widely consumed psychoactive substance. It is estimated that more than 50% of the world's adult population consume caffeine on a daily basis (Fredholm et al. 1999). One of the main reasons for such prevalent use is the well-known psychostimulant (cognitive enhancement) effect of caffeine. Caffiene's psychostimulant effect is most obvious at low doses (Nehlig et al. 1992; Daly et al. 1999; Fredholm et al. 1999). At higher doses, the effects of caffeine vary among individuals and may lead to various untoward effects (Fredholm et al. 1999). Human intake of coffee/caffeine appears to decline during aging in Western cultures. This is in part due to undesirable side effects of high doses of caffeine, including increased anxiety, increased blood pressure, headache, and confusion (Nehlig et al. 1992; Daly et al. 1999; Fredholm et al. 1999), and in part due to caffeine-intake restrictions suggested by health care professions. Such restrictions appear unwarranted, based on the comprehensive literature review here indicating that regular human consumption of caffeine does not impose significant adverse effects on the cardiovascular system, bone status, or the incidence of cancer (Fredholm et al. 1999; Winkelmayer et al. 2005; Higdon and Frei 2006; van Dam et al. 2006; Cadden et al. 2007; Daly 2007), and in fact regular caffeine consumption may be associated with reduced risk for some neurodegenerative disorders such as PD and AD during aging (Fredholm et al. 1999; Ross et al. 2000; Daly 2007; Ritchie et al. 2007).

In this review, we will first provide a general background on the signaling systems by which caffeine and methylxanthine modulate brain activity and their role in the relationship to the development and treatment of neurodegenerative disorders. This is followed by an outline of the major mechanism underlying neuroprotection against neurodegeneration offered by caffeine and adenosine receptor agents. In the second part, we discuss the current understanding of caffeine/xanthine and its major target adenosine receptors in the development of individual neurodegenerative disorders, including PD, stroke, AD, HD, TBI, and multiple sclerosis (MS).

2 Molecular Targets of Methylxanthenes, Including Caffeine

2.1 Non-adenosine Receptors

Pharmacologically, caffeine produces complex actions through multiple molecular targets (Nehlig et al. 1992; Daly et al. 1999; Fredholm et al. 1999). Historically, four different molecular responses to caffeine have been proposed to underlie its psychostimulant effects: calcium release, phosphodiesterase (PDE) inhibition, $GABA_A$ receptor inhibition, and antagonism of adenosine receptors. Initially, calcium release was thought to be the major mechanism for the action of caffeine and other methylxanthine-based compounds, because caffeine stimulates calcium release from intracellular storage at a threshold concentration of 250 μM (with maximal effect at 5–20 mM) (McPherson et al. 1991). Somewhat later it was found that these compounds also inhibit PDE, the enzyme which degrades cyclic AMP (cAMP), and it was proposed that caffeine operates by elevating intracellular cAMP concentrations (Choi et al. 1988). However, both calcium release and PDE inhibition are unlikely to account for the effects of caffeine in humans because PDE inhibition requires caffeine concentrations 10–100 times higher than those achieved by typical dietary intake (Daly et al. 1999), and caffeine is even less potent as a releaser of calcium. In the 1970s, caffeine was also found to inhibit binding of benzodiazepines to sites on $GABA_A$ receptors, raising a third possibility for the mechanism of caffeine's actions in humans (Marangos et al. 1979). The interaction between caffeine and $GABA_A$ receptors might explain the anxiogenic and convulsant effects induced by high doses of caffeine. However, the low affinity of caffeine for $GABA_A$ receptors ($K_i = 280$ μM) suggested that normal human caffeine consumption is unlikely to produce caffeine doses high enough to inhibit $GABA_A$ receptors. Consistent with multiple molecular targets of caffeine, a novel drug target validation strategy coupled with microarray profiling recently showed that a high dose of caffeine (50 mg kg^{-1}) induced complex expression patterns with *three* distinct sets of striatal genes associated with three distinct molecular targets: adenosine A_{2A} receptor ($A_{2A}R$), non-$A_{2A}R$, and a group requiring interaction of the $A_{2A}R$ inactivation and non-$A_{2A}R$ targets (Yu et al. 2009). Furthermore, the involvement of

PDE and $GABA_A$ receptor are also indicated as components of the non-$A_{2A}R$ targets by the overlapping of the striatal gene sets elicited by the PDE inhibitor rolipram or by the $GABA_A$ receptor antagonist bicucullin with distinct subsets of striatal genes elicited by caffeine (50 mg kg^{-1}) administered to $A_{2A}R$-knockout mice. This supports the findings of earlier work suggesting there are multiple molecular targets for caffeine, including the contribution of the $A_{2A}R$ and non-$A_{2A}R$ targets such as PDE and $GABA_A$ receptors. More recently, other novel targets for caffeine in mediating the modulatory effect of caffeine on neuronal survival, including ryanodine receptor channels (Guerreiro et al. 2008) and nuclear enzyme poly(ADP-ribose)polymerase-1 (Geraets et al. 2006; Szabo et al. 2006), are suggested.

2.2 Adenosine Receptors

In the late 1970s, it became increasingly obvious that caffeine's actions in the brain are better explained by its antagonism of adenosine receptors (Fredholm 1980). Adenosine receptor blockade takes place at concentrations many times lower than those required for calcium release, PDE inhibition, or inhibition of $GABA_A$ receptor binding (Fredholm et al. 1999; Daly 2007). The caffeine concentrations required for adenosine receptor blockade can be attained after drinking even a single cup of coffee (containing 40–180 mg of caffeine), which can produce plasma concentrations of 2–10 μM (Fredholm et al. 1999; Daly 2007). Thus, of these possible mechanisms, only adenosine receptor blockade occurs at an affinity (2–50 μM) compatible with the caffeine plasma concentrations attained by normal human caffeine consumption (i.e., 250 mg day^{-1}). In addition, caffeine's psychostimulant effects are best correlated with its blockade of brain adenosine receptors (Fredholm et al. 1999; Daly 2007). It is now the accepted view that caffeine, in doses consumed habitually by humans, binds both adenosine A_1 receptors (A_1Rs) and $A_{2A}Rs$ and, by blocking these receptors at pre- and postsynaptic sites, respectively, relieves the largely inhibitory tone of endogenous adenosine and enhances brain dopaminergic activity, resulting in psychostimulant action (Fredholm et al. 1999; Daly 2007). This is supported by pharmacological evidence and by behavioral studies performed in $A_{2A}R$-knockout mice (Nehlig et al. 1992; Daly et al. 1999; Fredholm et al. 1999; El Yacoubi et al. 2000; Chen et al. 2001). The requirement of the $A_{2A}R$ for caffeine's action in the brain is validated by the finding that caffeine-induced striatal gene expression is abolished in $A_{2A}R$-knockout mice (Yu et al. 2009). Interestingly, despite caffeine's similar affinities for the A_1R and the $A_{2A}R$ in brain, both pharmacological (Nehlig et al. 1992; Fredholm 1995; Fredholm et al. 1999) and genetic knockout (Fredholm et al. 1999; El Yacoubi et al. 2000; Chen et al. 2001) studies have revealed that caffeine's psychostimulant effect is better correlated with its blockade of brain $A_{2A}R$. It must be emphasized that although this evidence is suggestive, the conclusion is not yet firm and there is evidence that other targets may also play a role, particularly at the higher end of the range of doses taken by humans.

2.2.1 Interactions Between Adenosine Receptors and Other G-Protein-Coupled Receptors

Adenosine receptors are capable of forming oligomers among themselves or with other G-protein-coupled receptors (GPCRs). The A_1R forms an oligomer with the D_1 dopamine receptor which can be regulated by agonist binding (Gines et al. 2000). The $A_{2A}R$ can form higher-order oligomers with itself (Vidi et al. 2008), and has multiple binding partners, including the A_1R (Ciruela et al. 2006), the D_2 dopamine receptor (D_2R) (Hillion et al. 2002; Kamiya et al. 2003), the D_3 dopamine receptor (Torvinen et al. 2005), the metabotropic glutamate type 5 receptor (mGlu5R) (Diaz-Cabiale et al. 2002; Kachroo et al. 2005), and the cannabinoid CB_1 receptor (Carriba et al. 2007). With use of high-resolution immunoelectron microscopy and immunoprecipitation, higher-order oligomer complexes containing the $A_{2A}R$, the D_2R, the mGlu5R, or CB1 receptors were found to exist in native tissues (Navarro et al. 2008; Cabello et al. 2009; Ferre et al. 2009). Formation of these homo- or hetero-GPCR oligomers usually affects the pharmacological properties, intracellular signaling, and/or surface expression of both receptors (Vidi et al. 2008; Cabello et al. 2009; Navarro et al. 2009). Years after the initial discovery of these receptor oligomers, evidence began to emerge from different laboratories to support their physiological relevance. For example, with use of competitive peptides in brain slices, the $A_{2A}R$ was found to suppress D_2R-mediated regulation of membrane potential transitions and firing patterns in striatal neurons through the formation of $A_{2A}R$–D_2R heteromerization (Azdad et al. 2009). Direct protein–protein interactions between the $A_{2A}R$ and the D_2R may provide a mechanistic basis for the involvement of the D_2R in caffeine's action (Zahniser et al. 2000) and the ability of caffeine to affect the action of antipsychotic drugs that target the D_2R, including haloperidol (Varty et al. 2008; Salamone et al. 2009). It will of great interest to further characterize the pharmacological properties of these adenosine-receptor-containing heteromers toward adenosine drugs and methylxanthine in vivo. Along this line, a few drugs targeted toward receptor dimers or oligomers were reported in the past few years. For example, a dendrimer of the $A_{2A}R$-selective agonist CGS21680, which molecular modeling analysis predicts will bind to the homodimer of the $A_{2A}R$, was shown to effectively inhibit platelet aggregation (Ivanov and Jacobson 2008; Kim et al. 2008). Heterobivalent reagents composed of an $A_{2A}R$ antagonist and a D_2R agonist bound to membranes containing both receptors with higher affinities than monovalent ligands, and were implicated in drug development for PD (Soriano et al. 2009).

2.2.2 Interacting Accessory Proteins

Besides binding to GPCRs, the C terminus of the $A_{2A}R$ is also directly associated with a handful of interacting proteins, including the F-actin-cross-linking protein (α-actinin), which is important for $A_{2A}R$ internalization (Burgueno et al. 2003), a deubiquitination enzyme (Usp4) that controls $A_{2A}R$ expression level in plasma membranes (Milojevic et al. 2006), a binding protein of Translin (Trax) that is known to transport the brain-derived neurotrophic factor (BDNF) transcript in neuronal processes (Sun et al. 2006; Chiaruttini et al. 2009), a nucleotide exchange factor (ARNO) which mediates sustained mitogen-activated protein kinase (MAPK) signaling (Gsandtner et al. 2005), a calcium-binding protein (calmodulin) critical for the formation of the $A_{2A}R$–D_2R heterodimer (Navarro et al. 2009), and the fibroblast growth factor (FGF) receptor (Flajolet et al. 2008). These binding proteins have different natures and are involved in various novel functions of the $A_{2A}R$. Further studies are required to demonstrate how these binding proteins function in in vivo pathophysiological condition and to determine whether they might be useful targets for therapeutic development. In particular, the finding that coactivation of the $A_{2A}R$ and the FGF receptor plays a crucial role in the synaptic plasticity of the corticostriatopallidal pathway may have important clinical implications for major neurodegenerative diseases (Flajolet et al. 2008).

The multiplicity of adenosine receptor subtypes (particularly A_1Rs and $A_{2A}Rs$) and the complex interactions between adenosine receptors, other GPCRs, and accessory signaling proteins in the brain may partly explain the heterogeneity and complexity of caffeine's action, and may underlie the biphasic motor and cardiovascular responses to increasing doses of caffeine in rodents (Svenningsson et al. 1995, 1999).

3 Molecular and Cellular Mechanisms of Neuroprotection by Caffeine and Adenosine Receptors

3.1 Control of Glutamate Release at Presyanptic Sites

Adenosine receptors may influence the outcome of brain injury by modulating glutamate and aspartate release in the brain. A_1Rs are detected at high density at presynaptic nerve terminals and their activation efficiently inhibits the action of almost all neurotransmitters via G-protein-mediated inhibition of calcium channels in nerve endings (Dunwiddie and Masino 2001). The inhibition appears most prominent in excitatory glutamatergic systems where synaptic neurotransmission is almost completely blocked (Dunwiddie et al. 1981). Thus, by blocking A_1R-mediated presynaptic inhibition of excitatory neurotransmission, caffeine increases the general excitability of systems in the brain. On the other hand, $A_{2A}R$ agonists enhance the release of glutamate under ischemic and nonischemic conditions (O'Regan et al. 1992; Simpson et al. 1992; Sebastiao and Ribeiro 1996; Dunwiddie and Masino 2001). For example, the $A_{2A}R$ agonist CGS21680 can enhance glutamate release at concentrations as low as 10^{-12} M in synaptosomal and slice preparations (Sebastiao and Ribeiro 1996; Marchi et al. 2002). Of note, the concentration needed to enhance glutamate release ($EC_{50} = 1$ pM) is almost 1,000-fold lower than is required for GABA release ($EC_{50} = 1$ nM) (Cunha and Ribeiro 2000).

In vivo studies have shown that the neuroprotective effect of SCH58261 is observed only at a very low dose (0.01 mg kg^{-1}), 100–1,000 fold-lower than the dose used to stimulate motor activity (1–10 mg kg^{-1}) (Popoli et al. 2002, 2003). The enhancement of glutamate release by the $A_{2A}R$ may be due to its positive coupling to the cAMP–protein kinase A (PKA) signaling pathway, leading to increased Ca^{2+} influx (Gubitz et al. 1996; Dunwiddie and Fredholm 1997). This facilitating effect of $A_{2A}R$ agonists has been attributed to either an effect on glutamatergic terminals (Nikbakht and Stone 2001; Rosin et al. 2003) or an indirect effect via downregulation of A_1R-mediated inhibition (Lopes et al. 2002). Recent studies have indicated that glial $A_{2A}Rs$ may also play an important role in the control of glutamate efflux by regulation of a specific glial glutamate transporter (GLT-1). The $A_{2A}R$ agonist CGS21680 enhances glutamate efflux on cultured astrocytic glial cells from the cortex or brainstem (Li et al. 2001; Nishizaki et al. 2002). Voltage-clamp recording suggests that this effect occurs without affecting presynaptic glutamate release or postsynaptic glutamatergic conductance (Nishizaki et al. 2002). Thus, $A_{2A}Rs$ significantly modulate glutamate release by presynaptic and glial mechanisms.

3.2 Modulation of Cellular Survival Signals at Postsynaptic Sites

Protection by adenosine receptor activity may also result from direct action on receptors at postsynaptic sites on neurons. While adenosine-mediated inhibition of neural activity in the central nervous system (CNS) is believed to be largely a product of A_1R-mediated presynaptic inhibition, activation of A_1R also results in G-protein-dependent activation of inwardly rectifying potassium channels at postsynaptic sites, leading to hyperpolarization of the resting membrane potential of postsynaptic neurons (Dunwiddie 1997). This could potentially explain how caffeine, via blockade of A_1Rs, increases firing of different neurons, including the cholinergic neurons regulating sleep–wakefulness (Rainnie et al. 1994; Oishi et al. 2008). In addition, this effect may be why high doses of caffeine can induce seizures. Such a view is supported by in vitro data demonstrating that caffeine acting at A_1Rs on glutamatergic neurons produces epileptiform activity in vitro (Dunwiddie 1980; Dunwiddie et al. 1981). On the other hand, activation of $A_{2A}Rs$ inhibits NMDA-receptor-mediated synaptic currents in rat neostriatal neurons (Norenberg et al. 1997, 1998; Wirkner et al. 2000). This inhibition of NMDA receptor activity is mediated by the PKA pathway (Wirkner et al. 2000). Consequently, blockade of postsynaptic $A_{2A}Rs$ is likely detrimental to striatal neurons. Consistent with this notion, recent studies have demonstrated that the $A_{2A}R$ antagonists ZM241385 and SCH58261 potentiate quinolinic acid induced intracellular calcium efflux in striatal neurons (Popoli et al. 2002) and significantly amplify the excitotoxic effect of direct NMDA receptor stimulation, while reducing neurotransmitter release. Similarly, the $A_{2A}R$ antagonist 8-(3-chlorostyryl)caffeine (CSC) has been found to potentiate NMDA-induced hippocampal toxicity (Robledo et al. 1999).

In addition, stimulation of the postsynaptic $A_{2A}Rs$ was proposed to activate PKA to promote cell survival signals (Blum et al. 2003a). In PC12 cells, atypical protein kinase C was found to act downstream of PKA to prevent apoptosis induced by serum withdrawal (Huang et al. 2001). In addition, $A_{2A}R$ stimulation rescues the blockage of nerve growth factor (NGF)-induced neurite outgrowth by enhancing the phosphorylation of the cAMP-response element-binding (CREB) protein when the NGF-evoked MAPK pathway is damaged (Cheng et al. 2002), or by direct binding to TRAX when the p53-mediated pathway is blocked (Sun et al. 2006). Consistent with a protective role of the $A_{2A}R$, an A_{2A}-R-selective agonist (CGS21680) effectively ameliorates several major symptoms of HD in a transgenic mouse model (R6/2) of HD (Chou et al. 2005). At least part of the beneficial effect of CGS21680 in HD mice can be attributed to the PKA-mediated enhancement of ubiquitin proteasome activity (Chiang et al. 2009).

3.3 Control of Neuroinflammation in the CNS

Vascular and inflammatory effects, including $A_{2A}R$-mediated vasodilation (Winn et al. 1985; Ngai et al. 2001), inhibition of platelet aggregation (Sandoli et al. 1994; Ledent et al. 1997), and suppression of superoxide species generation by neutrophils (Cronstein et al. 1983, 1990) may underlie the protection offered by $A_{2A}R$ agonists. Particularly, activation of $A_{2A}Rs$ inhibits the production of proinflammatory cytokines such as tumor necrosis factor α, interleukin-6, and interleukin-12 (Hasko et al. 2000; Mayne et al. 2001; Ohta and Sitkovsky 2001; Day et al. 2003, 2004; Sitkovsky et al. 2004). This effect could be responsible for the neuroprotection afforded by $A_{2A}R$ agonists in cerebral hemorrhage (Mayne et al. 2001), spinal cord injuries (Cassada et al. 2002b), and other tissue-damaging insults (Ohta and Sitkovsky 2001; Day et al. 2003). Largely on the basis of $A_{2A}R$ action in peripheral tissues, extracellular adenosine acting at $A_{2A}Rs$ has been proposed as an endogenous circuit breaker that inhibits inflammation and limits extensive inflammatory tissue damage (Sitkovsky et al. 2004). On the other hand, in a recent study using chimeric mice, selective inactivation of $A_{2A}Rs$ in bone marrow cells (generated by transplantation) attenuated ischemia-induced expression of proinflammatory cytokines and reduced ischemic brain injury (Yu et al. 2004). This may reflect differential effects of $A_{2A}R$ activation on inflammation in the peripheral nervous system versus the CNS. It may also reflect complex (both potentially deleterious and neuroprotective) effects of $A_{2A}R$ activation in glial cells, including regulation of glutamate efflux (as described above), upregulation of cyclooxygenase 2, modulation of the activities of nitric oxide synthase, production of proinflammatory prostaglandins and cytokines, as well as microglial activation (Fiebich et al. 1996; Brodie et al. 1998). The precise influence of $A_{2A}R$ activation on brain glial cells remains to be determined. Recently, it was proposed that caffeine metabolites can inhibit the nuclear enzyme poly(ADP-ribose)polymerase-1 at concentrations attainable by normal human caffeine consumption (Geraets et al. 2006), suggesting another possible mechanism for caffeine modulation of the inflammatory response in the brain.

3.4 Interaction with Neurotrophic Factors

The Trk receptors for neurotrophic factors (such as NGF, BDNF, and glial-cell-line-derived neurotrophic factor, GDNF) are an important class of membrane receptors that involve autophosphorylation in tyrosine residues as a result of ligand binding, triggering a signaling cascade associated with regulation of cell death, survival, and differentiation (Hu and Russek 2008). The cross talk between adenosine receptors, particularly the $A_{2A}R$, and the receptor for neurotrophic factors involving tyrosine receptor kinase, has been demonstrated (for a review, see Sebastiao and Ribeiro 2009b). The $A_{2A}R$ was also shown to transactivate neurotrophin receptors and enhance their trophic functions (Lee and Chao 2001; Wiese et al. 2007; Sebastiao and Ribeiro 2009a) on BDNF-mediated synaptic transmission (Diogenes et al. 2004; Tebano et al. 2008) and long-term potentiation (Fontinha et al. 2008) and GDNF-mediated action in striatal dopaminergic terminals (Gomes et al. 2006). Indeed, systemic administration of an A_{2A}-selective antagonist (SCH58261) decreased the level of BDNF in the brain (Domenici et al. 2007). These findings collectively warrant further evaluation of the complex roles of adenosine drugs and methylxanthine in neurodegenerative diseases in the future.

3.5 Regulation of Blood–Brain Barrier Integrity

The blood–brain barrier (BBB) is a physical, immune, and metabolic barrier to protect the microenvironment of brain from the systemic circulation. BBB breakdown occurs in a variety of neurological disorders, including stroke (Latour et al. 2004), TBI (Hoane et al. 2006), AD (Zipser et al. 2007), PD (Kortekaas et al. 2005; Rite et al. 2007), and MS (Minagar and Alexander 2003), and may contribute to PD pathogenesis (Rite et al. 2007). Thus, improving BBB integrity represents an important mechanism by which neuroprotective agents such as caffeine may exert neuroprotective effects. Indeed, caffeine has been shown to protect against BBB dysfunction induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and high cholesterol in animal models of PD and AD (Chen et al. 2008a, b).

4 Methylxanthine/Caffeine and Neurodegenerative Disorders

4.1 Stroke

During ischemic insults, the level of extracellular adenosine increases rapidly and markedly (Hagberg et al. 1987; Matsumoto et al. 1992; Latini and Pedata 2001). An increased level of extracellular adenosine is generally considered an important neuroprotective mechanism against ischemic injury (Rudolphi et al. 1992; de Mendonça et al. 2000; Latini and Pedata 2001; Boison 2006; Cunha et al. 2006;

Chen et al. 2007). Indeed, reduction of the level of extracellular adenosine by transgenic overexpression of adenosine kinase – the primary adenosine-metabolizing enzyme – rendered the mouse brain more susceptible to ischemic cell death (Pignataro et al. 2007).

4.1.1 Human Studies of Methylxanthine/Caffeine

Given the widespread and extensive use of dietary caffeine, the impacts of coffee consumption on stroke have been extensively investigated in many different settings, with somewhat inconsistent findings. Some of the ambiguity as to whether caffeine is a risk factor for stroke might result from poor estimation of dietary caffeine consumption and/or a lack of information on systemic levels of caffeine and its metabolites in the body (James 2004). The potential contribution of caffeine to stroke is of particular interest because caffeine at dietary doses enhances blood pressure owing to direct vasoconstriction caused by adenosine receptor blockage (Pincomb et al. 1988; James 2004), and therefore caffeine is considered a cardiovascular risk factor (Greenland 1993). Interestingly, an earlier study of 45,589 men showed that total caffeine consumption was not associated with the risk of coronary heart disease or stroke (Grobbee et al. 1990). Consistent with this notion, a recent report of a 24-year follow-up study of 83,076 women also suggested that chronic coffee consumption is not a risk factor for stroke in women. However, a higher dose of coffee intake by hypertensive men (55–68 years old, 499 patients) was associated with a higher risk of thromboembolic stroke, but not of hemorrhagic stroke (Grobbee et al. 1990). In addition, heavy caffeine use (five drinks per day or pharmaceutical products) was found to be linked to intracerebral hemorrhage (18–49 years old, 217 patients; Feldmann et al. 2005) and subarachnoid hemorrhage (18–49 years old, 312 cases; Broderick et al. 2003). Since caffeine at dietary doses suppresses cerebral blood flow, caution may be required for patients recovering from acute ischemia stroke (Ragab et al. 2004). However, these effects of caffeine on cerebral blood flow may not occur after long-term use (Addicott et al. 2009).

Owing to their vascular effects, a combination of caffeine and ethanol (the two most commonly consumed substances by humans, designated caffeinol) was shown to reduce cortical infarct areas in rat ischemia models (Strong et al. 2000; Aronowski et al. 2003). Although the protection by caffeinol in cortical areas is effective, caffeinol exerts no beneficial effects in subcortical regions, where most human strokes occur (Belayev et al. 2004; Hoyte et al. 2004). In a rabbit embolic ischemia model which is more similar to human stroke than the rat model (Lapchak et al. 2002), caffeinol by itself exhibited no neuroprotective function (Lapchak et al. 2004). This discrepancy between the effects of caffeinol in rat and rabbit models might result from the fact that the stroke induced in rabbits was more heterogeneous than that in the rat model. Moreover, when caffeinol was combined with a plasminogen activator (t-PA, an FDA-approved treatment for acute ischemic stroke; Lapchak et al. 2002), the risk of hemorrhage increased, which somewhat dampened the enthusiasm for using caffeinol in stroke patients

(Lapchak et al. 2004). Fortunately, in pilot studies of patients with acute ischemia, caffeinol alone, caffeinol plus t-PA, or caffeine plus tissue-type plasminogen activator plus hypothermia evoked no adverse side effects, and therefore might be safe for patients with acute stroke (Piriyawat et al. 2003; Martin-Schild et al. 2009). Further studies are required to evaluate the efficacy of the above-mentioned combined treatments for acute stroke. Theophylline and its synthetic analog aminophylline, when administered alone to stroke patients, did not produce significant beneficial effects on ischemic stroke (Geismar et al. 1976; Britton et al. 1980).

4.1.2 Experimental Studies on Methylxanthine

A major part of methylxanthine's function can be attributed to adenosine receptors (particularly the A_1R and $A_{2A}R$) (Fredholm et al. 2001; Yang et al. 2009a). Indeed, mice expressing half the normal levels of the A_1R and the $A_{2A}R$ behave very similarly to those exposed to chronic caffeine (Yang et al. 2009b). In a rat cerebral ischemia model created by occlusion of the carotid arteries, theophylline worsened injury in the hippocampus with both normoglycemic and hyperglycemic ischemia (Zhou et al. 1994; Higashi et al. 2002). This experimental paradigm is of great interest because the amount of adenosine released during hyperglycemic ischemia is much lower than that released during normoglycemic ischemia (Hsu et al. 1991). Note that theophylline is a nonselective antagonist of A_1R and $A_{2A}R$. In the same ischemia model, an A_1R-predominant agonist (cyclohexyladenosine) markedly reduced hippocampal injury under normoglycemic and hyperglycemic ischemia (Zhou et al. 1994), while an $A_{2A}R$-selective antagonist (ZM 241385) reduced brain damage and memory function loss in hyperglycemic ischemia (but not in normoglycemia) (Higashi et al. 2002). The authors speculated that the A_1R is likely to exert a protective function against ischemic damage, and that the beneficial effects of $A_{2A}R$ blockage during hyperglycemic ischemia might be the result of potentiating the activity of the A_1R which attenuates glutamate release during ischemia (Heron et al. 1993; Pedata et al. 2001; Higashi et al. 2002). Effective protection against overall ischemia by other $A_{2A}R$ antagonists (e.g., SCH58261, CSC, and 4-amino-1-phenyl[1,2,4]-triazolo[4,3-*a*] quinoxaline) was reported in rodent ischemia models (Phillis 1995; Monopoli et al. 1998). Conversely, antagonists (e.g., 8-cyclopentyl-1,3- dipropylxanthine, DPCPX) of the A_1R were found to exacerbate ischemia-evoked damage (Phillis 1995), which suggested a protective role of the A_1R in ischemia. Nonetheless, although glutamatergic transmission is markedly affected by antagonists of the A_1R, elimination of the A_1R did not affect ischemia outcome owing to compensatory effects (Olsson et al. 2004). In addition, glutamate release and neuroprotection by (-)-N^6-(*R*)-(phenylisopropyl) adenosine do not correlate well. Glutamate release, therefore, might not be the sole pathway underlying the neuroprotective effect of the A_1R (Heron et al. 1994). An intriguing finding is that chronic treatment with A_1R agonists or antagonists produced effects opposite to those of acute treatment (Von Lubitz et al. 1994). In a gerbil forebrain ischemia model, acute treatment with an A_1R-selective agonist

(N^6-cyclopentyladenosine, CPA) protected neurons in the hippocampal CA1 region, while chronic treatment with CPA (15 days) shortened lifespan and decreased neuronal survival (Von Lubitz et al. 1994). The molecular mechanisms underlying the distinct effects of acute and chronic treatment with A_1R agonists are currently unknown. The change in response to chronic treatment might reflect compensatory adaptations. In contrast, the acute and chronic effects of $A_{2A}R$ drugs on stroke/ischemia are similar (Von Lubitz et al. 1995), probably owing to the relatively stable expression of the $A_{2A}R$ during chronic treatment with agonists or antagonists (Lupica et al. 1991; Abbracchio et al. 1992; Chern et al. 1993; Adami et al. 1995). Collectively, these data indicate that adenosine receptors are major players in modulating ischemic injury in the brain and in mediating the actions of methylxanthines during ischemia.

The $A_{2A}R$ is considered critically important in stroke because genetic inactivation of the $A_{2A}R$ attenuates cerebral infarction and decreases the severity of neurological deficits caused by focal ischemia (Chen et al. 1999, 2007). Acute administration of two $A_{2A}R$ antagonists (CSC and CGS15943) (Gao and Phillis 1994; Von Lubitz et al. 1995) was found to significantly suppress neurological deficits in a global hypoxia model in gerbils. Similarly, another $A_{2A}R$ antagonist (SCH58261) attenuated the release of excitatory amino acids, reduced infarct volume, and ameliorated turning behavior (Monopoli et al. 1998; Melani et al. 2003) in a rat focal ischemia model. In addition to inhibiting the release of glutamate, blocking the $A_{2A}R$ modulated the activities of other brain cells. For example, inhibition of the $A_{2A}R$ in microglia after ischemia blunted activation of MAPK and might reduce the damaging inflammatory response evoked by ischemia (Melani et al. 2006). Moreover, blocking the $A_{2A}R$ in oligodendrocytes suppresses the c-Jun N-terminal kinase MAPK pathway, obstructs the activity of glial scar inhibitor molecules, and might consequently prohibit myelin disorganization (Melani et al. 2009). Surprisingly, brain cells are not the only or even the major cell types responsible for the beneficial functions of $A_{2A}R$ inhibition during ischemia. Using $A_{2A}R$-knockout mice, Yu et al. (2004) demonstrated that the $A_{2A}R$ on bone-marrow-derived cells plays the key role in the detrimental function of the $A_{2A}R$ during ischemia, further strengthening the primary role of proinflammatory mediators in stroke.

Because of the importance of both the A_1R and the $A_{2A}R$ in stroke, the expression and function of both adenosine receptors have been actively investigated. In young animals (7 days old) subjected to unilateral ischemia expression of both receptors was markedly reduced in the cortex and hippocampus of the damaged hemisphere (Aden et al. 1994). This would clearly affect the efficacy of adenosine drugs for treating ischemia in young animals. In adult animals, expression of A_1Rs during ischemia appears to depend on the experimental paradigm and the extent of ischemic damage. Brief hypoxia evoked internalization and desensitization of the A_1R in hippocampal slices (Coelho et al. 2006), but there was no significant change in A_1R function or protein levels in the hippocampus following a four-vessel-occlusion ischemia model (Shen et al. 2002). It should also be pointed out that chronic treatment with caffeine alters the expression profiles of many important signaling molecules, including the A_1R and several non-adenosine receptors (Shi et al. 1993; Justinova et al. 2009), which might also contribute to the long-term impact of caffeine consumption.

As suggested in the previous paragraph, the function, regulation, and expression of the $A_{2A}R$ in young animals might be distinct from what has been observed in adult animals. This is an important issue because cerebral hypoxic ischemia is one of the major brain injuries of newborns. Genetic inactivation of the $A_{2A}R$ exacerbates the brain damage evoked by cerebral hypoxic ischemia in young mice (7 days old), suggesting a protective role for the $A_{2A}R$ in young animals (Aden et al. 2003). This finding is consistent with an earlier study showing that caffeine (50 mg kg^{-1} by intraperitoneal injection three times a day) caused apoptosis in multiple brain areas in young rats (7 days old) and in primary cortical cell culture (Kang et al. 2002). Similarly, in contrast to the protective role of the A_1R in adult animals, the A_1R in the brain of neonatal mice mediates the hypoxia-induced ventriculomegaly (Turner et al. 2003). Studies on the distribution and signaling pathways of adenosine receptors (particularly the A_1R and the $A_{2A}R$) in the brain of neonatal animals are required to evaluate whether there is any potential therapeutic use for adenosine drugs and methylxanthines in treating hypoxic–ischemic brain damage in newborns.

4.2 Traumatic Brain Injury

TBI remains one of the leading causes of disability and death in modern society (Marshall 2000; Rutland-Brown et al. 2006; Moppett 2007). Despite extensive efforts, no effective neuroprotective therapy is currently available for TBI. The neurological deficits found following TBI result from both primary and secondary (delayed) events (Graham et al. 2000; Leker and Shohami 2002; Moppett 2007). Primary damage to the brain cells and tissues is not reversible, while secondary damage is reversible and potentially preventable. The potential for reversing damage closely correlates with prognosis. Three possible mechanisms have been proposed for secondary brain injury in TBI: glutamate release, cytokine production, and Ca^{2+} overloading (Graham et al. 2000; Marshall 2000; Leker and Shohami 2002). Interfering with any of these pathological processes may reduce brain damage.

Adenosine levels are markedly increased both in brain interstitial fluid and in cerebrospinal fluid (CSF) after severe TBI in humans (Clark et al. 1997; Bell et al. 1998; Robertson et al. 2001). Adenosine has complex effects in experimental models of CNS trauma – both neuroprotection and neurotoxicity have been reported (Cassada et al. 2002a; Varma et al. 2002).

4.2.1 Human Studies

In clinical studies, Sachse et al. (2008) assessed concentrations of caffeine and its metabolites (theobromine, paraxanthine, and theophylline) in 97 ventricular CSF samples from an established bank of samples from 30 adults with severe TBI. Nine of the 24 TBI patients with detectable caffeine levels had levels of 1 μM or greater (194 ng mL^{-1}), the concentration chosen as the clinically significant threshold. The study found that the caffeine levels in CSF correlated with prognosis, with higher caffeine level predicting better clinical outcome.

4.2.2 Experimental Studies

In experimental TBI, caffeine and adenosine receptor agents can be neuroprotective or neurotoxic, depending on the dose, model, and timing. In rat models, acute administration of extremely large doses of caffeine (100–150 mg kg^{-1}, which equates with a human acutely consuming more than cups of caffeinated beverage) immediately before injury worsened the outcome (Al Moutaery et al. 2003). In contrast, in mice, chronic pretreatment with caffeine (5–50 mg kg^{-1}) reduced hippoccampal neuronal cell death after experimental TBI (Washington et al. 2005). Similarly, Li et al. (2008) demonstrated that at 24 h after a cortical impact brain injury, neurological deficits, cerebral edema, and inflammatory cell infiltration were all significantly attenuated in mice pretreated chronically (for 3 weeks) with caffeine in their drinking water. By contrast, acute treatment with caffeine (30 min before TBI) either had no effect (Li et al. 2008) or exacerbated TBI-induced brain injury (Al Moutaery et al. 2003). Thus, acute caffeine treatment adversely affects outcome after TBI, while chronic treatment provides a consistent neuroprotective effect. Lastly, the combination of caffeine and ethanol ("caffeinol") produces a beneficial effect after experimental TBI (Dash et al. 2004), mirroring its effect on stroke (Aronowski et al. 2003; Belayev et al. 2004) and indicating the importance of the interaction between caffeine and ethanol. Thus, chronic caffeine consumption may confer some neuroprotection against TBI and an epidemiological investigation into the possible association between human caffeine intake and TBI outcome is warranted.

It is suggested that the neuroprotective effect of caffeine may be due to long-term upregulation of A_1Rs or acute inhibition of A_{2A}Rs, along with other potential mechanisms. The attenuation of brain injury by chronic (but not acute) caffeine treatment suggests that enhanced A_1R-mediated suppression of excessive glutamate release and production of inflammatory cytokines may be responsible for this effect. In support of this notion, chronic caffeine treatment attenuated glutamate release and the production of inflammatory cytokines and upregulated A_1R messenger RNA in the brain (Li et al. 2008). Additional support for the role of the A_1R comes from the demonstration that administration of an A_1R agonist attenuates the behavioral deficits and contusion volume associated with brain injury, while an A_1R antagonist exacerbates them (Varma et al. 2002). Similarly, A_1R-knockout mice develop lethal acute status epilepticus after experimental TBI (Kochanek et al. 2006). On the other hand, the A_{2A}R activity may also contribute to the protection against TBI. Administration of an A_{2A}R agonist conferred beneficial effects on outcome and cerebral blood flow after experimental spinal cord injury or TBI (Cassada et al. 2002a; Reece et al. 2004). However, genetic inactivation of

A_{2A}Rs reduced neuronal apoptosis in a moderate cortical impact model (Li et al. 2009) and in a spinal cord compression injury model of mice (Li et al. 2006). Thus, both activation and inactivation of A_{2A}Rs have been shown to produce a neuroprotective effect in the early stage of TBI. The mechanism underlying these apparent paradoxical effects of the A_{2A}R activity in TBI remains unclear.

4.3 Alzheimer's Disease

AD is the most common cause of dementia, afflicting about 5% of the population older than 65 years and 10–20% at age 80 (Braak and Braak 1997; Alloul et al. 1998). While a small fraction of autosomal dominant, early onset forms of AD are caused by genetic mutations of genes encoding the amyloid precursor protein (APP), presenilin 1 (PS-1), and presenilin 2 (PS-2), the cause of the most common form of AD – sporadic, late-onset AD – remains unknown (Selkoe 2004; Bertram and Tanzi 2008). Polymorphisms of other genes, notably apolipoprotein E (APOE ε4), have been associated with the late form of AD (Strittmatter et al. 1993; Bertram and Tanzi 2008). These predisposing gene variations likely interact with environmental factors, including dietary factors, in the pathogenesis of AD. There are now treatments to ameliorate the cognitive symptoms of AD, but no interventions yet exist that slow or reverse the progressive pathogenic changes of AD (Mattson 2004; Selkoe 2004). Recent efforts to identify potential risk factors, as well as factors that that may decrease risk or prolong autonomy, have provided epidemiological evidence that caffeine, which is known to have positive effects on vigilance, attention, mood, and arousal, may also be neuroprotective in AD.

4.3.1 Human Studies

Early studies of brain tissue from patients who died with a confirmed diagnosis of AD revealed a loss of A_1R in the hippocampus, a brain region critically involved in learning and memory (Jansen et al. 1990; Jaarsma et al. 1991; Ulas et al. 1993), suggesting possible involvement of the adenosine receptor in the development of AD. This reduction of A_1R in the hippocampus was recently confirmed by a PET study of AD patients using the A_1R antagonist ^{11}C-8-dicyclopropylmethyl-3-propylxanthine as a ligand (Fukumitsu et al. 2008). In contrast, the levels of A_1R and A_{2A}R appear to be increased in the frontal cortex in AD, in parallel with increased functional activity of these receptors (Albasanz et al. 2008). Finally, a strong negative correlation between increased plasma levels of homocysteine and reduced levels of adenosine has been reported in AD (Selley 2004). This is due at least in part to formation of *S*-adenosylhomocysteine, suggesting the possibility that a deficiency of adenosine may contribute to neurological manifestation of increased homocysteine levels.

Several longitudinal studies have reported recently that a daily caffeine intake equivalent to three or more cups of coffee reduces cognitive decline in "non-demented" elderly men and women. For example, a significant association between regular coffee intake and improvement of cognitive performance in older subjects

(55+) and in women was found in two large cross-sectional population studies (Jarvis 1993; Johnson-Kozlow et al. 2002). This relationship is further substantiated by the prospective population study, Maastricht Aging Study (MAAS Study) in southern Netherlands (van Boxtel et al. 2003). Despite inadequate adjustment for other potential causes of cognitive change, the study found a cross-sectional association between caffeine consumption and improvements in psychomotor speed long term (Hameleers et al. 2000; van Boxtel et al. 2003) and verbal memory performance (van Boxtel et al. 2003), supporting the enhancement of cognitive function by caffeine in the “nondemented” elderly population.

Maia and de Mendonca (2002) first reported in a small case-control study that 78 AD patients had consumed markedly less caffeine during the 20 years preceding AD diagnosis than age-matched individuals without AD. Four recent prospective studies of large cohorts also support the inverse relationship between caffeine consumption and reduced risk of developing AD. The Canadian Study of Health and Aging (CSHA) is a large, nationwide, multicenter, longitudinal study of dementia in elderly people focusing on risk factors for AD. After surveying 4,615 subjects at 5-year follow-up, including 194 with AD and 3,894 cognitively normal controls, this population-based, prospective study found that regular consumption of caffeine is associated with a reduced risk of developing AD (Lindsay et al. 2002). In addition, the 10-year follow-up of the FINE study, involving 667 healthy men born in Finland, Italy, and the Netherlands between 1900 and 1920, suggests that consuming coffee reduces cognitive decline in elderly men, with the least cognitive decline for men consuming three cups of coffee per day. This inverse relationship has now been confirmed by yet another large population-based prospective study, the French Three Cities study involving 4,197 women and 2,820 men (Ritchie et al. 2007). This large prospective study examined the impact of caffeine use on cognitive functioning over time, taking into account multiple possible codeterminants of cognitive decline and thus clarifying previous small cross-sectional control studies. The study found that women (but not men) drinking three cups or more of coffee exhibited less decline in verbal retrieval and visuospatial memory over 4 years, even adjusting for other multiple factors contributing to cognitive decline. Lastly, the Cardiovascular Risk Factors, Aging, and Dementia (CAIDE) study addresses the possible relationship between coffee/tea drinking at midlife and development of dementia later in life (Eskelinen et al. 2009). This study involved 1,409 individuals aged 65–79 after an average 21-year follow-up (total of 61 cases identified as demented, 48 with AD) and found that individuals identified as coffee drinkers at midlife were 65% less likely to develop dementia and AD later in life compared with those drinking little or no coffee. This association is observed after adjusting for demographic, lifestyle, and vascular factors, as well as APOE ε4 allele and depressive symptoms. Thus, caffeine consumption may represent a potential prevention strategy for dementia/AD.

Interestingly, the xanthine derivative propentofylline has been specifically developed for and tested in clinical trials of AD. Propentofylline, a weak antagonist at adenosine receptors and an effective inhibitor of adenosine reuptake and of PDE, has been shown to enhance cognition in patients with vascular dementias (Mielke et al. 1996a, b). The initial study suggested that propentofylline might attenuate progression of AD (Kittner et al. 1997; Marcusson et al. 1997), but a subsequent large clinical trial has not confirmed these encouraging findings (Propentyfylline Plus Study). The lack of efficacy of this drug may be due to its relatively short-term use in this study. In AD, neurodegeneration may evolve for many years before the emergence of clinical symptoms and short-term treatment with neuroprotective agents is unlikely to markedly modify the progression of neurodegeneration.

4.3.2 Experimental Studies

Despite the considerable strength of the correlation in these large, longitudinal studies, epidemiological investigations cannot definitively isolate caffeine intake from other lifestyle choices that potentially affect cognition. Studies in cellular and animal models of AD have now provided evidence to address the causal relationship between caffeine's protective effect and reduced cognitive decline in humans. The first evidence of neuroprotection by caffeine in AD models was in cultured cerebellum granular cells where caffeine and the $A_{2A}R$ antagonist SCH58261 reduced amyloid β (Aβ) peptide induced aggregation, a key event associated with AD pathogenesis (Dall'lgna et al. 2003). This protective effect was substantiated in an animal study in which caffeine and $A_{2A}R$ antagonists protected against loss of learning and memory induced by intracerebroventricular infusion of Aβ peptide (Dall'lgna et al. 2003; Cunha et al. 2008). These findings are also in agreement with pharmacology studies showing that caffeine and $A_{2A}R$ antagonists reverse memory loss induced by aging (Prediger et al. 2005) and by spontaneous hypertension (Prediger and Takahashi 2005) and also attenuate neurochemical modifications in the hippocampus of streptozotocin-induced diabetic rats (Duarte et al. 2009). Studies of aged AD transgenic (APPsw, Swedish mutation) mice with high levels of brain Aβ and widespread cognitive impairment provide evidence for the therapeutic benefit of caffeine. The study found that long-term (between 4 and 9 months) administration of a 1.5 mg daily dose of caffeine (equivalent to 500 mg in human) to APPsw mice can reduce brain Aβ levels and protect against certain cognitive impairments (Arendash et al. 2006). This protective effect of caffeine is associated with reduced expression of both PS-1 and β-secretase (BACE1), thereby suppressing production of Aβ in the brain (Arendash et al. 2006). Furthermore, in aged (18–19-month-old) APPsw mice, which already exhibit decreased cognitive function, caffeine treatment enhanced working memory compared with nontreated APPsw mice (Arendash et al. 2009). Moreover, acute caffeine treatment rapidly reduces production of Aβ in both brain interstitial fluid and plasma without affecting Aβ elimination (Cao et al. 2009). Long-term oral caffeine treatment not only sustainably reduces the plasma levels of Aβ, but also decreases the levels of both soluble and deposited Aβ in hippocampus and cortex in aged AD mice. Intriguingly, caffeine's ability to improve cognitive performance in individual aged AD mice did not correlate with reduced plasma Aβ levels, but was closely associated with reduced inflammatory cytokine levels in hippocampus (Arendash et al. 2009; Cao et al. 2009). Lastly, caffeine was recently shown to reduce disruption of the BBB induced by a high-cholesterol diet (Chen et al. 2008b), suggesting additional mechanisms for the therapeutic effect of caffeine in AD and other neurodegenerative disorders.

4.4 Parkinson's Disease

PD is the second most common neurodegenerative disorder, affecting 1% of the world population aged 65 and older (Lang and Lozano 1998a, b). Dopamine replacement, such as L-dopa, remains the mainstay in treatment to control motor symptoms even 40 years after its discovery (Rascol et al. 2002; Lang and Obeso 2004; Goetz et al. 2005). As a consequence, the clinical problems associated with treatment, particularly with long-term management of PD, remain largely unchanged. After 5–10 years of L-dopa treatment, loss of L-dopa/dopamine agonist efficacy and the onset of debilitating motor complications (dyskinesia, wearing off, and on–off) are almost inevitable (Ahlskog and Muenter 2001). Other side effects of the dopamine therapy, including psychosis and dopamine dysregulation syndromes (e.g., compulsive gambling, hypersexuality), are difficult to manage (Stamey and Jankovic 2008; Yamamoto and Schapira 2008). Furthermore, nonmotor symptoms of PD (e.g., cognitive dysfunction, fatigue, balance impairment, sleep disturbance, autonomic dysfunction) are recognized as a key component of the illness and present another unmet therapeutic need (Chaudhuri et al. 2006). There is no effective therapy to slow or halt progression of PD.

The $A_{2A}R$ recently emerged as a leading nondopaminergic therapeutic target in PD. This is primarily the result of three lines of experimental and clinical investigation. First, the unique colocalization of $A_{2A}R$–D_2R in striatopallidal neurons and the antagonistic interaction between $A_{2A}R$ and D_2R, which may occur through $A_{2A}R$–D_2R heterodimer formation, provide a strong anatomical and molecular basis for the motor benefit of $A_{2A}R$ antagonists in PD (Ferre et al. 1991; Richardson et al. 1999; Schwarzschild et al. 2006). Second, on the basis of decade-long preclinical studies, $A_{2A}R$ antagonists such as KW-6002 (istradefylline) and SCH420814 have now completed clinical phase II–III trials, successfully confirming a motor benefit in advanced PD patients and leading to FDA filing of KW-6002 for PD patients (April, 2007, see http://www.kyowa-kpi.com). Third, the recent convergence of epidemiology and animal studies strongly suggests that $A_{2A}R$ antagonists also confer a neuroprotective effect in PD.

4.4.1 Human Studies

Relief of Motor Symptoms

The initial results showed that the $A_{2A}R$ antagonist KW-6002 (istradefylline, 20–80 mg day^{-1}) enhanced motor activity and potentiated the motor stimulant effect elicited by low doses of L-dopa, as evidenced by reduced "off" time and increased “on” time in advanced PD patients (Bara-Jimenez et al. 2003; Hauser et al. 2003). To confirm these initial findings, several double-blind, placebo-controlled, clinical phase IIB and III trials of KW-6002 in advanced PD patients were conducted. Four clinical IIB and III trials (Stacy et al. 2008; Jenner et al. 2009), with a total of about 1,500 advanced PD patients, have been reported so far, with a reduction in the average “off” time of about 1.7 h, compared with the “optimal” L-dopa dose regime (Stacy et al. 2008; Jenner et al. 2009). Thus, these results demonstrate the motor benefits of KW-6002 in advanced PD patients. As a result of these clinical findings, KW-6002 has been filed for FDA (USA) approval for the treatment of advanced PD. Similarly, a preliminary report by the Schering-Plough Research Institute at the conference “Targeting the $A_{2A}R$ to PD and Other CNS Disorders” (Boston, May, 2006) showed that the $A_{2A}R$ antagonist SCH420814 also produced motor benefits in advanced PD patients in a clinical phase IIA trial (see http://www.a2apd.org for the abstract). Despite some limitations of these clinical trials, such as a relatively large drop-off rate, and some unrealistic clinical trial settings, and the admittedly modest effects (Jankovic 2008), these studies support the concept that selective $A_{2A}R$ antagonists can stimulate motor activity by potentiating the L-dopa effect in advanced PD patients. Recent clinical trials indicate that KW-6002 administered with the existing L-dopa treatment appears to increase the incidence of dyskinesia in advanced PD patients; however, this increased dyskinesia was reported as benign by most patients (Stacy et al. 2008). Thus, it is not clear whether $A_{2A}R$ antagonists can modify L-dopa-induced dyskinesia (LID).

Potential Disease Modifying Effect

The most exciting prospective role for $A_{2A}R$ antagonists as a novel therapy for PD is their potential to attenuate dopaminergic neurodegeneration, as suggested by convergent epidemiological and experimental evidence. Ross et al. (2000) reported an inverse relationship between consumption of the nonselective adenosine antagonist caffeine and the risk of developing PD in a 30-year follow-up study in a large prospective study of 8,004 Japanese-American men in the Honolulu Heart Program. The age- and smoking-adjusted risk of PD was 5 times higher among men who reported no coffee consumption compared with men who reported a daily consumption of 28 oz or more of coffee. This finding was substantiated by a similar inverse relationship between the consumption of caffeinated (but not decaffeinated) coffee and the risk of developing PD in two larger, more ethnically diverse cohorts – the Health Professionals’ Follow-Up Study and the Nurses’ Health Study – involving 47,351 men and 88,565 women (Ascherio et al. 2001) and also more recently in the Finnish Mobile Clinic Health Examination Survey involving 19,518 men and women (Saaksjarvi et al. 2007). These studies firmly established a relationship between increased caffeine consumption and decreased risk of developing PD in men.

Despite the strong epidemiological evidence for a neuroprotective effect of caffeine, two recent pilot studies did not detect a positive correlation between caffeine consumption and PD progression in 1-year clinical trials involving a total of 413 early PD subjects (Simon et al. 2008). However, these studies are limited by short duration (1 year) of the follow-up and relatively small numbers of PD cases. In another study, no significant association was noted in a small case-control study of PD (94 cases) and $A_{2A}R$ gene polymorphism ($A_{2A}R$ 1976T>C) (Hong et al. 2005). Additional clinical studies are clearly warranted to assess this critical issue.

4.4.2 Experimental Studies

Over the last decade, several specific $A_{2A}R$ antagonists (such as KW-6002 and SCH58261, ST1535) have been developed and shown to enhance motor activity in animal models of PD (Richardson et al. 1997; Schwarzschild et al. 2002; Chen 2003; Tronci et al. 2007; Varty et al. 2008; Trevitt et al. 2009). In rodents depleted of dopamine by MPTP or reserpine treatment or rendered cataleptic by haloperidol, the administration of selective $A_{2A}R$ antagonists increases motor activity. Animals treated with $A_{2A}R$ antagonists following unilateral lesion with 6-hydroxydopamine (6-OHDA) (hemiparkinsonian) demonstrated an increase in contralateral rotation (Ferre et al. 1992, 1997; Shiozaki et al. 1999; Tronci et al. 2007). Similarly, $A_{2A}R$ antagonists stimulate motor activity in MPTP-treated nonhuman primates (Kanda et al. 1998a; Grondin et al. 1999; Varty et al. 2008). Thus, $A_{2A}R$ antagonists can stimulate motor activity in dopamine-depleted animals either alone or in synergy with L-dopa and other dopaminergic agonists. These preclinical studies set the stage for clinical trials to evaluate the ability of $A_{2A}R$ antagonists to relieve motor symptoms in PD patients.

To establish the causal relationship between caffeine consumption and neuroprotection against dopaminergic neurodegeneration, studies with animal models of PD provide a compelling clue about the potentially protective effects of caffeine by demonstrating that pharmacological blockade (by caffeine or selective $A_{2A}R$ antagonists) or genetic depletion of the $A_{2A}R$ attenuates dopaminergic neurotoxicity and neurodegeneration (Chen et al. 2001; Ikeda et al. 2002; Xu et al. 2002). Administration of caffeine following MPTP treatment attenuates the MPTP-induced reduction in dopamine content and loss of dopaminergic terminals in the striatum (Chen et al. 2001; Xu et al. 2002) as well as the loss of dopaminergic neurons in the substantia nigra (Ikeda et al. 2002; Oztas et al. 2002). This neuroprotection was seen after acute coadministration and after repeated injection of caffeine (Xu et al. 2002). Various $A_{2A}R$ antagonists (including SCH58261, KW-6002, 3,7-dimethyl-1-propargylxanthine, and CSC), attenuate MPTP-induced dopaminergic neurotoxicity, suggesting that the protective effects of caffeine are due to its action at the $A_{2A}R$ (Chen et al. 2001; Alfinito et al. 2003; Pierri et al. 2005). In contrast, there is no protective effect of the A_1R antagonist DPCPX (Chen et al. 2001), despite an early report that adenosine and an A_1R agonist protect against methamphetamine-induced neurotoxicity (Delle Donne and Sonsalla 1994). Finally, genetic inactivation of A_{2A}Rs also reduces MPTP-induced dopaminergic neurotoxicity (Chen et al. 2001). These studies provide a neurobiological basis for the inverse relationship between increased caffeine consumption and reduced risk of developing PD. The convergence of epidemiological evidence and findings from animal studies also raises the exciting possibility that A_{2A}R antagonists, including caffeine, may slow or halt dopaminergic neuronal degeneration.

Despite the consistent demonstration that A_{2A}R antagonists afford neuroprotection against dopaminergic neurotoxicity, the mechanism by which A_{2A}R inactivation protects against the loss of dopaminergic neurons remains unknown. The particular challenge lies in explaining the apparent dichotomy between restricted expression of the A_{2A}R in striatopallidal neurons and neuroprotection against degeneration of dopaminergic neurons in the substantia nigra, where only scattered expression of A_{2A}Rs is detected. A partial answer to this mechanistic question come from a study by Chen's group, who employed a forebrain-neuron-specific A_{2A}R-knockout model to demonstrate the distinct cellular mechanisms underlying motor stimulant and neuroprotective effects by A_{2A}R antagonists. While forebrain neuronal A_{2A}Rs are responsible for the motor effect, A_{2A}Rs in other cellular elements, such as microglial cells, may be associated with neuroprotection (Yu et al. 2008). An additional challenge is to identify the cellular mechanism which allows A_{2A}R inactivation to provide neuroprotection against a broad spectrum of brain insults, from ischemia to excitotoxicity to mitochondrial toxicity (for a recent review, see Chen et al. 2007). However, it should be emphasized that targets other than adenosine receptors should also be considered. For example, it was recently proposed that caffeine-mediated neuroprotection may be associated with expression of cytochrome P450 (Singh et al. 2009) or cytochrome oxidase (Jones et al. 2008) and with stimulation of ryanodine receptor channels (Guerreiro et al. 2008). Lastly, a recent study also suggested that caffeine may confer neuroprotection by increasing BBB integrity since MPTP-induced leakage in Evans blue dye and TITC-albumin in the striatum was attenuated by caffeine treatment (Chen et al. 2008a). This effect is further associated with increased expression of tight junction proteins (Chen et al. 2008a).

L-dopa-induced motor complications, dyskinesia in particular, are a major limiting factor in the management of advanced PD and are intrinsically linked with the chronic stimulation of dopamine receptors. Thus, nondopaminergic agents, such as A_{2A}R antagonists, may exert similar beneficial motor effects but have a low propensity to induce dyskinesia. Indeed, in L-dopa-sensitized, dopamine-depleted nonhuman primates, KW-6002 treatment reverses motor deficits but does not induce a dyskinesia score (Kanda et al. 1998b, 2000; Grondin et al. 1999). Genetic and pharmacology studies further suggest that the A_{2A}R antagonists may also modify development of LID. Genetic inactivation of the A_{2A}R attenuated the sensitization of rotational behavior induced by repeated L-dopa treatment, indicating that activation of A_{2A}Rs is required for the *development* of behavioral sensitization by chronic treatment with L-dopa (Fredduzzi et al. 2002) and with amphetamine (Xiao et al. 2006). Similarly, L-dopa-induced abnormal involuntary movements (AIMs) are attenuated by selective inactivation of A_{2A}Rs in forebrain neurons (Xiao et al. 2006) or by a *low* dose of KW-6002 (Bastia et al. 2005). Additionally, coadministration of KW-6002 and apomorphine (1 mg kg^{-1}) to MPTP-treated monkeys completely prevented the development of dyskinesia, while L-dopa alone produced typical dyskinesia (Bibbiani et al. 2002). The role of the A_{2A}R in the development of LID is supported by the increased level of A_{2A}Rs in striatum of 6-OHDA-lesioned rats, dyskinesic nonhuman primates, and dyskinesic PD patients after chronic L-dopa treatment (Morelli et al. 2007). However, a recent study measuring AIMs, showed that coadministration of KW-6002 at regular motor stimulant doses with L-dopa did not modify the AIM behavioral score in rats (Lundblad et al. 2003). Thus, it remains to be determined whether A_{2A}R antagonists can modify LID in PD models. Intriguingly, the A_{2A}R–D_2R model predicts that A_{2A}R antagonists should enhance rather than attenuate LID. However, a recent study has demonstrated the critical role of the A_{2A}R in forebrain outside the striatum in modulation of dopamine-mediated behavior (Shen et al. 2008). It is proposed that additional mechanisms (such as adenosine–glutamate interaction) in the cerebral cortex may be responsible for the possible antidyskinesic effects of A_{2A}R antagonists (Shen et al. 2008).

4.5 Huntington's Disease

HD (affecting one in 10,000 individuals) is an autosomal dominant disease characterized by chorea, dementia, psychiatric symptoms, and eventual death (Martin and Gusella 1986). The causative mutation is a CAG trinucleotide expansion in exon 1 of the huntingtin (Htt) gene (Group 1993). When the number of CAG repeats exceeds 36, Htt forms aggregates in the nuclei and cytoplasm of neurons, glia, and several different types of peripheral cells (e.g., liver, muscles, and adipocytes), hijacks a wide variety of proteins, and causes neuronal degeneration and metabolic dysfunction (Group 1993; Sugars and Rubinsztein 2003; Li and Li 2004; Chiang et al. 2005, 2009; Chou et al. 2005; Ryu et al. 2005; Chiang et al. 2007b; Wang et al. 2008b). Because the major clinical presentations predominantly appear in the CNS, HD was initially considered a neuronal degenerative disorder. Nonetheless, accumulating evidence suggests that defects in peripheral tissues also significantly contribute to HD pathogenesis (Sathasivam et al. 1999; Djousse et al. 2002; Ribchester et al. 2004; Chiang et al. 2007a; Mihm et al. 2007; Valenza et al. 2007; Bjorkqvist et al. 2008; Phan et al. 2009). To date, there is no effective treatment to prevent the progression of this dreadful disease (Mestre et al. 2009).

4.5.1 Human Studies

Given the concentrated expression of A_{2A}Rs in striatopallidal neurons, it is not surprising that a characteristic loss of A_{2A}R binding is detected in the very early stage of HD (grade 0) (Glass et al. 2000). Interestingly, $A_{2A}R$ expression and function in peripheral blood cells in HD was found to increase in 48 heterozygous and three homozygous patients compared with 58 healthy subjects (Varani et al. 2003). Furthermore, $A_{2A}R$ binding density in blood platelets of HD patients apparently correlates with age at onset anticipation (Maglione et al. 2006). It is suggested that these changes in $A_{2A}R$ binding in peripheral blood platelets likely reflect the status of inflammation and oxidative events associated with HD pathogenesis. Thus, if these findings can be confirmed, the $A_{2A}R$ in peripheral platelets could be a useful biomarker for HD.

A recent genetic association study of 791 unrelated HD patients found that the single nuclear polymorphism in the $A_{2A}R$ gene (1976C/T, rs5751876) is associated with the residual age at onset of the disease of 3.8 years (Dhaenens et al. 2009). This finding needs to be confirmed by a follow-up study with large cohorts.

4.5.2 Experimental Studies

The two primary targets of caffeine, the $A_{2A}R$ and the A_1R, have been investigated as targets for drug development for treatment of HD. The A_1R is of interest because of its well-established protective role in ischemia and epileptic conditions as described already herein. Consistent with the hypothesis that the A_1R might confer a protective role in HD, blockage of the A_1R using DPCPX exacerbated the damage to GABAergic neurons caused by a mitochondrial toxin (malonate; Alfinito et al. 2003) in a model of HD. In addition, with use of a 3-nitropropionic acid (3NP)-induced rat model of HD (Blum et al. 2001), an A_1R-selective agonist (adenosine amine congener, ADAC) devoid of cardiovascular side effects was shown to protect against striatal lesions and motor impairment caused by 3NP (Blum et al. 2002). Similar to what was observed in ischemia/stroke models, chronic use of ADAC in the test protocol did not have any beneficial effects, owing to desensitization of the A_1R (Abbracchio et al. 1992).

The $A_{2A}R$ has attracted much attention as a potential drug target for HD because of its expression in enkephalin-containing striatal neurons as well as at glutamatergic terminals in the corticostriatal pathway of the brain. In particular, stimulation of the presynaptic $A_{2A}R$ triggers glutamate release, and therefore is generally believed to be detrimental to neuronal survival. In contrast, the $A_{2A}R$ located on postsynaptic GABAergic terminals is considered protective (Corsi et al. 2000; Blum et al. 2003b; Fink et al. 2004). Mutant Htt suppresses CREB binding to its core promoter; thus, expression of the $A_{2A}R$ in the striatum is markedly decreased during HD progression (Ferre et al. 1993; Glass et al. 2000; Wyttenbach et al. 2001; Chiang et al. 2005). Nonetheless, the ability of the striatal $A_{2A}R$ in HD mice to evoke cAMP signaling is similar to that of wild-type mice (Chou et al. 2005), indicating aberrantly amplified signaling from the $A_{2A}R$ as was observed in peripheral blood cells of HD patients (Varani et al. 2001, 2003; Maglione et al. 2006). The $A_{2A}R$ is therefore a potential therapeutic target for HD despite its reduced expression.

In genetic mouse models of HD, both agonists and antagonists of the $A_{2A}R$ were tested for beneficial effects. Systemic delivery of an $A_{2A}R$ antagonist (SCH58261) for 1 week reduced NMDA-induced toxicity in a transgenic mouse model of HD (R6/2), but worsened motor coordination (Domenici et al. 2007). Intriguingly, in the same mouse model, chronic treatment (5 weeks) with an $A_{2A}R$ agonist (CGS21680) ameliorated several major symptoms [e.g., brain atrophy, striatal aggregates, deteriorated motor coordination, urea cycle deficiency, and poor ubiquitin proteasome system (UPS) activity] (Chou et al. 2005; Chiang et al. 2009). It appears that the disease stage, the drug administration protocol, and the clinical manifestations might play critical roles in evaluating the future therapeutic potential of $A_{2A}R$ drugs (Popoli et al. 2008). The ability of CGS21680 to enhance the UPS activity via a cAMP–PKA-dependent pathway is of particular interest (Chiang et al. 2009), because aggregate formation is a major hallmark of HD, and a dysregulated UPS is closely associated with the formation of Htt aggregates and HD pathogenesis (Zhou et al. 2003; Seo et al. 2004; Bennett et al. 2007; Hunter et al. 2007; Wang et al. 2008a). The proteasome-activating (or proteasome-modulating) capacity of $A_{2A}R$ agonists provides a new means of treating HD, and merits further evaluation. Because the $A_{2A}R$ is expressed in multiple tissues in which mHtt is present and forms aggregates, systematic administration of $A_{2A}R$ agonists is expected to boost the inferior UPS activities in both the CNS and peripheral tissues of HD patients, and might be more effective than interventions which only treat the CNS. Unfortunately, certain adverse effects of $A_{2A}R$ drugs on the cardiovascular system were reported (Gordi et al. 2006; Mingote et al. 2008), inevitably dampening the enthusiasm for their potential clinical application. Partial $A_{2A}R$ agonists with good BBB penetration might reduce or eliminate unfavorable side effects as has been recommended for other adenosine drugs (Gao and Jacobson 2004). Moreover, epidemiological investigation into the long-term effects of chronic caffeine consumption on the progress of HD patients is warranted.

4.6 Multiple Sclerosis

Multiple sclerosis (MS) is a common autoimmune disorder of the CNS with pathological characteristics that include lymphocyte and macrophage infiltration, CNS demyelination, and axonal damage, resulting in recurrent impairment of brain and spinal cord function (Prineas and Wright 1978; Hafler 2004). Its cause remains unknown, but MS is thought to be a prototypic autoimmune disease mediated by T lymphocytes with the participation of B lymphocytes targeted against myelin protein (Noseworthy et al. 2000; Keegan and Noseworthy 2002; Meinl et al. 2006). Current immunosuppression therapies such as glucocorticosteriods have significant side effects and their effectiveness is limited (a 30–60% reduction in the frequency of relapse). Thus, there is an ongoing search for a more effective treatment with fewer side effects.

4.6.1 Human Studies

Recently, several lines of clinical and experimental evidence have suggested that adenosine receptors may modulate neuroinflammation in MS. Adenosine levels in blood plasma decrease greatly in MS, accompanied by an increase in the level of tumor necrosis factor (Mayne et al. 1999). Furthermore, downregulation of A_1Rs has been reported in mononuclear cells in the peripheral blood and CD45-positive glial cells in the brain of MS patients (Johnston et al. 2001). These studies suggest that dysfunction of A_1Rs may contribute to the pathogenesis in MS patients.

4.6.2 Animal Studies

Myelin oligodendroglia glycoprotein (MOG) induces a combined autoimmune pathogenic T-cell and B-cell response (Adelmann et al. 1995), leading to demyelination in the brain, a hallmark of human MS lesions(Adelmann et al. 1995; Berger et al. 1997). Experimental autoimmune encephalomyelitis (EAE) induced by immunization with MOG is a widely used animal model of MS (Steinman and Zamvil 2006). A recent study showed that mice with a genetic deficiency in extracellular nucleotidase CD73, a molecule critical for generating extracellular adenosine in various cells, including T cells, are largely resistant to MOG-induced brain and spinal cord injury (Mills et al. 2008). Furthermore, genetic deficiency in A_1R exacerbated spinal cord injury with extensive inflammation and demyelination (Tsutsui et al. 2004). Conversely, treatment with the A_1R agonist ADAC reduces spinal cord inflammation and demyelination in EAE mice (Tsutsui et al. 2004). These results suggest that adenosine acting at the A_1R suppresses the inflammatory response, contributing to the pathogenesis of MS. The critical role of the A_1R in development and treatment of MS is further supported by the recent finding that suppression of EAE-induced neuroinflammation and neurobehavioral deficits by glucocorticoid treatment is accompanied by a concurrent increase in A_1R expression. This modulation in A_1R expression by glucocorticoids in the EAE model may be related to the reciprocal interactions between the A_1R and β-arrestin-1 reported in monocytoid cells (Tsutsui et al. 2008). In addition to these effects of the A_1R in experimental models of MS, pharmacological blockade of the A_{2A}R has been shown to attenuate EAE (Mills et al. 2008). Thus, adenosine acting at the A_{2A}R may facilitate inflammation and EAE. While the precise roles of adenosine and adenosine receptors in the development of EAE remain to be clarified, these findings clearly highlight the critical involvement of adenosine and adenosine receptors in modulation of EAE.

Two recent reports showed that caffeine can attenuate MOG-induced EAE in mice. Mice chronically treated with caffeine displayed significantly fewer clinical symptoms of motor impairment and reduced microglial activation in cerebral cortex after MOG immunization (Tsutsui et al. 2004; Mills et al. 2008). This finding has recently been extended to an EAE model induced by guinea pig spinal cord homogenates (GPSCH) with a more extensive inflammatory response, less extensive demyelination, but a more chronic disease course than MOG-induced EAE (Raine 1984; Smith et al. 2005). In the GPSCH model of EAE, chronic caffeine imparts neuroprotection against EAE in rats with both decreased incidence of EAE and attenuated EAE (Chen et al. 2010). Interestingly, chronic treatment with caffeine apparently exerts a neuroprotective effect against EAE through an A_1R-mediated shift from Th1 to Th2 cell function (Chen et al. 2010). These animal studies provide a neurobiological basis for epidemiological investigation into the possible relationship between caffeine consumption and development of MS in humans.

5 Concluding Remarks

Several decades after the earliest attempt to treat PD using caffeine(Shoulson and Chase 1975), tremendous amounts of information regarding the potential beneficial effect of caffeine as well as adenosine drugs on major neurodegenerative disorders have accumulated. In addition to the encouraging development of several $A_{2A}R$ selective antagonists which are now in advanced clinical phase III trials, detailed characterization of the primary targets of caffeine (A_1R and $A_{2A}R$) provides sound mechanistic bases for the action of caffeine. The exciting findings to date include the specific in vivo functions of adenosine receptors revealed by genetic mouse models, and the awareness of profound interaction between adenosine receptors and other GPCR and accessory proteins which might exhibit pharmacological properties distinct from those of adenosine receptor monomers. An increasing body of human and experimental studies provided encouraging evidence that regular human consumption of caffeine may, in fact, have several beneficial effects on neurodegenerative disorders, from motor stimulation to cognitive enhancement to potential neuroprotection. Importantly, neuroprotection by *chronic* treatment with caffeine can be demonstrated in animal models of PD, AD, TBI, stroke, and MS, highlighting a broad spectrum of neuroprotection with possibly common mechanisms of caffeine. Thus, with regard to neurodegeneration, these potential benefits of methylxanthines, caffeine in particular, strongly argue against the common practice by clinicians to discourage regular human consumption of caffeine. Additional studies are warranted to confirm neuroprotective and cognitive enhancement effects of caffeine in large, longitudinal clinical trials for these neurodegenerative disorders. This encouraging development invites further investigation into the action of caffeine and adenosine drugs to define the molecular basis by which methylxanthines and adenosine exert their neuroprotective or cognitive effects. This knowledge not only provides a neurobiological basis for guidelines for healthy usage of caffeine as a stimulant to improve human performance, but also opens up a real and novel possibility to develop methylxanthine-based treatment for these neurodegenerative diseases.

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Methylxanthines and Pain

Jana Sawynok

Contents

Abstract Caffeine, an antagonist of adenosine A_1, A_{2A} and A_{2B} receptors, is known as an adjuvant analgesic in combination with non-steroidal anti-inflammatory drugs (NSAIDs) and acetaminophen in humans. In preclinical studies, caffeine produces intrinsic antinociceptive effects in several rodent models, and augments the actions of NSAIDs and acetaminophen. Antagonism of adenosine A_{2A} and A_{2B} receptors, as well as inhibition of cyclooxygenase activity at some sites, may explain intrinsic antinociceptive and adjuvant actions. When combined with morphine, caffeine can augment, inhibit or have no effect depending on the dose, route of administration, nociceptive test and species; inhibition reflects spinal inhibition of adenosine A_1 receptors, while augmentation may reflect the intrinsic effects noted above. Low doses of caffeine given systemically inhibit antinociception by several analgesics (acetaminophen, amitriptyline, oxcarbazepine, cizolirtine), probably reflecting block of a component of action involving adenosine A_1 receptors. Clinical studies have demonstrated adjuvant analgesia, as well as some intrinsic analgesia, in the treatment of headache conditions, but not in the treatment of

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postoperative pain. Caffeine clearly exhibits complex effects on pain transmission; knowledge of such effects is important for understanding adjuvant analgesia as well as considering situations in which dietary caffeine intake may have an impact on analgesic regimens.

Keywords Acetaminophen · Amitriptyline · Aspirin · Caffeine · Morphine · Non-steroidal anti-inflammatory drugs

1 Introduction

Caffeine has been a component of analgesic formulations containing aspirin (acetylsalicylic acid) for some time. Clinical studies published in the 1960s and 1970s indicated that caffeine-containing analgesics produced effects that were similar to those of the analgesics alone (Zhang 2001). However, in the mid 1980s, an analysis of 30 unpublished clinical studies over 20 years derived a relative potency of 1.4 for analgesics (aspirin, acetaminophen) containing caffeine compared with the analgesic alone (Laska et al. 1984). This analysis established caffeine's reputation as an adjuvant analgesic, an agent that augments the action of a known analgesic.

During the 1960s and early 1970s, with the discovery of cyclic AMP as a second messenger system and the appreciation that methylxanthines inhibit phosphodiesterase enzymes, the actions of caffeine were considered within the context of this system. In the late 1970s, the actions of caffeine and other methylxanthines as adenosine receptor antagonists came to be appreciated (Fredholm 1980), and, along with the recognition that adenosine receptors are involved in pain regulation, this added a further dimension to the interpretation of experimental observations with these agents. This shift in focus is clearly illustrated by studies that examined the effects of methylxanthines on morphine analgesia, tolerance and dependence over this interval (Ho et al. 1973; Ahlijanian and Takemori 1985). When methods for spinal delivery of drugs were introduced in 1976, this allowed for the spinal pharmacology of pain transmission and its regulation by methylxanthine-sensitive adenosine receptors to be elaborated. Spinal administration of methylxanthines consistently inhibited spinal analgesia by morphine (Jurna 1984; DeLander and Hopkins 1986; Sweeney et al. 1987a), and this led to the adenosine hypothesis of opiate action within the spinal cord (Sawynok et al. 1989).

The main established targets for caffeine at concentrations normally achieved by human consumption are as an antagonist of adenosine A_1, A_{2A} and A_{2B} receptors (Fredholm et al. 1999). The adjuvant and intrinsic analgesic properties of caffeine are largely considered in the context of such actions (Sawynok and Yaksh 1993). Other pharmacological actions (inhibition of phosphodiesterase, Ca^{2+} release, block of $GABA_A$ receptors) are unlikely to manifest themselves in humans through any form of normal dietary use, but such actions potentially contribute in cases of unusually high doses of caffeine (Fredholm et al. 1999). Adenosine is known to be involved in several aspects of pain regulation, with actions depending on the particular receptor subtype (A_1, A_{2A}, A_{2B}, A_3) and on the site of action (periphery, spinal cord, supraspinal sites, with systemic effects reflecting actions at several of these sites) (Sawynok 2006). Interest in exploring the adenosine system has been reflected in the potential development of novel analgesic agents (receptor agonists, antagonists or regulators, also indirectly acting agents such as inhibitors of adenosine kinase), as well as potential adjuvant agents that interact with other analgesics. Furthermore, because of the widespread dietary use of caffeine, it is important to know the potential impact that caffeine may have on a range of therapeutic agents, even procedures, used in pain management.

This chapter addresses several aspects of the effect of methylxanthines on pain. Initially, intrinsic antinociceptive properties of caffeine in a broad range of preclinical models of pain are considered. Subsequently, interactions with several different classes of analgesics (non-steroidal anti-inflammatory drugs, or NSAIDs, acetaminophen, opioids, antidepressants, anticonvulsants) in preclinical studies are reviewed. Finally, the actions of caffeine as an adjuvant analgesic in humans are addressed.

2 Preclinical Studies with Methylxanthines

2.1 Intrinsic Antinociception by Caffeine

There are several reports of intrinsic antinociceptive effects of systemically administered (by intraperitoneal injection) caffeine in the preclinical literature. The effect of caffeine depends on the nature of the test, on the dose of caffeine and on the species tested. Several generalities emerge.

2.1.1 Caffeine Is Often Inactive at Doses up to 50 mg kg^{-1}

There are several reports that caffeine has no intrinsic effects in several different nociceptive tests in which a range of doses of caffeine have been evaluated. These include (1) tail immersion test, rats (25, 50 mg kg^{-1}) (Malec and Michalska 1988); (2) hot plate test, rats (25, 50 mg kg^{-1}) and mice (10, 50 mg kg^{-1}) (Malec and Michalska 1988); (3) hot plate test, mice (20, 40 mg kg^{-1}) (Engelhardt et al. 1997); (4) writhing test, mice (5–50 mg kg^{-1}) (Fialip et al. 1989; Gayawali et al. 1991); (5) pain-induced functional impairment test (10–56 mg kg^{-1}) (Granados-Soto et al. 1993; Flores-Acevedo et al. 1995; Díaz-Reval et al. 2001; López et al. 2006); (6) spinal nerve ligation, thermal hyperalgesia, rats (1.5–7.5 mg kg^{-1}) (Esser and Sawynok 2000); and (7) partial sciatic nerve ligation, mechanical allodynia, rats (10, 20 mg kg^{-1}) (Wu et al. 2006). Several works, in which the main goal was to examine the interaction of caffeine with another agent, have also evaluated effects of single doses of caffeine within this range and reported no effect for caffeine using these tests (noted in subsequent sections).

Curiously, some studies noted hyperalgesia with systemic doses of caffeine in this range. Thus, caffeine led to hyperalgesia in the tail immersion test in mice at 10 mg kg^{-1} (Godfrey et al. 2006). The end point in this test is largely a spinal reflex (it is intact in spinalized animals), and hyperalgesia may reflect caffeine acting spinally to inhibit tonic activity at adenosine A_1 receptors. In rats, spinal (intrathecal) administration of the methylxanthines aminophylline (Jurna 1984), theophylline and 8-phenyltheophylline produced hyperalgesia in the tail flick test, particularly at a lower intensity of stimulation (Sawynok et al. 1986). In mice, intrathecal administration of caffeine and of theophylline were noted not to alter tail flick latencies, but no data were shown (Delander and Hopkins 1986).

2.1.2 Caffeine Produces Antinociception Generally at Doses of 35–100 mg kg^{-1}

There have been reports of intrinsic antinociceptive properties for caffeine at doses generally between 35 and 100 mg kg^{-1} in studies where several doses of caffeine were examined. These include (1) inflammatory hyperalgesia models, rats (50, 100 mg kg^{-1}) (Siegers 1973; Seegers et al. 1981); (2) tail compression test, rats (30, 100 mg kg^{-1}) (Person et al. 1985); (3) hot plate test, mice (75, 100 mg kg^{-1}) (Malec and Michalska 1988; Abo-Salem et al. 2004); (4) hot plate test, rats (50–100 mg kg^{-1}) (Sawynok et al. 1995); (5) 5% formalin test, rats (35–75 mg kg^{-1}) (Sawynok et al. 1995); and (6) partial sciatic nerve ligation, mechanical allodynia, rats (40, 80 mg kg^{-1}) (Wu et al. 2006).

A few studies have not observed intrinsic antinociceptive actions at higher doses of caffeine. For example, Fialip et al. (1989) saw no effect of caffeine at several doses (40, 80, 100, 160, 200 mg kg^{-1}) in the hot plate test; however, that study used a higher intensity of stimulation (56°C) than in many other studies. On occasion, studies that examined the effects of only a single high dose of caffeine also saw no effect (e.g. 50 mg kg^{-1}, mouse hot plate test, Oliverio et al. 1983; 100 mg kg^{-1}, rat tail flick test, Misra et al. 1985).

It is important to note that several studies have reported antinociceptive effects with low doses of caffeine. Antinociception occurs with 2.5–10 mg kg^{-1} in the 2% formalin test in rats (Sawynok and Reid 1996a), with 1–5 mg kg^{-1} in the hot plate and writhing tests in mice and in the paw pressure and tail flick tests in rats (Ghelardini et al. 1997), and with 10 mg kg^{-1} in the hot plate test in mice (Abo-Salem et al. 2004). Stimulus intensity can be important in revealing antinociception with caffeine, as the ED_{40} at 2% formalin is 10 times lower than at 5% formalin (Sawynok and Reid 1996a). There may also be species differences between mice and rats. For example, 10 mg kg^{-1} caffeine produces antinociception in the 2% formalin test in rats (Sawynok and Reid 1996a) but not in mice (Sawynok et al. 2008). A further factor to note is that there may well be strain differences in expression of analgesia in mouse studies (Wilson and Mogil 2001); this is known to be important in general studies on analgesics, but no systematic studies are available for caffeine.

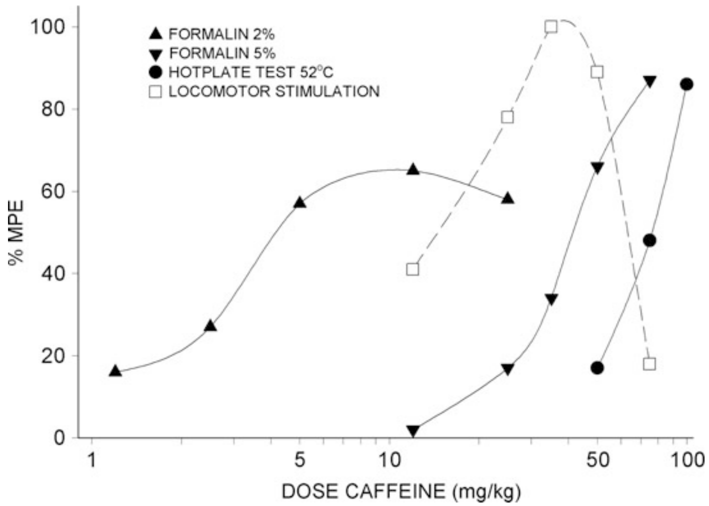

Fig. 1 Antinociception by caffeine in several nociceptive tests in rats from a single laboratory. Shown are (1) antinociception from 35 to 100 mg kg^{-1} in several tests, (2) the intensity dependence of antinociception in the same test (2 vs. 5% formalin) and (3) dissociation of antinociception from locomotor stimulation. (Data redrawn from Sawynok et al. 1995 and Sawynok and Reid 1996a)

Figure 1 is derived from data from a single laboratory using a single species and strain, and illustrates several aspects of the antinociceptive profile of caffeine. It shows (1) antinociceptive actions of caffeine at doses of 35–100 mg kg^{-1} in multiple nociceptive tests (thermal threshold test and a tonic chemogenic model of pain); (2) the dependence of antinociception on stimulus intensity (2 vs. 5% formalin); and (3) dissociation of antinociception from motor effects. With respect to the latter issue, locomotor stimulation can be a concern with respect to confounding end points commonly interpreted as antinociception. However, in rats, locomotor simulation is prominent up to 30–40 mg kg^{-1}, with higher doses of 75–100 mg kg^{-1} showing locomotor depression (Fredholm et al. 1999). Given that antinociception manifests itself in both motor stimulation and depression dose ranges, this suggests antinociception by caffeine is independent of motor effects. Locomotor stimulation by caffeine involves antagonism of adenosine A_{2A} receptors (Ledent et al. 1997).

2.1.3 Mechanisms of Antinociception

Adenosine A_1, A_{2A} and A_{2B} receptors are involved in antinociception, with the effects depending on the receptor subtype and the site of action (Sawynok 2006). Caffeine has a similar affinity as an antagonist of each of these receptors (Fredholm et al. 1999). Adenosine A_1 receptors at peripheral, spinal and supraspinal sites mediate antinociceptive actions, so the ability of caffeine to block these receptors cannot account for antinociception by caffeine. Adenosine A_{2A} and A_{2B} receptors on peripheral nerve terminals and other peripheral sites are recognized to play a pronociceptive role; while central actions are less well characterized, they also may be involved in pain facilitation. The selective adenosine A_{2A} receptor antagonist SCH58261 produces antinociception in several models (mouse writhing, hot plate, formalin tests) (Bastia et al. 2002; Godfrey et al. 2006; Hussey et al. 2007), while adenosine A_{2A} receptor knockout mice exhibit decreased pain responses (hot plate, tail flick, formalin tests) (Ledent et al. 1997; Hussey et al. 2007). In another study, the effects of PSB1115, a selective A_{2B} receptor antagonist, together with a series of other selective antagonists, produced antinociception (mouse hot plate test) mimicking both the antinociceptive action of caffeine and the augmentation of morphine (Abo-Salem et al. 2004). Both intrinsic and adjuvant effects of caffeine were suggested to result from adenosine A_{2B} receptor blockade (Abo-Salem et al. 2004).

Antinociception by higher doses of caffeine, where antinociception is observed more reproducibly, involves central noradrenergic mechanisms. Antinociception is potentiated by phentolamine (α-adrenergic receptor antagonist) in both the hot plate test and the formalin test, while depletion of spinal cord noradrenaline levels markedly reduced caffeine antinociception in the hot plate test (Sawynok et al. 1995). Depletion of central noradrenaline levels augmented antinociception by caffeine in the formalin test, but depletion of central 5-hydroxytryptamine levels exhibited no effect (Sawynok and Reid 1996b). Caffeine is known to alter the turnover of biogenic amines in several regions of the central nervous system (Nehlig et al. 1992), and these results indicate that central amine systems are important for antinociception by caffeine at medium (25–50 mg kg^{-1}, formalin test) and high (75–100 mg kg^{-1}, hot plate test) doses. However, there are some differences in effects between the two tests (e.g. depletion of spinal noradrenaline levels inhibits antinociception in one but not the other test), and a range of actions may be recruited.

There is one study that has demonstrated antinociception with low doses of caffeine (1–5 mg kg^{-1}) in several models in mice and rats (Ghelardini et al. 1997). This low-dose antinociception in the hot plate and writhing tests in mice involved central cholinergic mechanisms, because antinociception was blocked by muscarinic receptor antagonists as well as depletion of central cholinergic levels with hemicholinium-3 (Ghelardini et al. 1997). Such antinociception did not involve opioid or $GABA_B$ receptors, or central amines (Ghelardini et al. 1997). The latter observation distinguishes low-dose caffeine antinociception (5 mg kg^{-1}) from that seen at higher doses (35–100 mg kg^{-1}), as central amine mechanisms are not involved in the former, but are involved in the latter (Sawynok et al. 1995, Sawynok and Reid 1996b).

2.2 *Caffeine and NSAIDs/Acetaminophen*

The effects of caffeine on antinociception by aspirin and acetaminophen have been examined for some time in order to explore the rationale for the historical combination of caffeine with these agents in analgesic formulations. Several earlier preclinical studies demonstrated that caffeine augmented antinociception by both aspirin and acetaminophen in inflammatory hyperalgesia models in rats (Vinegar et al. 1976; Seegers et al. 1981; but see Engelhardt et al. 1997) and in the writhing model in mice (Gayawali et al. 1991). The doses of caffeine used in these studies were generally 10–50 mg kg^{-1}. In one study that extended the dose to 100 mg kg^{-1}, augmentation of antinociception was no longer observed at the highest dose (Gayawali et al. 1991).

The effects of caffeine on acetaminophen and several NSAIDs have been explored in a series of studies using the pain-induced functional impairment model in rats. In this model, uric acid is injected into the knee joint and walking activity on a revolving cylinder is examined; the test is sensitive to both NSAIDs and opioids. Caffeine, at 10–56 mg kg^{-1}, was inactive alone in this model (Granados-Soto et al. 1993). Sixteen acetaminophen–caffeine dose combinations were examined (acetaminophen 100, 178, 316, 562 mg kg^{-1} with caffeine 10, 18, 32, 56 mg kg^{-1}) and augmentation of the action of acetaminophen was observed only at certain doses. The 316 mg kg^{-1} dose of acetaminophen was augmented by all doses of caffeine, with a maximal effect at 32 mg kg^{-1}. The maximally effective dose of caffeine did not change the plasma levels or kinetics of acetaminophen. The selective augmentation of a mid-range dose of acetaminophen by caffeine was explained by considering a three-zone model of action for a sigmoidal function whereby only the mid zone exhibits sensitivity (Granados-Soto and Castenada-Hernández 1999). This analysis emphasized the need for examining several dose combinations in order to more fully explore the potential for significant drug interactions.

In a subsequent series of studies using the pain-induced functional impairment model, caffeine was also shown to augment the action of aspirin (Castenada-Hernández et al. 1994), tolmetin (Flores-Acevedo et al. 1995), ketolorac (López-Munoz et al. 1996; Aguirre-Banuelos et al. 1999), ibuprofen (López et al. 2006) and ketoprofen (Díaz-Reval et al. 2001). The doses of caffeine that were most effective were 32 mg kg^{-1} (aspirin), 32–56 mg kg^{-1} (tolmetin), 18–32 mg kg^{-1} (ketolorac), 10–18 mg kg^{-1} (ketoprofen) and 18–32 mg kg^{-1} (ibuprofen). Studies that examined the widest range of dose combinations (Díaz-Reval et al. 2001; López et al. 2006) also showed that only certain dose combinations showed augmented activity. These observations support the zone model previously proposed (Granados-Soto and Castenada-Hernández 1999).

With regard to the mechanism by which caffeine augments antinociception by acetaminophen and other NSAIDs, several studies determined the effect of caffeine on plasma levels and kinetics, and observed no effect on these parameters for acetaminophen (Granados-Soto et al. 1993), aspirin (Castenada-Hernández et al. 1994) or tolmetin (Flores-Acevedo et al. 1995). Functional changes, therefore, represent pharmacodynamic rather than pharmacokinetic interactions. Caffeine had no effect on cyclooxygenase (COX) activity alone and did not affect inhibition of COX activity in the brain by acetaminophen or aspirin (Engelhardt et al. 1997). However, when examined for activity in rat primary microglial cells, caffeine and acetaminophen both inhibited microglial prostaglandin E_2 (PGE_2) synthesis alone, and augmented the ability of aspirin to inhibit lipopolysaccharide-induced PGE_2 synthesis (Fiebich et al. 2000). Caffeine also was shown to inhibit COX-2 protein synthesis (Fiebich et al. 2000). These actions of caffeine may result from inhibition of adenosine A_{2A} receptors on microglia, as these receptors cause upregulation of the COX-2 gene and release of PGE_2 in microglia (Fiebich et al. 1996). It was proposed that inhibition of COX in microglial cells contributes to the adjuvant analgesic activity of caffeine (Fiebich et al. 2000). Additional mechanisms considered to be involved in the intrinsic antinociceptive properties of caffeine, including block of adenosine A_{2B} receptors and recruitment of central noradrenergic mechanisms (Sect. 2.1), also may be involved in adjuvant analgesia by caffeine.

Some studies have noted that low doses of caffeine can inhibit antinociception by acetaminophen. Siegers (1973) reported that 10 mg kg^{-1} caffeine inhibited antinociception by acetaminophen in an inflammatory hyperalgesia model in rats. Granados-Soto et al. (1993) observed that 10 mg kg^{-1} caffeine appeared to inhibit the action of acetaminophen at doses of 100 and 562 mg kg^{-1} in the pain-induced functional impairment model in rats, and while this was not significantly different, the observation was noteworthy enough to merit mention in a subsequent analysis of the same data (Granados-Soto and Castenada-Hernández 1999). More recently, Godfrey et al. (2006) reported that 10 mg kg^{-1} caffeine inhibited the antinociceptive action of acetaminophen in the tail immersion and hot plate tests in mice; this effect was not mimicked by a selective adenosine A_{2A} (SCH58261) or A_{2B} (PSB1115) receptor antagonist. Curiously, this inhibitory effect of caffeine on acetaminophen mimics inhibitory effects reported with several different classes of analgesics; in those cases the inhibitory effect of caffeine is attributed to blockade of central adenosine A_1 receptors (Sect. 2.4).

2.3 *Methylxanthines and Morphine*

Methylxanthine interactions with morphine need to be considered in two distinct contexts, that of systemic interactions, whereby drugs are distributed to several compartments and the behavioural effect reflects the integrated action of several systems, and local interactions, whereby drug actions represent an interaction within a specific compartment (spinal, supraspinal, peripheral). Systemic interactions were originally explored to determine aspects of the mechanism of action of morphine, but they are also relevant to interactions that might occur owing to dietary intake of caffeine when opioids are given, or to drug combinations in which caffeine is added to an analgesic formulation consisting of a combination of codeine and an NSAID or acetaminophen.

2.3.1 Systemic Interactions

During the 1980s, caffeine was reported to potentiate antinociception by morphine (100 mg kg^{-1}, tail flick test, rats, Misra et al. 1985; 30 mg kg^{-1}, tail pressure test, rats, Person et al. 1985; 75, 100 mg kg^{-1}, hot plate test, mice, Malec and Michalska 1988), not to influence morphine (50 mg kg^{-1}, hot plate test, mice, Oliverio et al. 1983) or to antagonize morphine (40 mg kg^{-1}, tail flick test, mice, Ahlijanian and Takemori 1985; 25–50 mg kg^{-1}, tail flick and hot plate tests, mice, Malec and Michalska 1988). The Malec and Michalska (1988) study seemed to resolve disparate observations by demonstrating dose dependence and species dependence of interactions within the same study – in mice, both inhibition (low dose) and augmentation (high dose) of morphine by caffeine were observed, while low doses of caffeine augmented the action of morphine in rats. Figure 2 presents a compilation of data from that study, illustrating the variety of effects that can occur with a single drug combination (caffeine – morphine). Similar biphasic effects also were seen with theophylline and morphine (Malec and Michalska 1988). More recently, 5 mg kg^{-1} caffeine was shown to augment the action of morphine in the hot plate

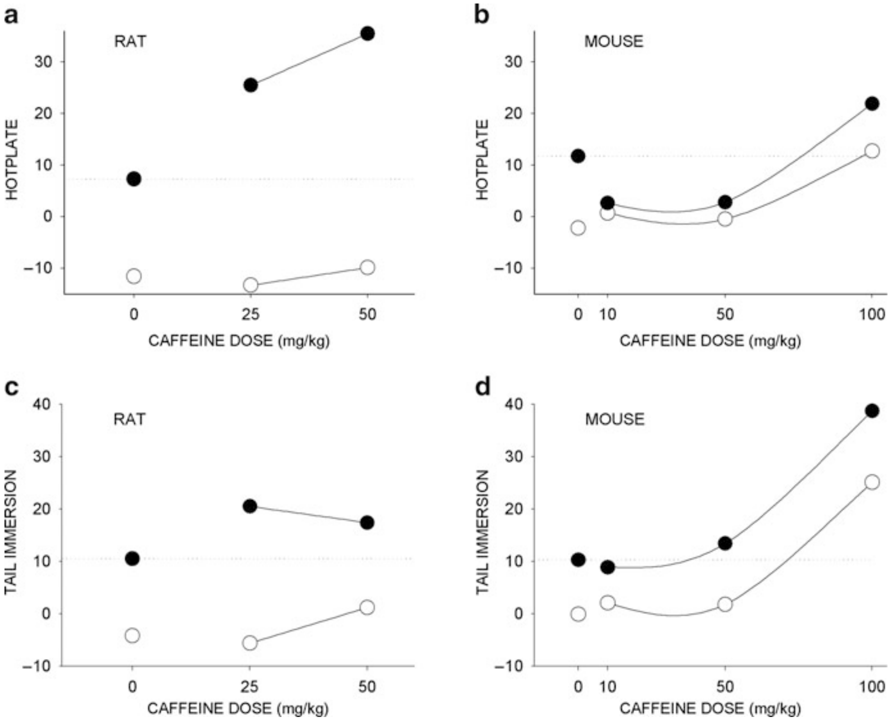

Fig. 2 Biphasic effects of caffeine on antinociception by morphine in (**a**, **c**) rats and (**b**, **d**) mice in two tests for nociception. Data points represent cumulative differences from the baseline; *hollow symbols* represent caffeine alone and *solid symbols* represent caffeine combined with 5 mg kg^{-1} morphine; *the dashed line* represents antinociception by morphine alone. In mice, there is (1) inhibition of morphine antinociception by low doses of caffeine, (2) augmentation of such antinociception by high doses of caffeine and (3) a lack of effect of intermediate doses of caffeine. In rats, low doses of caffeine augment the action of morphine, illustrating a species difference between mice and rats. (Data redrawn from Malec and Michalska 1988)

test in mice (Abo-Salem et al. 2004). In that study, both intrinsic antinociception seen with caffeine (10, 75 mg kg^{-1}; Sect. 2.1) and augmentation of the action of morphine (5 mg kg^{-1}) were mimicked by several selective adenosine A_{2B} receptor antagonists, and it was proposed that both actions of caffeine reflected adenosine A_{2B} receptor blockade.

2.3.2 Spinal Interactions

In 1981, it was shown that intraperitoneal administration of aminophylline inhibited the antinociceptive action of intraperitoneally administered morphine in the tail flick test in both intact and spinalized rats, which clearly implicated the spinal cord as the locus of the interaction (Jurna 1981). Subsequent studies showed that intrathecal administration of aminophylline, theophylline (non-selective adenosine receptor antagonists) or 8-phenyltheophylline (selective adenosine A_1 receptor antagonist) inhibited the action of intrathecally administered morphine in the tail flick and hot plate tests in rats (Jurna 1984; Sweeney et al. 1987a) and in the tail flick test in mice (DeLander and Hopkins 1986). When morphine was shown to increase the release of endogenous adenosine from in vitro and in vivo spinal cord preparations (Sweeney et al. 1987a, b), the methylxanthine blockade was understood to reflect block of spinal adenosine A_1 receptors activated by increased endogenous levels of adenosine. The characteristics and mechanisms of opioid-induced release of adenosine have been reviewed comprehensively (Sawynok and Liu 2003). A recent study has demonstrated that antinociception following intrathecal administration of morphine was reduced in adenosine A_1 receptor knockout mice, confirming involvement of spinal adenosine and A_1 receptors in the local action of morphine (Wu et al. 2005). The adenosine component of spinal opioid action, via adenosine A_1 receptors, also manifests itself prominently in nerve injury models of pain (Zhang et al. 2005). Finally, it is interesting to note that intrathecal, but not intravenous, opioids have been demonstrated to lead to spinal release of adenosine in humans (Eisenach et al. 2004).

2.3.3 Supraspinally Administered Morphine, Spinally Administered Methylxanthines

When morphine is given supraspinally by intracerebroventricular injection, antinociception is blocked by intrathecal administration of caffeine and theophylline in mice (DeLander and Hopkins 1986) and rats (Sweeney et al. 1991). In rats, supraspinally administered morphine results in spinal release of adenosine which is secondary to activation of descending serotonergic pathways; the adenosine that is released subsequently activates spinal adenosine A_1 receptors (Sweeney et al. 1991). In mice, spinal adenosine A_2 receptors appear to contribute to antinociception by supraspinally administered morphine (Suh et al. 1997).

2.3.4 Supraspinally Administered Morphine, Supraspinally Administered Methylxanthines

When opioids and caffeine were both given supraspinally, caffeine (inactive alone) inhibited antinociception by a μ-opioid and by a δ-opioid receptor agonist, but not by a κ-opioid receptor agonist (tail pinch and hot plate tests, mice) (Pham et al. 2003). The μ-opioid and δ-opioid receptor agonists inhibited adenosine uptake into synaptosomes, and the resultant increase in extracellular adenosine levels at supraspinal sites, with subsequent activation of adenosine receptors, was proposed to account for the methylxanthine effect (Pham et al. 2003).

2.4 Methylxanthines and Other Analgesics

2.4.1 Antidepressants

Tricyclic antidepressants are widely used in the treatment of neuropathic pain (Dworkin et al. 2007). Both caffeine and theophylline at 50 mg kg^{-1}, doses which were inactive alone, inhibited antinociception produced by systemic administration of the antidepressants amitriptyline and imipramine (tail flick test, mice) (Pareek et al. 1994). Aminophylline (10 mg kg^{-1}), also inactive alone, inhibited antinociception by the antidepressants clomipramine, maprotiline, imipramine and zimelidine (writhing test, mice) (Sierralta et al. 1995). Caffeine, generally between 5 and 10 mg kg^{-1}, inhibits antinociception by amitriptyline in several further models of pain – in the spinal nerve ligation model (thermal hyperalgesia, rats) (Esser and Sawynok 2000), in the streptozotocin-induced diabetic model (mechanical allodynia, rats) (Ulugol et al. 2002) and in the formalin model (2% formalin, mice) (Sawynok et al. 2008). Caffeine (5 mg kg^{-1}) also inhibits the action of a newer antidepressant, venlafaxine (hot plate test, mice) (Yaba et al. 2006). Collectively, these studies provide a coherent body of information indicating block of antidepressant actions by low doses of caffeine.

In all of these studies, antagonism by caffeine was considered to indicate an involvement of endogenous adenosine and adenosine A_1 receptors in the actions of antidepressants. A recent study provides direct support for this concept. While 10 mg kg^{-1} caffeine clearly blocked the ability of amitriptyline to produce antinociception in wild-type mice, this action was no longer observed in adenosine A_1 receptor knockout mice (Sawynok et al. 2008). In the latter situation, amitriptyline was still able to produce antinociception, and this was necessary in order to observe the differential effect of caffeine. Antidepressants are complex agents with several pharmacological actions, many of which contribute to antinociception (Micó et al. 2006), and this multiplicity of action may be what allows for intrinsic effects of amitriptyline to occur in A_1 receptor knockout mice.

Antidepressants also produce antinociception following local peripheral administration to the rodent hind paw, and this action is inhibited by local peripheral administration of caffeine. Local administration of caffeine inhibits antinociception produced by peripherally administered amitriptyline in the formalin test in rats (as does 8-phenyltheophylline) (Sawynok et al. 1999), in the spinal nerve ligation model in rats (thermal hyperalgesia) (Esser and Sawynok 2000) and in the streptozotocin-induced diabetes model in rats (mechanical allodynia) (Ulugol et al. 2002). Caffeine was no longer able to inhibit the peripheral action of amitriptyline in adenosine A_1 receptor knockout mice (Sawynok et al. 2008). Peripherally administered amitriptyline has been shown to increase extracellular levels of adenosine using microdialyis, perhaps by inhibiting adenosine uptake, and this increased tissue availability of adenosine is believed to contribute to its actions (Sawynok et al. 2005). Such studies clearly implicate adenosine and A_1 receptors as one component of the peripheral action of amitriptyline.

2.4.2 Carbamazepine/Oxcarbazepine

Carbamazepine is an anticonvulsant drug used to treat neuralgia and neuropathic pain, and oxcarbazepine is an analogue (10-keto derivative) better tolerated than the parent drug (Ambrósio et al. 2002). Caffeine (5 mg kg^{-1}) inhibited antinociception by carbamazepine in stressed rats (tail flick test) (Mashimoto et al. 1998). Caffeine (5–20 mg kg^{-1}; inactive alone) also inhibited the antinociceptive effects of both carbamazepine and oxcarbazepine administered systemically in an inflammatory hyperalgesia model in rats (Tomić et al. 2004). 1,3-Dipropyl-8-cyclopentylxanthine (DPCPX), a selective adenosine A_1 receptor antagonist, also inhibited the action of both drugs in a manner similar to caffeine (Tomić et al. 2004). Several reports indicate that carbamazepine and its derivatives can bind to adenosine A_1 and A_2 receptors, although it is not clear whether they function as agonists or antagonists (Ambrósio et al. 2002).

Peripheral administration of oxcarbazepine also produces antinociception in rats (inflammatory hyperalgesia), and this action is blocked by peripheral administration of caffeine (Tomić et al. 2006). Peripheral antagonism of oxcarbazepine by caffeine is mimicked by DPCPX, suggesting involvement of adenosine A_1 receptors in peripheral as well as systemic actions (Tomić et al. 2006). Agonist actions at adenosine A_1 receptors appear to contribute to peripheral, as well as systemic, actions of oxcarbazepine, although the nature of such involvement remains unclear.

2.4.3 Cizolirtine

Cizolirtine is a novel analgesic drug with a wide profile of activity in several preclinical pain models (Alvarez et al. 2000). The mechanism by which this agent produces antinociception is unclear as it does not interact with several receptors or ion channels (Alvarez et al. 2000). Cizolirtine produces antinociception in a preclinical model of neuropathic pain (streptozotocin-induced diabetes, rats), and its actions are blocked by 5 mg kg^{-1} caffeine (Aubel et al. 2007). Cizolirtine does not bind to adenosine receptors, and it was suggested that this agent leads to increased extracellular levels of endogenous adenosine (Aubel et al. 2007).

2.4.4 Summary of Analgesia-Modifying Properties of Caffeine

There are several general observations that can be made with respect to the ability of caffeine to modify antinociception by other analgesic agents. (1) *Low doses of caffeine* (*up to 10 mg* kg^{-1}) *inhibit antinociception by several analgesics.* Such doses of caffeine inhibit antinociception by acetaminophen (Siegers 1973; Godfrey et al. 2006), amitriptyline (Esser and Sawynok 2000; Ulugol et al. 2002; Yaba et al. 2006, Sawynok et al. 2008), carbamazepine and oxcarbazepine (Tomić et al. 2004) and cizolirtine (Aubel et al. 2007). This antagonism may reflect block of adenosine A_1 receptors recruited by increased endogenous levels of adenosine. The ability of 5 mg kg^{-1} caffeine to augment analgesia by morphine is proposed to be due to block of adenosine A_{2B} receptors (Abo-Salem et al. 2004). (2) *Moderate doses of caffeine* (*10–35 mg* kg^{-1}) *augment antinociception.* This is seen with acetaminophen (Gayawali et al. 1991; Granados-Soto et al. 1993) and several different NSAIDs (Gayawali et al. 1991; Castenada-Hernández et al. 1994; Flores-Acevedo et al. 1995; López-Munoz et al. 1996; Aguirre-Banuelos et al. 1999; Díaz-Reval et al. 2001; López et al. 2006). Augmentation is more readily observed within a medium-effect range (Granados-Soto and Castenada-Hernández 1999). Pharmacokinetic explanations for the positive interactions were excluded in several of these studies. Augmentation of antinociception may involve inhibition of COX in microglia (Fiebich et al. 2000). Mechanisms involving central amine systems may also be involved at moderate doses of caffeine (Sawynok et al. 1995). While some studies have reported potentiation of antinociception by morphine at such doses (Person et al. 1985), others have noted inhibition of antinociception by morphine (Malec and Michalska 1988; Ahlijanian and Takemori 1985); the latter interaction probably reflects actions at spinal sites. (3) *High doses of caffeine* (*75, 100 mg* kg^{-1}) *often show intrinsic antinociceptive effects* (Sect. 2.1) and augment antinociception by morphine (Misra et al. 1985; Malec and Michalska 1988), perhaps simply reflecting this intrinsic activity. The effects of 50 mg kg^{-1} caffeine can be variable, depending on the species and the test system, and may reflect biphasic effects by different doses of caffeine (Malec and Michalska 1988; Gayawali et al. 1991).

3 Clinical Studies with Caffeine

In the mid 1980s, an analysis of 30 trials indicated that caffeine (at least 65 mg) added to analgesia formulations containing aspirin and acetaminophen reduced the amount of analgesic needed by 40% (relative potency 1.4) (Laska et al. 1984). These adjuvant effects of were confirmed in several subsequent trials (Schachtel et al. 1991; Migliardi et al. 1994; Lipton et al. 1998; Diener et al. 2005) and extended to include combination with ibuprofen (Diamond et al. 2000). Further meta-analysis indicates that adding caffeine to analgesics increases the number of patients who experience pain relief from headache (rate ratio 1.36); it also leads to more patients with nervousness and dizziness (relative risk 1.60) (Zhang 2001). While there are some reports of adjuvant analgesic effects of caffeine in other pain conditions, meta-analysis shows no appreciable adjuvant effect with acetaminophen (Zhang and Po 1996), aspirin (Zhang and Po 1997) or ibuprofen (Po and Zhang 1998) for postoperative pain.

There are several issues to be considered with regard to caffeine and headaches. In some studies, caffeine also displayed intrinsic analgesic properties for treating tension-type headaches in doses of 130–200 mg caffeine (Ward et al. 1991; Diamond et al. 2000). Caffeine given orally at 300 mg relieved postdural puncture headache (Camann et al. 1990) but repeat oral doses of 75–125 mg caffeine (with acetaminophen) did not provide a prophylactic effect (Esmaoglu et al. 2005); an intravenous dose of 500 mg caffeine, however, did provide significant pain relief from postdural puncture headaches (Yucel et al. 1999). With chronic administration, caffeine withdrawal can lead to headaches (onset within 24 h of interruption of daily caffeine intake), although there is variability in the dose/duration requirements (Shapiro 2008). Postoperative headaches may be a manifestation of caffeine withdrawal resulting from perisurgical fasting, and perisurgical administration of caffeine is useful for relieving postoperative headaches (Fennelly et al. 1991; Hampl et al. 1995; Weber et al. 1997). Vascular effects (vasoconstriction) are considered to account for some of the pain relief that occurs in headaches (Shapiro 2008). On the other hand, habitual caffeine consumption or analgesic overuse can also be associated with the development of headache, migraine and chronic daily headache, but there are geographic and cultural differences in the prevalence of this (Shapiro 2008). While consumption of caffeine in children may be less than the adult rate, a case study noted resolution of headaches in 33 of 36 children when caffeine-containing beverages were withdrawn (Hering-Hanit and Gadoth 2003).

4 Summary and Conclusions

Interest in methylxanthine effects on pain arose from the presence of caffeine in many analgesic formulations, particularly those containing acetaminophen and aspirin. Caffeine (and other methylxanthines) subsequently became an investigative tool to determine the potential involvement of adenosine receptors in the mechanism of action of other analgesics. In preclinical studies, caffeine has been shown to exhibit several effects on nociception, depending on dose, nociceptive test, stimulus intensity and species. At low doses (up to 10–12.5 mg kg^{-1}) caffeine is usually inactive alone but inhibits the antinociceptive action of acetaminophen, amitriptyline, carbamazepine/oxcarbazepine and cizolirtine. A few studies have reported antinociception with caffeine at these low doses. At higher doses (approximately 15–45 mg kg^{-1}), it produces intrinsic effects in some preclinical models of antinociception, and augments antinociception by acetaminophen and NSAIDs even in tests where it lacks intrinsic effects. Doses in this range generally produce locomotor stimulation. At even higher doses (50–100 mg kg^{-1}), caffeine leads to intrinsic antinociception in a wide range of tests, locomotor suppression and diverse effects on the action of morphine depending on the test and species. Mechanisms implicated in *antinociception and augmentation of antinociception* involve inhibition of central adenosine A_{2B} (and possibly A_{2A}) receptors, inhibition of COX in microglia, engagement of central cholinergic systems and involvement of central noradrenergic systems. The mechanisms implicated in *inhibition of antinociception* involve block of central adenosine A_1 receptors. Clinical studies with caffeine have indicated adjuvant analgesic actions in combination with acetaminophen and NSAIDs and intrinsic analgesia in several headache conditions. Efficacy in headache states may involve alterations in central blood flow. The consistent preclinical observation of blockade of antinociception by several different classes of analgesics may be relevant to dietary intake of caffeine in humans; there are, however, no clinical studies that have addressed this issue.

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Methylxanthines and Sleep

Tarja Porkka-Heiskanen

Contents

Abstract Caffeine is widely used to promote wakefulness and counteract fatigue induced by restriction of sleep, but also to counteract the effects of caffeine abstinence. Adenosine is a physiological molecule, which in the central nervous system acts predominantly as an inhibitory neuromodulator. Adenosine is also a sleep-promoting molecule. Caffeine binds to adenosine receptors, and the antagonism of the adenosinergic system is believed to be the mechanism through which caffeine counteracts sleep in humans as well as in other species. The sensitivity for caffeine varies markedly among individuals. Recently, genetic variations in genes related to adenosine metabolism have provided at least a partial explanation for this variability. The main effects of caffeine on sleep are decreased sleep latency,

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shortened total sleep time, decrease in power in the delta range, and sleep fragmentation. Caffeine may also decrease the accumulation of sleep propensity during waking, thus inducing long-term harmful effects on sleep quality.

Keywords Adenosine · Adenosine receptors · Caffeine · Sleep · Sleep deprivation

1 What Is Sleep?

In humans, sleep is characterized by lack of consciousness: we are no longer able to evaluate ourselves and we lose contact with the surrounding reality. An important feature in brain function during sleep is decreased cortical reactivity: we are unable to respond to external stimuli as we would during wakefulness.

In mammals, sleep is defined through an electroencephalogram (EEG): in waking, cortical activity is desynchronized, leading to high-frequency, low-amplitude waveforms. When sleep is initiated, the EEG becomes synchronized, expressed as low-frequency, high-amplitude waveforms (Fig. 1). Sleep is further divided into two main stages: non-REM sleep and REM sleep. In REM sleep cortical activity is as desynchronized as in waking, and only on the basis of the

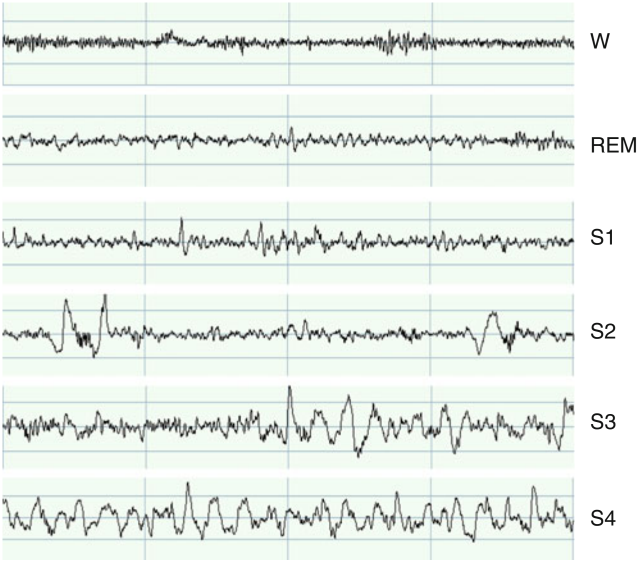

Fig. 1 Human vigilance states defined by an electroencephalogram (EEG). Waking (W) is characterized with a high-frequency but low-amplitude waveform, similarly to REM sleep (REM). Non-REM sleep is divided into stages 1–4, with deepening of sleep from stage 1 to stage 4. Upon deepening of sleep, the EEG wave frequency decreases while the amplitude increases

EEG, these stages cannot be distinguished. Non-REM sleep is characterized by slowing down of the body and brain functions: synchronized cortical activity, decreased heart rate and blood pressure, as well as breathing. In REM sleep, heart rate, blood pressure ,and breathing become irregular, with large and sudden variations. Muscle tone is almost completely lost in body muscles, but is maintained in the muscles of the head and in muscles used in breathing. However, the unconsciousness that is so characteristic to sleep is maintained.

The electric activity of the brain (cortex) can be measured using an electroencephalograph and can be either described visually as "brain waves" (Fig. 1) or quantized as a power spectrum over the frequencies of the EEG. A power spectrum is a mathematical measure of the intensity of brain electric activity, and it is calculated from the original EEG signal using Fourier transformation.

Low EEG frequencies are typical for sleep and high frequencies are typical for waking. The more power there is in the low-frequency range (typically 0.75–4 Hz, called delta range), the deeper the sleep is, while during waking, delta power is low. Powers in other frequency ranges also have significance for the vigilance state – in this chapter power in the theta range will be assessed in addition to that in the delta range. Theta activity is high during waking, and it increases during brain activation. In rodents, theta activity is characterized by exploratory behavior, meaning that the animals have motor activity (Vyazovskiy and Tobler 2005; Wigren et al. 2009). In humans, theta power increases in the course of waking, and it has been suggested to be a marker of sleep homeostasis during waking, while delta power is a marker of sleep homeostasis during sleep (Finelli et al. 2000).

An important feature of sleep is its homeostatic regulation, which means that a long waking period is followed by a correspondingly longer and deeper sleep period. This feature has been characterized from human (and animal) EEG power spectra and formalized mathematically in the two-process model of sleep regulation (Borbely 1982; see also Fig. 2). Sleep homeostasis is often described as accumulation of sleep propensity, or "sleep pressure" during the waking period, but what, at

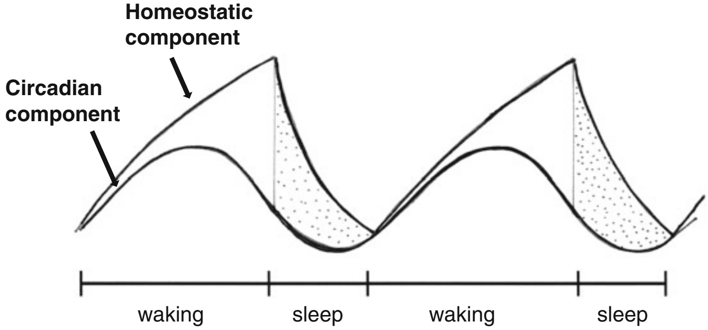

Fig. 2 The two-process model of sleep regulation. Sleep is regulated by two processes: the circadian component and the homeostatic component. During waking the homeostatic component increases until sleep is initiated. If waking is prolonged, the homeostatic component keeps increasing, while the circadian component continues its cycling variation. (Modified from Borbely 1982)

the molecular level, is accumulating is less clear. The accumulation of sleep pressure is necessary for initiation of sleep – if this mechanism is disturbed during the waking period, there will be difficulties in falling asleep and the sleep period may be terminated early. Also, the deep-sleep phases may be short, or absent.

Adenosine has been suggested to be one of the molecular correlates of sleep pressure and an inducer of sleepiness (Porkka-Heiskanen et al. 1997). Caffeine is an adenosine receptor blocker, counteracting sleepiness through this action.

2 Regulation of Sleep

2.1 Waking Mechanisms

Sleep and wakefulness are mutually exclusive states of vigilance and thus need to be regulated in concert. As a consequence, we cannot understand the regulation of sleep without understanding the regulation of wakefulness.

Cortical excitability underlies all cortical activity, and its level is maintained and regulated by several, partly overlapping neuronal networks with very similar anatomical structure. The key elements of these networks are small cell groups, lying either in the brainstem or in the hypothalamus and sending widespread projections to the cortex and other brain areas. The level of cortical excitability is defined by the level of activity of these cell groups, which release neurotransmitters that regulate the reactivity of cortical neurons (predominantly glutamatergic): during wakefulness the activity in the cell groups is high and a lot of neurotransmitters are released into the cortex, while in sleep the activity of the cell groups decreases or ceases and only small amounts of neurotransmitters are released, leading to low cortical reactivity (for a review, see Stenberg, 2007). The waking-maintaining cell groups in the brainstem consist of serotonin neurons in the raphe nuclei, noradrenaline neurons in the locus coeruleus, and acetylcholine neurons in the laterodorsal tegmental and pedunculopontine tegmental nuclei. In addition, in the hypothalamus/basal forebrain (BF) area, histamine neurons in the tuberomamillary nuclei, orexin neurons in the lateral hypothalamic nucleus, and cholinergic neurons in the BF serve this function (for a summary, see Fig. 3). The activity of all these cells is high during waking and decreases upon falling asleep. Initiation of sleep is not possible if these neurons continue their activity. The activity of these cells continues to decrease during deep-sleep phases, but interestingly, during REM sleep one group of neurons, the cholinergic neurons, increase their activity to the level of waking, while other waking-maintaining cell groups virtually cease firing.

All these neurons have adenosine receptors on them, thus making them targets for caffeine and offering it an opportunity to modulate the vigilance state through these neurons.

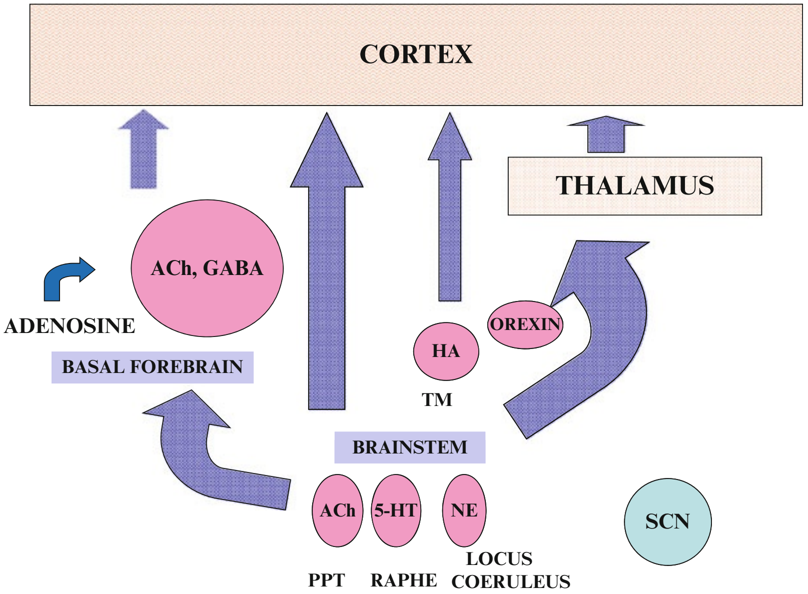

Fig. 3 Summary of the cell groups that regulate the vigilance state. All vigilance-regulating cell groups are located in the old parts of the brain: brainstem, basal forebrain and hypothalamus. The groups in the brainstem – pedunculopontine tegmental nucleus (PPT) with cholinergic cells (Ach), raphe nuclei with serotoninergic cells (5-HT) and locus coeruleus with noradrenergic cells (NE) – project either directly or via the thalamus or via the basal forebrain to the cortex. In the hypothalamus tuberomamillary nuclei (TM) histaminergic cells (HA) and in the lateral hypothtalamus orexinergic cells (OREXIN) send projections to the cortex, as do cholinergic cells (Ach) and GABAergic cells (GABA) in the basal forebrain. *SCN* suprachiasmatic nucleus – the site of the inner clock

2.2 Sleep Induction Mechanisms

So far only one area in the brain has been shown to contain neurons that are more active during sleep than during waking – sleep-active neurons: the preoptic area of the hypothalamus, particularly the ventrolateral preoptic area. The cells of this nucleus are GABAergic and send inhibitory projections to all waking nuclei. This system forms the core of the mutually exclusive vigilance state regulation: when sleep-active neurons are on, they send inhibitory signals to waking-active neurons, and vice versa (McGinty and Szymusiak, 2000; Saper et al. 2001).

What about induction of sleep homeostasis? Several molecules have been proposed to take part in this regulation, including adenosine (Porkka-Heiskanen et al. 1997), nitric oxide (Kalinchuk et al. 2006), prostaglandins (Hayaishi 1988), and cytokines (Krueger and Fang 1997). This chapter concentrates on the role of adenosine in this regulation.

The adenosine concentration increases during prolonged wakefulness in the BF (Porkka-Heiskanen et al. 1997), and as adenosine is predominantly an inhibitory neuromodulator (through activation of A_1 receptors), it will decrease neuronal activity. The inhibition of the BF wake-active cholinergic neurons will result in increased sleepiness and sleep propensity and initiation of recovery sleep. If the adenosine receptors are blocked during waking, also the accumulation of sleep pressure is attenuated (Gass et al. 2009; Landolt et al. 2004). This has relevance for the mechanisms by which caffeine reduces sleep: it can modulate the accumulation of sleep pressure.

2.2.1 Adenosine

The Effects of Adenosine in the Central Nervous System with Relevance for Regulation of the Vigilance State

Adenosine is predominantly an inhibitory neurotransmitter in the central nervous system (CNS), acting through A_1 receptors. There is evidence that the release of excitatory transmitters is inhibited by adenosine more strongly than that of inhibitory neurotransmitters (Fredholm and Dunwiddie 1988). Adenosine inhibits neuronal activity through several mechanisms, e.g., through decreasing neurotransmitter release (Dunwiddie 1985) and inhibiting the firing rate of neurons (Phillis and Edstrom 1976). The BF cholinergic neurons, which are part of the waking-maintaining system, are tonically inhibited by adenosine (Rainnie et al. 1994), and adenosine inhibits the tuberomamillar histaminergic neurons via A_1 receptors (Oishi et al. 2008), giving rise to the hypothesis that adenosine may promote sleepiness by inhibiting activity and neurotransmitter release of wakefulness-promoting neurons.

Adenosine Receptors in the CNS: Types and Distribution

There are four types of adenosine receptors in the CNS (for details, see Chap. 6, in this volume). Of the four receptor types, A_1 and A_{2A} appear to be relevant in regulation of vigilance states.

A_1 receptors are located in all parts of the CNS and on both inhibitory and excitatory neurons, while the A_{2A} receptor location appears to be limited to the striatum (for details, see Chap. 6, in this volume). The result of A_1 receptor stimulation is neuronal inhibition, and the functional outcome depends on whether inhibitory or excitatory neurons were inhibited. The result of A_{2A} receptor stimulation is neuronal activation.

Adenosine and Sleep

Circadian variation in the enzymes that metabolize adenosine suggests that also adenosine levels may undergo circadian- and/or vigilance-state-related variation (Chagoya de Sanchez et al. 1993). In rats, adenosine levels were high during the active (dark) period of this species both in the cortical areas (Chagoya de Sanchez et al. 1993; Huston et al. 1996) and in the BF (Murillo-Rodriguez et al. 2004). In freely moving cats, adenosine levels were lower in samples that were collected during sleep than in those collected during wakefulness (Porkka-Heiskanen et al. 1997). Adenosine, acting on A_1 receptors, promotes non-REM sleep by inhibiting the histaminergic neurons in the tuberomamillar nucleus (Oishi et al. 2008) (Fig. 3).

Prolonged waking increased adenosine levels in the BF of cats (Fig. 4) and increasing adenosine concentration experimentally in the BF induced sleep (Porkka-Heiskanen et al. 1997), while blocking the A_1 receptors during prolonged wakefulness prevented recovery sleep (Gass et al. 2009). Taken together, these data imply that adenosine is a mediator of sleep homeostasis.

Genetic Variations in the Human Adenosinergic System Relevant for Sleep

A functional genetic variation in adenosine deaminase (ADA) modulates sleep (Retey et al. 2005): both the duration and the intensity of slow-wave sleep were increased in individuals with ADA G/A genotype as compared with individuals with G/G genotype (Retey et al. 2005). The polymorphism (G to A transition at nucleotide 22) is functional, since the enzymatic activity of the G/A variant ADA is

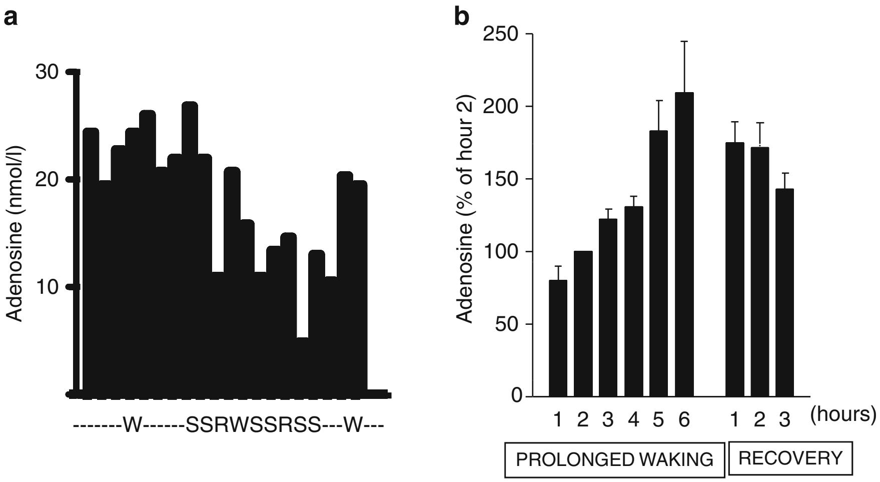

Fig. 4 **(a)** Variation in the basal forebrain adenosine concentration during a spontaneous sleep–wake cycle. Adenosine concentrations were measured during waking (W), sleep (S), and REM sleep (R). During waking the adenosine levels are high, decrease during sleep, and increase during REM sleep. **(b)** Adenosine concentration in the basal forebrain during prolonged wakefulness. Adenosine concentrations were measured during a 6-h wakefulness period from samples collected in the basal forebrain. The adenosine concentration increased steadily during the wakefulness period and started to decrease upon falling asleep (recovery). (Modified from Porkka-Heiskanen et al. 1997)

20–30% lower than that of the G/G variant (Battistuzzi et al. 1981), presumably leading to higher tissue adenosine levels.

3 Caffeine and Sleep

3.1 General Actions of Caffeine in the CNS with Potential Significance in the Regulation of Sleep

In general, the inhibition of CNS A_1 receptors will increase neuronal firing, while inhibition of A_{2A} receptors will decrease it.

In addition to the adenosine-receptor-mediated actions of caffeine (discussed in the next sections), caffeine can affect vigilance through other known mechanisms in the CNS. Caffeine increases the turnover of several monoamine neurotransmitters, including 5-hydroxytryptamine (5-HT) (Fernstrom et al. 1984), dopamine, and noradrenaline (Fredholm et al. 1984; Hadfield and Milio 1989) and it inhibits release of neurotransmitters (Fredholm and Dunwiddie 1988). High doses of caffeine can also activate phosphodiesterase, but this is usually not the case with normal human caffeine consumption (Fredholm et al. 1999).

The tonic inhibition of neurons by adenosine is inhibited by caffeine, which elevates the excitability of rat hippocampus slices (Dunwiddie et al. 1981; Greene et al. 1985) and activates the theta rhythm in rabbit hippocampus (Popoli et al. 1987). High caffeine doses (100 mg/kg or above) provoke seizurelike activity in the hippocampus.

3.2 Mechanisms of Caffeine Action on Sleep

There is consensus that caffeine acts through adenosine receptors to reduce sleepiness/sleep, but the question of whether this action takes place through A_1 or A_{2A} receptors is still debated (for a review, see Fredholm et al. 1999). This question has been intimately related to the question of whether adenosine acts on vigilance states through A_1 or A_{2A} receptors (Fredholm et al. 1999; Basheer et al. 2004) but it should be appreciated that these are two related, but separate questions.

Probably, both adenosine A_1 and A_{2A} receptors are involved in producing the sleep-promoting effects of adenosine, but these effects appear to be exerted in different parts of the brain. Consequently, several adenosine-receptor-mediated mechanisms may contribute to the sleep-reducing effects of caffeine, depending on the location of the receptors (whether they lie on waking-active or sleep-active cells) and whether they are predominantly of the inhibitory A_1 type or the activating A_{2A} type. One hypothesis states that caffeine induces waking through inhibition of the cholinergic waking-active neurons (Rainnie et al. 1994). This view has recently been challenged by results showing that caffeine does not affect vigilance in A_1-receptor-knockout mice, but significantly reduces sleep in A_{2A}-receptor-knockout mice (Huang et al. 2005), indicating that the effects of caffeine on the vigilance state are mediated through A_{2A} receptors.

In addition to this immediate arousing effect, caffeine may also inhibit the buildup of sleep homeostasis (Landolt et al. 2004). This effect is less well studied and generally neglected in discussions on the effect of caffeine on sleep, and particularly regarding its role in inducing sleeping problems (mainly prolonged sleep latency and/or premature awakenings). The mechanisms of this inhibition – whether A_1 or A_{2A} receptors are involved and which neuronal groups are involved – remain to be determined.

3.2.1 A_1 Receptors

Studies indicating that adenosine acts through A_1 receptors to reduce sleepiness/sleep are numerous. Early studies by Ticho and Radulovacki (1991) showed that local injections of adenosine A_1 receptor agonists in the preoptic area of the rat induced sleep, while an A_{2A} receptor agonist did not. Also, systemic as well as intracerebroventricular administrations of A_1 receptor agonists induced sleep (Benington and Heller 1995; Schwierin et al. 1996), while blocking the A_1 receptors pharmacologically (Strecker et al. 2000) or by inactivating them by using an antisense oligonucleotide targeted at the A_1 receptor (Thakkar et al. 2003) decreased sleep and increased waking. The administration of the adenosine A_1-receptor-selective agonist cyclopentyladenosine mimicked the EEG effects of sleep deprivation (Benington et al. 1995) and non-REM sleep (Schwierin et al. 1996).

The role of adenosine in induction of recovery sleep after sleep restriction has been convincingly shown in a series of experiments where an increase in BF adenosine concentration through either sleep restriction or pharmacological manipulation increased sleep (Porkka-Heiskanen et al. 1997; Porkka-Heiskanen et al. 2000), while blocking the A_1 receptors during the deprivation, either pharmacologically (Gass et al. 2009) or using an A_1-specific antisense oligonucleotide (Thakkar et al. 2003), decreased it. Interestingly, the number of A_1 receptors increased both in rat (Basheer et al. 2007) and in human (Elmenhorst et al. 2009) brain during sleep deprivation, further suggesting a role for A_1 receptors in regulation of sleepiness.

The site(s) where adenosine increases sleep through A_1 receptors is not known, but inhibition of wake-active neurons appears a plausible mechanism. The BF wake-active cholinergic neurons are tonically inhibited by adenosine through A_1 receptors (Rainnie et al. 1994), and the removal of this inhibition by blocking these receptors would increase their firing and cortical arousal. There is evidence that the activity of the cholinergic neurons can be regulated by adenosinergic manipulations: both theophylline (Murray et al. 1982) and caffeine can modify acetylcholine levels and metabolism in the brain (Phillis and Wu 1981; Murray et al. 1982; Carter et al. 1995). Caffeine increases the level of cortical acetylcholine dose dependently and even at doses that can be induced by ordinary caffeine consumption (Carter et al. 1995).

Evidently the induction of recovery sleep is connected with the BF cholinergic cells, since recovery sleep remained absent in animals that had been lesioned of these cells (Kalinchuk et al. 2008). In the lesioned animals, the increase in BF adenosine concentration during sleep restriction also remained absent.

3.2.2 A_2 Receptors

Several studies also suggest a role for A_{2A} receptors in regulation of vigilance states, and that caffeine may act through these receptors when reducing sleep. The key structure in this action appears to be the subarachnoid space, below the rostral BF, where prostaglandin D_2 receptor activation releases adenosine, which induces sleep through activation of A_{2A} receptors (for a review, see Urade and Hayaishi 1999; Hayaishi 2002).

Pharmacology studies showed that infusion of the A_{2A} receptor agonist CGS 21680 into the subarachnoid space increased slow-wave sleep. This effect was blocked by the A_{2A} receptor antagonist KF17837 (Satoh et al. 1996; Ram et al. 1997; Satoh et al. 1999). Injection of the selective adenosine A_{2A} receptor agonist CGS 21680 into the subarachnoid space mimicked the sleep-promoting effects of prostaglandin D_2, whereas an A_1 receptor agonist did not (Satoh et al. 1996). The authors stated that the adenosine A_{2A} receptors in the tuberculum olfactorium/ventral nucleus accumbens are a likely site of action (Satoh et al. 1996).

The strongest evidence to support a role for A_{2A} receptors in caffeine-mediated increase in waking comes from a study with A_{2A}-receptor-knockout mice. In this study caffeine was administered to mice that had been genetically engineered so that they had either no A_{2A} receptors or no A_1 receptors, as well as to normal mice with both receptors intact. Caffeine increased waking in the wild-type mice as well as in A_1-receptor-knockout mice, but not in A_{2A}-receptor-knockout mice, indicating that the effects of caffeine are mediated through A_{2A} receptors (Huang et al. 2005). This view is supported by human data: A single c.1083T>C polymorphism in the A_{2A} receptor gene ($ADORA_{2A}$) modulates individual sensitivity to subjective and objective effects of caffeine on sleep (Retey et al. 2007).

Interestingly, a recent study conducted on fruit flies showed that chronic administration of caffeine reduces and fragments their sleep (Wu et al. 2009), while mutants lacking the fly adenosine receptor (with sequence similarity to mammalian A_{2A} receptor) had normal amounts of baseline sleep, as well as normal homeostatic responses to sleep deprivation. Surprisingly, these mutants respond normally to caffeine. However, a phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX), mimicked the effects of caffeine in the mutant flies, while the effects of caffeine on sleep were blocked in flies that had reduced neuronal phosphodiesterase (PKA) activity. The authors concluded that chronic administration of caffeine promotes wakefulness in *Drosophila*, at least in part by inhibiting cyclic AMP PKA activity, but not through adenosine receptors. Whether such mechanisms contribute to wakefulness induced by chronic caffeine intake also in mammals remains to be clarified through further studies.

3.3 Effects of Caffeine on Sleep in Animals

Caffeine decreases sleep and increases wakefulness in all animal species tested so far, e.g., cats (Sinton and Petitjean 1989), (Yanik et al. 1987) and mice (Stenberg et al. 2003). In rats, the effects of caffeine on recovery sleep have also been addressed: caffeine reduced attempts to sleep during sleep deprivation and slowed the rate of recovery sleep, but did not prevent it (Wurts and Edgar 2000).

The effects of caffeine on the vigilance state are not restricted to mammals. A vigilance state that resembles sleep (characterized by immobility, increased arousal threshold, and signs of homeostatic regulation) can also be defined in species that do not have a CNS, which would allow EEG recording. Caffeine decreases sleep in the fruit fly (*Drosophila melanogaster*) (Shaw et al. 2000). A sleeplike state has also been characterized in zebra fish (*Danio rerio*) (Zhdanova et al. 2001; Yokogawa et al. 2007). Recently we showed that caffeine increases wakefulness and decreases "sleep" in this species (unpublished data).

3.4 Effects of Caffeine on Sleep in Humans

Sleep in humans is readily affected by caffeine. Epidemiology studies have reported an association between daily caffeine intake and sleep problems and daytime sleepiness (for a review, see Roehrs and Roth 2008).

The most prominent effects of caffeine on sleep are prolonged sleep latency, shorter total sleep time, increases in light sleep, and decreases in deep sleep. REM sleep is less affected but the latency to the first REM period is shortened. Subjective sleep quality is also decreased reflecting the longer sleep latency, increased awakenings, and the shorter total sleep time. Remarkably, these effects are obvious not only when caffeine is ingested just before bedtime, but also when caffeine is ingested in the morning and the effects on sleep are similar on the following night.

When caffeine is ingested shortly before bedtime, experimental findings include a dose-dependent decrease in total sleep time, an increase in sleep latency and a decrease in deep sleep (Karacan et al. 1976; Nicholson and Stone 1980), impaired sleep efficacy and a decrease in slow-wave sleep (Landolt et al. 1995a, b), as well as decreased power in the delta frequency range, but an increase in power in the spindle frequency range (Landolt et al. 1995a, b, see Fig. 5).

Caffeine ingested even in the morning has prominent effects on sleep during the subsequent night: when modest coffee drinkers ingested 200 mg caffeine in the morning, their total sleep time was reduced by about 10 min, the latency to stage 2 sleep was prolonged, and sleep efficiency (ratio of time asleep to time in bed) was reduced by about 3% (Landolt et al. 1995a, b). The changes in the EEG

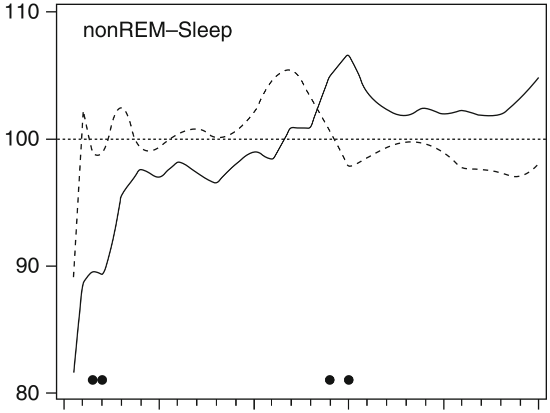

Fig. 5 Effect of caffeine on human sleep EEG. Caffeine decreases the EEG power density at low frequencies and increases it at theta frequencies. *Solid line* EEG recorded after caffeine intake, *dashed line* before-caffeine EEG. The *Y*-axis is the EEG power density (%). (Modified from Landolt et al. 1995a, b)

were similar to those observed after night ingestion of caffeine. Caffeine had already metabolized, as evidenced by low saliva concentrations, but it is possible that the psychoactive metabolites induced some of the changes (Retey et al. 2007; Landolt et al. 2004).

The caffeine-induced EEG changes in non-REM sleep are comparable in rested and sleep-deprived subjects. They are consistently found irrespective of whether the study participants maintain habitual morning caffeine consumption (Landolt et al. 1995a, b) or abstain from caffeine for days (Landolt et al. 1995a, b) (Landolt et al. 2004) and weeks (Retey et al. 2007; Landolt et al. 2004) before the experiments. It is thus improbable that reversal of withdrawal syndromes would explain these changes (James and Keane 2007).

Studies that compare the effects of caffeine on sleep between high- and low-coffee consumers have shown that caffeine intake in low consumers will induce prolonged sleep latency, disturbances in different sleep phases, and shorter total sleep time (Curatolo and Robertson 1983), while the effects in habitual coffee drinkers are smaller (Colton et al. 1968).

Not only caffeine itself but also abstinence from caffeine will induce sleep difficulties (for a review, see James and Keane 2007). In a field study, regular caffeine drinkers were assigned to groups that received either regular coffee or decaffeinated coffee for 9 days, or to a group that at 2-day intervals was switched between regular and decaffeinated coffee. Caffeine abstinence resulted in increased heart rate, decreased motor activity, subjective wakefulness, and an increased number of headaches and use of analgesics. The subjective effects and headaches disappeared after a few days of abstinence and weakened over successive, separated abstinence periods. The intermittent onset of caffeine consumption resulted in increased wakefulness (Höfer and Bättig 1994).

Caffeine has even been used as a model of insomnia in different experimental designs (Okuma et al. 1982).

Insomnia was mimicked in volunteers by administering to them 400 mg caffeine three times a day for 7 days. The treatment decreased the total sleep time and increased the sleep latency, while stage 4 sleep was reduced. Partial tolerance to the effects of caffeine developed in the course of the experiment (Bonnet and Arand 1992).

In summary, the sleep-disruptive effects of caffeine, even at doses equivalent to a single cup of coffee, have been well documented.

3.5 Caffeine and Performance

Caffeine restores performance and mood under sleep loss, when sleep has been previously restricted owing to, e.g., shift work or jet lag. Several studies, including laboratory and field studies, have documented the beneficial effect of caffeine on performance impairment associated with shift work (Bonnet et al. 2005; Schweitzer et al. 2006).

Naps are frequently used, and have been documented to be useful, in ameliorating the feeling of sleepiness and fatigue as well as a decrease of performance. The benefits of naps can be improved by combining them with caffeine intake (Bonnet 1991; Bonnet et al. 1995; Bonnet and Arand 1996).

However, does caffeine improve performance when ingested without a previous increase in sleep propensity? A study conducted on 36 healthy volunteers found no evidence of beneficial effects of caffeine on human performance (James 1998) although the subjects reported being more alert and feeling less tired when ingesting caffeine. The initially improved feeling of increased alertness disappeared when caffeine was used chronically (in the course of 6 days). It is possible that caffeine has only small, if any, beneficial effects on performance under sleep satiety but it is effective in improving performance under sleep loss. There are also methodological issues that cast doubts regarding the results on caffeine's effect on performance and mood (see Sect. 3.7 for details).

3.6 Individual Sensitivity to Caffeine

Human sensitivity to the effects of caffeine is variable. The basis of this variability has been widely debated. Physiological differences in the development of tolerance to the effects of caffeine may explain some of the differences (Colton et al. 1968; Curatolo and Robertson 1983; Bonnet and Arand 1992).

The plasma concentration of caffeine induced by ingestion of the same amount of caffeine by different people can vary between individuals by a factor of 15.9 (Birkett and Miners 1991), suggesting that slow metabolizers may be more sensitive to caffeine.

A genetic difference in caffeine sensitivity has been also suggested, and recently a distinct c.1083T $>$ C polymorphism in the A_{2A} receptor gene ($ADORA_{2A}$) has been found to modulate individual sensitivity to subjective and objective effects of caffeine on sleep (Retey et al. 2007), giving a firm biological explanation at least partly for the differences in caffeine sensitivity (Landolt 2008). Furthermore, another polymorphism in the same receptor gene, 1976T/T 2592 Tins/Tins polymorphism, induces differences in caffeine-induced anxiety: individuals with the polymorphism reported more anxiety after caffeine intake than individuals without the modification (Alsene et al. 2003).

There is also a large variability in how individuals respond to sleep deprivation: in some individuals a short deprivation period reduces performance, while others can maintain their performance level for prolonged periods of sleep loss (Haavisto et al. 2010). The relation of these individual differences to the adenosinergic system was assessed in a study where young volunteers stayed awake for one night. The subjects rated themselves as either caffeine-sensitive or caffeine-insensitive, and ingested either caffeine (200 mg) or a placebo twice during the waking period. In the placebo condition, those who had rated themselves as caffeine-sensitive showed a greater decrease in performance than those who had rated themselves as caffeine-insensitive. However, caffeine improved the performance particularly in caffeine-sensitive individuals (Hotta et al. 2009). The behavioral findings were supported by EEG findings: in those subjects in whom prolonged waking induced the largest increase in the frontooccipital power ratio, caffeine most potently reduced this ratio. In other words,there is negative association between the effects of sleep deprivation and caffeine. These findings suggest that adenosinergic mechanisms could at least partly explain differences in responses to sleep restriction.

3.7 Sleep During Caffeine Withdrawal

Caffeine induces dependency, even after a relatively short period of exposure with doses that are commonly used: withdrawal syndromes have been documented after three to seven daily uses of 100 mg caffeine (Juliano and Griffiths 2004). The withdrawal syndromes include headache, fatigue, drowsiness, decreased alertness, depressed mood, and irritability. Sleepiness commonly increases in the course of abstinence (Roehrs and Roth 2008).

The withdrawal syndrome introduces a serious confounding factor to human experiments with caffeine: most subjects habitually consume caffeine in variable daily amounts, but before the experiments they should stop caffeine use to ensure a zero level of caffeine in the blood. The half-life of caffeine is 3–7 h, while the withdrawal symptoms appear 12–24 h from the beginning of the abstinence. If overnight abstinence is used, the experiments start in the middle of the abstinence syndrome, including increased sleepiness. It has been argued that most of the reported effects of caffeine on mood and performance are the reversal of withdrawal symptoms (James and Keane 2007).

3.8 Effects of Other Methylxantines on (Human) Sleep

Methylxanthines include, in addition to caffeine, aminophylline, IBMX, paraxanthine, pentoxifylline, theobromine, and theophylline. The affinity of these substances for adenosine receptors varies (see Chap. 6, in this volume), as well as their efficacy in promoting wakefulness. Common beverages, such as tea and chocolate, contain, in addition to caffeine, other methylxanthines. Tea contains caffeine at a level of about 3% of its dry weight and, in addition, small amounts of theobromine and theophylline. Dry tea has more caffeine by weight than coffee, but as more dry coffee is used than dry tea to prepare a beverage, a cup of brewed tea usually contains significantly less caffeine than a cup of coffee of the same size. Chocolate contains theobromine, which has physiological effects similar to those of caffeine.

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Methylxanthines and Reproduction

Alba Minelli and Ilaria Bellezza

Contents

Abstract Reproduction is the process by which organisms create descendants. In human reproduction, two kinds of sex cells, or gametes, are involved. Sperm, the male gamete, and egg, or ovum, the female gamete, must meet in the female reproductive system to create a new individual and both the female and the male reproductive systems are essential to the occurrence of reproduction. Scientific reports dealing with the effects of methylxanthines on reproduction are mostly centred on the use of these compounds as phosphodiesterase inhibitors that, by maintaining high intracellular levels of cyclic AMP (cAMP), will affect the gametes differently. High cAMP levels will sustain sperm maturation while they hold the oocytes in mitotic arrest. Caffeine, being the methylxanthine most widely consumed by every segment of the population, has been the subject of greatest interest among health professionals and researchers. Conflicting results still seem to characterize the association between male/female caffeine consumption in adult life

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and semen quality/fertility, although moderate daily caffeine consumption of levels up to 400–450 mg/day (5.7–6.4 mg/kg/day in a 70-kg adult) do not seem to be associated with adverse effects, i.e. general toxicity, effects on bone status and calcium balance, cardiovascular effects, behavioural changes, increased incidence of cancer, or effects on male fertility. A clear stimulation of egg-laying by the coffee leaf pest *Leucoptera coffeella* was recently reported, providing support for the hypothesis that caffeine, in a dose-dependent way, in insects stimulates egg-laying, thus leading to the death of coffee trees.

Keywords Acquisition of sperm fertility · Assisted reproductive techniques · Oocytes maturation · Spermatogenesis

Abbreviation

AC	Adenylyl cyclase
AR	Acrosome reaction
cAMP	Cyclic AMP
Cdk1	Cyclin-dependant kinase
dbcAMP	Dibutyryl cyclic AMP
FSH	Follicle-stimulating hormone
GIFT	Gamete intra-Fallopian transfer
IBMX	3-Isobutyl-1-methylxanthine
IVF	In vitro fertilization
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
MI	Metaphase I
MPF	Maturation/meiosis or mitosis promoting factor
NOEL	No-observed-effect level
PDE	Phosphodiesterase
PKA	Protein kinase A
PKB	Protein kinase B
UPP	Ubiquitin-proteasome pathway

1 The Male Reproductive System

The male reproductive system consists of a number of sex organs that are part of the reproductive process. The testes are responsible for production of sperm and androgens, i.e. sex hormones essential to development and functional maintenance of the entire male reproductive tract. Each testis comprises two tissue compartments, which are functionally related but structurally separate: the seminiferous tubule compartment, lined with a complex epithelium of highly specialized Sertoli cells and developing spermatogenic cells, and the interstitial tissue compartment, which contains the androgen-producing Leydig cells, as well as the testicular vasculature, lymphatic and immune cells. The seminiferous tubules are connected through a structure called the rete testis, via a series of efferent ducts, to the adjacent epididymis, which concentrates and facilitates the maturation of the sperm. At ejaculation, epididymal fluid and sperm are propelled along the muscular vas deferens to the urethra, where they are combined with the secretions of the accessory glands to form the seminal plasma.

1.1 Spermatogenesis

Spermatogenesis is the process by which male spermatogonia develop into mature spermatozoa in sexually reproducing organisms. In mammals this process occurs in the male testes and epididymis in a stepwise fashion, and for humans takes approximately 64 days (Heller and Clermont 1963). Starting at puberty, it usually continues uninterrupted until death, although a slight decrease can be discerned in the quantity of the sperm produced with increase in age. The entire process can be broken up into several distinct stages, i.e. spermatocytogenesis, spermatidogenesis, and spermiogenesis. The initial stages occur within the testes and progress to the epididymis, where the developing gametes mature and are stored until ejaculation. The seminiferous tubules of the testes are the starting point for the process, where stem cells adjacent to the inner tubule wall divide in a centripetal direction to produce immature sperm. Maturation occurs in the epididymis and involves the acquisition of a tail and hence motility.

In spermatocytogenesis, a diploid spermatogonium in the basal compartment of seminiferous tubules divides mitotically to produce two diploid intermediate cells called primary spermatocytes.

On the basis of the appearance of the nuclei, three functionally separate spermatogonial cell types are recognized: type A dark spermatogonia, type A pale spermatogonia, and type B spermatogonia. The population of spermatogonia is maintained by type A dark spermatogonia, which do not directly participate in producing sperm and simply ensure a supply of stem cells. Type A pale spermatogonia repeatedly divide mitotically to produce identical cell clones. When repeated division ceases, the cells differentiate into type B spermatogonia. This stage is referred to as the spermatogonial phase. Type B spermatogonia undergo mitosis to produce diploid primary spermatocytes.

Each primary spermatocyte then moves into the adluminal compartment of the seminiferous tubules, duplicates its DNA and subsequently undergoes meiosis I to produce two secondary spermatocytes. This division implicates sources of genetic variation, such as random inclusion of either parental chromosome, and chromosomal crossover, to increase the genetic variability of the gamete.

Secondary spermatocytes rapidly enter meiosis II and divide to produce haploid spermatids in spermatidogenesis . Owing to the brevity of this stage, secondary spermatocytes are rarely seen in histological preparations. Each cell division from a spermatogonium to a spermatid is incomplete; the cells remain connected to one another by bridges of cytoplasm to allow synchronous development.

During spermiogenesis, the spermatids begin to grow a tail, and develop a thickened mid-piece, where the mitochondria gather and form an axoneme. Spermatid DNA also undergoes packaging, becoming highly condensed. The DNA is packaged firstly with specific nuclear basic proteins, which are subsequently replaced with protamines during spermatid elongation. The resultant tightly packed chromatin is transcriptionally inactive. The Golgi apparatus surrounds the now condensed nucleus, becoming the acrosome. One of the centrioles of the cell elongates to become the tail of the sperm. Maturation then takes place under the influence of testosterone, which is involved in the removal of the remaining unnecessary cytoplasm and organelles. The excess cytoplasm, known as residual bodies, is phagocytosed by surrounding Sertoli cells in the testes. The resulting spermatozoa are now mature but lack motility, rendering them sterile. The mature spermatozoa are released from the protective Sertoli cells into the lumen of the seminiferous tubule in a process called spermiation. The non-motile spermatozoa are transported to the epididymis in testicular fluid secreted by the Sertoli cells by peristaltic contraction. While residing in the epididymis, they acquire motility and become capable of fertilization. However, transport of the mature spermatozoa through the remainder of the male reproductive system is achieved via muscle contraction rather than the spermatozoon's recently acquired motility. During spermatogenesis, Sertoli cells support the developing gamete by maintaining the environment necessary for development and maturation via the blood–testis barrier, secreting the substances initiating meiosis, the androgen-binding protein, and inhibin, by phagocytosing residual cytoplasm left over from spermiogenesis (Fig. 1).

1.1.1 Methylxanthines and Spermatogenesis

Studies on the teratogenic or sperm-injuring potential of methylxanthine started nearly 40 years ago by investigating the effects of caffeine on spermatogenesis (Ax et al. 1976). It is to note that all animal studies have demonstrated that, depending on the method of administration and the species, the developmental no-observed-effect level (NOEL) is approximately 30 mg/kg per day, the teratogenic NOEL is 100 mg/kg per day, and the reproductive NOEL approximately 80–120 mg/kg per day (Christian and Brent 2001). Roosters, fed 0.1% caffeine mixed by weight into a standard ration (about 145 mg/day), after 14 days of treatment showed a significant decrease in fertility. Semen output and sperm concentration were markedly reduced after 17–21 days of treatment, and no semen could be collected from the roosters after a 30-day treatment. Testicular histological investigation showed interruption of spermatocyte divisions and abnormal spermiogenesis, but removal of dietary

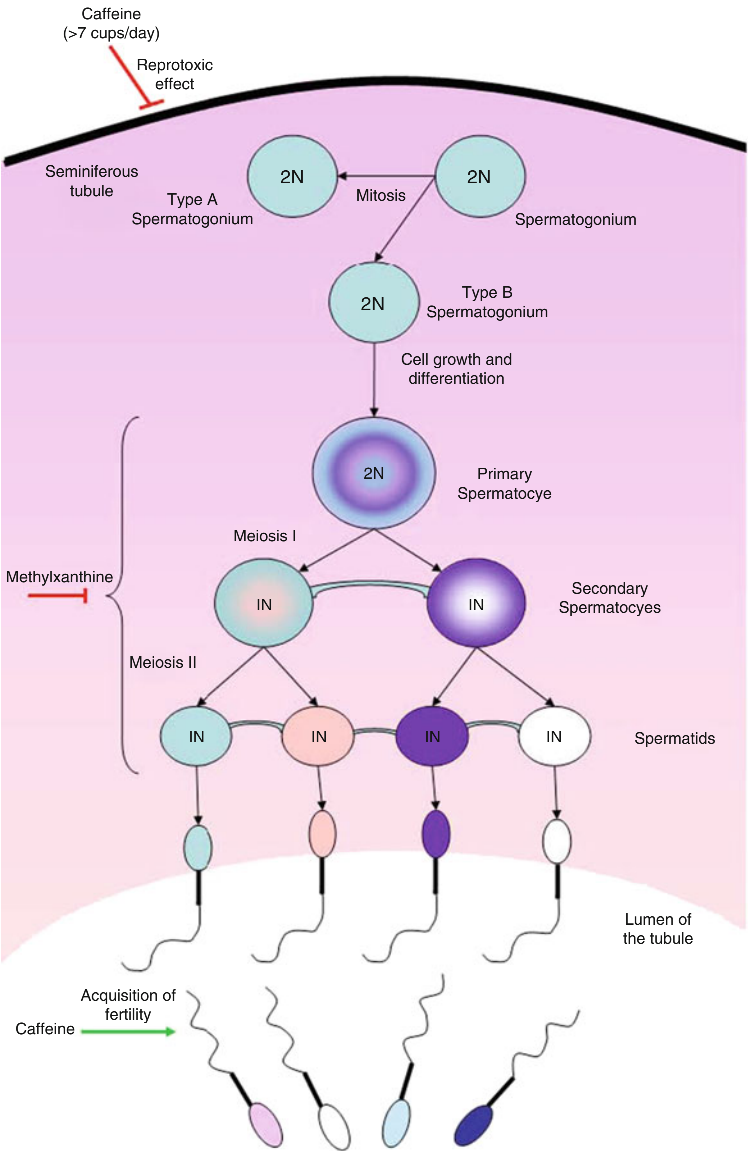

Fig. 1 Spermatogenesis and site of action of methylxanthines

caffeine resulted in resumption of semen production and a return of fertility to the control level. In rats fed caffeine, theobromine, or theophylline at a very high dietary level of 0.5% by weight into a standard ration ($LD_{50} = 200$ mg/kg, Fredholm et al. 1999) for periods ranging from 14 to 75 weeks, a significant positive finding was the occurrence of severe bilateral testicular atrophy with aspermatogenesis or oligospermatogenesis in 85–100% of the rats (Weinberger et al. 1978;

Friedman et al. 1979). The relative testicular toxicity of the methylxanthines was reported as caffeine being the most potent, theobromine slightly less potent, and theophylline considerably less potent. Somewhat variable atrophic changes of the accessory sexual organs (epididymis, prostate, and seminal vesicles) accompanied the testicular changes. Cytogenetic analysis of testes from caffeine- or theophylline-fed rats revealed a significantly reduced number of mitotic cells in the caffeine-treated group. Plasma testosterone concentrations were significantly elevated in the theobromine group and were elevated in the caffeine-treated group; this correlated morphologically with an apparent hyperplasia of interstitial cells in severely atrophied testes in these groups. Plasma cholesterol concentrations were significantly increased in the caffeine and theobromine groups (Gans 1982; Ettlin et al. 1986; Ezzat and El-Gohary 1994; Funabashi et al. 2000). Studies of the toxicities of theobromine and cocoa extract on the reproductive tract of male rats showed that theobromine and high-dose cocoa extract caused vacuolation within the Sertoli cell, abnormally shaped spermatids, and failed release of late spermatids in treated animals. However, the frequencies of some parameters of testis alterations were significantly lower in the high-dose cocoa-extract-treated group compared with the theobromine-treated group, demonstrating the ability of a cocoa extract containing theobromine to alter testis structure in a similar pattern but with reduced intensity compared with that observed after oral exposure to pure theobromine (Wang et al. 1992; Wang and Waller 1994).

The effects of caffeine at a concentration of 0.5% and fed to male rats for 7 weeks were compared with those of 0.8% dietary theobromine. Both dietary methylated xanthines produced significant decreases in food consumption and body-weight gain in rats when compared with their respective control groups. The theobromine-fed rats showed severe testicular atrophy with extensive spermatogenic cell degeneration and necrosis, while the testes of rats fed caffeine showed only scattered vacuolar degeneration of spermatogenic cells. Caffeine appeared to be more potent than theobromine as an anorexic agent in rats, but to be equivalent to theobromine in its potential for inducing thymic atrophy and spermatogenic cell destruction with testicular atrophy (Gans 1984). Long-term intake of caffeine caused suppression of spermatogenesis mainly through inhibition of the release of follicle-stimulating hormone (FSH). Daily administration of caffeine (30 or 60 mg/kg) to mature male rabbits for four consecutive weeks caused an increase in the plasma FSH level and a decrease in the luteinizing hormone (LH) level. A light microscope study revealed reduced size of the seminiferous tubules, inhibition of spermatogenesis, fatty degeneration of the liver, and hepatic lesions, whereas the adrenal glands exhibited signs of stimulated steroidogenesis (Ezzat and El-Gohary 1994). Caffeine, when administered to the rat (30 mg/kg/day) during pregnancy, affected certain aspects of normal sexual differentiation of the fetal gonads (Pollard et al. 1990). In the male fetus, caffeine significantly inhibited differentiation of the interstitial tissue and Leydig cells, with a significant consequent reduction in testosterone biosynthesis in the fetal testes. Caffeine also had an effect on the earlier morphogenic organization of the seminiferous cords. In the female fetus, caffeine did not modify ovarian differentiation nor the morphology of the ovaries, tissue arrangement, and overall appearance. These results indicating that caffeine, when administered during pregnancy, significantly inhibited the differentiation of the seminiferous cords and subsequent Leydig cell development in the interstitium, prompted the investigation of whether the observed effects were caused either by direct effects of caffeine or by intermediary secondary toxic effects of metabolites, i.e. theophylline and theobromine. Explants of 13-day-old fetal testis were cultured for 4 days in vitro in the presence of graded doses of caffeine, theophylline, or theobromine. Fetal testes exposed to caffeine or theobromine differentiated normally, developing seminiferous cords made up of Sertoli and germ cells, soon followed by the differentiation of functionally active Leydig cells appearing in the newly formed interstitium. In contrast, explants exposed to theophylline failed to develop seminiferous cords and, as a consequence, Leydig cells (Pollard et al. 2001). Recently, theophylline was shown to induce infertility by causing germ cell apoptosis in the testicular seminiferous epithelium. Theophylline exposure altered the expression of the genes within the ubiquitin–proteasome pathway (UPP), implicated in spermatogenesis and epididymal sperm quality control (Tengowski et al. 2007). Results suggest that the reprotoxic exposure alters the tissue-specific expression of UPP genes in the testis and epididymis, which may contribute to the aberrant spermatogenesis and epididymal processing of both normal and defective spermatozoa. Moreover, theophylline induced infertility by incapacitating the nurturing Sertoli cells, thus resulting in the premature release of late-differentiating spermatogenic cells, round spermatids (Weinberger et al. 1978). This leads to the depletion of spermatids and mature spermatozoa from the adluminal compartment of the seminiferous epithelium, ultimately causing testicular atrophy (Strandgaard and Miller 1998;Tengowski et al. 2005).

Other authors have suggest a beneficial effect of caffeine in the regulation of male gamete maturation since, acting as an agonist of ryanodine receptors, which induce release of Ca^{2+} from intracellular stores in spermatogonia, pachytene spermatocytes, and round spermatids, caffeine modulates calcium mobilization and plays a fundamental role in spermatozoa maturation (Chiarella et al. 2004). Conflicting results still seem to characterize the association between male caffeine consumption in adult life and semen quality, whereas the association between prenatal coffee consumption and semen quality and levels of reproductive hormones seems to be responsible for a small to moderate effect on semen volume and the levels of reproductive hormones (Ramlau-Hansen et al. 2008) .

1.2 Acquisition of Sperm Fertility

Mammalian spermatozoa emerging from the male reproductive tract are incapable of fertilizing eggs. They acquire this ability either during transit in the female reproductive tract (Yanagimachi 1994) or during incubation in suitable in vitro media (Allegrucci et al. 2001). Such conditioning, called capacitation, renders the spermatozoa capable interacting with the oocyte and thereby inducing the acrosome reaction (AR). Capacitation and AR are related to many effectors and signal transduction pathways, but the molecular basis of these processes is still to be fully elucidated (Kopf and Wilde 1990; Florman et al. 1992; de Lamirande et al. 1997; Tulsiani et al. 1998, 2007; Thundathil et al. 2002; Morales et al. 2007; Salicioni et al. 2007; Aitken et al. 2007; Wassarman 2009; Abou-haila and Tulsiani 2009). Sperm capacitation is a multistep process that involves several biochemical and ultrastructural changes in the sperm membrane, ranging from modification of membrane lipid composition to an increased permeability to ions. The efflux of membrane cholesterol leads to bovine sperm capacitation (Visconti et al. 1999). Albumin, high-density lipoproteins, and follicular and oviductal lipoproteins are capacitation effectors of human and bovine spermatozoa (Moreau et al. 1998; Thérien et al. 2001). Capacitation is correlated with an increase of protein tyrosine phosphorylation (Visconti et al. 1995a, b, 1999; Aitken et al. 1998) and the signal transduction pathway leading to protein tyrosine phosphorylation is thought to be central to either the attainment of the capacitative state (Visconti and Kopf 1998) or the concomitant expression of hyperactivated motility (Mahony and Gwathmey 1999; Si and Okuno 1999). As capacitation proceeds, several proteins undergo serine/threonine phosphorylation or threonine/tyrosine double phosphorylation (Thundathil et al. 2002). The AR is an exocytotic process by which lytic enzymes are released from the sperm acrosome and digest the zona pellucida so that spermatozoa can reach and fertilize the oocyte (Yanagimachi 1994; de Lamirande et al. 1997). Sperm AR occurs within minutes, cannot be reversed once it is induced, and can be triggered in vitro by different inducers, such as zona pellucida (Yanagimachi 1994), progesterone (Harrison et al. 2000), calcium ionophores, lysophosphatidylcholine (de Lamirande et al. 1997), follicular fluid (De Jonge et al. 1993), and ATP (Luria et al. 2002). Sperm AR takes place after fusion between the acrosome and the overlying plasma membrane and involves calcium influx, actin polymerization, a rise in intracellular pH, and protein activation (phospholipases, kinases, G proteins, etc.) (Yanagimachi 1994; Baldi et al. 2000; Liguori et al. 2005; Abou-Haila and Tulsiani 2009).

1.2.1 Methylxanthines and Acquisition of Sperm Fertility

The greatest part of the data in the literature reports the use xanthines/methylxanthines as phosphodiesterase (PDE) inhibitors that maintain intracellular levels of cyclic AMP (cAMP), thereby acting as motility-enhancing agents or capacitating effectors (Hong et al. 1981; Jiang et al. 1984; Depeiges and Dacheux 1985; Galantino-Homer et al. 1997; Leclerc et al. 1998; Jaiswal and Majumder 1998; Harayama et al. 1998; Leclerc and Goupil 2002; Buffone et al. 2005; Lachance et al. 2007; Yeste et al. 2008).

In a study finalized to clarify the role of the adenosine A_1 receptor in the acquisition of fertilizing capacity, caffeine was used as an adenosine A_1 receptor antagonist at low concentrations that binds to and inhibits half of the adenosine receptors (Fredholm et al, 1999). This dose of caffeine had very little, if any, effect

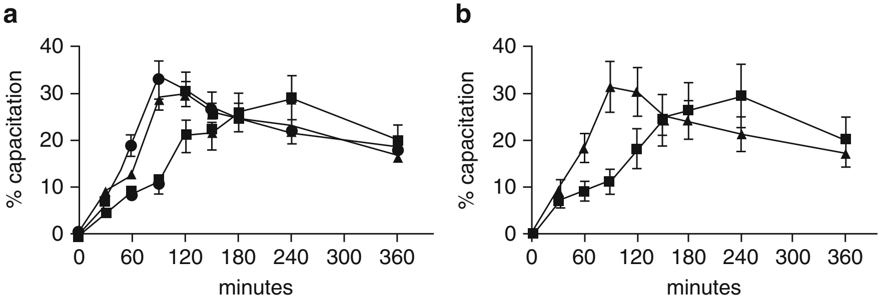

Fig. 2 **a** Percentage of capacitated cells as function of incubation time in $A_1R+/+$ (*filled circle*), $A_1R+/-$ (*filled triangle*), and $A_1R-/-$ (*filled square*) mouse spermatozoa (*filled square*). **b** Percentage of capacitated cells as function of incubation time of $A_1R+/+$ murine sperm in the presence of 15 μM caffeine (*filled triangle*) and 100 μM caffeine. (Adapted from Minelli et al. 2004)

on the acquisition of the capacitated status, whereas a very high dose of caffeine caused a significant reduction in sperm capacitation (Minelli et al. 2004) (Fig. 2). However, it is of note that high concentrations of caffeine are unlikely to be reached by caffeine consumers since strong side effects would preclude ingestion of this amount. Hence, the data suggest that regular caffeine consumption is unlikely to significantly affect spermatozoa function, thereby reassuring all coffee drinkers of the lack of negative effects of caffeine on male fertility. The scientific literature dealing with the AR contains references to the use of methylxanthines as PDE inhibitors (Kopf et al. 1983a, b; Carr and Acott 1990; Ain et al. 1999; Lachance et al. 2007) that maintain intracellular cAMP levels and induce the AR.

2 The Female Reproductive Tract

The female reproductive tract is a dynamic system cycling under the control of the key ovarian steroid hormones oestrogen and progesterone. It contains the uterus, which act as the receptacle for male sperm, and the ovaries, which produce the egg cells. The Fallopian tubes attach the uterus to the ovaries, which, at certain intervals, release an ovum, which passes through the Fallopian tube into the uterus. If, in this transit, the ovum meets with sperm, the sperm penetrate and merge with the egg, fertilizing it. The diploid zygote then implants itself in the wall of the uterus, where it begins the processes of embryogenesis and morphogenesis.

2.1 Oocyte Maturation

Despite the universal requirement of a haploid gamete for sexual reproduction, meiosis is regulated differently in oocytes and spermatocytes. As shown in Fig. 1, spermatocytes proceed through the meiotic divisions uninterrupted, whereas in female mammals, meiosis occurs over a prolonged period of time (Eppig et al. 2004). Mammalian oocytes are engaged in a complex meiotic cell division, characterized by several "stops and starts", and after resuming meiosis, they rely on maternal factors to sustain the subsequent developmental steps until the maternal-to-zygotic transition occurs .The process by which the oocyte completes the first meiotic division and undergoes other cytoplasmic changes and progresses to metaphase II is called oocyte maturation (Fig. 3). Because the mature, fertilizable oocyte

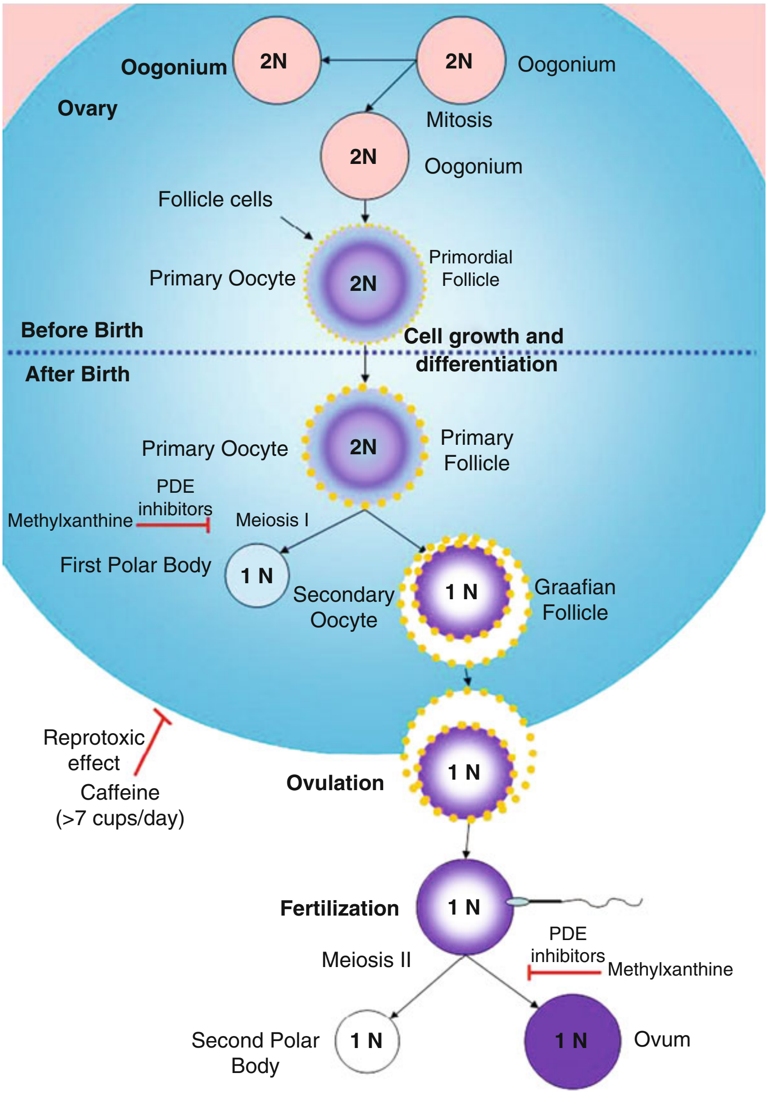

Fig. 3 Oogenesis and site of action of methylxanthines

has a relatively short lifespan in the female reproductive tract, the timing of oocyte meiotic arrest, as well as maturation, is tightly regulated (Mehlmann 2005). The functional unit within the ovary is the follicle, formed during embryonic development, and it comprises one or more layers of granulosa cells surrounding the oocyte (Gougeon 1996; Zeleznik 2004). During follicular growth, the somatic cells divide to form several layers, the oocyte enlarges, and a fluid-filled antrum begins to form. Some follicles at the early antral stage are "recruited" to continue growing; this growth is dependent on the pituitary gonadotropin FSH (Gougeon 1996; Zeleznik 2004). During this phase, the antrum divides the granulosa cells into two separate compartments: mural granulosa cells form the outer layers, while the cumulus cells surround the oocyte. The oocyte grows to its full size (about 75-μm diameter in the mouse, about 100 μm in the human), but remains arrested in prophase I. If an oocyte is removed from an antral follicle, it spontaneously resumes meiosis and progresses to second metaphase (Pincus and Enzmann 1935). This indicates that the follicle cells hold the oocyte in prophase arrest. Meiosis resumes in response to a surge of LH from the pituitary gland during the oestrous or menstrual cycle, shortly before ovulation. LH receptors (LHRs) are located on the mural granulosa cells but not on the cumulus cells or the oocyte (Peng et al. 1991; Richards et al. 2002), so the mechanism(s) by which LH stimulates oocyte maturation is indirect. Prior to the midcycle surge of LH, the growing oocyte acquires the ability to undergo oocyte maturation. The acquisition of meiotic competence occurs around the time of antrum formation (Mehlmann et al. 2004) and corresponds to a point at which the oocyte achieves a threshold level of maturation-promoting proteins, such as cyclin-dependant kinase (Cdk1) and cyclin B (Kanatsu-Shinohara et al. 2000). Meiotic arrest is regulated by cAMP levels within the oocyte (Conti et al. 2002; Eppig et al. 2004) since the cAMP level affects the activity of the Cdk/cyclin B protein complex, also known as maturation/meiosis or mitosis promoting factor (MPF). High cAMP levels result in the phosphorylation of Cdk1 on Thr14 and Tyr15, rendering it inactive (Duckworth et al. 2002), while a decrease in cAMP levels leads to the dephosphorylation of Cdk1 on Thr14 and Tyr15, the MPF complex becomes active, and the oocyte can re-enter meiosis. A hypothesis for how high levels of cAMP are maintained in competent, fully grown oocytes is that the oocyte produces its own cAMP through a G-protein-linked receptor in the oocyte plasma membrane that stimulates G_s and, subsequently, adenylyl cyclase (AC). Direct evidence for an essential role of G_s in the maintenance of meiotic arrest was obtained by microinjecting either a function-blocking antibody or a dominant negative form of the α-subunit of G_s into follicle-enclosed oocytes (Mehlmann et al. 2002; Kalinowski et al. 2003). This pathway was confirmed by the finding that oocytes from mice lacking the AC3 AC isoform, which is present in the oocyte, spontaneously undergo germinal vesicle breakdown within ovarian follicles (Horner et al. 2003).

Recently, it was shown that heat shock transcription factor 1, which triggers the transcription of several genes encoding heat shock proteins, is highly expressed in oocytes and plays an important role in normal progression of meiosis by directly regulating Hsp90α expression (Metchat et al. 2009).

2.1.1 Methylxanthines and Oocyte Maturation

As for male gametes, the largest part of the data in the literature reports the use xanthines/methylxanthines as PDE inhibitors that maintain the intracellular levels of cAMP responsible for the meiotic arrest. Several groups (Cho et al. 1974; Dekel and Beers 1978; Schultz et al. 1983a, b; Vivarelli et al. 1983; Bornslaeger and Schultz 1985; Törnell et al. 1990; Haider and Chaube 1996; Webb et al. 2000; Conti et al. 2002) showed that spontaneous maturation of oocytes isolated from their follicles can be prevented by including membrane-permeant cAMP analogues or cAMP PDE inhibitors, such as hypoxanthine and 3-isobutyl-1-methylxanthine (IBMX), in the culture medium. The cAMP levels decrease in oocytes following their removal from their follicles as well as in isolated oocytes after removal of IBMX. The decrease in the level of oocyte cAMP occurs within 2 h after washing out IBMX, a time during which the oocyte becomes committed to resuming meiosis and cAMP levels increase in isolated oocytes . When transported into oocytes from the cumulus cells via gap junctions, cAMP also plays an important role in the regulation of meiotic progression beyond the meiosis I (MI) stage (Shimada et al. 2002). Using selective PDE inhibitors, such as milrinone (a PDE3 inhibitor), cilostamide (a PDE3 inhibitor), and rolipram (a PDE4 inhibitor), studies focused on the differential regulation of cAMP levels within the oocyte and somatic (cumulus) cell compartments of the follicle showed that specific PDE subtypes are differentially localized within the two compartments of the follicle, i.e. the type 3 PDE in the oocyte and the type 4 PDE in the granulosa cells (Thomas et al. 2002). Moreover, oocyte cAMP levels are primarily regulated in oocytes by its degradation by PDE, whereas granulosa cell cAMP levels are controlled mainly by active AC, with both sources able to participate in oocyte meiotic regulation. IBMX does not interfere with the expression of LHR in cumulus cells surrounding oocytes, whereas the binding of LH to its receptor induces a further increase in cAMP level, progesterone production, and acceleration of meiotic progression to the metaphase I stage. The role of cAMP in the oocyte meiotic arrest was further supported by Laforest et al. (2005), who showed a fundamental significance of cAMP pathways in controlling meiotic resumption in porcine oocytes. This control is at two levels, the ability to synthesize cAMP via active AC, where the cyclase of porcine oocyte is sensitive to forskolin, and the degradation of cAMP via cilostamide-sensitive PDE. A more detailed study of the effects of IBMX on the oocyte meiotic block (Barretto et al. 2007) showed that IBMX is able to prevent resumption of meiosis by maintaining elevated cAMP concentrations in the oocyte, whereas roscovitine, a purine known to specifically inhibit MPF kinase activity, maintains bovine oocytes at the germinal vesicle stage, indicating that the meiotic inhibitors delay the progression of nuclear maturation without affecting cytoplasmic maturation. It was proposed that the inhibitory cAMP is synthesized within oocytes via a stimulatory α-subunit of G protein. After the presence of G_s-α molecules in porcine oocytes had been shown, an anti-G_s-α antibody was injected into porcine immature oocytes and this inhibition of ooplasmic G_s-α functions significantly promoted germinal vesicle breakdown of the oocytes, whose spontaneous meiotic resumption was prevented by IBMX treatment. Moreover, although cyclin B synthesis and

MPF activation were largely prevented until 30 h of culture in IBMX-treated oocytes, injection of anti-G_s-α antibody into these oocytes partially recovered cyclin B synthesis and activated MPF activity at 30 h, suggesting that meiotic resumption of porcine oocytes is prevented by ooplasmic G_s-α, which may stimulate cAMP synthesis within porcine oocytes, and that synthesized cAMP prevents meiotic resumption of oocytes through the signalling pathways involved in MPF activation (Morikawa et al. 2007) . More recently, Ozawa et al. (2008) focused their attention on cAMP content, gap-junctional communication status, and LHR expression in porcine cumulus–oocyte complexes treated with IBMX or with FSH. They found that the inhibition of PDEs in porcine cumulus–oocyte complexes makes the oocyte ready for release from meiotic arrest, whereas the maintenance of a moderate cAMP content may prolong gap-junctional communications and stimulate LHR expression. A recent paper (Pirino et al. 2009) showed that meiotic resumption requires activation of the MPF. Protein kinase A (PKA) activity sustains the prophase arrest by inhibiting Cdk1. Therefore, the inhibition of the activity of the Cdc25 protein required for MPF activation results in mitotic arrest. Phosphorylation of a highly conserved serine 321 residue of Cdc25B 21 plays a key role in the negative regulation and localization of Cdc25B during prophase arrest, suggesting that Cdc25B is a direct target of PKA.

2.2 Caffeine and Female Fertility

Caffeine is a subject of interest among consumers and health professionals because it is widely consumed in the diet by most segments of the population and can exert several pharmacological effects (Dews 1982; Fredholm 1995; Christian and Brent 2001; Mandel 2002; Derbyshire and Abdula 2008; Yu et al. 2009).

The medical literature contains many varied references indicating that human adverse reproductive/developmental effects are produced by caffeine. Although it is difficult to compare doses of caffeine in animals and humans, the medical literature dealing with developmental and reproductive risks of caffeine underwent a thorough revision by evaluating the biological plausibility of the epidemiological and animal findings. When comparing effects among different species, one can only accomplish dose equivalence be by considering the results of pharmacokinetics studies, metabolic studies, and dose-response investigations in the human and the species being studied. Moreover, the importance of dose within a species is of fundamental concern when determining developmental risks since most drugs/chemicals are potentially associated with developmental toxicity/teratogenicity only at some exposure level. The genetic constitution of an organism, i.e. both the maternal and the fetal genotypes, is also an important factor in the susceptibility of a species. Indeed, more than 30 disorders of increased sensitivity to drug toxicity or effects have been reported in the human owing to an inherited trait (McKusick 1988). Unlike human epidemiology studies, which are difficult to control and with multiple inherent flaws that prevent identification of causality, animal studies are conducted under conditions in which all the variables can be better controlled.

In addition, current non-clinical studies generally include identification of achieved blood levels/exposures in the maternal animal and the developing offspring, a critical factor because the severity of the effect is related to the ability of the conceptus to recover from the insult (Johnson and Christian 1984). Nevertheless, results of non-clinical animal studies provide excellent tools for predicting potential effects of caffeine on human reproduction and development. Indeed the LD_{50} of caffeine is fairly consistent across species, including *Homo sapiens* (Dews, 1982). The plasma level resulting from 1.1 mg/kg caffeine (a single cup of coffee containing 80 mg of caffeine ingested by a 70-kg human) ranges from 0.5 to 1.5 mg/L. A similar dose–concentration relationship is found in many species, including rodents and primates (Hirsh 1984). It is generally assumed that 10 mg/kg in a rat represents about 250 mg of caffeine in a human weighing 70 kg (3.5 mg/kg), and that this would correspond to about two to three cups of coffee.

Nawrot et al. (2003) reviewed the effects of caffeine on human health and concluded that for the healthy adult population, moderate daily caffeine consumption at levels up to 400–450 mg/day (5.7–6.4 mg/kg/day in a 70-kg adult, equivalent to four to five cups per day) was not associated with adverse effects, which include general toxicity, effects on bone status and calcium balance, cardiovascular effects, behavioural changes, increased incidence of cancer, and effects on male fertility. However, the authors also reported that children and women of reproductive age were “at risk” subgroups who might require dietary advice to moderate their caffeine intake. High levels of caffeine intake may delay conception among fertile women (Bolúmar et al. 1997). The effects of caffeine consumption on delayed conception were evaluated in a European multicentre study on risk factors of infertility in a randomly selected sample of 3,187 women aged 25–44 years. A significantly increased odds ratio for subfecundity in the first pregnancy was observed for women drinking more than 500 mg of caffeine per day (more than six cups), the effect being relatively stronger in smokers than in non-smokers. Women with the highest level of consumption had an increase in the time leading to the first pregnancy. In addition, women whose caffeine consumption was high had less than a third of the risk for a long menses (8 days or more) compared with women who did not consume caffeine. Those whose caffeine consumption was high also had a doubled risk for a short cycle length (24 day or less); this association was also evident in those whose caffeine consumption was high but did not smoke. However, caffeine intake was not strongly related to an increased risk for anovulation, short luteal phase (10 days or less), long follicular phase (24 days or more), long cycle (36 days or more), or measures of within-woman cycle variability (Fenster et al. 1999). The mean birth weight was reduced by high reported caffeine consumption, but this small decrease in birth weight, observed for maternal caffeine consumption, is unlikely to be clinically important except for women consuming 600 mg of caffeine daily (more than 7.2 cups) (Bracken et al. 2003). More recently, in a study finalized to determine whether smoking, alcohol, and caffeine may be related to the four indicators of ovarian age, i.e. antral follicle count, FSH, inhibin B, and oestradiol, and therefore to fecundability and fertility (Kinney et al. 2007), 188 women, aged 22–49, were investigated and least-squares was regression used to estimate differences in antral follicle count and hormone levels for women who smoke cigarettes or who drink alcohol or caffeine. Current smoking is related to elevated FSH levels, but not to the antral follicle count, inhibin B, or oestradiol. Neither alcohol nor caffeine was found to be related to any ovarian age indicator, suggesting that caffeine, at the dosage of 156 mg/day (1.9 cups), does not affect ovarian age indicators. On the basis of data from a large retrospective epidemiology study and from a large retrospective case-control study in humans, it appears that use of caffeine does not impair ovulation to the point of decreasing fertility and during pregnancy has little, if any, effect on the outcome of pregnancy. Nevertheless, although caffeine use during pregnancy does not appear to be associated with substantial risk and the association between soft drinks and ovulatory disorder infertility does not seem to be attributable to their caffeine content, most clinicians recommend that pregnant women limit their consumption of foods, beverages, and drugs containing caffeine, since caffeine crosses the placenta (Care Study Group 2008; Chavarro et al. 2009). Recent research (Björklund et al. 2008) has confirmed the concerns about caffeine consumption during pregnancy or the early postnatal period because there may be long-lasting behavioural changes after caffeine exposure early in life. Indeed, pregnant wild-type mice, given modest doses of caffeine (0.3 g/L in drinking water), gave birth to offspring that as adults exhibited increased locomotor activity in an open field. The offspring also responded to cocaine challenge with greater locomotor activity than mice not perinatally exposed to caffeine. The same behavioural experiments on mice heterozygous for adenosine A_1 receptor gene, where signalling via adenosine A_1 receptors is reduced to about the same degree as after modest consumption of caffeine, showed a behavioural profile similar to that of wild-type mice perinatally exposed to caffeine. It appeared that the mother's genotype was critical for behavioural changes in adult offspring, suggesting that perinatal caffeine, by acting on adenosine A_1 receptors in the

Table 1 Effects of male genotype

Parameter	A_1R +/+	A_1R +/−	A_1R −/−
In vivo fertility[a]			
Average number of pups	8 ± 2.1	8 ± 1.9	5 ± 1.7
Birthweight (g)	2.07 ± 0.8	2.05 ± 0.6	2.08 ± 0.4
Days between litters	45 ± 8	47 ± 8	53 ± 15
Reproductive parameters[b]			
Number of spermatozoa	$13 \times 10^6 \pm 2 \times 10^6$	$11 \times 10^6 \pm 3 \times 10^6$	$12 \times 10^6 \pm 1 \times 10^6$
Viability (%)	80 ± 10	76 ± 7	77 ± 9
Motility (%)	75 ± 7	72 ± 9	71 ± 11
Phenotype[c]			
Weight of adult animal (g)	33 ± 5	31 ± 7	32 ± 4
Weight of testis (mg)	132 ± 21	121 ± 15	122 ± 18

Adapted from Minelli et al. (2004)

[a] The values are the means ± the standard error of the mean (SEM) of 40 litters.

[b] The values are the means ± SEM of 20 male mice, $P < 0.05$.

[c] The values are the means ± SEM of 20 male mice, $P < 0.05$.

mother, causes long-lasting behavioural changes in the offspring that even manifest themselves in the second generation. Mice homozygous for genetic deletion of the adenosine A_1 receptor showed a reduction in number of offspring and increased time between litters (Table 1) (Minelli et al. 2004). Interestingly, caffeine and some of its derivatives present in coffee leaves affect egg-laying by the coffee leaf miner *Leucoptera coffeella*, one of the main coffee pests in the Neotropical region. In fact, increased leaf levels of caffeine favour egg-laying by the coffee leaf miner with a significant concentration–response relationship, providing support for the hypothesis that caffeine stimulates egg-laying by the coffee leaf miner in coffee leaves (Magalhães et al. 2008).

3 Assisted Reproductive Techniques

Since the birth of the first baby conceived with in vitro fertilization (IVF) and embryo transfer, assisted reproductive technology, an extremely successful form of therapy for many infertile couples, is currently practised all over the world.

3.1 Methylxanthines in Assisted Reproductive Techniques

The effects of AC, IBMX, and dibutyryl cAMP (dbcAMP) on porcine oocyte in vitro maturation, IVF, and subsequent embryonic development were investigated by Somfai et al. (2003). They showed that a change in the intracellular level of cAMP during oocyte collection does not affect the maturational and developmental competence of the oocytes and that synchronization of meiotic maturation using dbcAMP enhances the meiotic potential of oocytes by promoting the MI to metaphase II transition and results in high developmental competence by monospermic fertilization. In IVF experiments, IBMX in association with FSH and LH, was used to synchronize the oocytes. At 6 days after IVF, the blastocyst rate in oocytes matured under these conditions was significantly higher than that for oocytes cultured in the absence of LH. The results suggest that the treatment of oocytes with FSH and IBMX causes the expression of LHR in cumulus cells, holds the oocytes at the germinal vesicle II stage, and can be considered as a beneficial procedure to obtain in-vitro-matured oocytes with high developmental competence (Shimada et al. 2003a, b). Besides PKA, several protein kinases are involved in oocyte maturation, and studies of the mechanisms of protein kinase B (PKB) activation and its role in cumulus cells during in vitro meiotic resumption of oocytes showed that the addition of PDE inhibitors maintained the level of PKB activity in cumulus cells at levels comparable with those in cumulus cells just after collection from their follicles and that the inhibitory effect of hypoxanthine on spontaneous meiotic resumption was overcome by addition of a PKB inhibitor.

3.2 *Caffeine in Assisted Reproductive Techniques*

Recently, Maalouf et al. (2009) reported the effects of cumulus cell removal and caffeine treatment on the development of in-vitro-matured ovine oocytes. Whereas removal of cumulus cells and aging increases polyspermy, caffeine was effective in reducing this phenomenon, showing that caffeine treatment statistically increases the development to blastocyst and lowers the frequency of polyspermy.

Caffeine increases MPF and mitogen-activated protein kinase activities in ovine oocytes, prevents age-related changes, and increases cell numbers in blastocysts produced by somatic cell nuclear transfer (Lee and Campbell 2006, 2008). Used in experiments of nuclear remodelling of somatic cell nuclear transfer embryos and subsequent development and DNA methylation patterns, caffeine induces premature chromosome condensation at a high rate, a high blastocyst formation rate, and lowers the apoptotic cell index, suggesting that the nuclear remodelling type controlled by caffeine treatment can affect in vitro development and the methylation status of nuclear transfer in relation to nuclear reprogramming (Kwon et al. 2008). These results confirmed previous observations that showed that caffeine treatment promotes nuclear remodelling although it does not prevent the decrease in the developmental ability of cloned embryos caused by oocyte aging (Iwamoto et al. 2005; Kawahara et al. 2005). On the other hand, with use of a mouse model, it was shown that caffeine had no effect on the quality of oocytes matured in vivo, whereas it was detrimental to the quality of oocytes matured in vitro (Miao et al. 2007). However, in vivo studies with female mice administered 150 mg/kg caffeine at various times prior to metaphase I, showed non-significant differences in the frequencies of hyperploid, MI, diploid, premature centromere separation, single chromatids, and structural chromosome aberrations between the controls and each of the caffeine groups (Mailhes et al. 1996; Jaakma et al. 1997).

Studies of the effects of caffeine on male gametes showed stimulation of sperm capacitation and spontaneous AR (Funahashi 2003, 2005); therefore, during IVF procedures, supplementation with β-mercaptoethanol, which neutralizes the stimulatory effect of caffeine, has a beneficial effect in maintaining the function of gametes, the incidence of normal fertilization, and, consequently, the quality of IVF embryos. However, a limited exposure of gametes to caffeine significantly reduced the mean number of sperm cells that penetrated into the oocyte and asynchrony in the morphology of sperm nuclei in polyspermic oocytes (Funahashi and Romar 2004). Other epidemiology studies evaluated the timing and amount of caffeine intake by women and men undergoing IVF and gamete intra-Fallopian transfer (GIFT) on oocyte retrieval, sperm parameters, fertilization, multiple gestations, miscarriage, and live births. A prospective study of 221 couples was conducted between 1993 and 1998. "Usual" caffeine intake during the lifetime and 1 year prior to the study, caffeine intake during the week of the initial clinic visit, as well as caffeine intake during the week of the procedure were evaluated for beverages (coffee, soda, and tea) and chocolates. Not achieving a live birth was significantly associated with "usual" female caffeine consumption for an intake of more than 50 mg/day and consumption of 0–2 mg/day during the week of the initial visit. Infant gestational age decreased by 3.8 or 3.5 weeks for women who consumed more than 50 mg/day of caffeine "usually" or during the week of the initial visit. The odds of having multiple gestations increased by 2.2 and 3.0 for men who increased their "usual" intake or intake during the week of the initial visit by an extra 100 mg/day. Caffeine intake was not significantly associated with other outcomes. This was the first IVF/GIFT study to report any effect of caffeine on live births, gestational age, and multiple gestations and if these findings are replicated, caffeine use should be minimized prior to and while undergoing IVF/GIFT (Klonoff-Cohen et al. 2002).

4 Conclusion

Reports on methylxanthines and their effects on reproduction have mainly focused on their use as in vitro PDE inhibitors that, by maintaining high intracellular levels of cAMP, differently affect female and male gametes. Animal studies have largely shown that methylxanthines have toxic effects on gonads and gametogenesis of both sexes, although comparing doses of caffeine in animals and humans is for many reasons not an easy task. Caffeine is present in many beverages (coffee, tea, colas, and chocolate) and in over-the-counter medications. The medical literature contains many varied references that appear to indicate that human adverse reproductive/developmental effects are produced by caffeine. However, if caffeine causes such effects, the reproductive consequences could be very serious because caffeine-containing foods and beverages are consumed by most of the human populations of the world, and, as world "coffee culture" continues to grow, world caffeine intakes continue to increase. After revising the medical literature dealing with developmental and reproductive risks of caffeine on the basis of the biological plausibility of the epidemiological and animal findings and the methods and conclusions of previous investigators, clinical counsellors can inform prepregnant/pregnant women who do not smoke or drink alcohol and who consume moderate amounts of caffeine (5–6 mg/kg per day, five cups) that they do not have an increase in reproductive risks or adverse effects.

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Methylxanthines During Pregnancy and Early Postnatal Life

Ulrika Ådén

Contents

Abstract World-wide, many fetuses and infants are exposed to methylxanthines via maternal consumption of coffee and other beverages containing these substances. Methylxanthines (caffeine, theophylline and aminophylline) are also commonly used as a medication for apnea of prematurity.

The metabolism of methylxanthines is impaired in pregnant women, fetuses and neonates, leading to accumulating levels thereof. Methylxanthines readily passes

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the placenta barrier and enters all tissues and thus may affect the fetus/newborn at any time during pregnancy or postnatal life, given that the effector systems are mature.

At clinically relevant doses, the major effector system for methylxanthines is adenosine receptors. Animal studies suggest that adenosine receptors in the cardiovascular, respiratory and immune system are developed at birth, but that cerebral adenosine receptors are not fully functional. Furthermore animal studies have shown protective positive effects of methylxanthines in situations of hypoxia/ ischemia in neonates. Similarly, a positive long-term effect on lung function and CNS development was found in human preterm infants treated with high doses of caffeine for apneas. There is now evidence that the overall benefits from methylxanthine therapy for apnea of prematurity outweigh potential short-term risks.

On the other hand it is important to note that experimental studies have indicated that long-term effects of caffeine during pregnancy and postnatally may include altered behavior and altered respiratory control in the offspring, although there is currently no human data to support this.

Some epidemiology studies have reported negative effects on pregnancy and perinatal outcomes related to maternal ingestion of high doses of caffeine, but the results are inconclusive. The evidence base for adverse effects of caffeine in first third of pregnancy are stronger than for later parts of pregnancy and there is currently insufficient evidence to advise women to restrict caffeine intake after the first trimester.

Keywords Caffeine · Fetus · Methylxanthines · Neonatal · Newborn · Pregnancy · Theophylline

1 Introduction

The majority of expecting mothers in the Western world drink beverages containing methylxanthines during pregnancy and they continue their consumption during lactation. Many fetuses and infants are thus exposed to these substances. In addition, a large number of premature infants need pharmacological treatment for apnea of prematurity with methylxanthines.

There has been considerable concern over fetuses and infants being subjected to methylxanthines, with reported negative effects on pregnancy, perinatal, and long-term outcomes.

This chapter discusses how fetuses and newborns are subjected to methylxanthines, how the effects are mediated, and what information the current literature provides on the long-term effects of exposure to methylxanthines at clinically relevant doses.

2 Mechanisms of Action in the Neonate. Effector Systems and Their Maturation

2.1 Mechanisms of Action at Clinically Relevant Concentrations

The only known effect of caffeine at concentrations relevant to daily intake of coffee is blockade of adenosine A_1 and A_{2A} receptors that occurs at serum concentrations of 0.2–2 mg/L (0.001–0.01 mM; Fredholm et al. 1999). When pregnant women drink beverages containing caffeine, serum levels of caffeine soon become similar in the fetus and in the mother. When preterm infants are treated with caffeine for apnea of prematurity, more than 10 times higher serum levels are reached and then, in addition to adenosine A_1 and A_{2A} receptor blockade, an inhibitory effect on adenosine A_3 receptors, and a minimal blockade of phosphodiesterases, $GABA_A$ receptors, and Ca^{++} release can occur. The maturation of these effector systems may affect the way the fetus and the infant react to caffeine (see below).

It is now less common that fetuses and infants are exposed to theophylline than caffeine, since theophylline has been replaced in modern asthma therapy and for treatment of apneas in preterm infants, and caffeine is usually preferred to theophylline/aminophylline (see below). Theophylline is structurally related to caffeine and is thus a potent inhibitor of adenosine A_1, A_{2A}, and to some extent A_3 receptors at therapeutically relevant doses. The degree of inhibition of phosphodiesterases is minimal at these concentrations.

Methylxanthines have both pro- and anti-inflammatory properties. At therapeutic doses, most effects result from adenosine receptor antagonism and the proinflammatory actions may be more relevant (Haskó and Cronstein 2010; Ohta and Sitkovsky 2010), at least in adults. However, a recent study in umbilical cord blood monocytes indicated that caffeine inhibited TNF-α production in neonates, possibly via adenosine A_1 receptors (Chavez-Valdez et al. 2009).

Thus, some of the immunomodulatory mechanisms of methylxanthines are functioning at an early developmental stage, whereas other effects do not mature until later in infancy.

2.2 Maturation of Adenosine and $GABA_A$ Receptor Systems

As mentioned, the major effect of the methylxanthines caffeine and theophylline/aminophylline at therapeutic doses is antagonism of adenosine A_1 and A_{2A} receptors. The maturation of these receptor systems affects how the fetus and child react to methylxanthines. Data on the development of adenosine receptors are obtained from mice and rats, but there are no available data from human fetuses or infants to our knowledge.

Adenosine A_1 receptors are mainly present in the heart (conductive system and myocytes), brain (predominantly in cortex, hippocampus, cerebellum, pons, and medulla oblongata) kidney, testis, and adipose tissue. Adenosine-A_1-receptor-knockout mice appear to develop normally (Johansson et al. 2001), indicating that this receptor has no main effect on fetal development, but recent studies have pointed out differences in heart rate, body temperature, and locomotion in adult life of these mice (Yang et al. 2007). Adenosine A_1 receptors are present in a small amount early in brain development (Rivkees 1995; Aden et al. 2000), but are not functionally coupled to G proteins in the brain until adolescence (2–3 weeks of age in a mouse) (Aden et al. 2001). An important exception is the medulla oblongata, where functional coupling to G proteins was confirmed in rats just before birth using guanylyl-5′-*O*-(γ-[^{35}S]thio)-triphosphate binding (Herlenius et al. 2002). To this end, it is known that adenosine and adenosine A_1 receptor agonists depress respiratory rhythmogenesis in vivo and in vitro (Lagercrantz et al. 1984; Eldridge et al. 1985). Accordingly, methylxanthines exert profound stimulatory effects on respiration in newborns by antagonism of adenosine A_1 receptors in the pons and medulla oblongata (Lagercrantz et al. 1984; Tilley 2010).

An early functional coupling of adenosine A_1 receptors to G proteins was also reported in the heart (Aden et al. 2001), which is in agreement with the clinical observation that tachycardia is a frequently seen side effect of methylxanthines in premature infants. There are data indicating that adenosine A_1 receptors function in umbilical cord blood monocytes (Chavez-Valdez et al. 2009) Adenosine A_{2A} receptors are present in the brain and (predominantly in the basal ganglia), at endothelial cells, platelets, and on inflammatory cells (neutrophils, platelets, macrophages/microglial cells, and T cells), where they exhibit important anti-inflammatory properties (Haskó and Cronstein 2010; Ohta and Sitkovsky 2010, 2001). The first targeted disruption of the adenosine A_{2A} receptor gene in mice was reported in 1997 (Ledent et al. 1997) and in agreement with previous pharmacology studies, these animals had increased blood pressure and platelet aggregation. There were no major malformations in adenosine-A_{2A}-receptor-knockout mice, indicating that the stimulation of this receptor is not necessary for normal fetal development. The major development of cerebral adenosine A_{2A} receptors takes place after birth, as pointed out by rat studies (Rivkees 1995; Aden et al. 2000). It is not known whether adenosine A_{2A} receptors on inflammatory cells are functional early in life.

Adenosine A_3 receptors have a low affinity for caffeine and are therefore not considered as its primary target when ingested in beverages. However, therapeutic levels of methylxanthines given as therapy for preterm apneas may reach concentrations where adenosine A_3 receptors are blocked. Adenosine A_3 receptors are very scarce in the brain, but can be detected at low levels in several regions in the adult rodent brain. The functionality of these receptors in neurons remains unclear. It is, however, now evident that functional adenosine A_3 receptors are present on microglial cells and astrocytes (Hammarberg et al. 2003; Abbracchio et al. 2001; see Haskó and Cronstein 2010), but developmental data are lacking. Recent data show that deletion of adenosine A_3 receptors (adenosine-A_3-receptor-knockout mice) during brain development leads to a reduced response to caffeine in adult life (Bjorklund et al. 2008a). Although the mechanisms have not been elucidated, a developmental role of adenosine A_3 receptors might therefore be indicated, in particular during circumstances when there is fetal exposure to caffeine.

$GABA_A$ receptors may be a target for high doses of methylxanthines, for example, when given postnatally for the treatment of apnea of prematurity. This receptor system develops in the brain early during fetal life (Aden et al. 2000), but whereas activation of $GABA_A$ receptors in mature neurons results in membrane hyperpolarization, $GABA_A$ receptor activation during early stages of brain development (up until the first postnatal week in rodents, corresponding to term age in a human baby) causes depolarization of the postsynaptic membrane. The GABA-mediated depolarization is thought to regulate neurogenesis, synaptogenesis, and final neuron number by regulating second-messenger systems (Varani et al. 2005) and modulating DNA synthesis (Leinekugel et al. 1997). Blockade of $GABA_A$ receptors with methylxanthines has, therefore, a theoretic potential to affect brain development. However, it is completely unknown whether the net effect on outcome would be positive or negative.

3 Exposure to Methylxanthines During Fetal Life

As mentioned, it is nowadays uncommon that fetuses are exposed to theophylline, but many fetuses are subjected to caffeine because the majority (75%) of expecting mothers drink beverages containing caffeine (Eskenazi 1999).

3.1 Metabolism of Methylxanthines in the Pregnant Woman and in the Fetus

Methylxanthines are rapidly and completely absorbed from the gastrointestinal tract. There is only a minimal first-pass effect and once absorbed, methylxanthines pass blood–brain and placenta barriers, entering all tissues (Arnaud 1987). The half life of caffeine in pregnant women varies between 2 and 4.5 h during the first trimester, which is similar to that in nonpregnant women, but increases to 10 h at 17 weeks gestation and up to 18 h in the end of pregnancy, leading to an accumulation of caffeine in the mother and the fetus (Aldridge et al. 1981).

The main enzyme controlling caffeine metabolism is cytochrome P450 1A2 (CYP1A2). There are interindividual differences due to polymorphisms of this enzyme (Grosso and Bracken 2005). Another enzyme that regulates the metabolism to a lesser extent is *N*-acetyltransferase (Fenster et al. 1998). The activities of both of these enzymes are reduced during pregnancy (Tsutsumi et al. 2001), resulting in gradually increasing plasma concentrations (to about twice the prepregnancy levels) of caffeine during pregnancy, despite little change in reported consumption (Cook et al. 1996). Both the fetus and the placenta lack the enzymes needed to metabolize methylxanthines (Kalow and Tang 1991) and therefore elimination in the fetus is almost entirely dependent upon renal excretion. The urinary excretion rate increases with dose in both mothers and fetuses.

3.2 Exposure to Methylxanthines in Fetal Life and Perinatal Outcomes

Since caffeine and its metabolites can pass the placenta barrier, maternal coffee consumption may affect the fetus at any time throughout pregnancy, given that the effector systems are mature (see earlier).

Some studies have raised concern about fetuses being exposed to caffeine during pregnancy, but others have failed to find any associations between maternal caffeine intake and adverse perinatal outcomes.

There is some evidence from animal studies that high doses of caffeine may result in malformations of the fetus, including cleft palate and cardiovascular malformations (for a review, see Nehlig and Debry 1994). Epidemiology studies, though, have not been able to detect significant risks for teratogenic effects of caffeine exposure in humans (Browne 2006). Some studies have shown that excessive maternal consumption of caffeine in humans (more than 300 mg/day) may be related to reduced fertility (Marie-Soleil and Graham 2010) and was associated with increased rate of spontaneous abortions (Godel et al. 1992; Klebanoff et al. 1999; Cnattingius et al. 2000; Wen et al. 2001), intrauterine growth restriction (Bracken et al. 2003a; Klebanoff et al. 2002; Vlajinac et al. 1997; CARE Study Group 2008), and still birth (Wisborg et al. 2003). One theoretically possible explanation for some of these effects may be that caffeine increases the levels of catecholamines in the mother and in the fetus, which may induce uteroplacental vasoconstriction (Kirkinen et al. 1983).

On the other hand, several prospective epidemiology studies have shown no major effects of caffeine on ovulation (Chavarro et al. 2009; Chap. 21), intrauterine growth restriction, low birth weight, or preterm delivery (Bracken et al. 2003a).

The major limitations of the epidemiology studies in this field lie in their retrospective nature and the data on caffeine exposure are thus subject to recall and other types of bias and that some studies lack information on confounding variables such as smoking. Confounding due to pregnancy symptoms is another important issue that complicates the relation between caffeine consumption and spontaneous abortion. Nausea may influence the amount of caffeine consumed in early pregnancy and it is also a marker of fetal viability.

Only one randomized controlled study investigated the effect of caffeine versus restricted caffeine intake on pregnancy outcome (Bech et al. 2007). Caffeinated instant coffee was compared with decaffeinated coffee during the second and the third trimester. A moderate caffeine reduction of 182 mg/day did not affect birth weight or length of gestation. Thus, there is currently insufficient evidence for advising mothers to avoid caffeine during the last two thirds of pregnancy.

The few studies that have examined theophylline in pregnancy in relation to perinatal outcomes have found no association between theophylline and low birth weight (Schatz et al. 2004) or small size for gestational age (Schatz et al. 2004; Stenius-Aarniala et al. 1995; Bracken et al. 2003b).

4 Exposure to Methylxanthines in Neonates

4.1 Absorption, Metabolism, and Elimination

Methylxanthines, from breast milk or given orally to infants for treatment of apneas, are rapidly and completely absorbed from the gastrointestinal tract of the neonate with a minimal first-pass effect (Arnaud 1987). Once absorbed, they freely enter all body tissues, including the brain and gonads (Arnaud 1987).

Pharmacokinetic studies have shown that newborn infants (up to about term-equivalent age) have a prolonged half life of caffeine of around 100 h (see Table 1), which decreases with gestational age (Aranda et al. 1979), reflecting the immaturity of the hepatic CYP1A2 enzyme system. The enzyme capacity then gradually improves and reaches adult function at about 3 months (Aranda et al. 1979) after birth.

Like caffeine, theophylline is readily absorbed orally (Ogilvie 1978) and no dose adjustment is needed when switching from intravenous to oral administration. Theophylline is, like caffeine, metabolized by CYP1A2, but has variable pharmocokinetics during the first few days in newborns, which is why monitoring of the plasma concentrations is required. Although there is a faster metabolic clearance of theophylline relative to caffeine in neonates, because theopylline can be back-methylated to caffeine, the net effect of methylxanthines may be the same since caffeine and theophylline exert similar biological actions. The half life in premature infants is 20–30 h.

Even though caffeine and theophylline are excreted in the urine mainly unchanged in the neonate, significant methylation of theophylline to caffeine occurs and the latter may exert additional pharmacological effects (Dani et al. 2000).

4.2 Methylxanthines and Breast Feeding

As mentioned already, although caffeine is excreted to a limited extent in breast milk, the immature metabolism of methylxanthines in neonates and, in particular, in preterm infants (Aranda et al. 1979) makes them at risk of accumulating methylxanthines. Theophylline (e.g., given to the mother for asthma) and theobromine (from chocolate) are also present in breast milk after administration (Yurchak and Jusko 1976) and accumulate in the neonate (Aranda et al. 1976).

While some studies have suggested possible risk effects of methylxanthines in pregnancy (see Sect. 3.2), a growth-promoting effect has been demonstrated in breast-feeding rat pups (Hart and Grimble 1990), which was shown to be due to increased milk volume. By contrast, there are a few animal studies showing that theophylline (Milsap et al. 1980; Carnielli et al. 2000) and caffeine (Bauer et al. 2001) increase the metabolic rate, which would infer a negative effect on growth. Similarly, a minimal decrease in fetal growth was observed in humans after excessive maternal caffeine drinking (more than 600 mg/day, about six cups of coffee) (Bracken et al. 2003a).

There has also been concern over the use of caffeine in mothers at the particular time point around delivery. Adenosine acting via adenosine receptors has endogenous neuroprotective effects in the adult brain (Fredholm 2007; Müller and Jacobson 2010). Although results in the neonate are complex (for a review, see Millar and Schmidt 2004), it was anticipated that the presence of the antagonist caffeine would possibly not be beneficial in a situation of birth asphyxia or postnatal apnea (with subsequent hypoxia). It was therefore surprising that when rat dams were given caffeine in their drinking water during pregnancy and lactation, in a dose that produced plasma concentrations similar to those achieved after 300–400 mg/day in humans, hypoxic ischemic brain damage was reduced by about 30% (Bona et al. 1995). No major effects on adenosine receptors or $GABA_A$ receptors were found (Aden et al. 2000). A similar perinatal exposure to caffeine prevented periventricular white matter damage in mice reared in hypoxia by enhancing myelination (Back et al. 2006). Further studies in adenosine-A_1-receptor-knockout mice (Turner et al. 2003) indicated that blockade of this receptor might contribute to the protective effect of caffeine in the immature brain. It is also possible that the anti-inflammatory effects of caffeine (Haskó and Cronstein 2010; Ohta and Sitkovsky 2010) may be partly instrumental for the beneficial effects of caffeine in the developing brain.

4.3 *Methylxanthines for Apnea of Prematurity*

More than three decades ago, it was demonstrated that methylxanthines can reduce the frequency of apneic episodes in premature infants (Kuzemko 1973; Aranda et al. 1977). Since then, methylxanthines have become part of the routine clinical management of apnea of prematurity. Apnea commonly occurs in premature infants below 34 weeks of gestational age and is a cessation of breathing due to immaturity of the respiratory drive, followed by decreased oxygen saturation in the blood and bradycardia. Recommended doses and desired plasma concentrations for

Table 1 Pharmacokinetics of methylxanthines in neonates. Data from Dani et'al. (2000) and Aranda et'al. (1976)

	Theophylline[a]	Caffeine
Plasma levels in neonates after moderate maternal coffee intake	No data available	0.2–1 mg/L (Neims and von Borstel 1983)
Route of administration	Intravenous, per os	Intravenous, per os
Dose (mg/kg)		
Loading	4–6	20
Maintenance	1.5-3 every 8-12 hr	5 (−10) every 24 hr
Plasma half life (h, range)	20–30	100 (40–230)
Therapeutic window (mg/L)	6–12	5–25
Adverse effects	Commonly include tachycardia, vomiting, hyperglycemia, irritability, sleeplessness	Usually mild, but includes tachycardia, vomiting restlessness

[a] Theophylline is often administered as the salt aminophylline, which consists of approximately 80% theophylline. If changing from intravenous administration of aminophylline to oral administration, the dose needs to be increased by 20%.

therapeutic use of caffeine and theophylline in premature neonates are shown in Table 1. It is important to note that the plasma concentration of caffeine used to treat this condition is 10–100 times higher than the plasma concentrations reached if the mother drinks moderate doses of coffee and the child is exposed via breast milk (Table 1). The methylxanthine therapy often goes on for several weeks until the premature child has matured.

Caffeine and theopylline exert similar effects, but have differences in pharmacokinetic properties (Table 1). In clinical practice, caffeine citrate has now become the methylxanthine of choice because it has a wider therapeutic range, has essentially complete full bioavailability, and can thus be given orally and routine measurements of blood concentrations are not needed.

As mentioned, given the neuroprotective effects of endogenous adenosine, there has been concern over the effects of high doses of methylxanthines in neonates at risk of apneas and postnatal hypoxic ischemia. It was therefore surprising that when rat pups were given theophylline in therapeutically relevant doses just before the induction of a hypoxia ischemia, the brain damage was reduced by almost 50% (Bona et al. 1997). The authors speculated that the mechanism for this protection might include anti-inflammatory effects of theophylline.

To this end, in a study where premature infants were randomized to either caffeine or a placebo for apnea of prematurity, it was shown that caffeine improved the rate of survival, decreased the number of children with severe respiratory sequelae (bronchopulmonary dysplasia), and reduced the incidences of cerebral palsy and cognitive delay at 18 months of age (Schmidt et al. 2007). With use of post hoc analysis, about half of the protective effect of caffeine was attributed to positive effects on the respiration, but half of the effect remained unexplained. These results indicate that caffeine has inherent direct or indirect neuroprotective effects.

5 Short-Term Effects of Methylxanthines in the Infant

5.1 Physiological Effects

Methylxanthines exert profound stimulatory effects on respiratory drive in neonates by antagonism of adenosine A_1 receptors in the pons and medulla oblongata (Lagercrantz et al. 1984; Tilley 2010), improved chemoreceptor sensitivity to CO_2 (Davi et al. 1978), mainly via blockade of adenosine A_{2A} receptors (Conde et al. 2006), increased oxygen consumption (Bauer et al. 2001), and increased cardiac output (Walther et al. 1990). As mentioned already, experimental data show that adenosine A_1 receptors are clearly G-protein-coupled (Aden et al. 2001) and capable of function at birth in the heart. Accordingly, a commonly seen clinical effect/side effect of methylxanthines is tachycardia.

Adverse effects are similar for theophylline and caffeine (Table 1) but are milder and occur less often for caffeine. One specific adverse effect of theophylline is that cerebral blood hemodynamics are transiently affected (Dani et al. 2000), possibly via blockade of cerebrovascular adenosine A_{2A} receptors

Renal effects of methylxanthines include increased diuresis (via tubular adenosine A_1 receptors) and increased calcium excretion (Bauer et al. 2001; McPhee and Whiting 1989; Rieg et al. 2005).

Unfavorable symptoms can also occur in neonates if a chronic administration of methylxanthines during pregnancy suddenly ceases, i.e., if breast feeding is not established early after birth.

5.2 Withdrawal Symptoms

There are case reports in the literature on transient withdrawal symptoms in full-term neonates exposed to high concentrations of caffeine after excessive maternal ingestion of coffee, mate, or cola drinks (450–1,800 mg/day). These symptoms include jitteriness, high-pitched cry, hypertonia in the limbs, brisk tendon reflexes, and vomiting and resolved spontaneously within 1–2 days (Khanna and Somani 1984; McGowan et al. 1988). In these cases the withdrawal symptoms were preceded by lack of breast feeding.

It is also possible that some apneic episodes encountered in neonates who were exposed to caffeine during pregnancy but then were not breast-fed, may be due to a central withdrawal effect of caffeine at the pontomedullary level. This speculation was supported by experimental data from neonatal rat pups where caffeine was given in the drinking water to pregnant and lactating dams. If caffeine was withdrawn at birth, apneas could be induced and subsequent caffeine exposure during breast feeding was able to prevent apneas in the rat pups (Bodineau et al. 2006).

6 Long-Term Effects of Methylxanthines for the Developing Organism

Even though the short-term effects of caffeine, in relevant doses, seem to be largely beneficial, there has been considerable concern over the long-term effects of methylxanthines in the developing brain and other organs.

6.1 CNS Function

Exposure to psychostimulant drugs during brain development may lead to long-term effects beyond the time point when the drug exposure is withdrawn (Andersen 2005). There is some experimental evidence that high doses of methylxanthines induce long-lasting behavioral changes in the offspring (Nehlig and Debry 1994; Henderson et al. 1991; Nakamoto et al. 1991). Also, when a modest dose of caffeine (similar to plasma levels achieved in moderate maternal coffee drinking) was given in the drinking water to mice throughout pregnancy and lactation, the adult offspring exhibited increased locomotor activity in an open field (Nehlig and Debry 1994). A similar behavioral profile was found in mice heterozygous for the adenosine A_1 receptor gene, where signaling via adenosine A_1 receptors was reduced to about the same degree as after modest consumption of caffeine (Bjorklund et al. 2008b). Furthermore, it appeared that the mother's genotype, not the offspring's, was critical for behavioral changes in adult offspring, thus indicating that perinatal caffeine acting on adenosine A_1 receptors in the mother caused a long-term effect in the offspring (Bjorklund et al. 2008b). Interestingly, these effects even manifested themselves in the second generation (Bjorklund et al. 2008b).

However, in humans, perinatal exposure to coffee per se was not related to any increased risk of having ADHD or hyperkinetic diagnosis (Linnet et al. 2009). Similarly, in a follow-up study of 500 pregnant women and their offspring, there was no association between perinatal caffeine exposure and IQ and attention tests at 7 years of age (Barr and Streissguth 1991). Furthermore in a large randomized controlled trial on caffeine (given postnatally in high doses) for apnea of prematurity, a reduced incidence of cerebral palsy and cognitive delay at 18 months of age was shown (Schmidt et al. 2007).

Although no detrimental long-term effects from perinatal methylxanthine exposure have been demonstrated in humans so far, studies on development of CNS functions require a very long follow-up period and complex neuropsychological testing; therefore, effects may nevertheless be present but difficult to detect.

6.2 Respiration and Cardiovascular Function

There is some experimental evidence that neonatal caffeine treatment with plasma levels comparable to these achieved with treatment for apnea of prematurity alters the ventilatory response to hypercapnia and hypoxia in adolescence and adulthood (Montandon et al. 2008). Mainly adenosine A_1 receptors were found to be involved in these plasticity changes, which speculatively might have implications for diseases implied in respiratory control dysfunction such as SIDS and sleep apnea (Montandon et al. 2008).

It is also conceivable that perinatal exposure to caffeine may have persisting effects on cardiovascular function, since adenosine is an important regulator thereof (Riksen et al. 2010). Effector systems for adenosine receptors actually mature earlier in the cardiovascular system than in the CNS, at least in rats (Aden et al. 2001), and there is some evidence that early caffeine exposure can alter gene expression of adenosine and dopamine receptors and thyrosine hydroxylase in the carotid body and adrenal glands of rats (Montandon et al. 2008). Another study showed adverse effects of a dose of caffeine relevant to daily intake of coffee on embryonic arteries with transiently decreased blood flow. These effects were adenosine A_{2A} dependent (Momoi et al. 2008). It is thus possible that early exposure to caffeine might have long-lasting cardiovascular effects, but there is currently a lack of long-term follow-up data of cardiorespiratory function and human data.

As mentioned, an important positive influence of perinatal caffeine on lung function was seen in a randomized controlled study of apnea of prematurity, where caffeine decreased the number of children with a chronic respiratory disease (bronchopulmonary dysplasia) at 18 months of age (Schmidt et al. 2007). The major protective effect of caffeine in this context was that ventilator-induced lung injury was avoided to a large extent, because the caffeine-treated infants came off the ventilator earlier than the controls (Schmidt et al. 2007). Another possibly contributing effect might be that caffeine and other methylxanthines have inherent immunomodulatory mechanisms (Haskó and Cronstein 2010; Ohta and Sitkovsky 2010) that might protect both from lung and brain injury.

7 Conclusions

Many fetuses and neonates are exposed to low levels of methylxanthines owing to maternal drinking of coffee. Much higher doses of caffeine and theophylline are used in long-term pharmacological treatment of apneas in preterm infants. Pregnant women, fetuses, and neonates have an inability to detoxify methylxanthines, rendering the developing organism with accumulating levels of methylxanthines, which implies that there is a risk for adverse effects. Brain development is of particular concern.

Animal studies, however, suggest that the major effector system for methylxanthines, adenosine receptors, is not fully developed in the brain at birth. Furthermore, animal studies have shown protective positive effects of methylxanthines in situations of hypoxia/ischemia in neonates. Similarly, a positive long-term effect on lung function and CNS development was found in human preterm infants treated with high doses of caffeine for apneas. There is now evidence that the overall benefits from methylxanthine therapy for apnea of prematurity outweigh potential risks in the short term.

Experimental studies, however, have indicated that long-term effects of low-dose caffeine during pregnancy and lactation may include altered behavior in the offspring, but there are currently no human data to support this.

Negative effects on pregnancy and perinatal outcomes have been reported in epidemiology studies, but the results are inconclusive. The evidence base for adverse effects of caffeine in first third of pregnancy is stronger than for later parts of pregnancy. Whereas it may be prudent for women in early pregnancy to limit caffeine intake (less than 300 mg/day), there is currently insufficient scientific evidence to advise mothers to avoid consuming caffeine during later parts of pregnancy.

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Methylxanthines and the Kidney

Hartmut Osswald and Jürgen Schnermann

Contents

Abstract This chapter describes the effects of the natural methylxanthines caffeine and theophylline on kidney function. Theophylline in particular was used traditionally to increase urine out put until more potent diuretics became available in the middle of the last century. The mildly diuretic actions of both methylxanthines are mainly the result of inhibition of tubular fluid reabsorption along the renal proximal

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tubule. Based upon the use of specific adenosine receptor antagonists and the observation of a complete loss of diuresis in mice with targeted deletion of the A1AR gene, transport inhibition by methylxanthines is mediated mainly by antagonism of adenosine A1 receptors (A1AR) in the proximal tubule. Methylxanthines are weak renal vasodilators, and they act as competitive antagonists against adenosine-induced preglomerular vasoconstriction. Caffeine and theophylline stimulate the secretion of renin by inhibition of adenosine receptors and removal of the general inhibitory brake function of endogenous adenosine. Since enhanced intrarenal adenosine levels lead to reduced glomerular filtration rate in several pathological conditions theophylline has been tested for its therapeutic potential in the renal impairment following administration of nephrotoxic substances such as radiocontrast media, cisplatin, calcineurin inhibitors or following ischemia-reperfusion injury. In experimental animals functional improvements have been observed in all of these conditions, but available clinical data in humans are insufficient to affirm a definite therapeutic efficacy of methylxanthines in the prevention of nephrotoxic or postischemic renal injury.

Keywords Adenosine · Caffeine · Diuresis · Natriuresis · Nephropathy · Renin · Theophylline

1 Introduction

The plant constituents caffeine and theophylline have been known to alter kidney function since the demonstration in 1864 that caffeine can increase urine production in patients with congestive heart failure and edema (Koschlakoff 1864). Interest in the renal actions of methylxanthines has remained acute although the clinical use of theophylline is largely restricted to the treatment of extra-renal diseases such as asthma. The focus of this chapter is a description of the renal effects of the natural methylxanthines, mainly of caffeine and theophylline, and a discussion of current understanding of the mechanisms underlying these effects. Important progress in mechanistic thinking has been made by using modified methylxanthines, and it will therefore sometimes be necessary to include data obtained with synthetic xanthine derivatives when this permits discrimination between the different targets of the natural compounds. This is especially relevant in the case of methylxanthines as antagonists of adenosine receptors, a predominant mechanism in many renal actions of methylxanthines. Nevertheless, a broad discussion of adenosine and its interaction with adenosine receptors is not the goal of this review, and adenosine will only be discussed in the context of understanding methylxanthine actions. We also will make reference to nonnatural xanthine compounds in cases where their actions are in conflict with those of caffeine or theophylline or where they shed light on likely mechanisms of action.

2 Diuresis

The natural methylxanthines caffeine and theophylline were used traditionally to increase urine output until more potent diuretics became available in the middle of the last century. In numerous studies in animal and humans the order of diuretic potency of the natural methylxanthines was found to be theophylline > caffeine > paraxanthine > theobromine. The actions of methylxanthines as diuretics have been extensively reviewed in an excellent chapter in the *Handbook of Experimental Pharmacology* in which the literature prior to 1970 was summarized and discussed in full detail (Fulgraff 1969). In the period since then, it has been confirmed repeatedly that caffeine and other methylxanthines can induce an increase in urine flow in humans and experimental animals. The dose of caffeine that elicits a significant acute diuresis has been reported to be on the order of 300 mg, the equivalent of about four to five cups of coffee (Grandjean et al. 2000; Passmore et al. 1987; Riesenhuber et al. 2006). As determined by impedance analysis, an acute intake of 642 mg of caffeine without a change in total fluid intake caused a measurable decrease in body weight corresponding to a 2.7% reduction of total body water (Neuhauser et al. 1997). Furthermore, a positive fluid balance prior to methylxanthine administration enhanced the diuretic efficacy of methylxanthines, while a negative fluid balance reduced the diuretic response (Fulgraff 1969). The diuretic potency of caffeine appears to be also modulated by age and habituation, with old age and previous exposure to caffeine causing further decreases in the diuretic effectiveness of caffeine (Izzo et al. 1983).

Overall, the relatively modest potency of caffeine to enhance water excretion and cause net water loss is consistent with studies in which caffeine (up to 6 mg/kg) given for 11 days did not affect 24-h urine volume and was not associated with symptoms of negative fluid balance (Armstrong et al. 2005). The same conclusion was drawn in a study where the diuretic effects of caffeinated and noncaffeinated electrolyte drinks were compared in individuals at rest and during moderate exercise (Wemple et al. 1997). While the ingestion of caffeine (25 mg/dl at 35 ml/kg) caused a higher urine flow in individuals at rest compared with ingestion of caffeine-free fluid, urine flow was reduced to the same level during exercise and this was associated with identical increments of plasma catecholamine concentrations. In a smaller study, consumption of tea as the major fluid source did not cause noticeable differences in hydration status compared with nontea fluid intake in a group of mountaineers at high altitude (Scott et al. 2004). Thus, the general advice against using caffeinated drinks for volume replacement may need to be qualified in that beverages containing moderate amounts of caffeine do not appear to cause significant fluid losses (Armstrong 2002).

Caffeine in high concentration supplied in a calcium-free medium has been shown to prevent the increase in calcium level caused by vasopressin in rat renal papillary collecting duct cells and this may blunt the increase of cyclic AMP (cAMP) level and the effect of vasopressin on water permeability (Ishikawa et al. 1992). Caffeine appears to exert this effect by depletion of endoplasmic calcium stores, suggesting that its action may be caused by interaction with ryanodine-sensitive calcium-release channels. Nevertheless, there is no evidence in support of the notion that methylxanthines in lower concentrations inhibit solute-free water absorption in the distal part of the nephron. In fact, theophylline has been observed to mimic the effect of vasopressin on water permeability in isolated perfused collecting ducts and in the bladder of the toad (Grantham and Orloff 1968; Orloff and Handler 1962). Both vasopressin and theophylline increased cellular cAMP levels, indicating that the theophylline effects are mediated by inhibition of phosphodiesterase (PDE) (Handler et al. 1965). Caffeine is unlikely to reduce collecting duct water reabsorption through inhibition of adenosine receptors since in perfused and nonperfused collecting ducts adenosine has been shown to inhibit AVP-stimulated water permeability through activation of A_1 adenosine receptors (Edwards and Spielman 1994; Yagil 1990). Since A_2-adenosine-receptor-mediated effects have not been identified (Edwards and Spielman 1994; Yagil 1990), caffeine would thus be expected to enhance, not inhibit, collecting duct water transport, just as inhibition of cAMP degradation by possible methylxanthine effects on PDE would not be predicted to inhibit water transport. Furthermore, there does not seem to be a direct effect of caffeine on vasopressin secretion since plasma vasopressin levels have been reported to be unaltered after caffeine ingestion (Izzo et al. 1983; Nussberger et al. 1990). One would conclude that the type of diuresis caused by methylxanthines is mostly or exclusively a solute diuresis.

3 Natriuresis

Increased urine flow caused by methylxanthines is accompanied by increased excretion of sodium, chloride, calcium, phosphate, magnesium, and other urinary solutes. Although methylxanthines have in some studies been found to increase the tubular sodium load, significant natriuresis can occur without changes in glomerular filtration rate (GFR) or renal blood flow (RBF), clearly indicating that the natriuresis caused by methylxanthines is predominantly the result of inhibition of tubular salt transport (Davis and Shock 1949; Ludens et al. 1970; Shirley et al. 2002). Natriuresis without hemodynamic changes was also caused by methylxanthines in premature infants and newborn rabbits (Gouyon and Guignard 1987; Mazkereth et al. 1997). The increased excretion of calcium caused by caffeine may have implications for calcium homeostasis. Abstinence from moderate daily caffeine consumption (200 mg or less) has been noted to significantly increase plasma concentrations of ionized calcium and to reduce PTH levels in women with a relatively low dietary calcium intake (Massey et al. 1994; Wise et al. 1996).

In addition to causing natriuresis and diuresis, the administration of theophylline to conscious rats (10–50 mg/kg oral) or anesthetized rabbits (15 mg/kg intravenously) was accompanied by increased urinary excretion of prostaglandin E_2 (PGE_2) and cAMP (Baer et al. 1983; Oliw et al. 1977). Pretreatment with indomethacin or meclofenamate prevented the natriuretic action of theophylline, and conversely, theophylline caused a transient reversal of the antinatriuresis and antidiuresis elicited by indomethacin in patients with rheumatic diseases (Oliw et al. 1977; Seideman et al. 1987). The possibility that some effects of methylxanthines on urine excretion could be indirect is also supported by the observation that plasma and kidney atrial natriuretic factor (ANF) activity increased following prolonged caffeine ingestion (Eggertsen et al. 1993; Lee et al. 2002). Nevertheless, no change in plasma ANF was seen 2 h after an oral intake of a single 250 mg dose of caffeine (Nussberger et al. 1990). That the natriuretic effect of methylxanthines is probably not strictly dependent on ANF is also suggested by the observation that prior exposure to theophylline did not modify the natriuretic action of ANF (Beutler et al. 1990).

There is considerable evidence to indicate that methylxanthine-induced natriuresis is predominantly a consequence of inhibition of salt transport along the proximal convoluted tubule. Administration of 400 mg of caffeine to healthy human subjects caused an about 1.5-fold increase in sodium excretion and this was associated with an increase in the clearance of lithium (Shirley et al. 2002). A reduction of proximal solute reabsorption in humans as assessed by lithium clearance was also caused by theophylline and aminophylline (Beutler et al. 1990; Brater et al. 1983). At the level of the single tubule, systemic administration of theophylline (20 mg/kg subcutaneously) caused an about 20% reduction in proximal tubular reabsorptive capacity as determined with the split-droplet technique in the rat (Fulgraff 1969). Tubular microperfusion of the loop of Henle with solutions containing theophylline or 3-isobutyl-1-methylxanthine did not significantly alter chloride reabsorption, indicating that methylxanthines do not affect salt absorption along proximal straight tubules and thick ascending limbs (Schnermann et al. 1977). The effect of methylxanthines on sodium transport in tubular segments beyond the proximal tubule and the loop of Henle has not been explored in detail, although on the basis of indirect evidence an inhibitory action in more distal parts of the tubule has been proposed (Brater et al. 1983; Shirley et al. 2002).

The mechanism by which methylxanthines inhibit proximal NaCl reabsorption is related to their properties as antagonists of adenosine receptors. Xanthine derivatives such as doxofylline and enprofylline with low affinity for adenosine receptors, but similar ability to inhibit PDE, have been noted to exert only marginal effects on natriuresis compared with aminophylline or theophylline, suggesting that inhibition of adenosine receptors is critical for the natriuretic action (Andersson et al. 1984; Cirillo et al. 1989; Franzone et al. 1988). Strong experimental evidence indicates that it is the A_1 adenosine receptor subtype whose inhibition results in natriuresis. In mice with targeted deletion of A_1 adenosine receptors, the diuretic and natriuretic effect of caffeine (45 mg/kg) or theophylline (45 mg/kg) was entirely absent (Rieg et al. 2005). The natriuresis caused by systemic administration of xanthine derivatives designed to selectively inhibit A_1 adenosine receptors such as CVT-124, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), and KW-3902 has been shown by lithium clearance and renal tubular micropuncture approaches to be accompanied by inhibition of proximal tubular fluid reabsorption (Knight et al. 1993; Kost et al. 2000; Mizumoto and Karasawa 1993; Wilcox et al. 1999).

DPCPX-induced natriuresis can be prevented by pertussis toxin, consistent with an involvement of the Gi-coupled A_1 adenosine receptors (Kost et al. 2000).

In view of the dominant role of Na/H exchange for sodium reabsorption in the proximal tubule, it is not surprising that modulation of NHE3 has been implicated in adenosine-dependent modulation of sodium reabsorption. In opossum kidney cells, low concentrations of an A_1 adenosine receptor agonist (less than 10^{-8} M) do in fact activate NHE3, an effect that is blocked by A_1 adenosine receptor antagonists and apparently mediated by inactivation of adenylyl cyclase (Di Sole et al. 2003). Downregulation of NHE3 and of α_1/β_1-NaKATPase protein expression was observed following a 1-day treatment with caffeine in rats (Lee et al. 2002). On the other hand, theophylline (1 mM) did not affect HCO_3 flux as assessed from the pH recovery in stationary microperfusion studies in the rat (Bailey 2004). In renal proximal tubular cell cultures and opossum kidney cells, A_1 adenosine receptor activation stimulated and inhibition of A_1 adenosine receptors by DPCPX or KW-3902 inhibited apical sodium/phosphate (Cai et al. 1994, 1995; Coulson et al. 1991) and Na/glucose cotransport (Coulson et al. 1991, 1996). Inhibition of phosphate uptake by A_1 adenosine receptor antagonists was associated with a dose-dependent increase of cellular cAMP production as well as an increase in protein kinase C activity (Cai et al. 1995; Coulson et al. 1991, 1996). Theophylline and A_1-adenosine-receptor- selective xanthine derivatives inhibited basolateral HCO_3 conductance in microperfused rabbit proximal convoluted tubules and this effect was mimicked by forskolin and chlorophenylthio-cAMP, suggesting that methylxanthines inhibit Na/HCO_3 cotransport activity by increasing intracellular cAMP levels (Takeda et al. 1993). To the extent that caffeine causes an increase in arterial blood pressure, a potential direct role of blood pressure in inhibiting tubular reabsorption and altering NHE3 distribution needs to be considered (Nussberger et al. 1990; Rachima-Maoz et al. 1998; Rakic et al. 1999). Finally, it should be pointed out that administration of methylxanthines only explores the impact of a reduction in adenosine-mediated effects, and that the consistent stimulatory effect of adenosine suggested by this intervention is therefore not in conflict with the possibility that high concentrations of adenosine may elicit inhibition of proximal tubular transport (Di Sole 2008; Di Sole et al. 2003).

4 Hemodynamics

Several studies in anesthetized dogs agree that the intrarenal infusion of methylxanthines does not affect RBF significantly, although renal vascular tone may decrease slightly because of small blood pressure reductions (Ibarrola et al. 1991; Osswald 1975; Premen et al. 1985). Furthermore, theophylline or caffeine do not alter renal plasma flow in humans to an extent that could be detected by clearance techniques (Beutler et al. 1990; Brater et al. 1983; Brown et al. 1993; Passmore et al. 1987). Changes of GFR in response to methylxanthines are sometimes more pronounced than those of RBF causing increases of filtration fraction (Fulgraff

1969). On the other hand, theophylline consistently inhibited adenosine-induced reductions of GFR and RBF in dogs and rats (Osswald 1975; Osswald et al. 1977; Pawlowska et al. 1987; Spielman 1984). Similarly, the vasodilator response of medullary blood flow to adenosine was blocked by 8-phenyltheophylline (Dinour and Brezis 1991). Thus, methylxanthines can affect renal hemodynamics by blocking the vascular actions of adenosine, at least in the range of supranormal adenosine levels. The absence of major effects of methylxanthines on renal hemodynamics could be due to low resting levels of adenosine. However, this seems unlikely since renal interstitial adenosine levels as determined by microdialysis are on the order of 50–200 nM, a range in which both A_1 and A_{2a} adenosine receptors should be partially occupied (Baranowski and Westenfelder 1994; Nishiyama et al. 2001; Siragy and Linden 1996; Zou et al. 1999). Thus, renal vascular tone under basal conditions appears to represent a state of balanced activation of A_1 and A_{2a} adenosine receptors. Vascular actions of theophylline could also result from a reduction of the inhibitory effect of adenosine on catecholamine release from renal sympathetic nerve terminals (Hedqvist and Fredholm 1976). In isolated perfused rabbit kidneys, theophylline did not affect norepinephrine release or RBF under basal conditions, presumably a reflection of the absence of a basal sympathetic tone (Hedqvist et al. 1978). However, the increased norepinephrine release following renal nerve stimulation was slightly potentiated by theophylline, and this was accompanied by a decrease in the vasoconstrictor response to nerve stimulation (Hedqvist et al. 1978). Similarly, theophylline attenuated the vasoconstrictor response to exogenous norepinephrine (Hedqvist et al. 1978; Yoneda et al. 1990). Finally, the reduction of GFR following dipyridamole and indomethacin administration in rheumatic patients was fully reversed by theophylline, suggesting that some of the vascular effects of indomethacin may be adenosine-mediated (Seideman et al. 1987).

In contrast to the negligible effects of methylxanthines on global renal vascular tone, theophylline has been found to fully inhibit the local tubuloglomerular feedback (TGF) response to changes of NaCl concentration in individual nephrons. Intratubular and intravenous administration of theophylline or 1,3-dipropyl-8-*p*-sulfophenylxanthine caused a dose-dependent blockade of the afferent arteriolar constriction induced by increases in NaCl concentration in the tubular fluid passing the macula densa segment (Franco et al. 1989; Osswald et al. 1980; Schnermann et al. 1977). This effect is the result of inhibition of A_1 adenosine receptors since the effect of theophylline on TGF was fully mimicked by subtype-specific adenosine receptor antagonists (Kawabata et al. 1998; Ren et al. 2002; Schnermann et al. 1990; Thomson et al. 2000; Wilcox et al. 1999). Infusion of hypertonic saline into the renal artery of anesthetized dogs has been shown to cause sustained vasoconstriction, and this has been considered a model of "whole kidney TGF" (Gerkens et al. 1983). Like single nephron TGF, vasoconstriction caused by hypertonic saline was inhibited by theophylline or aminophylline (Gerber and Nies 1986; Gerkens et al. 1983). Extracellular conversion of released cAMP by ectophosphodiesterases and 5′-nucleotidase has been suggested to constitute a significant source of adenosine in the kidney (Jackson et al. 1997). Thus, the PDE-inhibitory effects of xanthines may contribute to inhibition of TGF by reducing the interstitial adenosine levels (Mi et al. 1994).

5 Renin Secretion

It is remarkable that methylxanthines are able to cause natriuresis despite the fact that they also stimulate the strongly antinatriuretic renin–angiotensin system. Theophylline increased plasma renin activity in dogs, and this increase was shown to occur without changes in blood pressure or in the plasma levels of epinephrine and norepinephrine, indicating that it was not mediated by the renal baroreceptor mechanism or by adrenergic receptors (Reid et al. 1972). In fact, theophylline stimulated renin release even in dogs treated with propranolol. Likewise, oral administration of caffeine in rats for 10 days was associated with a marked rise of renin secretion (Tofovic and Jackson 1999). Even though it was thought that the theophylline effect may be mediated by inhibition of PDE and the resulting increase in cellular cAMP levels (Reid et al. 1972), it now seems likely that stimulation of renin by methylxanthines is at least in part, but probably predominantly, a consequence of inhibition of adenosine receptors. Inhibition of A_1 adenosine receptors by the selective antagonist FK-453 caused a significant increase of plasma renin levels (Balakrishnan et al. 1993), and DPCPX partially inhibited the stimulation of renin release caused by low NaCl concentration in a microperfused juxtaglomerular apparatus preparation (Weihprecht et al. 1990). Furthermore, infusion of caffeine or theophylline at a dose that did not change renal cortical cAMP levels has been reported to abolish the inhibitory effect of intrarenally infused adenosine on renin secretion, and this effect could be dissociated from hemodynamic changes (Arend et al. 1987; Choi et al. 1993; Spielman 1984). In support of the notion that endogenous adenosine exerts a general inhibitory “brake” function in renin release, caffeine or theophylline as well as specific A_1 adenosine receptor antagonistic xanthines have been reported to augment the renin-stimulatory effects of a low renal artery pressure, a low-sodium diet, furosemide, isoproterenol, and vasodilators such as diazoxide and hydralazine (Brown et al. 1991; Langard et al. 1983; Paul et al. 1989; Pfeifer et al. 1995; Tofovic et al. 1991; Tseng et al. 1993). Despite the convincing effect of methylxanthines in experimental animals, studies in normotensive human subjects examining the effect of caffeine (250 mg) or coffee drinking have reported plasma renin concentration to increase, decrease, or remain unchanged, indicating that caffeine in moderate doses may not consistently induce the plasma caffeine levels needed to stimulate renin release (Nussberger et al. 1990; Robertson et al. 1978; Smits et al. 1983). Similarly, no increases of plasma renin concentration were observed in response to caffeine or coffee drinking in hypertensive patients (Eggertsen et al. 1993; Palatini et al. 1996; Robertson et al. 1984) or in patients with autonomic failure (Onrot et al. 1985).

6 Disease and Therapeutic Aspects

6.1 Polycystic Kidney Disease

Generation of cAMP has been shown to play a role in the secretion of fluid that is thought to be partly responsible for the accumulation of fluid in renal cystic disease (Belibi et al. 2002). Thus, by augmenting cAMP levels, methylxanthines could contribute to the progression of cyst formation. In fact, in primary cultures of renal cysts from patients with autosomal dominant polycystic kidney disease (ADPKD), caffeine (10–1,000 μM) increased levels of cellular cAMP and this was associated with an increase of transepithelial chloride secretion. Furthermore, caffeine greatly potentiated the augmentation of cAMP induced by Gs-coupled agonists such as vasopressin and PGE_2. The increase of cAMP levels by caffeine was in part mediated by inhibition of PDE since rolipram, a PDE inhibitor that does not interact with adenosine receptors, also caused a marked elevation of cAMP levels. However, an elevation in cAMP level was also seen with adenosine (10 mM), and this effect was attenuated, but not fully blocked by caffeine, suggesting that stimulation of A_2 adenosine receptors contributed to the accumulation of cAMP in cyst cells (Belibi et al. 2002). Cyst formation has also been related to impaired mechanosensation by primary cilia since both polycystin 1 (PC1) and polycystin 2 (PC2) are found in association with cilia. In fact, the increase in cytosolic calcium concentration caused by a flow stimulus in renal cells of wild-type mice was not seen in cells from PC1- or PC2-deficient mice or after treatment of wild-type cells with blocking antibodies against PC2 (Nauli et al. 2003). The increase in cytosolic calcium concentration by flow was blocked by high concentrations of caffeine, suggesting that it was caused by release of stored calcium across ryanodine-sensitive receptors in response to an initial calcium entry through PC2 cation channels. Nevertheless, caffeine ingestion did not accelerate cyst formation in the Han–Sprague-Dawley rat model of ADPKD although it was associated with the generation of hypertension (Tanner and Tanner 2001).

6.2 Nephropathies

In several experimental disease models, chronic caffeine administration has been found to exacerbate the development of hypertension and renal disease, perhaps through the effect of caffeine on renin secretion. For example, the presence of caffeine (0.1%) in the drinking water of rats augmented the blood pressure increase caused by renal arterial constriction and this was associated with a greater increase of plasma renin concentration (Choi et al. 1993; Kost et al. 1994; Ohnishi et al. 1986). Similarly, a 10-day caffeine exposure enhanced renin secretion to a markedly greater extent in spontaneously hypertensive heart failure (SHHF/Mcc-fa) rats than in control rats (Tofovic et al. 1999). In association with the increase of renin secretion, prolonged caffeine ingestion caused a faster decline of renal function and a significant enhancement of urinary protein excretion (Tofovic and Jackson 1999). An adverse effect of chronic caffeine intake on renal function was also observed in puromycin aminonucleoside induced nephrosis in rats. Both the decline of renal function assessed as creatinine clearance and the increase of renin secretion were enhanced in puromycin-treated rats receiving caffeine in comparison with nephrotic rats receiving tap water (Tofovic et al. 2000). In addition, caffeine potentiated the development of interstitial fibrosis and glomerulosclerosis caused by puromycin (Tofovic et al. 2000). Finally, long-term treatment with caffeine reduced renal function and augmented proteinuria in obese diabetic ZSF1 rats despite improving glucose tolerance. Caffeine-induced renal deterioration was paralleled by enhanced fibrosis, proliferation, and inflammation (Tofovic et al. 2002, 2007). In addition to stimulating the release of renin, the effects of caffeine may be mediated through interference with the direct anti-inflammatory effects of adenosine (Tofovic et al. 2007).

In view of this solid body of evidence in support of caffeine as a risk factor in renal disease it is unexpected that the methylxanthine pentoxifylline has been found to produce exactly opposite outcomes. Pentoxifylline has only a low affinity for adenosine receptors and is generally considered a PDE inhibitor with some selectivity for PDE4 (Daly 2007). In animal studies, pentoxifylline markedly reduced the functional decline, proteinuria, fibrosis, and inflammation in rats following 5/6 nephrectomy or treatment with anti-GBM antiserum, and this was associated with an attenuation of the stimulated expression of mitogenic and profibrotic gene products (Chen et al. 2004; Lin et al. 2002). This effect was probably independent of the renin–angiotensin system since a combination of pentoxifylline with an ACE inhibitor further diminished disease progression (Lin et al. 2002), and since the plasma renin level was found to be unchanged in a human study (Chen et al. 2006). Furthermore, pentoxifylline protected against endotoxin-induced renal failure in mice and reduced plasma levels of TNF-α, IL-1β, and nitric oxide (Wang et al. 2006). In relatively small human trials, pentoxifylline reduced proteinuria and slowed the GFR decline in patients with chronic renal failure (Lin et al. 2008; Perkins et al. 2009), and it reduced proteinuria in patients with primary glomerular diseases in association with a reduction of urinary MCP-1 excretion (Chen et al. 2006). In nonhypertensive type 2 diabetic subjects pentoxifylline was as effective in reducing microalbuminuria as the ACE inhibitor captopril (Rodriguez-Moran and Guerrero-Romero 2005). A meta-analysis of ten randomized controlled studies in adult patients with diabetic kidney disease suggested comparable efficacies of pentoxifylline and captopril in reducing proteinuria (McCormick et al. 2008). The mechanism of action of the protective effects of pentoxifylline or its metabolite lisofylline is unclear.

6.3 Radiocontrast Nephropathy

Iodinated radiocontrast agents used in a number of radiological imaging procedures can cause acute renal failure. The incidence is very low in the absence of complicating factors, but it increases considerably in patients with pre-existing renal conditions or in other circumstances that represent a risk factor for developing acute renal failure in general, such as dehydration or low cardiac output. Since radiocontrast-induced renal failure is accompanied by a reduction of RBF and GFR, methylxanthines have been among a number of vasodilator agents that have been assessed in regard to their preventive potential. In sodium-depleted dogs, radiocontrast agents reduced RBF and GFR, and both of these effects were attenuated by prior administration of theophylline (Arend et al. 1987; Deray et al. 1990). Furthermore, theophylline partially prevented the reduction of medullary blood flow induced by iodixanol (Lancelot et al. 2002). Thus, theophylline appears to act by antagonizing vasoconstriction mediated by A_1 adenosine receptor activation, and this hypothesis was corroborated in studies in which the A_1-adenosine-receptor-selective antagonist KW-3902 was even more effective than theophylline in attenuating iohexol-induced renal functional impairment in dogs with pre-existing renal insufficiency (Arakawa et al. 1996). Acute renal failure and cytotoxicity following iohexol administration were found to be more pronounced in wild-type than in A_1-adenosine-receptor-deficient mice, and a similar protective effect could be seen when wild-type mice were pretreated with the A_1-adenosine-receptor-selective antagonist DPCPX (Lee et al. 2006). In rats pretreated chronically with N^{ω}-nitro-L-arginine methyl ester, but not in control rats, sodium diatrizoate caused a decrease of GFR and RBF that could be fully prevented by pretreatment with theophylline, DPCPX, or KW-3902 (Erley et al. 1997; Yao et al. 2001). It could also be prevented by extracellular volume expansion, the standard preventive strategy (Yao et al. 2001). In the majority of studies, prophylactic administration of theophylline or aminophylline has also been reported to provide protection against radiocontrast renal failure in humans (Erley et al. 1994; Huber et al. 2001, 2003; Kapoor et al. 2002; Katholi et al. 1995; Kolonko et al. 1998). Protection by theophylline was similar to that afforded by oral or intravenous hydration (Erley et al. 1999). Two recent meta-analyses, one including 480 patients and the other including 585 patients, concluded that theophylline or aminophylline appear to attenuate the radiocontrast-induced decline of renal function (Bagshaw and Ghali 2005; Ix et al. 2004). On the other hand, an analysis of 41 studies that used radiocontrast agents in combination with theophylline, *N*-acetylcysteine, fenoldopam, dopamine, iloprost, statins, furosemide or mannitol showed that only *N*-acetylcysteine provided significant renoprotection, whereas the risk reduction provided by theophylline was not significant (Kelly et al. 2008). Greater protection by *N*-acetylcysteine than by theophylline was also observed in a study in dehydrated rats (Efrati et al. 2009). Exactly how methylxanthines exert their limited protective effect is unclear in view of the well-recognized multifactorial pathophysiological mechanisms of radiocontrast-induced nephropathy (Cox and Tsikouris 2004; Persson et al. 2005). Because of similar effects of other vasoactive agents such as ANP, dopamine, endothelin antagonists, calcium channel blockers, and PGE_2, nonspecific vasodilatation of the renal vascular bed is likely to play a major contributory role. Nevertheless, despite some promising results, the overall clinical experience does not support the use of methylxanthines as a first-line defense against the induction of contrast nephropathy (Lin and Bonventre 2005).

6.4 Calcineurin Inhibitors

The main complication of immunosuppression by calcineurin inhibitors is nephrotoxicity manifesting itself as a decline of renal function associated with vasoconstriction and a reduction of RBF. In early studies in rats, theophylline failed to ameliorate cyclosporine-induced renal vasoconstriction, indicating that it was not caused by adenosine (Churchill et al. 1990). Subsequently however, caffeine, theophylline, and pentoxifylline were observed to reduce the acute contractile response to cyclosporine of isolated glomeruli and mesangial cells in culture (Potier et al. 1997). Furthermore, the acute reduction of GFR and RBF caused in rats by a single dose of tacrolimus was completely reversed by theophylline given 1 h after the drug (McLaughlin et al. 2003a). Concomitant chronic administration of theophylline together with tacrolimus prevented the decrease of creatinine clearance that was caused by tacrolimus in the control group (McLaughlin et al. 2003b). In contrast, chronic administration of theophylline did not protect against cyclosporine-induced renal failure in rabbits and even enhanced its cytotoxic effects, whereas functional recovery was seen when theophylline was given as a single dose following a 5-day treatment with cyclosporine (Prevot et al. 2002). Follow-up studies that would shed light on the reasons for these divergent observations are not available. In children with nonrenal transplants who showed signs of tacrolimus nephrotoxicity, such as an increase in serum creatinine levels and oliguria despite treatment with loop diuretics, a single dose of aminophylline (5 mg/kg) caused a doubling of urine flow rate and osmolar clearance as well as a moderate increase of renal function (McLaughlin and Abitbol 2005).

6.5 Cisplatin

Cisplatin-based chemotherapy is another treatment modality that is often associated with nephrotoxicity. In a placebo-controlled trial on 36 patients, the administration of theophylline before and for 5 days following cisplatin treatment completely prevented the fall of inulin clearance that was seen in the placebo group, in which GFR fell by 21% (Benoehr et al. 2005). This study in humans is thus consistent with observations in rats in which aminophylline ameliorated cisplatin-induced renal failure when given in the maintenance phase although it did not prevent the decline of renal function when administered prophylactically (Heidemann et al. 1989). Since enprofylline did not mimic the protective action of aminophylline, adenosine receptor activation is the likely cause for the decline of renal function (Heidemann et al. 1989). Specifically, the protective action seems related to inhibition of A_1 adenosine receptors because the A_1-adenosine-receptor-specific antagonists

DPCPX and KW-3902 were effective both in preventing and in treating the nephrotoxic effects of cisplatin (Knight et al. 1991; Nagashima et al. 1995). A higher dose of cisplatin in vivo as well as exposure of LLC-PK1 cells to cisplatin caused upregulation of A_1 adenosine receptor expression, and this was associated with cytoprotection on the basis of the finding that nonselective and A_1-adenosine-receptor-selective antagonists exacerbated cisplatin-induced nephrotoxicity (Bhat et al. 2002; Pingle et al. 2004; Saad et al. 2004). Thus, the potential of methylxanthines to exert both protective and injurious effects may be a reflection of the wide spectrum of adenosine actions in fundamental processes such as tissue oxygen supply and inflammation.

6.6 Glycerol

Theophylline and other methylxanthines have been consistently found to ameliorate the experimental acute renal failure caused by intramuscular injection of glycerol when treatment was started at the time of injury (Bidani and Churchill 1983; Bowmer et al. 1986, 1988). The protective effect appears to be a consequence of inhibition of A_1 adenosine receptors since subtype-specific antagonists mimic the action of the natural methylxanthines (Ishikawa et al. 1993; Panjehshahin et al. 1992; Suzuki et al. 1992) and since their effect can be seen in a dose range that has no discernible effect on renal PDE activity (Panjehshahin et al. 1992). In contrast to the protective action of methylxanthines in myoglobinuric acute renal failure, theophylline did not improve renal function in the renal failure caused by mercury chloride or gentamicin (Kellett et al. 1988; Rossi et al. 1990).

6.7 Ischemia–Reperfusion

Interest in the potential of methylxanthines to improve renal function following postischemic acute renal failure has arisen from the fact that ischemia is associated with increases of adenosine tissue content in the kidney as well as in other organs (Osswald et al. 1977). Thus, it was conceivable that methylxanthines may exert beneficial effects by preventing the renal vasoconstriction caused by excess adenosine. In rats, theophylline, administered as a single dose of 100 μmol/kg 10 min before the release of a 1-h renal artery occlusion, increased GFR and electrolyte excretion threefold to sixfold within 3 h of the postischemic period compared with vehicle-treated animals (Osswald et al. 1979), an observation that was later confirmed in both rats and rabbits (Gouyon and Guignard 1988; Lin et al. 1986). The protective mechanism of methylxanthines in the initiation phase of renal injury following ischemia–reperfusion is likely related at least in part to inhibition of vasoconstrictive adenosine receptors. Support for this notion comes from a recent study in which the A_1 adenosine receptor antagonist DPCPX infused prior to and following a 30-min period of bilateral renal artery obstruction was observed to significantly improve creatinine clearance over the initial 4 h following reperfusion (Moosavi et al. 2009). Furthermore, the immediate postischemic reduction of GFR was enhanced by dipyridamole, an inhibitor of adenosine uptake through equilibrative nucleoside transporters, and this effect was abrogated by theophylline (Lin et al. 1987). In clinical studies, a single dose of theophylline given early after birth in asphyxiated full-term infants elicited beneficial effects by reducing the renal involvement and fall in GFR as determined over the first 5 days (Bakr 2005; Bhat et al. 2006; Eslami et al. 2009; Jenik et al. 2000).

The role of methylxanthines in the maintenance phase following renal ischemia is an area of considerable controversy. Early studies have shown that pretreatment of rats with a single dose of theophylline during a 30-min renal artery occlusion was associated with higher RBF and GFR during the maintenance phase of acute renal failure after 5 days, suggesting that theophylline administration in the acute phase affected the severity of renal failure in the maintenance phase (Lin et al. 1988). Furthermore, theophylline administered 5 days after ischemia acutely increased RBF and GFR in previously untreated rats (Lin et al. 1988). Theophylline also caused an increase of GFR measured 5 days after experimental renal transplantation in rats without affecting the inflammatory response (Grenz et al. 2006). On the other hand, in a small study with limited statistical power, theophylline was not found to afford protection against acute renal failure during cardiac surgery (Kramer et al. 2002). It is uncertain whether theophylline exerts these effects by antagonizing vasoconstrictor effects of adenosine. In fact, adenosine itself given immediately after a renal ischemia of 45 min provided renoprotection after 24 h, an effect mimicked by CGS-21680 and therefore apparently mediated by activation of A_{2a} adenosine receptors (Lee and Emala 2001). Similarly, the rise of serum creatinine level assessed 1 and 2 days following renal ischemia was found to be reduced by chronic administration of the selective A_{2a} agonist DWH-146 and enhanced in A_{2a}-adenosine-receptor-deficient mice (Day et al. 2003; Okusa et al. 1999). Relative renoprotection 24 h following renal ischemia was also provided by A_1 adenosine receptor agonists, and a worsening of the outcome was observed in A_1-adenosine-receptor-deficient mice (Kim et al. 2009; Lee et al. 2004a, b). Comparable renoprotective effects of A_{2a} and A_1 adenosine receptor activation suggest that the functional improvement after extended reperfusion is unrelated to the vascular actions of adenosine since the vascular effects of activating A_{2a} or A_1 adenosine receptors are opposite. The common denominator may be a dominant anti-inflammatory action of adenosine that is exerted by both receptor subtypes.

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The Cardiovascular Effects of Methylxanthines

Niels P. Riksen, Paul Smits, and Gerard A. Rongen

Contents

Abstract In the concentration range that is normally achieved in humans, e.g., after the drinking of coffee or in patients treated with theophylline, the cardiovascular effects of methylxanthines are primarily due to antagonism of adenosine A_1 and A_2 receptors. Inhibition of phosphodiesterases or mobilization of intracellular calcium requires much higher concentrations. In conscious humans, acute

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exposure to caffeine results in an increase in blood pressure by an increased total peripheral resistance, and a slight decrease in heart rate. This overall hemodynamic response is composed of direct effects of caffeine on vascular tone, on myocardial contractility and conduction, and on the sympathetic nervous system. Caffeine is the most widely consumed methylxanthine, mainly derived from coffee intake. Regular coffee consumption can affect various traditional cardiovascular risk factors, including a slight increase in blood pressure, an increase in plasma cholesterol and homocysteine levels, and a reduced incidence of type 2 diabetes mellitus. Although most prospective studies have not reported an association between coffee consumption and coronary heart disease, these findings do not exclude that the acute hemodynamic and neurohumoral effects of coffee consumption could have an adverse effect in selected patient groups who are more vulnerable for these effects, based on their genetic profile or medication use.

Keywords Adenosine · Blood pressure · Caffeine · Cardiovascular effects

1 Introduction

Systemic administration of methylxanthines can profoundly affect hemodynamic parameters, such as blood pressure and heart rate. These hemodynamic changes result from direct effects on myocardial contractility and conduction, on vascular tone, and on the sympathoadrenal system. The net effect on blood pressure and heart rate can differ substantially between the various methylxanthines. Thorough knowledge of these cardiovascular effects of methylxanthines is important for three reasons. Firstly, methylxanthine derivatives have been used in patients for their distinct cardiovascular effects, such as pentoxifylline in patients with peripheral artery disease. Secondly, these cardiovascular effects can explain some of the side effects of methylxanthine derivatives that are used in other patient group, e.g., as bronchodilators in patients with obstructive pulmonary disease. But most importantly, the cardiovascular effects of caffeine, which is the most widely consumed methylxanthine derivative, can have important effects on cardiovascular morbidity and mortality in the general population (Riksen et al. 2009).

In this chapter, we will discuss in detail the separate effects of methylxanthines on the heart, the blood vessels, the sympathoadrenal and renin–angiotensin system, and the integrative hemodynamic effects. We will discuss effects observed in *in vitro* experiments, and in animal experiments, but our main focus is on the effects observed in humans *in vivo* in concentrations that are reached in daily clinical practice. We will start with a brief summary of the molecular targets of the methylxanthines, which are relevant to their cardiovascular effects. Finally, we will discuss the potential impact of the cardiovascular effects of dietary caffeine, mainly derived from coffee consumption, on cardiovascular morbidity and mortality in the general population.

2 The Molecular Targets of the Methylxanthines

The molecular mechanisms of methylxanthines are diverse, and include inhibition of cyclic nucleotide phosphodiesterases, mobilization of intracellular calcium, and adenosine receptor antagonism. Francis et al. (2010), Guerreiro et al. (2010), and Müller and Jacobson (2010) are devoted to describing these mechanisms in detail. Here, we will briefly review the molecular targets that are relevant to the cardiovascular effects of the methylxanthines, particularly when they are given in concentrations within the therapeutic range.

Caffeine can increase the free intracellular calcium concentration of muscle cells by activation of ryanodine receptors, which are calcium-release channels located in the endoplasmic reticulum, but only in concentrations of more than 1 mM, which is much higher than the concentration reached after coffee consumption (Fredholm et al. 1999; McPherson et al. 1991). Butcher and Sutherland (1962) reported in the early 1960s that theophylline, caffeine, and theobromine inhibited bovine cyclic adenosine monophosphate (cAMP) phosphodiesterase with EC_{50} values of approximately 200–300 μM for theophylline, and 1,000 μM for caffeine and theobromine. These values are in the same range as those obtained in more recent studies (Fredholm 1984). Therefore, at the therapeutic range of 20–80 μM for theophylline there is relatively little phosphodiesterase inhibition. It is even more obvious for caffeine that the concentration required to inhibit phosphodiesterase is by far higher than the concentration reached after regular coffee consumption (Fredholm 1984).

As mobilization of intracellular calcium stores and inhibition of phosphodiesterase activity require millimolar and high micromolar concentrations of caffeine, these molecular targets do not explain the cardiovascular effects of caffeine in humans *in vivo*. In contrast, caffeine effectively antagonizes adenosine receptors in the concentration range reached after regular coffee consumption. Also, other methylxanthines, such as theophylline, have been shown to antagonize adenosine receptors in the therapeutic concentration range (Sattin and Rall 1970). Currently, four adenosine receptor subtypes have been identified, designated as adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors (Fredholm et al. 2001a). Traditionally, the adenosine receptors are classified on the basis of their ability to decrease or increase intracellular cAMP concentration: adenosine A_1 and A_3 receptors are coupled to G_i proteins and stimulation will decrease the intracellular cAMP level. In contrast, adenosine A_{2A} and A_{2B} receptor stimulation increases cAMP levels via G_s proteins (Fredholm et al. 2001a). Functional studies on human adenosine receptors using intracellular cAMP concentrations have shown that caffeine blocks adenosine receptors with K_b values of approximately 30, 10, 15, and more than 100 μM for the adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors, respectively, which are similar to the potencies reported earlier in studies using binding assays (Fredholm et al. 2001b). The effect of competitive antagonists is dependent on the occupancy of the receptors with the endogenous ligand. The levels of adenosine under baseline normoxic conditions are sufficient to activate the adenosine A_1, A_{2A}, and A_3 receptors. Therefore, caffeine is generally believed to act on the adenosine A_1 and A_{2A}

receptors to exert most of its effects. Theophylline and paraxanthine have slightly higher affinities than caffeine for the adenosine A_1, A_{2A}, and A_{2B} receptors and are also weak antagonists for the adenosine A_3 receptor (Fredholm et al. 2001b; Klotz et al. 1998). The affinities of other methylxanthines, including theobromine and pentoxifylline, are lower than that of caffeine (Fredholm and Persson 1982; Schwabe et al. 1985). Enprofylline has an affinity profile that differs from that of the other methylxanthines in that it has very low affinities for adenosine A_1, A_{2A}, and A_3 receptors, whereas it is as potent as theophylline in blocking the adenosine A_{2B} receptor (Feoktistov and Biaggioni 1995). Additional evidence that the hemodynamic effects of caffeine are indeed due to adenosine receptor antagonism has recently been obtained in a study in mice heterozygous for both the adenosine A_1 receptor gene and the adenosine A_{2A} receptor gene. In female control mice, 7 days of caffeine administration reduced the heart rate. In A_1/A_{2A} heterozygous mice, however, the baseline heart rate was lower, and was not affected by caffeine administration (Yang et al. 2009).

Two additional molecular targets of methylxanthines related to purine pharmacology have been reported, but, given the high concentrations required to activate these targets, both mechanisms are unlikely to contribute significantly to any cardiovascular effect of methylxanthines *in vivo*. At a concentration of 0.5 mM, theophylline, caffeine, and theobromide inhibit 5′-nucleotidase, isolated from rabbit kidney (Fredholm et al. 1978) and rat brain (Tsuzuki and Newburgh 1975). In addition, theophylline and caffeine can inhibit the rat nucleoside transporter with IC_{50} values of approximately 1 and 3 mM (Plagemann and Wohlhueter 1984).

On the basis of these pharmacology studies, it has been argued that adenosine receptor antagonism is the single most important mechanism underlying the cardiovascular effects of caffeine and theophylline in humans *in vivo* (Fredholm 1980; Fredholm et al. 2001a). On the other hand, other methylxanthines, which are more potent phosphodiesterase inhibitors, might act primarily though this mechanism (Kamphuis et al. 1994). Indeed, the hemodynamic effects of adenosine in humans *in vivo* are blunted by concomitant administration of caffeine (Smits et al. 1987, 1989) and theophylline (Biaggioni et al. 1991; Smits et al. 1990), but not enprofylline (Smits et al. 1989).

3 The Cardiovascular Effects of Adenosine Receptor Stimulation

To facilitate accurate prediction of the cardiovascular effects of methylxanthines, the effects of adenosine receptor stimulation by its endogenous ligand, and the potential effects of methylxanthines on adenosine receptor expression and function will be briefly discussed in this section. A series of experiments on human adenosine receptors using changes in intracellular cAMP levels revealed EC_{50} values for adenosine of approximately 0.3, 0.7, 24, and 0.3 μM for the adenosine A_1, A_{2A},

A_{2B}, and A_3 receptors, respectively (Fredholm et al. 2001b). As the endogenous adenosine concentration during normoxia is in the range 20–300 nM (Ramakers et al. 2008), the adenosine A_1 and A_3 and to a lesser extent the A_{2A} receptor can be activated by physiological concentrations of adenosine, but this is also dependent on the receptor density of the target cells (Fredholm et al. 2001b). In contrast, the low-affinity adenosine A_{2B} receptor is only activated in situations in which the adenosine concentration is increased, such as during ischemia or inflammation.

Interestingly, in addition to direct adenosine receptor antagonism, methylxanthines have also been reported to affect adenosine receptor density and the endogenous adenosine concentration. In humans, administration of caffeine (750 mg per day for 1 week) induced an upregulation of the adenosine A_{2A} receptor on thrombocytes accompanied by an increased potency of an adenosine A_{2A} receptor agonist to increase intracellular cAMP levels (Varani et al. 1999). In rats, administration of caffeine and 8-sulfophenyltheophylline was reported to increase the circulating adenosine concentration, but this finding has not been confirmed since then (Conlay et al. 1997).

It has been well established that systemic administration of adenosine in conscious humans elicits a typical hemodynamic response, consisting of an increase in heart rate and systolic blood pressure, and a slight drop in diastolic blood pressure (Biaggioni et al. 1986; Smits et al. 1987). This response is the overall result of various separate effects of adenosine on the myocardium, the vascular tone, and the activity of the sympathetic nervous system (Riksen et al. 2008; Rongen et al. 1997). In isolated heart preparations, adenosine has a negative inotropic, dromotropic, and chronotropic effect by activation of adenosine A_1 receptors (Belardinelli et al. 1989). In addition, adenosine can attenuate ischemia-, and catecholamine-induced cardiac arrhythmias, probably by preventing catecholamine-induced release of calcium from the sarcoplasmic reticulum via adenosine A_1 receptor stimulation (Song et al. 2001). With regard to vascular tone, adenosine induces vasodilation in most vascular beds via activation of the adenosine A_{2A} receptor (Belardinelli et al. 1998). Also in humans, selective activation of the adenosine A_{2A} receptor increases coronary blood flow (Lieu et al. 2007). Adenosine A_{2B} receptors can also contribute to the vasodilator effect of adenosine in humans (Kemp and Cocks 1999). In addition, experimental animal studies have provided evidence that adenosine A_1 receptor stimulation can attenuate the vasodilator effect of adenosine A_{2A} receptor activation (Tawfik et al. 2005) and that the adenosine A_{2B} and A_3 receptors can also contribute to coronary vasodilation in rats (Hinschen et al. 2003).

In addition to direct effects on the heart and vascular tone, adenosine can modulate the activity of the sympathetic nervous system. In conscious humans, adenosine has two distinct and opposite actions. Firstly, adenosine induces a reflex activation of the sympathetic nervous system by stimulation of adenosine-sensitive chemoreceptors located in the carotid body (Biaggioni et al. 1987; Timmers et al. 2004), and sympathetic afferents from the heart (Cox et al. 1989), kidney, and forearm skeletal muscle (Costa and Biaggioni 1994). Secondly, stimulation of presynaptic adenosine receptors can inhibit norepinephrine release from sympathetic nerve terminals (Rongen et al. 1996). *In vivo* studies have suggested that the adenosine A_{2A} and A_3

receptors are responsible for this prejunctional effect (Barraco et al. 1995; Donoso et al. 2006). The augmentation of sympathetic nervous system traffic by stimulation of the carotid chemoreceptors appears to explain most of the hemodynamic effects of systemic administration of adenosine in conscious humans: administration of adenosine into the aorta proximal to the carotid chemoreceptors increases blood pressure and heart rate, whereas injection distal to these receptors decreases blood pressure (by direct unopposed vasodilation) (Biaggioni et al. 1987). Also, systemic administration of adenosine in patients after bilateral carotid body tumor resection decreases blood pressure and does not affect muscle sympathetic nerve activity, in contrast to the effects in healthy volunteers (Timmers et al. 2004).

In addition to these hemodynamic effects, adenosine receptor stimulation affects the function of platelets and inflammatory cells, and modulates intrinsic tolerance against ischemia and reperfusion. Activation of adenosine A_{2A} receptors on human platelets inhibits their aggregation (Varani et al. 2000). The adenosine A_{2A} receptor is also the primary and dominant adenosine receptor subtype responsible for the potent anti-inflammatory effects of adenosine (Hasko et al. 2008). Finally, endogenous adenosine is an important mediator of myocardial ischemic pre- and postconditioning, which is defined as a reduction of lethal ischemia–reperfusion injury by a brief sublethal period of ischemia and reperfusion immediately before or after the lethal insult (Hausenloy and Yellon 2007). This protective effect is initiated by adenosine A_1 and A_3 receptor stimulation (Carr et al. 1997). In addition, stimulation of the adenosine A_{2A} receptor at the moment of myocardial reperfusion can also limit infarct size.

4 The Cardiovascular Effects of Methylxanthines

4.1 Overview of the Cardiovascular Actions of Methylxanthines

In healthy volunteers, acute exposure to caffeine in a dose that is regularly encountered after intake of two to three cups of coffee increases blood pressure by increasing total peripheral resistance without relevantly affecting cardiac output (Casiglia et al. 1991; Farag et al. 2005; Pincomb et al. 1985), although the heart rate often decreases slightly (Rongen et al. 1995). Exposure to extremely high plasma concentrations of methylxanthines, as occurs in theophylline intoxication, induces tachyarrythmias and hypotension (Woo et al. 1984).

Discussion of the mechanisms involved in these cardiovascular actions of methylxanthines should take into account the various targets of these compounds that are concentration-dependently engaged in the overall effect of methylxanthine exposure. Furthermore, a distinction between *direct actions* on the heart, blood vessels, and regulatory systems (in particular, the autonomic nervous system, adrenal glands and renin–angiotensin system) and *indirect or secondary effects* that may result, for example, from metabolic vasodilation in the coronary system in response to increased afterload or from baroreflex modulation of the autonomic nervous system or the renin–angiotensin system in response to blood pressure changes should be appreciated. Finally, since the adenosine receptors are an important target of methylxanthines at relevant concentrations that occur during daily life, the availability of endogenous extracellular adenosine, which may differ between organs, the conditions studied, and the experimental setups used should be taken into account.

To disentangle all these different factors that could potentially modulate the cardiovascular response to a methylxanthine, we will first describe the action of methylxanthines in isolated organs. Subsequently, we will discuss the actions of methylxanthines on the autonomic nervous system and the renin–angiotensin system.

4.2 Effect of Methylxanthines on Blood Vessels and Organ Perfusion

Most *in vitro* studies on the direct vasomotor action of xanthine derivatives in isolated vessel preparations have revealed a vasodilator action (Bardou et al. 2002; Brodmann et al. 2003; Grossmann et al. 1998; Harada et al. 1995; Lo et al. 2005; Sekiguchi et al. 2002). The explanation for the vasodilator response to methylxanthines in these isolated vessels is probably twofold: firstly, the concentrations used in these studies are relatively high, resulting in significant phosphodiesterase inhibition as reflected by increases in cAMP and cyclic guanosine monophosphate, two important vasodilating second messengers; secondly, in these denervated nonperfused vessels with low metabolic activity, extracellular adenosine formation is probably low, resulting in a reduced baseline contribution of adenosine receptor stimulation to vascular tone.

In humans, the vasomotor action of methylxanthines has been studied in the forearm model, a method to explore direct actions of drugs on vasomotor tone without relevant systemic exposure that could otherwise bias results by triggering central actions or reflex (counter) regulations. In these studies, both caffeine and theophylline induce a vasodilator response at relatively high doses without any effect on vascular tone at lower doses. Although both methylxanthines antagonize adenosine-induced vasodilation in this model, the vasodilator effect of methylxanthines on baseline tone cannot be explained by adenosine-receptor antagonism. Therefore, it is generally assumed that this action is mediated by phosphodiesterase inhibition. Alternatively, caffeine may augment endothelial nitric oxide release, resulting in vasodilation. However, although this phenomenon may occur in response to acetylcholine, an effect of caffeine on basal nitric oxide release in the forearm vasculature has not been demonstrated yet (Umemura et al. 2006). Furthermore, this observation contrasts with observations from others who reported a decrease in acetylcholine-mediated vasodilation in response to acute caffeine exposure (Papamichael et al. 2005). The lack of a vasoconstrictor response in the forearm suggests that in this particular vascular bed adenosine does not importantly contribute to basal vascular tone. Most likely, this is explained by the low metabolic rate of resting muscle, although there is evidence for extracellular adenosine formation in the forearm as shown by the vasodilator response to inhibition of the equilibrative nucleoside transporter by either draflazine or dipyridamole (Bijlstra et al. 2004; Rongen et al. 1996). The extracellular adenosine concentration is mainly determined, however, by the rapid uptake of extracellular adenosine by neighboring cells. As such, extracellular adenosine concentrations can still be very low despite significant extracellular adenosine formation. Caffeine inhibited vasodilation in response to exercise without affecting baseline blood flow to muscle, supporting the concept of a differential contribution of adenosine to vascular tone in resting and exercising muscle (Daniels et al. 1998).

In the heart, the metabolic rate is much higher than in resting skeletal muscle. In the human coronary circulation, theophylline reduces basal coronary blood flow, which has been taken as evidence for a role for adenosine in the basal regulation of coronary vascular tone (Edlund et al. 1995; Edlund and Sollevi 1995). However, to our knowledge, these observations have not been confirmed by other research groups. Furthermore, evidence in animals suggests that methylxanthines only reduce coronary flow in conditions of increased metabolic demand or reduced oxygen supply (Ishibashi et al. 1998; Melchert et al. 1999; Phillis et al. 1998), although others have found a vasoconstrictor response to aminophylline in coronary arteries *in vivo* in baseline conditions at low doses that did not affect myocardial contractility (Paoloni and Wilcken 1975).

In the kidney of hypertensive patients, caffeine inhibits adenosine-induced vasodilation but has no effect on basal renal flow. In anaesthetized rabbits, injection of the adenosine receptor antagonist 8-sulfophenyltheophylline into the renal artery did not affect baseline renal flow either (Eppel et al. 2006). Adenosine has a complex action in the renal circulation to serve the tubuloglomerular feedback: in the afferent arterioles adenosine induces vasoconstriction (adenosine-A_1-receptor-mediated), whereas in the efferent arterioles adenosine induces vasodilation by activation of adenosine A_2 receptors. Therefore, caffeine may not affect overall renal blood flow but may still affect glomerular circulation, resulting in disruption of the tubuloglomerular feedback system and an increase in the glomerular filtration fraction (Persson 2001). This action of caffeine and theophylline may contribute to their mild diuretic action.

Thus, despite an increase in total peripheral resistance after acute exposure to caffeine, investigations of the effect of methylxanthines on isolated vessels or organ perfusion have not revealed a consistent vasoconstrictor effect. An explanation for this apparent discrepancy may involve the lack of sensitivity to detect minimal vasoconstriction in individual vascular beds, or a significant vasoconstrictor effect in a vascular bed in which the effect of methylxanthines has not been extensively studied, such as the mesenteric circulation (Rutherford et al. 1981).

Apart from an effect on vascular tone in resistance vessels, caffeine has also been shown to affect conduit vessels: it adversely affects arterial stiffness (Karatzis et al. 2005; Mahmud and Feely 2001; Vlachopoulos et al. 2003) and reduces flow-mediated dilation in the brachial artery (Papamichael et al. 2005). Aortic pulse wave velocity, a measure of aortic stiffness, was associated with a higher incidence of coronary heart disease and cardiovascular mortality in a prospective study among healthy older subjects (Sutton-Tyrrell et al. 2005). The drinking of caffeinated coffee, but not decaffeinated coffee, acutely increased pulse wave velocity in healthy subjects (Mahmud and Feely 2001). Moreover, coffee drinking resulted in an increased augmentation index of the aortic pressure waveform, indicating increased wave reflection (Karatzis et al. 2005; Mahmud and Feely 2001). The intake of 250 mg of caffeine has been demonstrated to induce similar effects on arterial stiffness (Vlachopoulos et al. 2003), suggesting that caffeine is the compound in coffee responsible for the observed effects.

4.3 *Actions of Methylxanthines on the Heart*

Caffeine and theophylline antagonize the effects of endogenous adenosine on cardiac rhythm and conduction. Thus, in theory, these substances could increase the firing rate in the sinus node and increase atrioventricular nodal conduction velocity. At higher concentrations, methylxanthines also inhibit phosphodiesterases, resulting in augmentation of the β-adrenergic rise in intracellular cAMP levels. On top of that, through interactions with adenosine receptors in the autonomic nervous system, norepinephrine release in the heart may be increased (see Sect. 4.4). Therefore, it does not come as a surprise that abundant use of methylxanthines has been associated with cardiac arrhythmias (Agwunobi et al. 1996; Cannon et al. 2001; Ishida et al. 1996), in particular when combined with cocaine or amphetamines (McNamara et al. 2007; Mehta et al. 2004). However, in an unselected population the regular use of caffeine did not significantly modulate the risk for arrhythmias such as atrial fibrillation (Frost and Vestergaard 2005). Methylxanthines have been used successfully in the treatment of bradyarrhythmias, in particular in settings of increased extracellular adenosine appearance (Cawley et al. 2001; DeLago et al. 2008).

Aminophylline, caffeine, and theophylline have a direct positive inotropic action on myocardial cells (Dimarco et al. 1985; Paoloni and Wilcken 1975; Rutherford et al. 1981). The mechanism of this action depends on the concentration of the methylxanthine and the location (atrial versus ventricular tissue). In atrial tissue, adenosine A_1 receptor stimulation results in sarcolemmal hyperpolarization, which prevents calcium influx and subsequent contractility. This response is independent of sympathetic tone (Urquhart and Broadley 1992a, b). Blockade of these adenosine receptors with methylxanthines in the presence of endogenous adenosine will result in increased contractility. In ventricular myocardial cells, adenosine A_1 receptor stimulation only reduces contractility in the presence of β-adrenergic receptor stimulation by inhibition of β-adrenoceptor-induced activation of adenylate cyclase (Belardinelli et al. 1995). Therefore, blockade of these ventricular adenosine receptors by methylxanthines will only result in increased contractility in the presence of sympathetic tone. At higher methylxanthine concentrations, this positive inotropic action of methylxanthines will be augmented by inhibition of phosphodiesterase, resulting in a further increase in cAMP levels. When the concentrations are raised to high (toxic) levels, caffeine and theophylline increase cellular calcium influx by activating the ryanodine receptor (Kong et al. 2008), with a positive effect on contractility (Rasmussen et al. 1987)

Only recently, the effect of coffee and caffeine on ischemia–reperfusion injury of the myocardium has been investigated. It has consistently been shown in experimental studies that a brief period of ischemia increases the tolerance against a subsequent prolonged ischemic insult, a phenomenon which has been termed "ischemic preconditioning" (Yellon and Downey 2003). This mechanism has been implied to explain the observation that the infarct size is smaller in patients who had experienced angina in the hours preceding the acute myocardial infarction (Yellon and Downey 2003). It transpired that several drugs, including hydroxymethylglutaryl-CoA reductase inhibitors (statins), could mimic this infarct size-limiting effect. Indeed, it has recently been demonstrated that treatment with atorvastatin improved the outcome in patients undergoing primary percutaneous intervention for an acute myocardial infarction (Patti et al. 2007). Interestingly, the cardioprotective effect of both ischemic preconditioning and statins is mediated by adenosine receptor stimulation (Sanada et al. 2004; Yellon and Downey 2003). Consequently, consumption of caffeinated coffee, but not decaffeinated coffee, appeared to abolish the cardioprotective effect of atorvastatin in a rat model of myocardial infarction (Ye et al. 2008). Recently, similar results were obtained with rosuvastatin and intravenous caffeine administration in a human forearm model of ischemia–reperfusion injury (Meijer et al. 2009). Likewise, in two human experimental models of ischemia–reperfusion injury, the intravenous administration of a single dose of caffeine (4 mg/kg) completely blocked the protective effect of ischemic preconditioning (Riksen et al. 2006).

4.4 Action of Methylxanthines on the Autonomic Nervous System and the Renin–Angiotensin System

Acute exposure of humans to caffeine results in a doubling of the plasma epinephrine concentration without significantly affecting the plasma norepinephrine concentration (Smits et al. 1986), although a relatively small rise in plasma norepinephrine concentration has also been observed (Robertson et al. 1978). Comparison of the hemodynamic response to caffeine between healthy controls and bilaterally adrenalectomized patients (in whom plasma epinephrine remained undetectable) revealed that the adrenal release of epinephrine is only partially involved in the pressor response to caffeine (Smits et al. 1986).

Intravenous administration of caffeine reduces muscle sympathetic nerve activity in the human peroneal nerve of healthy volunteers, probably due to a baroreflex inhibition of sympathetic outflow in response to the increase in blood pressure (Notarius et al. 2001). Taken together, these results suggest that caffeine facilitates norepinephrine release from sympathetic nerve endings. However, it should be realized that plasma norepinephrine concentrations do not directly reflect norepinephrine release form nerve endings. Owing to an active norepinephrine reuptake mechanism and metabolism, only a small fraction of the released norepinephrine appears in the circulation. Nevertheless, this proposed action of caffeine is supported by the observation that both endogenous as well as exogenous adenosine reduce forearm norepinephrine release in humans as measured with a tracer technique that partially circumvents the problems in the interpretation of plasma norepinephrine levels (Rongen et al. 1998a, 1996). Furthermore, a wealth of preclinical evidence indicates that adenosine inhibits norepinephrine release from sympathetic nerve endings by activation of a presynaptic adenosine receptor (see Sect. 3). By blocking these receptors, caffeine could facilitate norepinephrine release, thus preventing a baroreflex reduction in plasma norepinephrine concentration. Presynaptic facilitation of norepinephrine release by a caffeine-induced release of epinephrine provides an alternative explanation for the unaffected or even increased plasma norepinephrine concentration in the setting of a baroreflex-mediated inhibition of sympathetic nerve traffic (Newton et al. 1999). This is supported by the observation in adrenalectomized patients that the plasma norepinephrine concentration decreased in response to caffeine, whereas it remained unaffected in healthy controls (Smits et al. 1986).

In patients with heart failure, a condition that increases the endogenous adenosine formation, methylxanthines either do not affect baseline muscle sympathetic nerve activity or increase this measure of postganglionic sympathetic activity (Andreas et al. 2004; Notarius et al. 2001), an observation, however, that has been disputed by Notarius et al. (2003). Interestingly, the exercise pressor reflex in patients with heart failure is strongly increased. Caffeine inhibited this exaggerated response in patients with heart failure, suggesting that in patients with heart failure, caffeine may prevent some adverse actions of endogenous adenosine, namely, the activation of metaboreceptors in exercising muscle (Notarius et al. 2001).

The reported effect of caffeine on the plasma renin activity varies from a fall in plasma renin activity (Smits et al. 1986) to an increase (Robertson et al. 1978). Direct infusion of caffeine into the renal artery did not affect plasma renin concentration (Wierema et al. 2005), suggesting that the previous observations after systemic exposure were confounded by the blood pressure response to caffeine, which could reduce renal renin release, by the diuretic action of caffeine on renal tubules or by caffeine-induced epinephrine release, which could potentially increase renal renin release.

In theory, methylxanthines could modulate the vascular release of or response to angiotensin II by blocking adenosine receptors (Lai et al. 2006; Taddei et al. 1991). Whether these actions have any relevance for the cardiovascular effects of systemic exposure to methylxanthines remains to be established. In the human forearm, angiotensin II receptor blockade did not affect the vasomotor response to adenosine, suggesting that adenosine–angiotensin II interactions are not relevant in peripheral vessels in humans (Rongen et al. 1998a). However, in the human renal circulation, the vasoconstrictor response to angiotensin II was reduced by oral caffeine administration (Brown et al. 1993), which is in agreement with observations in animals using 8-cyclopentyltheophylline, a methylxanthine with selectivity for the adenosine A_1 receptor (Lai et al. 2006).

5 The Cardiovascular Effects of Dietary Caffeine

Caffeine is by far the most widely consumed methylxanthine. Although caffeine is also present in tea, soft drinks, energy drinks, and chocolate, coffee is the most important source of dietary caffeine in adults. Experimental studies in healthy volunteers have demonstrated that the drinking of two cups of regular coffee acutely raises systolic and diastolic blood pressure and slightly lowers heart rate (Nurminen et al. 1999). It appears that caffeine is responsible for this pressor effect, as the same response is observed after administration of caffeine (Robertson et al. 1978), but not after the administration of decaffeinated coffee (Smits et al. 1985a).

5.1 Tolerance to the Cardiovascular Effects of Caffeine

When extrapolating these acute hemodynamic effects of coffee to the effects of its sustained consumption in daily life, one has to take into account tolerance to these acute effects. The pressor response and the increase in (nor)epinephrine levels in response to the administration of 250 mg of caffeine in healthy subjects was no longer present after 3 days of daily caffeine administration (Robertson et al. 1981). In addition, the daily drinking of caffeinated coffee acutely raised the blood pressure only in the first 5 days of the experiment (Ammon et al. 1983). More recent studies, however, have reported that only approximately half of all subjects showed complete tolerance to the acute pressor effect of caffeine, and that the others continued to show a significant pressor response with repeated dosing (Farag et al. 2005; Lovallo et al. 2004). In addition, after 1 week of regular coffee consumption (more than two cups per day), the pressor response to the administration of adenosine was still significantly attenuated 6 h after the last coffee intake (Rongen et al. 1998b). Furthermore, caffeinated coffee has been reported to consistently induce an acute increase in blood pressure in healthy regular coffee consumers, when administered after four to five half lives of caffeine in each subject (Smits et al. 1985b). Moreover, in this study, the increase in blood pressure appeared to be inversely related to the basal plasma caffeine concentration. These findings suggest that differences in the half life of caffeine could account for the observed interindividual variation in the development of tolerance to the acute effects of caffeine, although in another study the baseline saliva caffeine concentrations did not differ between the tolerant and nontolerant groups (Lovallo et al. 2004).

5.2 *The Effects of Long-Term Coffee Consumption on Cardiovascular Risk Factors*

5.2.1 Long-Term Effect on Blood Pressure

Evidence for a sustained effect of coffee consumption on blood pressure can be derived from randomized controlled trials, usually performed in small groups of healthy subjects for a maximum of approximately 14 weeks (Jee et al. 1999; Noordzij et al. 2005), or from cross-sectional or longitudinal observational studies in large populations. Two meta-analyses of these randomized controlled trials have concluded that regular coffee intake slightly increases blood pressure by 1.2/0.5 mmHg (Noordzij et al. 2005) or 2.4/1.2 mmHg (Jee et al. 1999) for systolic/diastolic blood pressure, respectively. Table 1 summarizes the randomized controlled studies on coffee intake versus no coffee intake on blood pressure which were included in these meta-analyses. Interestingly, the blood pressure elevations were larger in the studies using caffeine tablets then in the studies on coffee consumption, suggesting that other compounds in the coffee could potentially counterbalance the pressor effect of caffeine. Cross-sectional epidemiology studies have provided conflicting results, but these studies are notoriously prone to confounding as patients with hypertension may have been advised to moderate their coffee intake. Four longitudinal cohort studies have investigated the impact of coffee consumption on blood pressure and the incidence of hypertension (Hu et al. 2007; Klag et al. 2002; Uiterwaal et al. 2007; Winkelmayer et al. 2005). Klag et al. (2002) have reported that consumption of one cup of coffee is associated with a blood pressure increase of 0.19/0.27 mmHg; however, after adjustment for confounding factors, coffee consumption was not associated with incident hypertension in this cohort. In 155,594 women participating in the Nurses' Health Study, coffee consumption was not associated with the incidence of hypertension

Table 1 Summary of randomized controlled studies on the effect of long-term coffee intake versus no coffee intake on blood pressure

Reference	No. of subjects	Duration (days)	Type of coffee	Coffee (mL)	Caffeine (mg)	SBP change (mmHg)	DBP change (mmHg)
Bak and Grobbee (1990)	66	63	Filtered	700	469	6.1 (2.27)	3.0 (1.56)
Bak and Grobbee 1990)	62	63	Boiled	700	441	6.0 (2.17)	2.8 (1.77)
Burr et al. (1989)	54	28	Instant	1,235	741	2.9 (1.4)	−0.9 (1.20)
van Dusseldorp et al. (1991)	43	79	Boiled	900	774	3.5 (1.18)	0.9 (0.92)
van Dusseldorp et al. (1991)	42	79	Filtered	900	774	0.4 (0.98)	0.4 (0.90)
MacDonald et al. (1991)[a]	50	14	Instant	450	225	−0.7 (1.45)	0.1 (0.88)
Rakic et al. (1999)[b]	27	14	Instant	750	300	3.6 (1.60)	4.7 (1.20)
Rakic et al. (1999)	21	14	Instant	750	300	−1.6 (6.87)	−0.2 (4.18)
Rosmarin et al. (1990)	21	56	Filtered	540	270	2.1 (2.15)	−2.4 (2.45)
Superko et al. (1991)	120	56	Filtered	1,067	615	1.3 (1.57)	0.2 (1.18)
Superko et al. (1994)	99	56	Filtered	1,067	615	1.4 (1.51)	0.7 (1.29)

SBP systolic blood pressure, *DBP* diastolic blood pressure, Numbers in parenthesis indicate standard error values

[a] In patients with mild/moderate untreated hypertension

[b] In treated hypertensive patients

(Winkelmayer et al. 2005). More recently, Hu et al. (2007) have shown in a Finnish population that moderate coffee consumption (two to seven cups per day), but not heavy coffee consumption (8 cups or more per day), was associated with an increased risk for the initiation of antihypertensive drug treatment. In a Dutch population, coffee abstainers had a lower risk of hypertension than those with a coffee intake of more than zero to three cups per day (adjusted odds ratio 0.54, 95% confidence interval 0.31–0.92) (Uiterwaal et al. 2007).

5.2.2 Long-Term Effect on Plasma Cholesterol

In 1966 a significant correlation between coffee consumption and serum lipid concentrations was reported in men with coronary heart disease (Little et al. 1966). This possible association was subsequently studied in many cross-sectional observational studies (Thelle et al. 1987). The brewing method appeared to be a crucial factor in the cholesterol-raising effect of coffee. The diterpenes cafestol and kahweol, which are present in nonfiltered coffee, including boiled coffee, cafetière coffee, and Turkish coffee, but which are largely removed by filtering the coffee, appeared to be responsible for the increase in plasma cholesterol concentration (Urgert and Katan 1997). Indeed, only studies performed in populations drinking mainly boiled coffee or Turkish coffee showed an association between coffee consumption and the plasma cholesterol concentration (Jansen et al. 1995; Lindahl et al. 1991; Pietinen et al. 1990). A meta-analysis of randomized controlled trials in healthy subjects showed an average increase in total cholesterol of 0.31 mmol/L, particularly with consumption of six or more cups per day (Jee et al. 2001). Also in these studies, filtered coffee only slightly increased total cholesterol concentration, and did not increase LDL cholesterol concentration, in contrast to the studies using nonfiltered coffee. More recently, however, the drinking of 600 mL of filtered coffee daily for 4 weeks was shown to also significantly increase total cholesterol concentration (Strandhagen and Thelle 2003) and coffee abstinence for 6 weeks lowered total cholesterol concentration in healthy subjects normally consuming more than four cups of filtered coffee daily (Christensen et al. 2001), indicating that a cholesterol-raising effect is not absolutely restricted to unfiltered coffee.

5.2.3 Long-Term Effect on Plasma Homocysteine

Several cross-sectional studies have reported a positive dose-dependent relation between coffee consumption and the total plasma homocysteine concentration, with an increase of approximately 20% in the subjects with the highest coffee consumption (Nygard et al. 1997; Panagiotakos et al. 2004; Stolzenberg-Solomon et al. 1999). In addition, coffee abstinence for 6 weeks decreased the total plasma homocysteine concentration by approximately 1.5 μmol/L in subjects who were used to drinking four or more cups per day, indicating a causal relationship (Christensen et al. 2001). Subsequent randomized controlled trials in healthy volunteers have obtained some insight into the possible mechanism of the homocysteine-raising effect of coffee. Consumption of both filtered coffee (Verhoef et al. 2002) and unfiltered coffee (Grubben et al. 2000) for 2 weeks significantly increased plasma homocysteine levels. The observation that taking caffeine-filled capsules for 2 weeks also increased plasma homocysteine levels, but to a lesser extent than by drinking coffee, suggests that caffeine is partly, but not solely, responsible for this effect (Verhoef et al. 2002). Indeed, Olthof et al. (2001) demonstrated that a 7-day treatment with chlorogenic acid, one of the other components of coffee, also increased plasma homocysteine levels.

5.2.4 Long-Term Effect on Type 2 Diabetes Mellitus

In contrast to the potentially deleterious effect of coffee on plasma homocysteine and cholesterol levels, coffee consumption can also have beneficial effects with regard to cardiovascular health by reducing the incidence of type 2 diabetes mellitus. It was reported that subjects consuming seven or more cups of coffee per day had a relative risk of 0.50 (95% confidence interval 0.35–0.72) of developing type 2 diabetes mellitus compared with individuals drinking two or fewer cups per day (van Dam and Feskens 2002). A systematic review of nine prospective and seven cross-sectional cohort studies also showed a dose-dependent risk reduction for the development of type 2 diabetes, with a relative risk of 0.65 (95% confidence interval 0.54–0.78) and 0.72 (95% confidence interval 0.62–0.83) for drinking seven or more or five to six cups of coffee per day compared with two or fewer cups (van Dam and Hu 2005). More recent prospective studies have confirmed this beneficial effect of coffee intake on the incidence of type 2 diabetes mellitus (Paynter et al. 2006; Pereira et al. 2006; van Dam et al. 2006). Interestingly, in these latter studies, consumption of decaffeinated coffee protected to a similar extent against the development of diabetes as caffeinated coffee, which suggests that caffeine is not responsible for this observed effect (Pereira et al. 2006; van Dam et al. 2006). It has been speculated that chorogenic acid and antioxidants present in coffee could mediate this protective effect (Riksen et al. 2009).

5.3 Association Between Coffee Consumption and Coronary Heart Disease

As described in Sect. 4, administration of a single dose of caffeine induces various hemodynamic effects (Fig. 1). In addition, long-term caffeine administration can also affect hemodynamic parameters, as well as other independent cardiovascular risk factors (Fig. 1). As coffee is one of the most widely consumed beverages in the world, any possible association with the development of coronary heart disease might have a tremendous impact on the overall burden of disease. An impressive number of epidemiology studies have examined coffee as a potential risk factor for coronary heart disease. Four comprehensive meta-analyses of these trials have

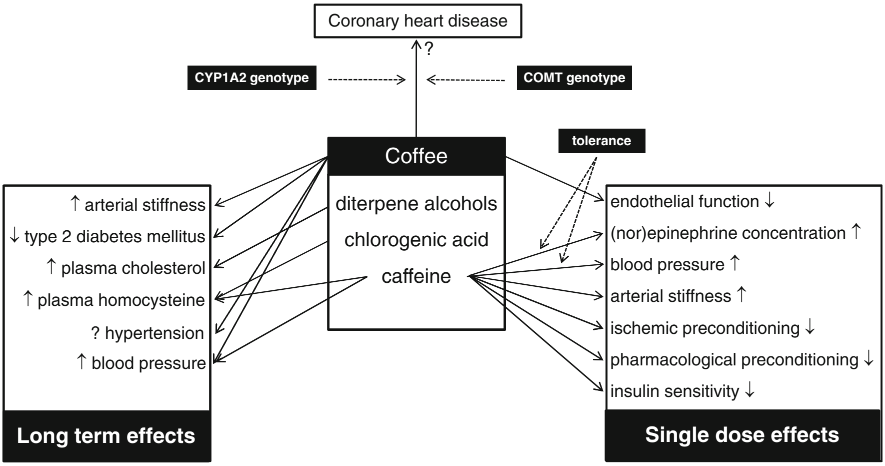

Fig. 1 Summary of the individual effects of coffee on cardiovascular parameters and risk factors, which could all contribute to an association between coffee consumption and cardiovascular disease. The effects of administration of a single dose of coffee are depicted on the *right*, whereas the effects of long-term coffee consumption are depicted on the *left*. It is indicated with *arrows* when an effect is attributed to one single component of coffee. The *text in the filled boxes* and the *dotted arrows* indicate effect modulators of specific effects of coffee (see the text). (Reproduced with permission from Riksen et al. 2009)

reported a positive association between coffee consumption and coronary heart disease in case-control studies, whereas there appeared to be no association, or a weaker association, in prospective cohort studies (Greenland 1993; Kawachi et al. 1994; Myers and Basinski 1992; Sofi et al. 2007). More recently, three large prospective studies concluded that coffee consumption does not increase the risk of an acute myocardial events and cardiovascular mortality (Lopez-Garcia et al. 2008; Rosner et al. 2007; Zhang et al. 2009). In contrast, case-control studies did show an association between caffeine intake and nonfatal myocardial infarction, with an estimated population attributable risk of 12.8% (95% confidence interval 5.9–25.7%) (Kabagambe et al. 2007; Klatsky et al. 2008).

In conclusion, prospective studies have not shown a consistent positive association between coffee intake and coronary heart disease, whereas retrospective studies in general do report such an association. This apparent discrepancy has often been explained in terms of the retrospective studies being more likely to suffer from bias and confounding, in particular recall bias (Kawachi et al. 1994; Sofi et al. 2007). However, this discrepancy could also be interpreted in favor of an acute rather than a chronic adverse effect of coffee on coronary heart disease: obviously, retrospective studies might provide a more accurate assessment of coffee intake in the period immediately before the coronary event, in contrast to the prospective studies, in which coffee consumption is generally assessed years before the event. Therefore, the discrepancy between the results of the case-control and prospective studies might also be compatible with the hypothesis that coffee has an acute adverse effect (i.e., triggering a coronary event) rather than a long-term adverse effect (i.e., promoting the development of atherosclerosis). The recent observation, in a cohort study of older subjects, that there is an inverse correlation, particularly in women, between coffee consumption and coronary calcifications, which are a marker for atherosclerosis in coronary arteries, also suggests that chronic coffee consumption does not adversely affect the development of atherosclerosis (van Woudenbergh et al. 2008). In contrast, experimental studies have consistently shown that coffee or caffeine acutely raises blood pressure, circulating levels of (nor)epinephrine, and arterial stiffness, and impairs endothelium-dependent vasodilation and ischemic and pharmacological preconditioning. It has generally been appreciated that acute coronary events can be triggered by physical and emotional stressors, which cause similar acute physiological hemodynamic and neurohumoral changes as coffee drinking. As such, coffee consumption could act as a trigger for coronary events. The recent finding, in a retrospective study, that there was an increased risk for acute myocardial infarction in the first hour after coffee consumption favors this hypothesis (Baylin et al. 2006).

Another factor that has to be taken into account when studying the association between coffee drinking and coronary heart disease is that several studies have suggested that there is a marked interindividual variation in the susceptibility to the adverse effects of coffee. In experimental studies, only half of all subjects show a complete tolerance for the acute hemodynamic and neurohumoral effects of coffee. Therefore, in the setting of chronic daily coffee consumption, some subjects could be more susceptible to the acute effects of coffee consumption than others. Interestingly, in a case-control study, coffee consumption was only associated with an increased risk of acute myocardial infarction in patients with the cytochrome P450 1A2 genotype, which predicts slow hepatic metabolism of caffeine, suggesting that caffeine plays a role in this association (Cornelis et al. 2006). Previous studies have suggested also that the effect of coffee on the plasma cholesterol and homocysteine concentrations shows interindividual variation, which is genetically determined (Strandhagen et al. 2004; Weggemans et al. 2001a, b). Finally, it has been examined whether the relation between coffee intake and coronary heart disease is dependent on the metabolism of circulating catecholamines. In a prospective study, the risk of an acute myocardial infarction in heavy coffee consumers with a low activity of catechol *O*-methyltransferase was higher than in participants with a high catechol *O*-methyltransferase activity (odds ratio 3.2, 90% confidence interval 1.2–8.4) (Happonen et al. 2006), suggesting that the acute effect of coffee on circulating catecholamines might be involved in the adverse cardiovascular effect of coffee.

In conclusion, a wealth of evidence demonstrate that chronic coffee consumption does not increase the risk of coronary heart disease in the general population. These findings do not exclude, however, that the acute hemodynamic and neurohumoral effects of coffee consumption could have an adverse effect in selected patient groups who are more vulnerable for these effects, based on their genetic profile or medication use (Riksen et al. 2009).

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Methylxanthines in Asthma

Stephen L. Tilley

Contents

Abstract Methylxanthines represent a unique class of drugs for the treatment of asthma. The methylxanthine theophylline has demonstrated efficacy in attenuating the three cardinal features of asthma – reversible airflow obstruction, airway hyperresponsiveness, and airway inflammation. At doses achieving relatively high serum levels in which toxic side effects are sometimes observed, direct bronchodilatory effects of theophylline are recognized. At lower serum concentrations, theophylline is a weak bronchodilator but retains its capacity as an immunomodulator, anti-inflammatory, and bronchoprotective drug. Intense investigation into the molecular mechanisms of action of theophylline has identified several different points of action. Phosphodiesterase inhibition and adenosine receptor antagonism have both been implicated in promoting airway smooth muscle relaxation and bronchodilation.

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Similar mechanisms of action may explain the inhibitory effects of theophylline on immune cells. At lower concentrations that fail to inhibit phosphodiesterase, effects on histone deacetylase activity are believed to contribute to the immunomodulatory actions of theophylline. Since anti-inflammatory and immunomodulatory effects of methylxanthines are realized at lower serum concentrations than are required for bronchodilation, theophylline's predominant role in asthma treatment is as a controller medication for chronic, persistent disease.

Keywords Adenosine receptors · Airway smooth muscle · Asthma · Mast cell · Methylxanthines · Phosphodiesterase · Theophylline

Abbreviations

ASM	Airway smooth muscle
cAMP	Cyclic AMP
cGMP	Cyclic GMP
HDAC	Histone deacetylase
ICS	Inhaled corticosteroids
PDE	Phosphodiesterase
PPARγ	Peroxisome-proliferator-activated receptor γ
RyR	Ryanodine receptor

1 Introduction

The methylxanthine theophylline is one of the most widely prescribed medications for the treatment of asthma worldwide. Guidelines for the treatment of asthma, however, recommend that theophylline be used as a second- and third-line agent owing to its potential for toxicity, and the availability of effective alternatives, namely, inhaled corticosteroids (ICS) and β-agonists. For patients who do not achieve optimal asthma control on ICS and β-agonists, or those without access to these medications, theophylline represents an important component of our therapeutic armamentarium.

2 Historical Background

As early at the eighteenth century, physicians recognized that strong coffee was effective at improving symptoms in asthmatics. In 1860, medical textbooks recommended coffee for the treatment of dyspnea in bronchial asthma (Salter 1860). In the early 1900s, the bronchodilating effects of 1,3,7-trimethylxanthine (caffeine) and 3,7-dimethylxanthine (theobromine) were demonstrated experimentally in bovine bronchial smooth muscle and guinea-pig lung (Trendelenburg 1912; Baehr and Pick 1913; Pal 1912). Shortly thereafter, pharmacologists at Johns Hopkins University set out to study the antispasmodic actions of a number of popular remedies for asthma, including methylxanthines, using bronchial smooth muscle strips from pigs. Macht and Ting (1921) published their findings in 1921, noting that 1,3-dimethylxanthine (theophylline) was a much more effective bronchodilator than caffeine. The first clinical description of theophylline use in asthmatics was reported by Hirsch (1922) from Germany in 1922, when he described four patients who responded well to the rectal administration of "Spasmopurin," a mixture of 66.7% theophylline and 33.3% theobromine. Hirsch also tested his theophylline/theobromine combination on bovine bronchial smooth muscle strips, noting smooth muscle relaxation, and thus concluded that dimethylxanthines act by producing relaxation of bronchial smooth muscle (Schultze-Werninghaus and Meier-Sydow 1982). Despite his recommendation in his publication that theophylline be considered for clinical use both acutely and prophylactically in asthma, methylxanthines did not receive further attention for this indication until 1936, when numerous antidotal reports emerged touting the efficacy of theophylline in patients with asthma. In 1937, two concurrent but independent clinical trials reported, as did Hirsch in 1922, that methylxanthines were efficacious in asthma. Interestingly, both investigations were initially focused on the use of aminophylline, the more soluble ethylenediamine salt of theophylline, as a diuretic for the relief of dyspnea in heart failure, only incidentally noting its efficacy in asthmatics. Herrmann et al. (1937 reported the "prompt, complete, persistent relief" of extreme dyspnea in 14 of 16 patients with status asthmaticus, while Greene et al. (1937) reported the relief of asthma in 11 allergic asthmatics treated with aminophylline, and showed improvement in pulmonary function following its administration. After the "rediscovery" of theophylline for bronchial asthma by Herrmann et al. and Greene et al. in the 1930s, numerous additional reports emerged on the subject, and the Food and Drug Administration approved the use of theophylline for asthma in the USA in 1940.

Among the available drugs for the treatment of asthma at the time (methylxanthines, anticholinergics, adrenergic agonists), the methylxanthines were effective bronchodilators that acted quickly and had a reasonable duration of action. As concerns about the safety of systemic use of adrenergic agonists rose, methylxanthines became the cornerstone of asthma therapy worldwide. Clinical trials clearly demonstrated that theophylline could reduce the symptoms of chronic asthma, improve lung function, improve exercise tolerance, reduce the need for rescue medication, and facilitate the withdrawal of oral corticosteroids (reviewed in McFadden 1985; Weinberger and Hendeles 1996)). Slow-release formulations of theophylline were developed, allowing the dosing interval to be increased from 6 to 12 h, and eventually to 24 h, thus improving patient compliance. The golden age of methylxanthines had been reached. However, owing to the development of more effective therapies, and rising concerns about potential toxicities of methylxanthines, the use of theophylline in industrialized nations began to fall.

Declines in theophylline use began with the development of selective β_2-adrenergic agonists, and studies demonstrating the inferiority of methylxanthines to these new agents in acute asthma exacerbations. In the 1980s, several studies from emergency departments reported that aerosolized albuterol, a selective β_2-agonist, consistently outperformed aminophylline and theophylline (Rossing et al. 1980; Fanta et al. 1986; Siegel et al. 1985), resulting in dramatic reductions in the use of methylxanthines for acute exacerbations of asthma.

With an increased appreciation that airway inflammation plays a critical role in the pathogenesis of asthma, potent ICS have been developed and have largely replaced theophylline as the mainstay of treatment for chronic persistent asthma in developed countries. In addition, continued investigation into the pathogenesis of asthma has resulted in the development of therapies targeting the leukotriene pathway (e.g., monteleukast, zileuton) and IgE binding to mast cells (omaluzimab). As a result, the use of theophylline as a controller therapy for chronic asthma has declined in parts of the world with access to these anti-inflammatory and immunomodulatory drugs.

3 Actions of Methylxanthines in the Lung

The primary effect of methylxanthines in the human asthmatic lung was for many years assumed to be their capacity to relax airway smooth muscle (ASM). More recent observations, however, suggest that additional mechanisms may be responsible for the therapeutic effects of this class of drugs. These mechanisms include inhibition of immune cell activation and proliferation, effects on mucociliary transport, and reduction in proinflammatory gene expression via the induction of histone deacetylase (HDAC) activity.

The bronchodilating effects of methylxanthines on ASM from animals was first reported in 1912, and shortly thereafter the efficacy of theophylline to relax ASM in bronchial strips from pigs was recognized (Trendelenburg 1912; Baehr and Pick 1913; Pal 1912). In the early 1980s, independent laboratories in France and Sweden demonstrated that theophylline relaxed ASM in both large and small airways from ex vivo human lung samples obtained from patients undergoing lung surgery (Guillot et al. 1984; Finney et al. 1985), suggesting a mechanism for the beneficial clinical effects of theophylline observed in asthmatics. Interestingly, these studies showed that approximately 25% of the specimens failed to relax with β-agonists, but demonstrated universal relaxation with theophylline. Theophylline is a relatively weak bronchodilator, with an EC_{50} for relaxation of human ASM in vitro ranging from 14 to 67 μg/mL (Guillot et al. 1984; Cortijo et al. 1993). Pharmacodynamic studies in humans have demonstrated dose-dependent bronchodilation with serum theophylline concentrations between 5 and 20 μg/mL, with maximal effects requiring the maintenance of serum levels at the upper end of this range (Mitenko and Ogilvie 1973a, b). Observations that lower serum concentrations of theophylline were effective at preventing exercise-induced bronchoconstriction and airway inflammation suggested that ASM-independent effects may be responsible for its efficacy. These observations were particularly important since the serum levels required for direct bronchodilation are difficult to achieve owing to dose-limiting toxicities of nausea, vomiting, and tachycardia.

Theophylline can inhibit bronchoconstriction induced by several stimuli, including exercise, methacholine, histamine, and antigens (Magnussen et al. 1987, 1988; McWilliams et al. 1984; Pauwels et al. 1985). For many of these stimuli, the bronchoprotective effects of theophylline are realized at low serum levels (4–10 μg/mL), and do not correlate with the degree of acute bronchodilation. These observations suggest that the bronchodilation and bronchoprotection afforded by theophylline may occur by independent mechanisms.

Mast cells are central to the pathogenesis of asthma, and bronchoconstriction resulting from a variety of stimuli occurs indirectly as a result of mast cell activation. The clinical observations of bronchoprotection by theophylline to challenge by numerous provoking stimuli suggest that theophylline may be acting on mast cells to produce its beneficial clinical effects. Indeed, studies with both rodent and human mast cells have shown that theophylline can dose-dependently inhibit mediator release by cells activated by antigens and other stimuli (Sydbom and Fredholm 1982; Pearce et al. 1982; Louis and Radermecker 1990; Weston et al. 1997).

Theophylline has demonstrated inhibitory effects on many other cell types important to asthma pathogenesis, including eosinophils, neutrophils, lymphocytes, and macrophages, as well as in animal models of asthma. Theophylline can inhibit eosinophil chemotaxis to a number of stimuli (Numao et al. 1991), reduce leukotriene C_4 release (Tenor et al. 1996), and reduce superoxide production by eosinophils (Yasui et al. 2000a). Theophylline induces apoptosis of activated eosinophils from asthmatics (Takeuchi et al. 1999). Similar to observations in eosinophils, theophylline can promote apoptosis and reduce chemotaxis and superoxide production by neutrophils (Yasui et al. 2000b). T-lymphocyte proliferation and activation can be suppressed by theophylline at concentrations that are used therapeutically in patients with asthma (Scordamaglia et al. 1988; Singer et al. 1992; Rosenthal et al. 1992). Theophylline can also suppress mitogen-induced secretion of proinflammatory cytokines from human peripheral blood lymphocytes (Scordamaglia et al. 1988; Prieur and Granger 1975). Finally, theophylline displays a number of inhibitory effects on macrophage function, including chemotaxis and migration into tissues and production of reactive oxygen species, arachidonic acid products, and proinflammatory cytokines (Stephens and Snyderman 1982; Godfrey et al. 1987; Calhoun et al. 1991; Bailly et al. 1990; Prabhakar et al. 1993). Very low concentrations of theophylline (1.8 μg/mL) can suppress endotoxin-induced TNF-α production by monocytes in vivo (Spatafora et al. 1994).

Methylxanthines have been shown to reduce allergic inflammation in many species, including rat, guinea pig, and rabbit (Pauwels 1987; Manzini et al. 1993; Ali et al. 1992). While the inhibitory actions of theophylline on each type of immune cell in vitro and in animal models strongly suggested that methylxanthines might reduce inflammation in asthmatics, the doses used in most of these investigations exceeded clinically achievable doses in humans.

A number of clinical studies have established that theophylline has clinically relevant anti-inflammatory properties in asthmatics in doses that produce little to no toxicity. In a double-blind, placebo-controlled study of chronic treatment of mild allergic asthmatics for 6 weeks using a theophylline dose that produced plasma levels of 6.6 μg/mL, biopsies following antigen challenge showed significantly reduced numbers of EG2+ eosinophils and CD4+ lymphocytes in the bronchial wall (Sullivan et al. 1994). In a similarly designed study, theophylline treatment resulted in reduced IL-4 expression and a decrease in the number of epithelial CD8+ cells in bronchial biopsies (Finnerty et al. 1996). Improved asthma control was observed in the theophylline-treated group. In patients with nocturnal asthma, low-dose theophylline has been shown to inhibit the early morning influx of neutrophils and eosinophils into the lung (Kraft et al. 1996). Finally, low-dose theophylline can reduce the number of CD4+ and CD8+ T cells in bronchoalveolar lavage fluid after allergen challenge (Jaffar et al. 1996). These reductions in lymphocyte numbers were observed in patients already using high-dose ICS, suggesting that the molecular mechanisms responsible for the anti-inflammatory effects of theophylline are different from those of corticosteroids.

4 Molecular Mechanisms of Action

Several mechanisms have been proposed for the observed bronchodilatory and immunomodulatory effects of theophylline in the asthmatic airway. Phosphodiesterase (PDE) inhibition and adenosine receptor antagonism are the most established. Effects on endogenous catecholamine release, calcium ion flux, modulation of HDAC activity, and induction of peroxisome-proliferator-activated receptor γ (PPARγ) expression have also been reported.

4.1 ASM and Bronchodilation

4.1.1 PDE Inhibition

Elevation of intracellular cyclic AMP (cAMP) concentration, and to a lesser extent cyclic GMP (cGMP) concentration, is a major mechanism promoting ASM relaxation and resultant bronchodilation. Theophylline is a nonselective inhibitor of PDE, the enzymes which break down cyclic nucleotides. As a result, theophylline treatment can increase intracellular cAMP and cGMP concentrations in ASM cells and by this mechanism induce bronchodilation (Fig. 1). Dose-dependent ASM relaxation and concomitant cAMP elevation has been demonstrated in ex vivo airways from both animals and humans (Rabe et al. 1995; Fredholm et al. 1979). Theophylline is a weak PDE inhibitor, and the degree of PDE inhibition is low at therapeutically achievable serum levels. For example, total PDE activity in

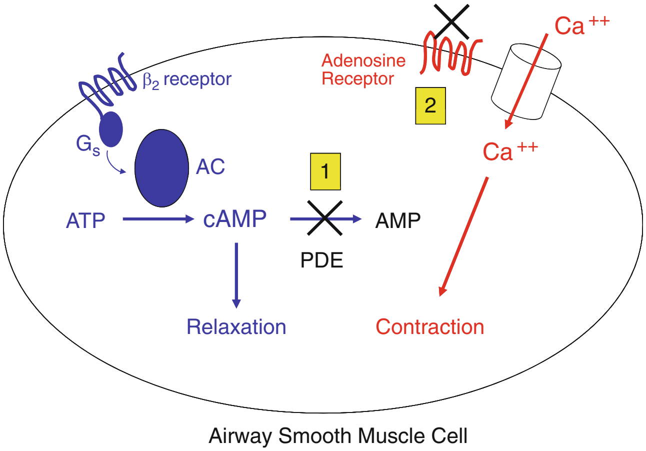

Fig. 1 Effects of methylxanthines on airway smooth muscle function. (1) Phosphodiesterase inhibition by methylxanthines inhibits cyclic AMP (*cAMP*) degradation and promotes airway smooth muscle (ASM) relaxation by raising intracellular cAMP levels. (2) Adenosine receptor antagonism. Activation of adenosine receptors increases intracellular calcium levels. In some species, adenosine activates adenosine receptors on ASM; in other species, the effect is indirect via activation of adenosine receptors on mast cells and neurons, resulting in paracrine signaling to ASM by additional mediators, promoting ASM contraction (leukotrienes, histamine). By antagonizing adenosine receptors, methylxanthines block calcium-activated ASM contraction

human lung extracts is inhibited by only 5–10% at therapeutic concentrations of theophylline (Polson et al. 1978). However, some PDE isoenzymes appear to be more sensitive, with selected isoforms demonstrating 50% inhibition by theophylline concentrations in the high-therapeutic range (18 μg/mL) (Bergstrand and Lundquist 1978). These data are consistent with clinical observations of increased bronchodilation if plasma theophylline levels are pushed to greater than 15 μg/mL. While the effects of theophylline on airway tone at lower plasma levels may be due in part to some degree of PDE inhibition, it is believed that its actions on other cell types may indirectly influence airway caliber.

4.1.2 Adenosine Receptor Antagonism

In addition to their actions as PDE inhibitors, methylxanthines also act as adenosine receptor antagonists (Fredholm et al. 1979; Fredholm and Persson 1982). Since exogenous adenosine produces bronchoconstriction in asthmatics, it has been postulated that theophylline's bronchodilatory actions may result from antagonism of adenosine receptors (Cushley et al. 1983a, b). The specific adenosine receptors involved in adenosine-induced bronchoconstriction have been extensively investigated. In rabbits, activation of A_1 receptors elicits ASM contraction ex vivo and bronchoconstriction in vivo (Ali et al. 1992, 1994a, b; Abebe and Mustafa 1998). In mice, adenosine-induced bronchoconstriction occurs indirectly through activation of A_1 receptors on neurons and A_3 receptors on mast cells (Hua et al. 2007; Tilley et al. 2003). ASM contraction occurs as a result of acute increases in intracellular calcium levels. Thus, methylxanthines can inhibit these actions in ASM directly through antagonism of adenosine receptors on ASM (Fig. 1), and indirectly by decreasing the levels of paracrine mediators released by adjacent or embedded mast cells through inhibition of mast cell activation by adenosine, as described in more detail below.

The pathways mediating adenosine-induced bronchoconstriction in human asthmatics have not been fully elucidated. The mechanism appears to be indirect through mast cell activation, since the response can be largely attenuated my mast-cell-stabilizing drugs and antihistamines (Phillips et al. 1987, 1989a, b). In contrast to rodents, in vitro studies with the malignant human mast cell line HMC-1 have implicated the A_{2B} receptor in adenosine-induced mast cell activation (Feoktistov and Biaggioni 1995; Feoktistov et al. 2001). Clinical studies have shown enprofylline to have superior efficacy to theophylline in the treatment of asthma (Persson et al. 1986). While initially felt to be devoid of antagonist activity at adenosine receptors, enprofylline is now believed to be a reasonably selective A_{2B} receptor antagonist (Fredholm and Persson 1982; Feoktistov and Biaggioni 1995; Auchampach et al. 1997). Collectively, these data have suggested the A_{2B} adenosine receptor as an attractive therapeutic target in asthma (Feoktistov et al. 1998).

4.1.3 Endogenous Catecholamine Release

Animal studies have demonstrated that methylxanthines provoke the release of epinephrine from the adrenal gland (Peach 1972; Poisner 1973; Berkowitz and Spector 1971). In normal humans 3 h following the administration of aminophylline (5 mg/kg), epinephrine levels increased twofold (Higbee et al. 1982). Similar effects of aminophylline were found in asthmatic children in a study which examined the relationship between pulmonary function and plasma catecholamine levels (Ishizaki et al. 1988). During a constant 72-h infusion of aminophylline, peak expiratory flow progressively increased during the first 48 h. In contrast, epinephrine levels rose rapidly but returned to normal levels within 24 h. These results suggest that methylxanthine-induced epinephrine release may contribute to the immediate bronchodilation observed by raising the cAMP concentration in ASM, via stimulation of β_2 adrenergic receptors but that the sustained and continued bronchodilatory effects of methylxanthines occur by an alternative mechanism.

4.1.4 Ryanodine Receptors and Calcium

A fourth mechanism by which methylxanthines may influence airway caliber is through their effects on intracellular calcium in ASM cells. Intracellular calcium regulates both ASM contraction and relaxation. While acute, step increases in intracellular calcium levels elicit ASM contraction via phosphorylation of myosin light-chain kinase, slower increases in intracellular calcium levels can dephosphorylate myosin light-chain kinase and induce relaxation. In 1977, it was suggested that calcium ion uptake into storage sites in smooth muscle cells could be influenced by theophylline (Kolbeck et al. 1979). Caffeine is now a well-recognized ryanodine receptor (RyR) agonist inducing calcium release from internal stores (Herrmann-Frank et al. 1999; Dettbarn et al. 1994; Cheek et al. 1993). Caffeine, aminophylline, and theophylline have all been demonstrated to potentiate luminal calcium activation of RyRs, reduce the threshold for spontaneous calcium release, and increase basal activity of RyRs in cardiac myocytes (Kong et al. 2008). While it has been postulated that these effects of methylxanthines on calcium homeostasis may be responsible for their arrhythmogenic potential, this same mechanism, if present in ASM, might explain the beneficial effects of these drugs on bronchomotor tone.

4.2 Molecular Mechanisms in Immune Cells

While PDE inhibition and cAMP level elevation results in inhibition of immune cell proliferation and activation, a number of lines of evidence suggest that the immunomodulatory effects of theophylline occur predominantly by alternative mechanisms. Unlike ASM, where relaxation in vitro is not observed following exposure to low concentrations of theophylline, inhibitory effects on many immune cells have been demonstrated at very low theophylline levels. Several mechanisms have been proposed for the immunomodulatory properties of low-dose theophylline.

4.2.1 Adenosine Receptor Antagonism

Adenosine can elicit both pro- and anti-inflammatory effects in immune cells as a result of signaling through G-protein-coupled receptors with different intracellular signaling pathways. A_1 and A_3 receptors couple to G_i, and their activation results in rises in intracellular calcium levels, which typically activates immune cells. As depicted in Fig. 2, antagonism of these adenosine receptors will inhibit immune cell activation. In contrast, A_{2A} and A_{2B} receptors couple to G_s, and their activation results in rises in intracellular cAMP levels, resulting in inhibition of immune cell function. In the HMC-1 mast cell line, A_{2B} receptors couple to G_s as well as G_q (Ryzhov et al. 2006). The complexity is further increased by the differential expression of each adenosine receptor by specific immune cells, changes in receptor expression during inflammation, differing ligand affinities of each receptor, and changing adenosine concentrations depending upon the intensity and duration of the asthmatic attack. In asthma, attention has been largely directed to the proinflammatory effects of adenosine.

In the asthmatic lung, adenosine acutely degranulates airway mast cells. Adenosine receptor antagonism by theophylline can block adenosine-induced mast cell

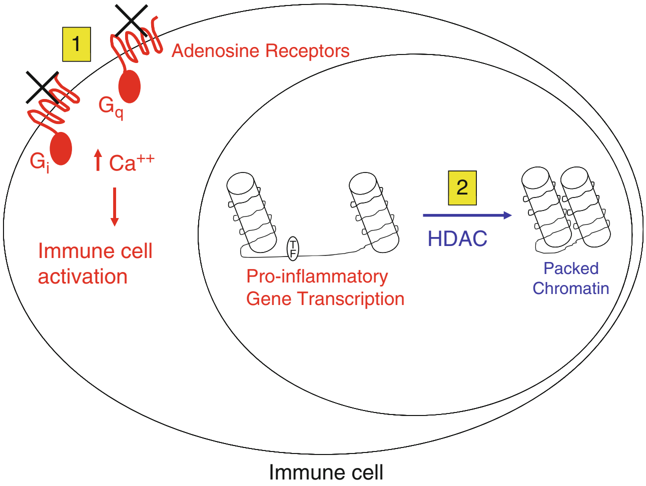

Fig. 2 Anti-inflammatory and immunomodulatory actions of methylxanthines in immune cells. (1) Methylxanthines antagonize cell-surface adenosine receptors, blocking the proinflammatory actions of adenosine on immune cells. (2) Methylxanthines increase histone deacetylase activity, promoting chromatin packing, preventing proinflammatory gene transcription

activation; thus, inhibition of mast cell function by blocking adenosine receptor–G_q–calcium signaling is one plausible mechanism of action of theophylline (Fig. 2). However, many immune cells, including mast cells, express G_s-coupled A_{2A} and A_{2B} adenosine receptors, and their activation results in the accumulation of intracellular cAMP, a potent inhibitor of immune cell activation and migration. Antagonism of these G_s-coupled receptors by theophylline would be expected to activate rather than inhibit the function of immune cells, including T-cells and eosinophils, suggesting that additional mechanisms may be responsible for the chronic, immunosuppressive effects of methylxanthines.

4.2.2 Modulation of HDAC Activity and Induction of PPARγ

The acetylation of core histones by coactivator proteins results in DNA “unwinding,” facilitating RNA polymerase binding and transcription of proinflammatory cytokines. Corepressor proteins that have HDAC activity suppress inflammatory gene transcription by promoting the repacking of chromatin (Fig. 2). In bronchial biopsies and alveolar macrophages from asthmatics, HDAC activity is reduced relative to that in normal subjects (Ito et al. 2002a; Cosio et al. 2004). Theophylline has been shown to directly increase HDAC enzymatic activity in airway epithelia and macrophages (Ito et al. 2002b). Neither PDE inhibition nor adenosine receptor antagonism mimicked the effects of theophylline on HDAC activity, supporting the conclusion that direct interactions of theophylline with HDAC proteins is a novel mechanism of action. While the exact means by which theophylline activates

HDAC is not certain, data from this study suggest that it may occur by allosteric action and/or phosphorylation. Since corticosteroids induce HDAC gene transcription, resulting in increased HDAC protein levels, theophylline and steroids may act synergistically to suppress proinflammatory mediator production by both resident structural cells and infiltrating immune cells in the asthmatic lung. Since low doses of theophylline can influence HDAC activity independent of PDE inhibition and adenosine receptor antagonism, clinically beneficial effects may be realized without the major recognized side effects of theophylline (nausea, vomiting, seizures, cardiac arrhythmias) which are believed to result from antagonism of these PDE enzymes and adenosine receptors. The human studies described below support the concept that these biochemical observations regarding HDAC activity may be clinically relevant.

PPARγ is a nuclear receptor regulating immune cell function. Synthetic agonists for PPARγ have been shown to attenuate factor-induced eosinophil survival and chemotaxis. In one study using human peripheral blood eosinophils, theophylline markedly enhanced both messenger RNA and protein levels of PPARγ in eosinophils (Usami et al. 2006). It is likely that several of the mechanisms described above act together to mediate the anti-inflammatory actions of methylxanthines.

5 Clinical Use of Methylxanthines for the Treatment of Asthma

5.1 Acute Severe and Near-Fatal Asthma

Methylxanthines are not generally recommended for patients presenting to the emergency department with acute exacerbations of asthma. Inhaled β-agonists in combination with early systemic steroids and anticholinergics are highly effective, and studies looking at the addition of intravenously administered aminophylline to this regimen have yielded mixed results (Fanta et al. 1986; Siegel et al. 1985; Wrenn et al. 1991; Ream et al. 2001; D’Avila et al. 2008). In a meta-analysis of 13 clinical trials comparing nebulized β-agonists with or without intravenous administration of aminophylline, no additional benefit from adding aminophylline was observed (Littenberg 1988). In addition to a potential lack of added efficacy, theophylline has a very narrow therapeutic/toxic index, and several studies have reported increased side effects when it is added to β-agonists therapy (Fanta et al. 1986; Siegel et al. 1985). In a study from England, out of 43 asthma deaths analyzed, there was a significantly greater percentage of toxic theophylline concentrations (21%) in patients who died compared with matched controls (7%) (Eason and Markowe 1989). Taken together, these studies suggest methylxanthines should not be used routinely for acute exacerbations of asthma. They may have a role, however, in patients presenting with status asthmaticus who are refractory to standard care or those with impending respiratory failure (Levy et al. 1998; Self et al. 2002; Aubier et al. 1981).

5.2 Chronic Asthma

Owing to the superior efficacy of ICS and long-acting β-agonists, methylxanthines are no longer recommended as first-line therapy for the treatment of chronic persistent asthma. However, numerous clinical trials with theophylline have demonstrated efficacy, and asthma guidelines continue to endorse the use of this class of drugs as an add-on therapy in noncontrolled asthmatics, and as alternatives to ICS and long-acting β-agonists (e.g., if access to these agents is restricted owing to financial or social barriers) (Bateman et al. 2008). Several trials have shown the efficacy of low-dose theophylline as an add-on therapy to ICS in chronic persistent asthma. Evans et al. (1997) reported that adding theophylline to low-dose ICS was as effective as doubling the ICS dose. Consistent with this study, subsequent trials have reported similar improvements in lung function and asthma control in subjects treated with low-dose ICS and theophylline compared with those taking high-dose ICS alone (Ukena et al. 1997; Lim et al. 2000; Spears et al. 2009).

6 Methylxanthines and Respiration

Methylxanthines are respiratory stimulants. They have been used clinically to treat infants with apnea of prematurity as well as adults with central sleep apnea and periodic breathing–Cheyne–Stokes respiration (Kelly and Shannon 1981; Espinoza et al. 1987; Dowdell et al. 1990). Apnea of prematurity occurs in 85% of infants born earlier than 34 weeks of gestation (Barrington and Finer 1991). Aminophylline, theophylline, and caffeine reduce the frequency of apnea and the need for mechanical ventilation, and are the mainstay of treatment for this common disorder (Henderson-Smart and Steer 2001).

The effect of theophylline on central apnea, periodic breathing, and obstructive sleep apnea has been examined in several clinical trials. A blinded, placebo-controlled study showed that theophylline administered intravenously overnight improved central, but not obstructive, apneas (Espinoza et al. 1987). In a small study of men with heart failure and periodic breathing with central apneas, orally administered theophylline decreased central apneas from a mean value of 26 per hour to six per hour (Javaheri et al. 1996). In contrast, studies in patients with obstructive sleep apnea have failed to show a significant improvement in apnea by methylxanthines (Guilleminault and Hayes 1983; Mulloy and McNicholas 1992).

The mechanism by which methylxanthines stimulate respiration has been extensively investigated, and several potential mechanisms have been proposed. These include augmentation of hypoxic and hypercapnic ventilatory responses, inhibition of the ventilatory depressant effect of adenosine, increasing metabolic rate, and improving respiratory muscle performance (Javaheri and Guerra 1990; Lakshminarayan et al. 1978; Murciano et al. 1987, 1984; Fredholm 1984; Eldridge et al. 1983). Relevant to sleep-disordered breathing associated with heart failure, theophylline exerts positive inotropic actions on the heart which may indirectly improve periodic breathing by decreasing circulation time.

7 Conclusions

Since their discovery, methylxanthines have cycled in and out of favor for the treatment of asthma in developed nations. Worldwide they continue to represent one of the most commonly used therapies for bronchial asthma. Recognition that methylxanthines have immunomodulatory effects at low serum concentrations, and that they may act synergistically with corticosteroids, suggests that they can be exploited as immunomodulators rather than bronchodilators – without systemic toxicity. Intense investigation into the cellular and molecular mechanisms by which caffeine and theophylline modulate airway inflammation in asthma has helped identify a number of novel biological pathways that may serve as future drug targets for asthma and other inflammatory diseases. Until such molecular scalpels are developed and tested, the continued use of theophylline will be important for relieving dyspnea and improving the quality of life for patients afflicted with this most common immune-mediated pulmonary disease.

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Methylxanthines and Inflammatory Cells

György Haskó and Bruce Cronstein

Contents

Abstract Both caffeine and theophylline have a variety of roles in regulating inflammatory responses. At pharmacologically relevant concentrations most of the effects of these commonly used methylxanthines are attributable to adenosine receptor blockade and histone deacetylase activation. In addition, at higher concentrations methylxanthines can suppress inflammation by inhibiting phosphodiesterases, thereby elevating intracellular cyclic adenosine monophosphate levels. In summary, methylxanthines regulate inflammation by multiple mechanisms.

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1 Introduction

Caffeine is a nonselective adenosine receptor antagonist, and it is believed that at concentrations achieved during normal human consumption, caffeine exerts its biological effects through antagonism of adenosine receptors (Fredholm et al. 1999). Similar to caffeine, theophylline is a nonselective albeit more potent antagonist of adenosine receptors (Fredholm et al. 2001). Adenosine is an extracellular purine nucleoside signaling molecule, which regulates cell and tissue function both in health and in disease (Hasko et al. 2008). Adenosine is generated following the degradation of its precursor, ATP, a process which can occur both extra- and intracellularly. ATP, a mostly intracellular molecule, is liberated from the cell following stressful and injurious events, and is metabolized to adenosine via a cascade of ectonucleotidases, including CD39 (nucleoside triphosphate diphosphorylase) and CD73 (5′-ectonucleotidase) (Yegutkin 2008). Adenosine that accumulates intracellularly following ATP metabolism is extruded from the cell via nucleoside transporters (Volonte and D'Ambrosi 2009). Cells of the immune/inflammatory system, including neutrophils, mast cells, endothelial cells, lymphocytes, and platelets, have been appreciated as the most prodigious sources of extracellular adenosine (Eltzschig et al. 2008; Hasko et al. 2008). In addition to serving as a source for adenosine release, immune cells are also among the most widely studied cell types targeted by the regulatory influences of adenosine (Deaglio et al. 2007; Erdmann et al. 2005; Feoktistov and Biaggioni 1995; Fozard et al. 1996; Hasko et al. 2007, 2008; Holgate 2005; Sitkovsky 2009).

2 Adenosine Receptor Expression in the Immune System

Adenosine produces its biological effects by binding to and activating one or more of four membrane-spanning adenosine receptors, designated A_1, A_{2A}, A_{2B}, and A_3. All four adenosine receptors contain seven transmembrane domains and couple to intracellular GTP binding proteins (G proteins). Adenosine elicits activation of A_1, A_{2A}, and A_3 receptors with EC_{50} values that range from 0.01 to 1 μM, and A_{2B} receptor activation occurs at adenosine levels that exceed 10 μM (EC_{50} 24 μM) (Fredholm et al. 2001). Because physiological adenosine concentrations are less than 1 μM, physiological levels of adenosine can activate only A_1, A_{2A}, and A_3 receptors, and A_{2B} receptor activation requires pathophysiological conditions (Fredholm 2007). In addition to adenosine concentrations at the cell surface, receptor density and the functionality of the intracellular signaling pathways coupled to adenosine receptors are also key factors in dictating the nature and magnitude of the effect of adenosine on the cell. For example, A_{2A} receptor activation inhibits production of the T helper (Th)-1-inducing cytokine interleukin (IL)-12 more potently by human monocytes that are pretreated with the proinflammatory cytokine IL-1 or tumor necrosis factor (TNF)-α, mediators that also increase A_{2A} receptor expression in these cells (Khoa et al. 2001). In addition, the effect of adenosine can also be affected by the polarized localization of adenosine receptors: A_3 adenosine receptors accrue at the leading edge of migrating neutrophils and are instrumental in directing the movement of cells in response to chemotactic mediators (Chen et al. 2006). Finally, it is important to keep in mind that results regarding adenosine receptor function in one species cannot be readily extrapolated to another one, because sequence differences in cloned adenosine receptors have been shown to be associated with differential pharmacological responses to selective agonists and antagonists. In this regard, A_3 receptors were not even discovered until they were cloned (Zhou et al. 1992), because the A_3 receptor, especially in rodents, is insensitive to caffeine and theophylline, antagonists which had been pivotal in identifying adenosine-receptor-mediated effects.

3 Adenosine Receptor Signaling in the Immune System

Adenosine receptors in general dictate cell function through coupling to G proteins, but some G-protein-independent actions have also been reported (Fredholm et al. 2007). Adenosine receptors were initially classified as A_1 [cyclic AMP (cAMP)-decreasing] or A_2 (cAMP-increasing) receptors (van Calker et al. 1979). Subsequently the cAMP-increasing A_2 receptors were divided into two groups: high-affinity A_{2A} receptors and low-affinity A_{2B} receptors (Bruns et al. 1986). More recent studies have revealed that in addition to A_1 receptors, A_3 receptors also decrease intracellular cAMP concentrations (Jin et al. 1997). In addition to signaling via the adenylyl cyclase–cAMP system, adenosine receptors can signal through a variety of other pathways. A_{2A} receptors, as other Gs-protein-coupled receptors, signal chiefly via the adenylate cyclase–cAMP–protein kinase A (PKA) canonical pathway, but they can also activate exchange factor directly activated by cAMP (Epac) (Fredholm et al. 2007). Signaling downstream from PKA occurs through phosphorylation of the transcription factor CREB on serine residue 133, leading to direct CREB-mediated transcriptional activation (Nemeth et al. 2003). Activated CREB can also modulate gene expression indirectly by competing with nuclear factor (NF)-κB or other transcription factors for an important cofactor, CBP (Fredholm et al. 2007). In other cell types, adenosine A_{2A} receptors stimulate collagen production via mitogen-activated protein (MAP) kinases (Che et al. 2007) and inhibit neutrophil superoxide production through activation of protein phosphatases (Revan et al. 1996). Furthermore, recent results implicated CEBPβ in the stimulatory effect of A_{2A} receptor agonists on IL-10 production by *Escherichia coli*-challenged macrophages (Csoka et al. 2007). A_{2B} receptor stimulation can induce both adenylyl cyclase activation via Gs and phospholipase C activation via

Gq (Feoktistov and Biaggioni 1997). Interaction between these two pathways is important for upregulation of IL-4 production by mast cells upon A_{2B} receptor activation (Ryzhov et al. 2006). Specifically, Gq-mediated activation of phospholipase Cβ causes calcium mobilization and an increase in NFATc1-dependent IL-4 transcription, whose response is further facilitated by Gs-mediated NFATc1 protein accumulation. Traditionally, A_3 receptor activation is linked to Gi-mediated inhibition of adenylyl cyclase and Gq-mediated stimulation of phospholipase C (Gessi et al. 2008) and A_3 receptors can activate phospholipase D, RhoA, WNT, MAP kinase and phosphatidylinositol 3-kinase pathways in governing cell function. For example, A_3-receptor-mediated augmentation of histamine released in sensitized murine mast cells was blocked by inactivating Gi proteins with pertussis toxin and by using pharmacological phosphatidylinositol 3-kinase inhibitors(Zhong et al. 2003). Caffeine and theophylline block A_1, A_{2A}, and A_{2B} receptors at pharmacologically relevant concentrations but it appears that these methylxanthines are not potent A_3 receptor antagonists (Fredholm et al. 1999). Although there is controversy regarding what constitutes relevant caffeine concentrations in the immune system (Horrigan et al. 2006), the inhibitory effect of adenosine on formyl-Met-Leu-Phe (fMLP)-stimulated respiratory burst in neutrophil leukocytes was reversed by 30 μM caffeine (Fredholm et al. 1996), a concentration that occurs in plasma following consumption of caffeine-containing beverages (Fredholm et al. 1999). In the same study, theophylline proved to be an antagonist of adenosine receptors and it was more potent than caffeine. In addition to its effect on neutrophil respiratory burst, caffeine inhibited the adenosine-receptor mediated accumulation of cAMP in rat thymocytes with a K_i value of approximately 20 μM (Fredholm and Sandberg 1983). Theophylline was slightly more potent and its K_i value was approximately 10 μM.

4 Caffeine and Theophylline Are Phosphodiesterase Inhibitors at High Concentrations

Higher concentrations of both caffeine and theophylline inhibit cAMP phosphodiesterase (PDE) (Beavo et al. 1971). Inhibition of PDEs by caffeine or theophylline generally requires concentrations of 100–1,000 μM, which typically exceeds the concentrations observed in blood following normal oral dosing and can be associated with toxicity (Sawynok and Yaksh 1993). Nevertheless, it is plausible that some of the biological effects of these methylxanthines are due to PDE inhibition.

5 Theophylline as a Histone Deacetylase Activator

Expression of inflammatory gene expression is governed by a balance between histone acetylation and deacetylation (Barnes 2006). Inflammatory stimuli activate transcription factors such as NF-κB, which leads to histone acetylation and increased inflammatory gene expression. This process is reversed by recruitment of histone deacetylases to the promoter of inflammatory genes. Recent studies have shown that theophylline at clinically relevant concentrations is an activator of histone deacetylases, which explains its anti-inflammatory effects especially in combination with glucocorticoids (Cosio et al. 2004).

6 Effect of Methylxanthines on Inflammatory Cells

6.1 Caffeine

On the basis of the fact that caffeine plasma concentrations rarely exceed 50–60 μM during normal human consumption (Fredholm et al. 1999), we will first consider studies that showed caffeine being efficacious at altering inflammatory cell function at these low concentrations. Sullivan et al. (1995) demonstrated that caffeine increased chemiluminescence and myeloperoxidase release by lipopolysaccharide (LPS)-primed, fMLP-stimulated mixed leukocyte cultures, and increases were already evident at 10 μM caffeine. Consistent with the fact that caffeine antagonizes adenosine receptors at this concentration, addition of the adenosine-degrading enzyme adenosine deaminase abolished the effect of caffeine, indicating that caffeine acted by altering an endogenous adenosine response. In addition, the possibility of PDE inhibition as a mechanism for the enhancing effect of caffeine on leukocyte activation was excluded by a lack of effect of caffeine on leukocyte cAMP levels. In a more recent study, 50 μM caffeine decreased TNF-α production by LPS-activated cord blood (neonatal) monocytes by 20% (Chavez-Valdez et al. 2009). In the same study, however, caffeine failed to affect TNF-α production by adult monocytes.

Caffeine at concentrations of 100 μM or higher appears to downregulate inflammatory cell function. Horrigan et al. (2004) found that caffeine suppressed TNF-α release by LPS-stimulated human whole blood at 100 μM by approximately 40% in a cAMP-PKA dependent fashion. In addition, the production of IL-1β, IL-12, and IL-10 was not affected by 100 μM caffeine (Horrigan et al. 2004). In another study, caffeine was shown to decrease colony formation in cultures of murine bone-marrow-derived macrophages, and again, 100 μM was the lowest effective concentration (Inouye and Wharton 1986).

6.2 Theophylline

Theophylline has been used as a bronchodilator in the therapy of asthma and chronic obstructive pulmonary disease (COPD) for several decades. Its therapeutic concentration is between 55 and 110 μM and it causes unacceptable side effects above 110 μM (Ito et al. 2002). It has long been suggested that theophylline may exert some of its beneficial effects in asthma and COPD by downregulating inflammation. One widely held view is that the beneficial effect of theophylline is secondary to its ability to block A_{2B} receptors (Hasko et al. 2009). This is based, in part, on recent in vivo evidence (Mustafa et al. 2007) documenting that selective antagonists of adenosine A_{2B} receptors inhibit airway inflammation and airway reactivity induced by allergen or AMP in a murine asthma model. The contribution of A_{2B} receptor blockade to the anti-inflammatory effects of theophylline is also underlined by the observation that at therapeutic concentrations, theophylline is a relatively potent A_{2B} receptor antagonist. Although A_{2B} receptors on mast cells seem to be major players in triggering the lung inflammatory response in asthma and COPD (Polosa and Holgate 2006), proinflammatory effects of A_{2B} receptor stimulation have also been observed with human bronchial smooth-muscle cells (Zhong et al. 2004), human bronchial epithelial cells (Zhong et al. 2006), and human lung fibroblasts (Zhong et al. 2005), which produce increased levels of IL-6 (Zhong et al. 2004, 2005) and IL-19 (Zhong et al. 2006) following A_{2B} receptor activation.

In addition to A_{2B} receptors, theophylline blocks A_1 and A_{2A} receptors at therapeutically relevant concentrations and thus it can reverse the effects of endogenously released adenosine, resulting in both pro- and anti-inflammatory effects depending on which adenosine receptors are expressed. For example, 10 μM theophylline enhanced TNF-α production by human monocytes stimulated with advanced glycation end products, an effect that was postulated to be secondary to blockade of anti-inflammatory adenosine (A_{2A} or A_3) receptors on monocytes (Meiners et al. 2004). Similar to TNF-α production by monocytes, theophylline at therapeutically relevant concentrations (maximal efficacy at 50 μM) augmented superoxide production by fMLP-activated neutrophils (Yasui et al. 2000b). Because 8-sulfophenyltheophylline, a nonselective adenosine receptor antagonist, reproduced the stimulatory effect of theophylline on superoxide production, it was concluded that theophylline acts by antagonizing adenosine receptors. Finally, 50 μM theophylline was shown to accelerate human granulocyte apoptosis (Yasui et al. 1997, 2000a), an observation consistent with an adenosine-receptor-mediated effect, because adenosine can delay the apoptosis of granulocytes (Walker et al. 1997) and other adenosine receptor antagonists can mimic the effect of theophylline (Yasui et al. 2000a).

In addition to blockade of adenosine receptors, recent studies have revealed a further mechanism that can explain the anti-inflammatory effects of theophylline in asthma. This new evidence is based on the idea that inflammatory gene expression is regulated by a balance between histone acetylation and deacetylation (Barnes 2006). It appears that theophylline is able to tip this balance in favor of histone deacetylation, resulting in decreased inflammatory gene expression (Ito et al. 2002).

Lastly, theophylline has anti-inflammatory effects in various in vitro cellular systems when used at therapeutically irrelevant, high concentrations. Theophylline inhibited TNF-α production by LPS-stimulated human mononuclear cells at 200–1,000 μM (Endres et al. 1991); this effect was mediated by inhibition of PDE. In addition, theophylline decreased arachidonate (Hichami et al. 1995), thromboxane B_2, (Baker and Fuller 1992), and lysosomal enzyme (Hichami et al. 1995) release by stimulated mononuclear cells at supratherapeutic concentrations.

7 Enprofylline is Anti-inflammatory by Blocking A_{2B} Receptors

On the basis of the efficacy of theophylline as an antiasthma drug (Holgate 2005), efforts to develop a similar xanthine-based compound with a better safety profile led to the development of enprofylline (3-propyl xanthine). It was subsequently demonstrated that similar to theophylline, enprofylline can weakly block A_{2B} receptors on mast cells, which might explain its efficacy as an antiasthma agent. The advantage of enprofylline over theophylline is that enprofylline is a selective A_{2B} antagonist, whereas theophylline blocks other adenosine receptors as well (Fozard and Hannon 1999).

8 Effects of Methylxanthines in Inflammatory States

8.1 Caffeine Exacerbates Acute Inflammatory Liver Injury by Blocking the Physiological Anti-inflammatory Effect of Endogenous Adenosine

Ohta and Sitkovsky (2001) demonstrated that endogenous adenosine by engaging A_{2A} receptors has a nonredundant role in the prevention of inflammatory liver damage induced by concanavalin A. They showed that mice deficient in A_{2A} receptors exhibited increased and protracted production of proinflammatory cytokines, including TNF-α and interferon-γ, which was paralleled by augmented biochemical and histological signs of liver injury. These results raised the possibility that caffeine acting as an A_{2A} receptor antagonist might interfere with the endogenous protective mechanism rendered by adenosine–A_{2A} receptor interaction. To address this possibility, Ohta et al. (2007) injected mice with 20 mg/kg caffeine, which corresponds with caffeine amounts ingested during normal human consumption (Fredholm et al. 1999), before inducing liver injury using concanavalin A. The results showed that caffeine exacerbated liver injury as determined by biochemical and histological analysis, and the increased liver injury coincided with increased production of harmful proinflammatory cytokines.

To investigate the role of A_{2A}-receptor-mediated signaling in the exacerbation of liver injury following caffeine administration, caffeine (10 and 20 mg/kg) was injected into A_{2A} receptor knockout and wild-type mice and liver injury was induced using concanavalin A (Ohta et al. 2007). While caffeine produced an exacerbated liver injury in wild-type mice, knockout mice failed to respond to caffeine with increased acute liver injury, confirming that caffeine enhances liver injury through blocking the action of endogenous adenosine. Importantly, when caffeine was administered at the supratherapeutic dose of 100 mg/kg, it no longer enhanced inflammation; it actually suppressed it. This anti-inflammatory effect is consistent with caffeine being a PDE inhibitor at high concentrations, because other PDE inhibitors have similar anti-inflammatory effects in general acute inflammation (Hasko et al. 1998; Nemeth et al. 1997).

8.2 *Caffeine and Theophylline Reverse the Anti-inflammatory Effects of Methotrexate*

Low-dose orally administered methotrexate is currently the gold standard therapy for the treatment of rheumatoid arthritis. Recent studies have demonstrated that most of the anti-inflammatory effects of methotrexate are mediated by enhanced adenosine release and activation of anti-inflammatory adenosine receptors (Cronstein 2005). Indeed, most of the genetic markers associated with response or lack of response to methotrexate are in enzymes inhibited by methotrexate polyglutamates that are involved in adenosine generation (Dervieux 2009; Dervieux et al. 2004, 2005, 2009; Weisman et al. 2006; Wessels et al. 2006a, b).

Because it is difficult to sample adenosine levels at inflamed sites in patients with rheumatoid arthritis and it is, therefore, difficult to validate the hypothesis that adenosine mediates the anti-inflammatory effects of methotrexate therapy, experiments were carried out with the adjuvant arthritis model of rheumatoid arthritis using adenosine receptor antagonists. In these studies, both theophylline and caffeine reversed the anti-inflammatory effects of methotrexate in rats with adjuvant arthritis (Montesinos et al. 2000) without affecting the course of the arthritis itself. This finding suggested that such commonly used adenosine receptor antagonists as caffeine might reverse the therapeutic effects of methotrexate therapy in patients as well. Indeed, a small prospective study by Nesher et al. (2003) demonstrated that patients started on methotrexate but told to abstain from caffeine responded significantly better than those who did not. Similar observations were made by Silke et al. (2001) of methotrexate in patients as well. In contrast, a larger retrospective study (Benito-Garcia et al. 2006) did not confirm the effect of caffeine consumption on the response to methotrexate; however, since all of the patients in this study had been taking methotrexate for longer than 2 years, it is likely that any patients who did not respond at all had already stopped methotrexate therapy by the time that the survey was done.

9 Conclusion

Both caffeine and theophylline play a variety of roles in regulating inflammatory responses. Although many of the effects of these commonly used methylxanthines are attributable to adenosine receptor blockade, these methylxanthines regulate inflammation by multiple mechanisms.

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Methylxanthines, Inflammation, and Cancer: Fundamental Mechanisms

Akio Ohta and Michail Sitkovsky

Contents

Abstract Methylxanthines are an integral part of everyday food and drink consumption even though the majority of humans do not identify them by their chemical name. The breakthrough in understanding the action(s) of methylxanthines was in large part due to the understanding that methylxanthines can function as antagonists of adenosine receptors. This represented an example of scientific search and was instructive in view of both new therapeutic options and alarming realizations. It was the subsequent demonstration of the in vivo critical role of A2A adenosine receptors in controlling excessive collateral inflammatory damage that attracted the attention

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of immunologists to the A2A-adenosine-receptor-antagonizing methylxanthines. We summarize here data showing that caffeine is capable of preventing the inhibition of antitumor T cells in a hypoxic tumor microenvironment. On the other hand, caffeine may exacerbate liver damage by weakening the tissue-protecting A2A adenosine receptor signaling during episodes of acute liver inflammation. However, methylxanthines may also prevent the excessive hepatic connective tissue deposition that is associated with the progression of chronic hepatitis to cirrhosis, which is one of the common causes of mortality.

Keywords Adenosine · Adenosine receptor · Autoimmunity · Caffeine · Hepatitis · Hypoxia · Tumor

Abbreviations

A2AR	A2A adenosine receptors
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
cAMP	Cyclic AMP
Con A	Concanavalin A
GGT	γ-Glutamyltransferase
LPS	Lipopolysaccharide

In this chapter we will provide an overview of both pro- and anti-inflammatory properties of caffeine. This is potentially important since caffeine is now consumed without appreciation that it may affect the course of ongoing inflammatory response. Caffeine has long been considered to be anti-inflammatory because of its activity as a phosphodiesterase inhibitor (Horrigan et al. 2006). To inhibit phosphodiesterase, however, caffeine must be present at a high concentration, which may not be achieved during normal consumption in humans (May et al. 1982; Smith et al. 1982; Tiffin et al. 1995). It was subsequently understood that the effects of caffeine at doses that reflect the routine levels of consumption are mediated by the ability to antagonize the adenosine receptors (Fredholm et al. 1999). Recent studies investigated adenosine receptor antagonism by clinically relevant dose of caffeine and found that caffeine rather promotes inflammation (Ohta et al. 2007), which is opposite to the effects as a phosphodiesterase inhibitor. In this chapter, we discuss the effects of this representative methylxanthine on inflammation, focusing on pathogenesis of inflammatory liver diseases. We also describe the possible use of caffeine as a proinflammatory anticancer drug to be combined with tumor immunotherapy.

1 Caffeine and Liver Inflammation

1.1 Possible Prevention of Liver Diseases by Habitual Coffee Consumption

Inflammation of the liver results from a variety of causes. The most prevalent cause of hepatic inflammatory diseases is hepatitis virus infection (Lai et al. 2003; Poynard et al. 2003). There are also many patients suffering from alcohol-associated hepatitis, autoimmune hepatitis, and drug-induced liver injury (Lee 2004; Czaja 2005). After the acute phase, different degrees of severity of inflammation may persist as chronic hepatitis in a considerable number of viral, autoimmune, and drug-induced hepatitis patients. The repeated liver tissue remodeling caused by frequent inflammation often leads to development of fibrosis and cirrhosis (Nakamoto et al. 1998; Freeman et al. 2003; Fattovich et al. 2004). Furthermore, repeated liver inflammation may result in the development of liver cancer (Nakamoto et al. 1998; Fattovich et al. 2004).

Inflammatory cells play a major role both in liver damage and in tissue repair (Chisari 1997; Rehermann 2000; Ohta et al. 2000). The immune response of viral antigen-specific T cells plays a pivotal role in the destruction of infected hepatocytes. Recent studies have suggested that even in cases of chemical hepatotoxicity, immune cells, including T cells, participate in expanding liver damage after initial chemical insult (Luster et al. 2001; Simeonova et al. 2001). Furthermore, T cells are suggested to regulate development of cirrhosis by affecting fibrogenesis after hepatic damage (Shi et al. 1997; Safadi et al. 2004).

During the last two decades, epidemiology studies consistently showed an inverse relation between habitual coffee consumption and the risk of liver diseases. Coffee consumption has been shown to inversely correlate with the risk of cirrhosis (Klatsky and Armstrong 1992; Klatsky et al. 1993; Corrao et al. 2001; Tverdal and Skurtveit 2003; Ruhl and Everhart 2005a). In 1992, it was reported that the risk of alcoholic cirrhosis decreased to 20% by consumption of four or more cups of coffee per day (Klatsky and Armstrong 1992). The same dataset also showed a lower risk of cirrhosis death in coffee drinkers (Klatsky et al. 1993). Subsequent studies from Italy showed a decreased risk by coffee drinking in both alcoholic and nonalcoholic cirrhosis (Corrao et al. 2001). The relative risk was correlated with the amount of coffee, e.g., 47% in those who drink one cup per day, 23% for two cups, 16% for four or more cups compared with noncoffee drinkers. A mortality follow-up study from Norway confirmed the existence of an inverse relation between coffee and cirrhosis (Tverdal and Skurtveit 2003). In a recent study from the USA, subjects who consumed more than two cups of coffee had a lower incidence of chronic liver diseases (relative risk 0.43) compared with those who consumed less than one cup per day (Ruhl and Everhart 2005a). Such a protective effect of coffee was clear in the high-risk group for liver diseases, i.e., heavy alcohol intake, overweight, diabetes, or high iron load.

The serum levels of γ-glutamyltransferase (GGT), a relevant marker of cirrhotic risk, were found to be lower in coffee drinkers. GGT levels decreased in association with coffee consumption in the general population (Casiglia et al. 1993), more evidently among heavy alcohol drinkers and heavy smokers (Tanaka et al. 1998). There is also an inverse relation between coffee and serum transaminase levels, aspartate aminotransferase (AST) and alanine aminotransferase (ALT). In the general population, the relative risk for elevated AST and ALT levels (more than 40 U/L) decreased in coffee drinkers, correlating with the daily coffee consumption (Honjo et al. 2001). A study from the USA showed that lower ALT levels (less than 43 U/L) were associated with increasing coffee consumption. The relative risk was 0.56 in persons who drank more than two cups compared with noncoffee drinkers (Ruhl and Everhart 2005b). The observed protective effect of coffee was independent of the type of risk for liver injury. These studies indicate that coffee consumption is associated with decreased hepatocyte damage in individuals at high risk of liver injury.

For caffeine-containing beverages other than coffee, a few studies have shown controversial results. The epidemiological relation between caffeine consumption and liver diseases, however, is not as clear as discussed above for coffee consumption. Initially, tea and other caffeine-containing beverages were reported to have no correlation with serum GGT levels and cirrhosis (Klatsky et al. 1993; Corrao et al. 2001). However, recent studies have shown an inverse relation between tea consumption and serum ALT levels and the incidence of chronic liver diseases in the high-risk population (Ruhl and Everhart 2005a, b). Indeed, tea consumption was as effective as coffee consumption in the latter studies.

1.2 Caffeine May Promote the Induction of Acute Hepatitis

Biological effects of caffeine have been studied for a long time (Fredholm et al. 1999; Foukas et al. 2002). Importantly, caffeine can modulate intracellular levels of cyclic AMP (cAMP), which is a regulator of immune cell functions. The increase of the level of cAMP inhibits activation of inflammatory effector cells such as T cells and granulocytes. The induction of cAMP strongly inhibits oxidative burst in neutrophils and suppresses inflammation (Nielson et al. 1990). In T cells, cAMP downregulates various cellular functions, including proliferation, cytokine production, and cytotoxicity mediated by Fas ligand and granule exocytosis (Sitkovsky et al. 1988; Sugiyama et al. 1997). Caffeine is capable of increasing cAMP levels through inhibition of cAMP phosphodiesterase (Fredholm et al. 1999). Caffeine can exert an anti-inflammatory effect, which is a property shared with many other cAMP phosphodiesterase inhibitors. When rats were injected with D-galactosamine and/or lipopolysaccharide (LPS), coffee and other caffeine-containing beverages decreased liver damage, but decaffeinated coffee was not hepatoprotective (He et al. 2001; Akashi et al. 2009). An anti-inflammatory effect of caffeine was directly implicated in the suppression of liver damage by a high dose of caffeine.

Anti-inflammatory action by phosphodiesterase inhibition is well established; however, the clinical relevance of this pathway might be limited since the inhibition of phosphodiesterase requires a higher concentration of caffeine than the concentration achieved by normal human caffeine consumption (Fredholm et al. 1999). A caffeine concentration as high as 100 μM was needed to inhibit production of LPS-induced cytokines in a cAMP/protein kinase A dependent manner (Horrigan et al. 2004). However, an acute dose of 2–2.5 mg/kg caffeine raised the blood concentration of caffeine only to 20–25 μM in humans; this dose corresponds to two cups of coffee (May et al. 1982; Smith et al. 1982; Tiffin et al. 1995).

It is accepted now that it is the antagonism of adenosine receptors that is mostly operational at clinically relevant concentrations (Fredholm et al. 1999; Foukas et al. 2002). The antagonism of adenosine receptors requires approximately 20 times lower concentration of caffeine than the inhibition of phosphodiesterase (Fredholm et al. 1999). Currently, the behavioral activation by caffeine is largely accounted for by the interference with tonic activation of adenosine receptor signaling, especially A2A adenosine receptors (A2AR).

There are four different cell-surface receptors for extracellular adenosine: A1, A2A, A2B, and A3 receptors (Fredholm et al. 2001). The high-affinity A1 receptor and the low-affinity A3 receptor are coupled to Gi protein. The cAMP-elevating Gs-protein-coupled A2 receptors are subdivided into high-affinity A2AR and low-affinity A2B adenosine receptors. Immune cells express A2AR at high levels, and adenosine binding to A2AR inhibits immune activation through cAMP–protein kinase A signaling pathway. Pharmacological stimulation by an A2AR agonist results in the inhibition of proliferation (Huang et al. 1997), production of cytokines (Lappas et al. 2005), cytotoxicity of T cells (Koshiba et al. 1997), and activation of monocytes (Link et al. 2000) and granulocytes (Cronstein et al. 1990). The anti-inflammatory action of A2AR agonists has also been shown in vivo, including in ischemia/reperfusion injury (Lappas et al. 2006), airway inflammation (Fozard et al. 2002), inflammatory bowel disease (Odashima et al. 2005), and acute hepatitis (Ohta and Sitkovsky 2001).

Adenosine is an abundant compound in cells, and an important source of extracellular adenosine accumulation is tissue hypoxia (Sitkovsky et al. 2004; Sitkovsky and Ohta 2005). Hypoxia is associated with the increase of ATP catabolism into adenosine by the functions of apyrase (CD39) and 5′-ectonucleotidase (CD73) (Eltzschig et al. 2004). ATP released into the extracellular space undergoes phosphohydrolysis by CD39, which converts ATP/ADP to AMP in tandem with CD73, which then converts AMP to adenosine. Interestingly, hypoxia induces these two cell-surface enzymes, CD39 and CD73, promoting an increase of extracellular adenosine accumulation (Eltzschig et al. 2004; Kobie et al. 2006; Deaglio et al. 2007). Hypoxia also inhibits conversion of adenosine into AMP by adenosine kinase activity (Morote-Garcia et al. 2008). Furthermore, uptake of extracellular adenosine to the intracellular compartment is inhibited under the hypoxic condition. Therefore, the overall outcome of tissue hypoxia will be an increase of extracellular adenosine levels and a subsequent increase of adenosine signaling (Sitkovsky et al. 2004; Sitkovsky and Ohta 2005).

The physiological relevance of endogenous adenosine in the downregulation of inflammation is proven to be tissue-protective by preventing the collateral damage in a negative-feedback manner. The excessive damage to endothelial cells and the microcirculation leads to the interruption of the normal blood and oxygen supply, resulting in tissue hypoxia (Sitkovsky et al. 2004; Sitkovsky and Ohta 2005). Tissue hypoxia caused by inflammatory damage to the blood circulation can induce an increase of local adenosine levels (Driver et al. 1993; Martin et al. 2000). In the inflamed tissue, ATP release from damaged/dead cells may also contribute to the elevation of extracellular adenosine levels (Trautmann 2009). The increase in adenosine–A2AR signaling subsequent to inflammatory tissue damage then downregulates immune reactions in local inflamed tissue. This hypothesis was proven in a study demonstrating exacerbated inflammation in A2AR-deficient mice (Ohta and Sitkovsky 2001). Induction of acute hepatitis in A2AR-deficient mice caused much more devastating liver damage than in wild-type mice. Administration of an A2AR antagonist to wild-type mice also showed the exacerbation of hepatitis (Ohta and Sitkovsky 2001). These results indicate that extracellular increase of the level of adenosine is of great importance in the control of inflammatory responses. Adenosine produced from damaged tissue is the negative-feedback signal to immune cells necessary to prevent continuing excessive inflammatory damage to the vital organ that can lead to critical tissue dysfunction. Augmented inflammation in A2AR-deficient mice was not limited to hepatitis. It was also observed in arthritis, ischemia-reperfusion damage, sepsis, and lung inflammation (Montesinos et al. 2003; Day et al. 2004; Thiel et al. 2005; Németh et al. 2006). There are many other endogenous anti-inflammatory molecules that play an important role in the resolution of inflammation (Lawrence et al. 2002); however, it is interesting that no other active anti-inflammatory pathway is able to compensate for the absence of A2AR (Ohta and Sitkovsky 2001).

The demonstration of adenosine–A2AR interaction as a critical pathway in physiological downregulation of acute inflammation and protection of tissues raised the possibility that A2AR antagonists may be able to enhance inflammation. Caffeine is a nonselective antagonist of various adenosine receptor subtypes and it can antagonize A2AR (Fredholm et al. 1999). This, in turn, suggested the possibility that caffeine might exacerbate tissue damage if consumed during an acute inflammation episode (Ohta et al. 2007).

To clarify this clinically important issue, we examined the effects of caffeine on inflammation using a concanavalin A (Con A)-induced murine hepatitis model. Intravenous injection of Con A into mice induces T-cell-dependent necroinflammatory liver damage, which resembles human viral/autoimmune hepatitis (Tiegs et al. 1992). Con A-induced liver injury has been studied extensively, and the involvement of T cells, natural killer T cells (Toyabe et al. 1997; Kaneko et al. 2000), and Kupffer cells (Schumann et al. 2000) as well as the production of cytokines have been shown to be indispensable to the induction of the liver damage. As expected, Con A-induced liver damage was strongly exacerbated by caffeine at a dose of 10–20 mg/kg body weight (Ohta et al. 2007). The liver damage in caffeine-coinjected mice accompanied upregulation of proinflammatory cytokines, including interferon-γ. Caffeine was confirmed to exacerbate liver damage through antagonism of A2AR because it failed to increase liver damage in A2AR-deficient mice (Ohta et al. 2007).

In these assays caffeine was tested using a clinically relevant dose, which is equivalent to two or three cups of coffee consumption in humans. Injection of 20 mg/kg caffeine into mice raised the serum caffeine concentration to 40 μM after 30 min (Ohta et al. 2007). This concentration of caffeine is sufficient to antagonize A2AR but not to inhibit cAMP phosphodiesterase (Fredholm et al. 1999). When caffeine was given at a high dose (100 mg/kg), which is not often achieved, the mice were strongly protected from Con A-induced liver damage accompanying downregulation of proinflammatory cytokines and a significant increase of anti-inflammatory cytokine IL-10 (Ohta et al. 2007).

This study showed that consumption of a normal amount of caffeine by humans may result in blockade of A2AR signaling in immune cells and may lead to exacerbation of acute inflammation. Because of multiple effects of caffeine on biological functions (Fredholm et al. 1999; Foukas et al. 2002), the effect of caffeine on inflammation is biphasic, dependent on the doses. Indeed, caffeine has opposite (pro- vs anti-inflammatory) effects on inflammation, with the dose determining the overall effect. In contrast to the inhibition of cAMP phosphodiesterase at high doses, a low dose of cafffeine regulates cAMP levels by a different mechanism, i.e., inhibition of adenosine-mediated cAMP induction by antagonism of A2AR. The exacerbation of inflammation by a low dose of caffeine may be more clinically relevant since humans habitually consume lower, “proinflammatory” doses of caffeine (May et al. 1982; Smith et al. 1982; Tiffin et al. 1995).

The proinflammatory role of caffeine may not be easily revealed in epidemiology studies, which consistently show a beneficial effect of coffee consumption for liver diseases. One of the reasons could be that besides caffeine, coffee contains many other components, such as polyphenolic compounds (chlorogenic acid, caffeic acid, protocatechuic acid), lignan (secoisolariciresinol), diterpenes (cafestol, kahweol), and trigonelline (George et al. 2008). It is possible that some of these compounds may be responsible for the protection against liver diseases.

While the studies described above defined adenosine as the endogenous negative regulator of acute inflammation, it should be noted that adenosine also plays an important role in the resolution of inflammation. It has been shown that A2AR signaling promotes remodeling of damaged tissue by promotion of angiogenesis through upregulation of vascular endothelial growth factor (Montesinos et al. 2004) and downregulation of antiangiogenic thrombospondin-1 (Desai et al. 2005). Moreover, adenosine can upregulate collagen production and promote fibrogenesis (Nakav et al. 2009). Recent studies showed that A2AR-deficient mice are less susceptible to hepatic fibrosis induction (Chan et al. 2006). Importantly, A2AR antagonists, including caffeine, also prevented promotion of hepatic fibrosis (Nakav et al. 2009; Chan et al. 2006; Fernández et al. 2008). Taken together, these findings show that adenosine not only inhibits induction of inflammation but also promotes tissue remodeling through angiogenesis and fibrogenesis. While it is still to be carefully investigated, it is possible to speculate that caffeine may prevent cirrhosis development by the inhibition of the A2AR-mediated fibrosis-promoting pathway.

2 Caffeine and Tumor Immunology

Proinflammatory effects and subsequent exacerbation of tissue damage by A2AR antagonists are detrimental if they take place in vital organs, but the same effects may be highly desirable if they enhance the antipathogen response and inflammatory damage in cancerous tissues. Here we will provide an overview of the path to improve the immunotherapy of cancer by taking advantage of the proinflammatory effects of caffeine.

Difficulties in the efficient induction of inflammatory damage in tumors have been a big obstacle in immunotherapy of cancer (Gajewski et al. 2006; Mellor and Munn 2008) even though there have been very impressive improvements in the induction of antitumor immunity, e.g., cancer vaccines, genetically engineered antitumor T cells, and immune modulatory molecules (Gattinoni et al. 2006; Dougan and Dranoff 2009). These failures are explained by the tumor's escape due to the tumor's fortification with many different immunosuppressisve mechanisms that can critically impair the efficacy of the induced antitumor effectors (Drake et al. 2005; Bai et al. 2008). The presence of regulatory T cells and myeloid suppressor cells may play a significant role in the intratumoral immunosuppressive mechanism. Anti-inflammatory cytokines such as IL-10 and TGF-β are secreted from tumors (and from the suppressor cells at least in part). Expression of programmed death ligand-1 can induce apoptosis of T cells. Glucose deprivation and L-arginine deficiency by the expression of indoleamine-2,3-dioxygenase causes nutrient deficits in antitumor effector cells (Gattinoni et al. 2006; Dougan and Dranoff 2009).

Another important immunosuppressive mechanism that caffeine could be interfering with is hypoxia and subsequent increase of the level of extracellular adenosine in the tumor microenvironment (Ohta et al. 2006). Many solid tumors are characterized by an insufficient oxygen supply and transient or chronic hypoxia (Harris 2002; Vaupel and Mayer 2007; Sitkovsky et al. 2008). Tumor hypoxia may contribute to the propagation of oncogenic signals in the tumor microenvironment as was shown in the switch to the angiogenic phenotype (Laderoute et al. 2000), and tumor hypoxia is associated with poor prognosis (Evans et al. 2000; Giatromanolaki et al. 2001). Reflecting local hypoxia in tumors, adenosine levels are high in the tumor microenvironment (Ohta et al. 2006). The tumor-protecting role of adenosine was demonstrated by a dramatic improvement of tumor rejection in A2AR-deficient mice (Ohta et al. 2006). In this study, A2AR-deficient mice, but none of the wild-type mice, could survive tumor challenge, suggesting that hypoxic tumors protect themselves from incoming antitumor immune cells by producing extracellular adenosine, which then inhibits immune cells through A2AR.

In this experiment, treatment with A2AR antagonists improved T cell-dependent tumor eradication in wild-type mice (Ohta et al. 2006). One such antagonist was caffeine, which enabled T-cell-mediated elimination of tumor nodules by interfering with adenosine–A2AR interactions. This immunopotentiating activity of caffeine is consistent with the exacerbation of inflammatory tissue damage in acute hepatitis described above. Furthermore, caffeine treatment decreased the number of microvessels in the tumor and increased the number of apoptotic tumor cells (Ohta et al. 2006). This suggests that caffeine not only served as an immunoenhancer but also improved antitumor responses through inhibition of adenosine-mediated angiogenesis.

3 Control of Inflammation by Methylxanthines

In summary, the use of methylxanthines is a promising approach to promote immune responses against pathogens and against tumors. These compounds interrupt the immunosuppressive adenosine–A2AR signaling and may enhance the antipathogen immunity and pathogen destruction by immune cells. It is expected that caffeine could be effective as an adjuvant in vaccination against many other pathogens and with cancer vaccines. However, although a strong antiviral immune response is beneficial to the elimination of pathogens, suppression of the tissue-protecting adenosine–A2AR pathway may cause the uncontrolled activation of immune cells and severe inflammation. For example, exacerbation of acute viral hepatitis can result in fulminant hepatitis, the serious and high-mortality complication. Consumption of A2AR-antagonizing methylxanthines may require caution in patients with ongoing acute inflammation. While viral fulminant hepatitis causes acute liver failure, the main cause of acute liver failure is the drug-induced hepatitis, most often because of acetaminophen overdose (Caraceni and Van Thiel 1995; Lee 2003). Since activated inflammatory cells are often found in drug-induced hepatitis (Luster et al. 2001; Simeonova et al. 2001), methylxanthines may also enhance the tissue damage during drug-induced hepatitis. In contrast, during chronic hepatitis, A2AR antagonists are expected to prevent excessive hepatic connective tissue deposition resulting from fibrogenesis. Methylxanthines may prevent excessive hepatic connective tissue deposition that is associated with the progression of hepatitis to cirrhosis, which is one of the common causes of mortality. Since methylxanthines can affect many of the events during acute/chronic hepatitis, it is important that studies are conducted to determine whether the use of A2AR-antagonizing methylxanthines should be informed by the individual's inflammation status.

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Methylxanthines and Drug Dependence: A Focus on Interactions with Substances of Abuse

Micaela Morelli and Nicola Simola

Contents

Abstract This chapter examines the psychostimulant actions of methylxanthines, with a focus on the consequences of their excessive use. Consumption of methylxanthines is pervasive and their use is often associated with that of substances known to produce dependence and to have abuse potential. Therefore, the consequences

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of this combined use are taken into consideration in order to evaluate whether, and to what extent, methylxanthines could influence dependence on or abuse of other centrally active substances, leading to either amplification or attenuation of their effects. Since the methylxanthine that mostly influences mental processes and readily induces psychostimulation is caffeine, this review mainly focuses on caffeine as a prototype of methylxanthine-produced dependence, examining, at the same time, the risks related to caffeine use.

Keywords Adenosine receptors · Amphetamine · Caffeine · Cocaine · Nicotine

Abbreviations

CPP	Conditioned place preference
D_2High	High-affinity D_2
DARPP-32	Dopamine- and cyclic-AMPregulated 32-kDa phosphoprotein
DSM IV	*Diagnostic and Statistical Manual of Mental Disorders*, fourth edition
IEGs	Immediate early genes
NGFI-A	Nerve growth factor I-A
NMDA	*N*-Methyl-D-aspartate

1 Methylxanthine Dependence

Methylxanthines, which include caffeine, theophylline, paraxanthine, and theobromine, are present in several dietary products, including coffee, tea, soft and “energy drinks”, maté, cakes, candies, and chocolate. Moreover, theophylline is used as an antiasthmatic, and caffeine is added to cold remedies and analgesic medications (Brice and Smith 2002). Owing to the large diffusion of these products, it can be assumed that the world population consumes methylxanthines every day in any of these forms. It must be noted that the majority of caffeine, the most popular of the methylxanthines, is degraded by the hepatic microsomal enzymatic system to paraxanthine and partially to theobromine and theophylline (Svenningsson et al. 1999).

Psychoactive substances are often associated with dependence phenomena, the definition of which identifies the presence of a pattern of behaviors focused on repetitive and compulsive seeking and taking of drugs. Compulsive and repetitive drug use may result in tolerance to the effect of the drug and withdrawal symptoms when use is reduced or stopped. The *Diagnostic and Statistical Manual of Mental Disorders*, fourth edition (DSM IV), by the American Psychiatric Association (1994) delineates seven criteria to define dependence (Table 1).

Table 1 Characterization of methylxanthines according to the criteria of drug dependence proposed by the *Diagnostic and Statistical Manual of Mental Disorders*, fourth edition (American Psychiatric Association 1994)

Criterion of drug dependence	Fulfillment by methylxanthines
Induction of tolerance	Caffeine partially fulfills this criterion, as it induces tolerance to some (e.g., cardiovascular effects), but not all, of its effects. Tolerance to caffeine usually disappears after a short period the use of the substance has been discontinued
Induction of withdrawal symptoms	Caffeine partially fulfills this criterion, as it can induce withdrawal symptoms such as fatigue, headache, irritability, and depressed mood. Although these symptoms can be severe in certain individuals, caffeine withdrawal syndrome is usually not harmful and resolves in a few days after use of the substance has been discontinued
Taking the substance in larger amounts or over a period longer than intended	Methylxanthines can partially fulfill this criterion. However, it has to be considered that methylxanthines are usually consumed in the form of coffee, tea, soft drinks, and chocolate. Besides their methylxanthine content, the organolectic properties of such dietary sources (e.g., flavor and sugar content), can greatly influence their prolonged intake
Persistent desire and unsuccessful efforts to cut down, or control, use	Methylxanthines, in general, do not fulfill this criterion. Similarly to what was observed for the previous criterion, excessive consumption of methylxanthine-containing dietary sources may be due to factors other than methylxanthines (see Sect. 4)
Spending a great deal of time in activities necessary to obtain the substance or recover from the effects of the substance	Methylxanthines do not fulfill this criterion
Giving up or reducing important social, occupational, or recreational activities because of substance use	Methylxanthines do not fulfill this criterion. On the contrary, conviviality may drive the consumption of methylxanthines
Continued use despite knowledge of persistent or recurrent physical or psychological problems exacerbated by the substance	Methylxanthines, in general, do not fulfill this criterion. On the other hand, the use of caffeine is often described as self-regulating, owing to the onset of unwanted aversive effects at high doses of the substance

Although possible dependence on caffeine has been considered by several research groups (Gilliland and Bullock 1984; Griffiths et al. 1990; Nehlig 1999), methylxanthines do not fulfill the criteria for drugs producing dependence, whereas caffeine partially fulfills these criteria (Table 1). The two symptoms of dependence quoted by the DSM IV that methylxanthines may be associated with more closely are tolerance and withdrawal. Tolerance to a drug and withdrawal do not define characteristics of dependence, although they typically accompany dependence on certain drugs.

Since caffeine use is associated with very limited physical and psychological consequences and with weak reinforcing properties and withdrawal symptoms, the definition of caffeine as an "atypical drug of dependence" given by Daly and Fredholm (1998) is still widely accepted. Methylxanthine consumption is often associated with a group of factors varying from social conviviality and relaxation to stimulation, as an aid to regulate sleep and to increase attention and concentration. All these factors may have an important role when considering the different aspects influencing dependence.

In addition to caffeine dependence, the DSM IV describes caffeine use as associated with several distinct psychiatric syndromes: caffeine intoxication, caffeine-induced sleep disorder, and caffeine-induced anxiety disorder. Moreover, caffeine produces biphasic effects, with low doses eliciting increased attention, concentration, a feeling of well-being, increased energy, desire to socialize, and motivation for work, and high doses resulting in anxiety, tension, restlessness, and sleeplessness. One of the interesting effects of caffeine ingestion is that it tends to be self-regulating. Unlike other psychoactive substances such as heroin, ethanol, and psychostimulants such as cocaine and amphetamine, the use of which tends to increase without bound, the amount of caffeine that people consume is limited by the onset of unwanted side effects.

2 Methylxanthine Tolerance

Tolerance is a pharmacological phenomenon where the dose of a drug needs to be continually increased in order to achieve and maintain the same effect. Tolerance has been clearly described for some of the effects of caffeine.

In humans, tolerance to the behavioral effects of caffeine is of low magnitude and incomplete (Fredholm et al. 1999; Watson et al. 2002). It develops to the central effects of caffeine such as increase in tension, anxiety, and jitteriness, as well as to peripheral effects such as modifications in blood pressure, heart rate, and diuresis (Fredholm et al. 1999; Nehlig 2004). In line with the widespread use of caffeine for its psychostimulant effects, minimal tolerance develops to caffeine-induced attention and wakefulness.

In experimental animals, tolerance develops to the motor-stimulant effects of caffeine and theophylline and to the cerebral electrical activation, seizures, and disruption of operant behavior for food reward produced by caffeine (Finn and Holtzman 1988; Lau and Falk 1995) but not to the increase in brain 2-deoxyglucose uptake (Nehlig 2004). Of particular interest is the finding that long-term caffeine administration decreases the susceptibility of mice to *N*-methyl-D-aspartate (NMDA)-induced seizures (Georgiev et al. 1993).

Tolerance does not appear to be related to modifications in the total level of active metabolites of methylxanthine in the brain. Instead, disruption of dopaminergic functions by downregulated levels of adenosine A_{2A} receptors, which largely interact with dopamine receptors, and increased expression of adenosine A_1 receptors in specific brain areas were found in caffeine-tolerant rats (Svenningsson et al. 1999).

3 Methylxanthine Withdrawal

The general definition of withdrawal takes into consideration the presence of symptoms that occur upon the abrupt discontinuation or a decrease in dosage of a drug. In addition, in order to experience the symptoms of withdrawal, dependence on the drug must be present.

In humans, the caffeine withdrawal syndrome, characterized by headache, fatigue, drowsiness, irritability, depressed mood, and anxiety, starts after 12–24 h of abstinence, peaks 20–48 h later, and does not appear to be related to the quantity of caffeine ingested (Fredholm et al. 1999; Griffiths et al. 1990; Nehlig 2004). Interestingly, there is a relationship between headache and cerebral blood flow (Couturier et al. 1997). Symptoms of caffeine withdrawal are not necessarily manifested all at the same time and their intensity may vary considerably between different individuals. Caffeine withdrawal syndrome is usually not harmful, it is self-limiting, and no reliable modifications in social behavior have been observed (Comer et al. 1997).

In accordance with the vast and increasing number of adolescents who drink caffeinated beverages, caffeine dependence and withdrawal symptoms are increasingly being reported in teenagers (Bernstein et al. 2002). Withdrawal symptoms were also apparent in newborns of mothers who were heavy drinkers of coffee or maté, which contain both caffeine and theobromine (McGowan et al. 1988; Martín et al. 2007).

In the adult North American population, coffee accounts for three quarters of daily methylxanthine consumption, whereas in the teenage population the majority of caffeine (two thirds) is consumed from soft drinks (Bernstein et al. 2002).

Theophylline withdrawal has been reported in some adult asthmatic patients in whom exacerbation of asthma associated with a significant decrease in peripheral blood monocytes, activated CD4+ T lymphocytes, and CD8+ T cells was observed without the presence of psychotropic symptoms (Kidney et al. 1995).

In experimental animals, withdrawal induces a decrease in locomotor activity (Kaplan et al. 1993; Nikodijević et al. 1993) and disrupts operant behavior (Mumford et al. 1988). In rodents, the magnitude and duration of withdrawal appear to be a function of the amount of caffeine assumed.

4 Methylxanthine Abuse Potential

Reinforcement has a major role in the abuse potential of drugs. This term refers to the efficacy of a substance in establishing and maintaining a behavior on which the delivery of the substance is dependent.

In humans, methylxanthines – particularly caffeine, on which the majority of studies are focused – have mild reinforcing properties, which maintain the self-administration of beverages containing these substances. Reinforcement occurs in both moderate and heavy consumers, although at different rates. Moreover, a clear U-shaped response exists, with high doses associated with aversive effects (Fredholm et al. 1999; Nehlig 2004). The presence of reinforcing properties is the principal but not the only determinant of caffeine ingestion, since in some studies its intake has been shown to be driven by the need to avoid withdrawal symptoms (Schuh and Griffiths 1997). Interestingly, however, humans discriminate caffeine and theophylline from a placebo or amphetamine without needing to be in a withdrawal state (Oliveto et al. 1993; Griffiths and Mumford 1995).

Although not yet clearly demonstrated, it has been suggested that factors unrelated to methylxanthine content – such as the smell and flavor of coffee, tea, and chocolate, together with the social environment that accompanies their consumption – may have an important role in their abuse potential (Benton 2004; Nehlig 2004).

In experimental animals, one of the first parameters examined by studies aimed at evaluating the abuse potential of methylxanthines was discrimination. The results of these studies showed that caffeine produced a slight increase in responding, which was approximately twice as high as with theophylline, while amphetamine produced a much greater increase (Modrow et al. 1981; Carney et al. 1985). Moreover, in rats able to discriminate caffeine from saline, theobromine did not evoke caffeine-like responses (Carney et al. 1985). Caffeine and theophylline were also compared with theobromine. The rank order of potency was caffeine > theophylline > theobromine (Carney 1982). In follow-up studies focused on caffeine and dopamine receptor agonist drugs, it was reported that low doses of caffeine produced cues resembling a weak dopaminergic stimulus (Harland et al. 1989). A similar type of generalization was found in nonhuman primates (Holtzman 1996). These studies demonstrated the presence of differences and similarities between methylxanthines and psychostimulants such as amphetamine and cocaine, and are in line with studies in humans that show low reinforcing properties of methylxanthines compared with other psychostimulants (Heishman and Henningfield 1992). More recent studies that focused on the abuse potential of methylxanthines have shown that while both caffeine and theophylline at low doses induce conditioned place preference (CPP) in rodents (Zarrindast and Moghadamnia 1997; Bedingfield et al. 1998), caffeine maintains self-administration behavior in all or a subset of animals depending on the protocol of administration, whereas theophylline is not self-administered

(Griffiths and Mumford 1995; Sahraei et al. 1999). An irregular pattern of caffeine self-administration was also observed in nonhuman primates, confirming that, in contrast to other known psychostimulants, caffeine acts as a reinforcer in limited conditions (Griffiths and Mumford 1995). In addition, it is important to emphasize that self-administration studies utilize intravenous administration, a way that magnifies the reinforcing effects of a drug, whereas caffeine is consumed orally. In line with this, intravenous administration of caffeine increased the amount of self-administered cocaine, whereas drinking caffeine reduced it (Kuzmin et al. 2000).

Regarding the role of methylxanthines in "chocolate addicts," different issues should be considered. Chocolate is often consumed to relieve distress; however, the amount of cocoa consumed, although chronic, remains moderate. Chocolate addicts rarely display other addictive behaviors (Dallard et al. 2001) and do not seem to suffer from eating disorders, but may represent a population vulnerable to depression or anxiety. Methylxanthines contained in chocolate – caffeine and primarily theobromine – may contribute to the popularity of chocolate. However, their content is far too low to reach levels high enough to produce stimulation. Other attributes are probably much more important in determining chocolate's special appeal and in explaining related self-reports of chocolate cravings and "chocoholism" (Smit et al. 2004). Moreover, the reinforcing properties of chocolate, which are similar to those of palatable food (Maccioni et al. 2008), have never been demonstrated to depend on theobromine, which does not appear to have abuse potential.

Several studies have investigated the biochemical basis of methylxanthine abuse potential and, similar to behavioral studies, concluded that methylxanthines, and particularly caffeine, differ profoundly from other psychostimulants recognized to have abuse potential. All these studies suggest that functional interactions between adenosine and dopamine receptors, whose activation is critically involved in promoting psychostimulation, are crucial to caffeine-elicited abuse effects (Ferré et al 1997). Interactions between adenosine and neurotransmitters other than dopamine, such as glutamate, serotonin, and acetylcholine, may be also important to caffeine-induced central effects. In particular, adenosine–glutamate interactions have been suggested to participate in caffeine-elicited psychostimulation (Ferré 2008).

Particularly important to this subject are the studies examining the response in dopamine release and cerebral glucose utilization in the shell of the nucleus accumbens, an area deeply involved in addictive properties of drugs (Di Chiara 2002). Caffeine – differently from drugs having overt abuse potential such as cocaine, amphetamine, and nicotine – increases dopamine release or glucose utilization in the nucleus accumbens shell only at high doses, while it increases dopamine release in the medial prefrontal cortex at doses corresponding to those assumed to result from its recreational consumption (Nehlig and Boyet 2000; Quarta et al. 2004; De Luca et al. 2007).

5 Interactions Between Methylxanthines and Other Psychoactive Drugs

Several investigations in experimental animals have demonstrated the ability of methylxanthines, and in particular of caffeine, to modulate the psychopharmacological effects of many psychoactive substances, including drugs of abuse (Tuazon et al. 1992; Shoaib et al 1999; Kunin et al. 2000; Gasior et al. 2002; Green and Schenk 2002) (Fig. 1). Similar effects of methylxanthines have also been observed in humans, and epidemiology studies have shown that caffeine consumption is often a correlate in drug dependence (Istvan and Matarazzo 1984; Kozlowski et al. 1993). On the basis of these findings, concerns have emerged about the possibility that intake of methylxanthines may facilitate either the development of dependence on other substances or relapse in former drug addicts. Such concerns have grown ever since the introduction to the market of the so-called "energy drinks", which contain caffeine at quite high concentrations and are being increasingly consumed, often in combination with substances with abuse potential. This habit could favor the pharmacological interactions between caffeine and addictive substances,

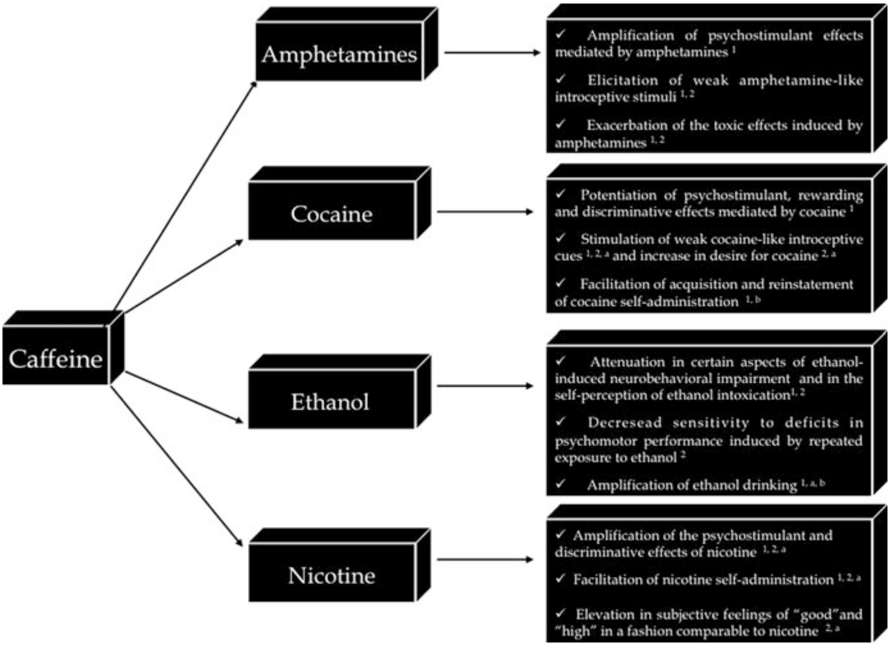

Fig. 1 General overview of the main pharmacological interactions between the methylxanthine caffeine and substances bearing abuse potential. *1* an effect observed in experimental animals, *2* an effect observed in humans, *a* an effect for which contradictory reports are present in the literature, *b* an effect observed in experimental animals and the occurrence of which is hypothesized to take place also in humans

potentially influencing the effects of the latter. Furthermore, unintentional consumption might also play a role in the interactions between methylxanthines and substances of abuse. Several chemical determinations have in fact demonstrated that methylxanthines are present at significant levels, as either contaminants or additives, in several illicit drugs (Fucci and De Giovanni 1998; Cheng et al. 2006).

Similarities and differences in the effects and interactions between methylxanthines and other psychoactive substances have both been described. Moreover, it has been ascertained that these often involve effects other than those related to dependence phenomena, potentially leading to harmful, unwanted consequences.

This part of the chapter summarizes current knowledge on the interactions between methylxanthines, in particular caffeine, and other psychoactive substances, including addictive, recreational, and prescribed drugs, describing the most representative ones. The implications of these interactions are discussed, with particular emphasis on drug dependence.

5.1 Amphetamines

Several studies have addressed the issue that methylxanthines and amphetamines may influence each other's actions.

Drug-discrimination studies performed in experimental animals have demonstrated that caffeine can, to some extent, engender introceptive stimuli resembling those triggered by amphetamines (Holtzman 1986; Jain and Holtzman 2005). Such a similarity with amphetamines can also be envisioned for other methylxanthines, as some substances in this class are known to share the discriminative properties of caffeine (Carney et al. 1985). Methylxanthines can also potentiate the effects of amphetamines. However, while such a facilitatory influence does not seem to involve enhancement of the rewarding properties of amphetamines (Tuazon et al. 1992), it has nevertheless been observed for several effects, such as stimulation of motor activity, food-maintained operant behavior, and expression of immediate early genes (Jaszyna et al. 1998; Tronci et al. 2006). It must be emphasized here that the facilitation of amphetamine-mediated effects by methylxanthines observed in experimental animals can persist even in the absence of concomitant exposure to caffeine (Cauli et al. 2003; Tronci et al. 2006). Moreover, it is also worth recalling that evidence in experimental animals indicates that the amphetamine-like stimulus effect of caffeine does not undergo tolerance (Jain and Holtzman 2005). Taken together, the previous findings are particularly interesting when considering the possible role that consumption of methylxanthines may have in amphetamine abuse (Fig. 1).

Methylxanthines exert their central effects chiefly by antagonizing the A_1 and A_{2A} receptors (Fredholm et al. 1999). It is well known that A_{2A} receptors and dopamine D_2 receptors interact in opposite functional ways (Ferré et al. 1997). In line with this, investigations in rodents have clearly shown, on the one hand, that many of the behavioral and neurochemical effects of caffeine have a dopaminergic component, as dopamine receptor blockade can suppress them (Garrett and Griffiths 1997). On the

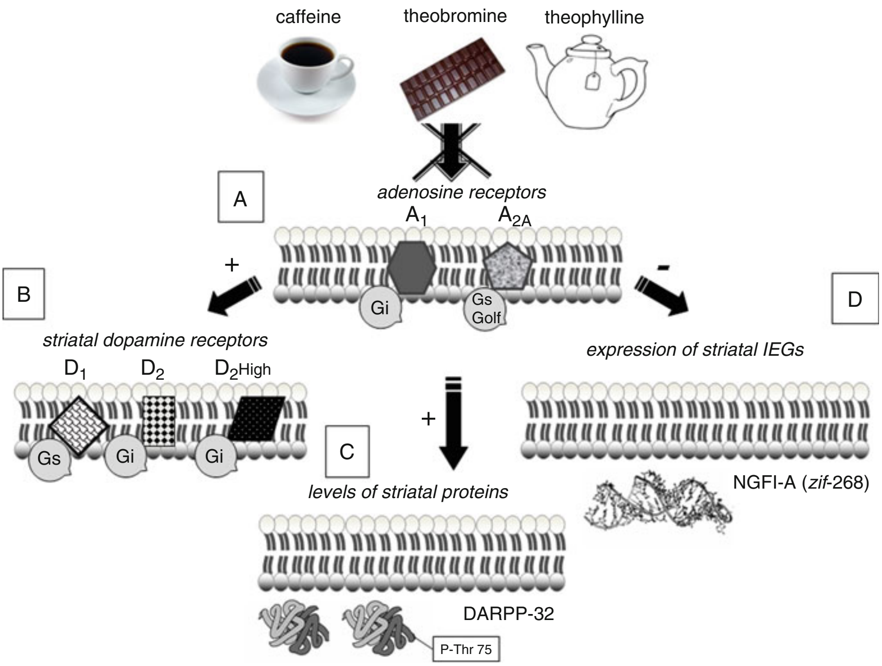

Fig. 2 General overview of the molecular mechanisms involved in the interactions between methylxanthines and psychoactive substances having abuse potential. Methylxanthines act as competitive antagonists of both A_1 and A_{2A} receptors (**A**). Such antagonism of adenosine receptors can potentiate striatal dopamine transmission by indirectly facilitating the signal mediated by D_1 and D_2 receptors and by elevating the relative proportion of high-affinity D_2 receptors (D_2High) (**B**). Additional mechanisms of this pharmacological interactions related to methylxanthine-mediated antagonism of adenosine receptors may involve the elevation of the levels of dopamine- and cyclic-AMP-regulated 32-kDa phosphoprotein (DARPP-32), and of its Thr-75 phosphorilated isoform (**C**) and the depressed expression of the immediate early gene (IEG) NGFI-A (or *zif-268*) (**D**) in the corpus striatum. Effects on dopamine receptors, striatal proteins, and IEGs have chiefly been demonstrated for caffeine, but can also be hypothesized for other methylxanthines, on the basis of the antagonism of adenosine receptors these substances may exert

other hand, it has been determined that exposure to caffeine can engender facilitation of dopamine transmission in the corpus striatum of rodents. This effect has been shown to involve proteins such as dopamine-and cyclic-AMP-regulated 32-kDa phosphoprotein (Lindskog et al. 2002; Vaugeois 2002; Hsu et al. 2009a), immediate early genes such as nerve growth factor I-A also known as *zif-268* (Tronci et al. 2006), and high-affinity D_2 (D_2High) receptors (Simola et al. 2008) (Fig. 2). Therefore, modulation of dopamine transmission could be an important mechanism underlying the interactions between amphetamines and methylxanthines, and it may also be involved in the influence methylxanthines exert on the effects of other psychostimulants (see Sect. 5.2).

Future studies should also focus on another major issue in methylxanthine–amphetamine interactions, namely, the ability of methylxanthines to enhance the toxic effects of amphetamines. To date, this phenomenon has been characterized with regard to the interactions between caffeine and 3,4-methylenedioxymethamphetamine (also known as MDMA and ecstasy) and methamphetamine. In rodents, combined administration of caffeine powerfully exacerbates the toxic effects of these amphetamines (Delle Donne and Sonsalla 1994; McNamara et al. 2006).

Studies performed in humans have also evaluated interactions and similarities between methylxanthines and amphetamines. In this regard, drug-discrimination studies with caffeine are particularly interesting as they confirm what has been observed in experimental animals by showing that caffeine is capable of triggering amphetamine-like introceptive stimuli, though with weaker intensity (Heishman and Henningfield 1992) (Fig. 1). Since introceptive cues are an important component in drug abuse (Stolerman 1993), such an effect could potentially favor either the development or the relapse of an amphetamine dependence in individuals jointly consuming amphetamines and methylxanthines. Further investigation is necessary and should also address the potential additive toxicological interactions involving methylxanthines and amphetamines, as the occurrence of such a phenomenon has been reported in humans as well (Lambrecht et al. 1993).

5.2 Cocaine

Individuals addicted to cocaine often consume it in combination with various psychoactive substances, either licit or illicit, in what is defined as “polydrug use” (Leri et al. 2003). On the basis of the popularity of methylxanthines and of the similarity in the human consumption of amphetamines and cocaine, several studies have examined the interactions between methylxanthines and cocaine, and explored whether consumption of methylxanthines may influence cocaine abuse.

Investigations in experimental animals demonstrated that methylxanthines may exert effects that are to some degree similar to those triggered by cocaine and that methylxanthines can potentiate cocaine-mediated effects, although such a potentiation has not always been observed. Drug-discrimination experiments have demonstrated that rats trained to discriminate cocaine generalize to caffeine, although in an incomplete fashion, and that caffeine can amplify cocaine-mediated discriminative effects (Harland et al. 1989). A facilitatory influence of methylxanthines has also been described on other effects mediated by cocaine in rodents, such as motor stimulation and eliciting CPP (Schenk et al. 1990; Bedingfield et al. 1998). This latter finding appears interesting, as it suggests that methylxanthines could enhance cocaine-mediated reward. Most notably, evidence in rodents shows that methylxanthines dramatically influence cocaine self-administration. Thus, a faster acquisition of cocaine self-administration has been observed in rats exposed to chronic caffeine (Horger et al. 1991). Such a facilitatory influence of caffeine has also been observed in rhesus monkeys trained to self-administer smoked cocaine (Comer and Carroll 1996). Furthermore, both caffeine and theophylline have been shown to efficiently reinstate cocaine self-administration in experienced rats in which this behavior was extinguished (Green and Schenk 2002) (Fig. 1). Although no consensus can be reached on the basis of the results of studies in experimental animals on the ability of methylxanthines to enhance cocaine-mediated reinforcement (Horger et al. 1991; Kuzmin et al. 2000), the previous findings are of interest. In fact, the facilitation and reinstatement of cocaine self-administration by methylxanthines provides straightforward evidence that consumption of methylxanthines might be causally linked to an increase in cocaine intake.

Akin to what is observed for amphetamines, studies in experimental animals have indicated that methylxanthines might amplify cocaine-mediated effects by facilitating dopamine transmission (Green and Schenk 2002). Hence, the elevation in the level of D_2High receptors in the striatum of rats exposed to caffeine for a long time could be an important mechanism (Simola et al. 2008), since an increase in the level of D_2High receptors has also been observed in rats trained to self-administer cocaine (Briand et al. 2008) (Fig. 2). In this regard, it has, however, to be mentioned that the existence of two interchangeable affinity states for the dopamine D_2 receptors (D_2High and low-affinity D_2 receptors, having high and low affinity for dopamine, respectively) has been clearly demonstrated only by in vitro experiments (George et al. 1985). On the other hand, whether such a phenomenon exists also in vivo is still controversial (McCormick et al. 2008), although evidence supporting this hypothesis has recently been obtained (Seeman 2009).

In line with the findings described above, investigations in humans have revealed the existence of similarities in the effects and reciprocal interactions between methylxanthines and cocaine. Cocaine addicts may consume more caffeine than the general population, suggesting a causal link could underlie the combined intake of these substances (Budney et al. 1993). Moreover, caffeine has been shown to elicit subjective effects resembling those of cocaine, although in a weaker fashion, and to increase cocaine “wanting” in cocaine abusers (Rush et al. 1995; Oliveto et al. 1998). These effects of caffeine, however, could not always be replicated (Liguori et al. 1997) (Fig. 1). It should be noted, however, that human studies addressing methylxanthine–cocaine interactions have often been performed under strikingly discrepant experimental conditions with respect to the procedures used and the participants recruited (e.g., former abusers, abusers, naïve individuals). Such methodological differences may have greatly influenced the outcomes observed in different investigations. Therefore, although conclusive evidence has not been obtained demonstrating that consumption of methylxanthines can promote cocaine abuse in humans, data demonstrating that methylxanthines can amplify certain effects of cocaine deserve full consideration. It is worth mentioning that development and relapse of cocaine dependence are powerfully modulated by environmental and pharmacological cues, including the consumption of psychoactive substances exerting subjective effects resembling those of cocaine (Spealman et al. 1999). Therefore, the ability of methylxanthines to engender cocaine-like introceptive stimuli may have particular relevance to cocaine abuse.

5.3 Cannabis Derivates

Although cannabis is one of the most popular drugs of abuse, little is known on the pharmacological interactions between methylxanthines and cannabis derivates, such as marijuana and hashish, and on the implications such interactions might have on drug dependence.

Studies in humans have shown that teenagers and college students often consume caffeine together with marijuana (Bernstein et al. 2002; Miller 2008). However, whether caffeine might be a gateway drug for marijuana dependence has not been proven.

Studies in experimental animals support this view, with evidence indicating that a functional interaction exists between A_{2A} and cannabinoid CB_1 receptors. Such an interaction takes place at both the neurochemical level (as a dependence of CB_1-mediated transmission on A_{2A} receptors has been reported in cotransfected cell lines) and the behavioral level (as antagonism of A_{2A} receptors counteracts the motor-depressant effects mediated by CB_1 receptor stimulation in experimental animals) (Carriba et al. 2007). Recent investigations support a role for adenosine transmission, and thus methylxanthines, in cannabis abuse. Studies in mice have shown that A_{2A} receptors are involved in rewarding effects and physical dependence induced by Δ9-tetrahydrocannabinol (Soria et al. 2004). Moreover, long-term exposure to caffeine has been shown to facilitate CB_1-mediated transmission in the rat corpus striatum (Rossi et al. 2009). On the basis of these findings, it is reasonable to hypothesize that consumption of caffeine, and/or other methylxanthines, may also have some relevance to cannabis use in humans.

5.4 Ethanol

Epidemiology studies have shown that a positive correlation may exist between the consumption of caffeine and that of ethanol (Istvan and Matarazzo 1984; Kozlowski et al. 1993). Moreover, anecdotal reports describing caffeine as a "hangover helper" have long provided an empirical justification for the combined consumption of these substances. A wealth of studies in both experimental animals and humans have been performed to elucidate the features of methylxanthine–ethanol interactions. This issue has been attracting even greater interest ever since the introduction to the market of the highly caffeinated "energy drinks." In fact, such beverages are increasingly being consumed in combination with ethanol, and evidence exists that this habit is often associated with health-threatening consequences (Marczinski and Fillmore 2006; O'Brien et al. 2008).

In experimental animals, both caffeine and theophylline ameliorate several behavioral parameters indicative of ethanol intoxication (Dar et al. 1987; Connole et al. 2004). Results showing a worsening of ethanol-induced impairment have, however, also been reported, depending on the specific methylxanthine and the effect of ethanol considered (Kuribara and Tadokoro 1992). Therefore, such studies do not definitively clarify the precise effects of methylxanthines on ethanol intoxication, and suggest that when an amelioration of intoxication by methylxanthines exists, it is often narrowed to certain aspects of the phenomenon. In addition, experiments in rats have shown that caffeine can promote ethanol drinking (Kunin et al. 2000) (Fig. 1). This finding would provide a direct link between caffeine intake and ethanol consumption, although it must be acknowledged that such an effect of caffeine has not always been observed (Potthoff et al. 1983).

It is still not clear what molecular mechanism could underlie the stimulation of ethanol drinking by caffeine. The mechanisms at the basis of the counteraction caffeine may exert on effects related to ethanol intoxication, however, appear to be more defined. In particular, antagonism of A_1 and A_{2A} receptors appears crucial to this action of caffeine, though the specific receptor involved varies according to the effect of ethanol considered. Hence, A_1 receptors seem to mediate caffeine-elicited reversal of deficits in motor coordination induced by ethanol (Barwick and Dar 1998; Connole et al. 2004), and it is reasonable to think this may be because of the high enrichment of these receptors in areas governing motor coordination such as the cortex and cerebellum (Ribeiro et al. 2002). However, blockade of A_{2A} rather than A_1 receptors seems to be involved in caffeine-mediated counteraction of hypnosis induced by ethanol (El Yacoubi et al. 2003).

Studies in humans examining methylxanthine–ethanol interactions have mostly focused on the influence caffeine exerts on ethanol intoxication, and have yielded mixed results (Liguori and Robinson 2001; Drake et al. 2003). Nevertheless, it is worth mentioning that these studies converge on the point that caffeine consumed in association with ethanol, rather than improving ethanol-induced impairments, would reduce the self-perception of ethanol intoxication (Fig. 1). This has been suggested to be a major risk associated with joint ethanol–caffeine consumption, since an altered perception of one’s psychophysical integrity could lead to the performance of hazardous activities. Notably, this view has received support from a recent investigation showing an increase in the performance of risky behaviors (e.g., driving under ethanol intoxication) by college students who reported combined drinking of caffeinated “energy drinks” and ethanol (O’Brien et al. 2008).

As previously mentioned, epidemiology studies suggest the existence of a positive correlation between the consumption of caffeine and the consumption of ethanol. Interestingly, caffeine can promote ethanol drinking in rats (Kunin et al. 2000), whereas human data show that caffeine enhances tolerance to ethanol (Fillmore 2003), the onset of which is critical to ethanol dependence (DSM IV). Hence, it can be hypothesized that a caffeine-mediated increase in ethanol tolerance could promote the escalation of ethanol consumption. On the other hand, the studies in ethanol consumers demonstrating that caffeine reduces the feelings of ethanol intoxication seem to suggest that joint ethanol–caffeine drinking might reflect a sort of self-medication, in which caffeine serves to counteract the adverse effects - of ethanol (Grattan-Miscio and Vogel-Sprott 2005). To date, little is known on the interactions between ethanol and methylxanthines other than caffeine. Investigation of this issue appears warranted, on the basis of studies in experimental animals showing that theophylline may interact with ethanol in a different way from caffeine (Dar et al. 1987).

5.5 Nicotine

A positive relationship between the consumption of nicotine in the form of tobacco smoking and caffeine in the form of drinking coffee has long been described by anecdotal reports and more recently by epidemiology studies (Istvan and Matarazzo 1984; Swanson et al. 1994). Several investigations have been performed to elucidate the features of this habit; however, very scarce information is available on the interactions between nicotine and methylxanthines other than caffeine.

Studies in experimental animals have demonstrated that caffeine can amplify many behavioral effects of nicotine (Shoaib et al. 1999; Gasior et al. 2002; Celik et al. 2006). The results obtained from drug-discrimination and self-administration experiments are particularly interesting and relevant to nicotine abuse. Although it does not engender nicotine-like subjective cues, caffeine has been shown to amplify the discriminative effects of nicotine when given in joint administration (Gasior et al. 2002). Moreover, a faster acquisition of nicotine self-administration has been reported in rats chronically exposed to caffeine, suggesting that caffeine may potentiate the reinforcing effects of nicotine (Shoaib et al. 1999) (Fig. 1). Notably, the regimen of caffeine exposure used in these studies was found not to significantly affect the pharmacokinetics of nicotine (Gasior et al. 2002). This, therefore, suggests that caffeine facilitates the effects of nicotine through pharmacodynamic mechanisms, and lends support for caffeine consumption being a risky habit for nicotine abuse (Gasior et al. 2002).

The precise molecular mechanisms through which caffeine amplifies nicotine-mediated effects are not yet completely understood. Caffeine is known to affect the dopaminergic component of the nicotine discriminative stimulus in the rat (Gasior et al. 1999), and so it is reasonable to hypothesize the involvement of the dopaminergic system in caffeine–nicotine interactions. Although caffeine and nicotine preferentially target adenosine and nicotinic receptors, respectively, one should bear in mind that both drugs have a very complex pharmacological profile (Fredholm et al. 1999; Barik and Wonnacott 2009). Therefore, different neurotransmitters could be involved in caffeine–nicotine interactions.

Studies in humans have extended preclinical findings by ascertaining both similarities and additive interactions between caffeine and nicotine. It has been reported that caffeine and nicotine elevate subjective ratings of "good effect", liking, and "high" in a comparable fashion (Garrett and Griffiths 2001). Moreover, caffeine has been found to potentiate the stimulant and reinforcing effects of nicotine, and it has been observed that some subjective effects of caffeine, such as feelings of comfort, may be more marked in smokers (Perkins et al. 2001; Jones and Griffiths 2003). However, differences between the subjective effects of caffeine and nicotine have also been reported (Garrett and Griffiths 2001). Further, potentiation of nicotine-reinforcing effects by caffeine has not always been observed (Perkins et al. 2005; Blank et al. 2007) (Fig. 1). It is worth noting that the use of different methodological approaches may have affected the outcomes of these studies. For example, the smoking history of the individuals investigated is known to critically influence caffeine–nicotine interactions, as long-term nicotine use may elicit tolerance to some of its effects (Sobel et al. 2004). Similarly, other factors, such as caffeine history, route of administration of either substance, drug use, and the specific effects evaluated, are known to influence the features of caffeine–nicotine interactions (Garrett and Griffiths 2001; Jones and Griffiths 2003). Mechanisms other than pharmacological potentiation of nicotine effects have also been proposed to underlie joint consumption of caffeine and nicotine. On the basis of the evidence that smoking hastens caffeine metabolism in humans, it has been suggested that high caffeine intake by smokers could stem from a self-adjustment of the caffeine dose owing to its increased metabolism (Benowitz et al. 1989). Avoiding nicotine abstinence has also been taken into question, as caffeine counteracts some symptoms of nicotine withdrawal (Cohen et al. 1994; Sobel et al. 2004). Finally, other studies hypothesize that joint consumption of caffeine and nicotine might arise from personality traits, rather than pharmacological interactions between the substances (Gurpegui et al. 2007).

5.6 *Methylphenidate*

Pharmacological similarities and interactions between methylxanthines and methylphenidate have been described.

Studies in experimental animals have shown, on the one hand, that caffeine amplifies the motor-stimulant effects of methylphenidate through a dopaminergic mechanism (Boeck et al. 2009) and, on the other hand, that caffeine and methylphenidate share discriminative properties (Holtzman 1986).

In human studies, the ability of caffeine to engender methylphenidate-like introceptive cues has been replicated (Oliveto et al. 1993). Together with preclinical data, this finding suggests that consumption of caffeine might influence and potentially amplify the effects of methylphenidate in humans. This consideration is particularly relevant as methylphenidate is being increasingly used without medical prescription as a performance enhancer, chiefly by college students (Dupont et al. 2008), and individuals who improperly use methylphenidate are at higher risk for abusing other drugs and performing risky behavior (McCabe et al. 2005).

5.7 *Antagonists of the Glutamate NMDA Receptors*

Substances acting as antagonists of glutamate NMDA receptors, such as phencyclidine and its derivative ketamine, have long been used as recreational drugs

(Wolff and Winstock 2006); however, systematic studies evaluating their effects in humans are lacking.

Behavioral studies in experimental animals have observed a modulation of NMDA-mediated transmission by methylxanthines (de Oliveira et al. 2005). In line with this, caffeine has been reported to potentiate the psychostimulant effects of ketamine and to exert additive effects with phencyclidine in rodents (Powell and Holtzman 1998; Hsu et al. 2009b). Interestingly, in the rat, caffeine has been found to potentiate the rewarding effects elicited by NMDA receptor blockade, suggesting that caffeine consumption might impact the addictive properties of antagonists at such receptors (Bespalov et al. 2006), although to date this has not been proven conclusively. Nevertheless, and irrespective of drug abuse, the combined consumption of methylxanthines and NMDA receptor antagonists, in particular ketamine, appears inadvisable. In fact, studies in experimental animals have demonstrated that caffeine can boost the toxic and lethal effects of ketamine (Hsu et al. 2009b), suggesting that joint consumption of these substances can be potentially dangerous.

5.8 Opiates

It is not clear whether there is a correlation between intake of methylxanthines and opiate abuse, and hardly any evidence is available on this issue.

Studies performed in experimental animals have demonstrated a potentiation of morphine-stimulated locomotion by acute caffeine in mice; however, an inversion of this effect has been observed over time (Kuribara 1995; Weisberg and Kaplan 1999). Mixed findings have also been reported with regard to the way methylxanthines influence the rewarding/reinforcing properties of opiates. Theophylline has been reported to attenuate morphine-induced CPP in rats, suggesting that this methylxanthine could blunt the rewarding properties of morphine (Sahraei et al. 2006). Theophylline has also been found to affect morphine self-administration in rats, suggesting an influence on the reinforcing properties of opiates. However, such an effect appears very complex, as theophylline has been reported to either facilitate or depress morphine self-administration (Sahraei et al. 1999). Interestingly, a depressant influence on morphine self-administration has also been observed in rats treated with caffeine (Sudakov et al. 2003).

Both A_1 and A_{2A} receptors seem to participate in the interactions between methylxanthines and opiates. The specific subtype involved, however, depends on the particular effect of the opiates considered, and this might explain the mixed outcomes from studies in experimental animals. In fact, while the A_1 receptors are mostly involved in opiate-stimulated locomotion, the A_{2A} receptors have a major role in opiate reinforcing properties (Sahraei et al. 1999; Weisberg and Kaplan 1999). Moreover, a role for dopamine transmission in the modulation of opiate effects by methylxanthines has been suggested (Kuribara 1995).

The data in experimental animals summarized above do not account for an overt facilitatory influence of methylxanthines on opiate-induced effects, and accordingly do not provide support for the consumption of methylxanthines being a major risk for opiate abuse in humans. Nevertheless, it is worth mentioning that, in experimental animals, blockade of adenosine transmission can precipitate the symptoms of opiate withdrawal (Khalili et al. 2001; Stella et al. 2003).

5.9 Anxiolytic–Hypnotics

Anxiolytic–hypnotics, including benzodiazepines and barbiturates, are often used without medical prescription or for a time longer than is required for them to exert their therapeutic effects (Licata and Rowlett 2008). Several investigations have examined the pharmacological interactions between methylxanthines and these compounds.

Investigations in experimental animals have shown that methylxanthines and anxiolytic–hypnotics interact at the pharmacokinetic level (Lau and Wang 1996). Conversely, negligible pharmacodynamic interactions seem to take place between these substances (Lau et al. 1997).

The findings of studies in humans are in line with what has been reported in experimental animals, showing the existence of pharmacokinetic interactions between methylxanthines and benzodiazepines and the occurrence of mixed pharmacodynamic effects following the joint consumption of these substances (Roache and Griffiths 1987; Cysneiros et al. 2007).

It has been reported that the improper use of benzodiazepines may be associated with heavy caffeine consumption (Lekka et al. 1997), but conflicting data on this issue also exist (Cooper et al. 2004). Notably, even when such an association was observed, it was not possible to ascertain whether caffeine consumption triggered the use of benzodiazepines or whether caffeine was consumed as self-medication to counteract some unwanted effect of benzodiazepines (Lekka et al. 1997). Similarly, hardly any data exist that suggest a causal correlation between consumption of methylxanthines and abuse of barbiturates. Therefore, on the basis of current evidence, consumption of methylxanthines does not appear to be a major risk factor for the improper use of anxiolytic–hypnotic drugs.

5.10 Miscellaneous Psychoactive Drugs

Studies in experimental animals have demonstrated that, in addition to the substances described already, methylxanthines can interact with diverse psychoactive drugs, such as antipsychotics, antidepressants, phenylethylamines, and volatile solvents (Young et al. 1998; Chan and Chen 2003; Enríquez-Castillo et al. 2008; Varty et al. 2008). The implications of such interactions in terms of drug dependence, however, appear very limited.

6 General Considerations on Methylxanthines and Drug Dependence Phenomena

The data summarized in this chapter indicate that consumption of methylxanthines, such as caffeine, may promote the onset of dependence – although the features of such dependence appear less marked than those typical of dependence on other psychoactive substances such as psychostimulants, ethanol, nicotine, and opiates. Dependence on caffeine is generally compatible with social and productive life, although, as suggested by studies in experimental animals, it might carry a risk of favoring the establishment of a dependence on other substances. In fact, as described already, several pieces of evidence demonstrate that methylxanthines can amplify the effects of diverse addictive substances, potentially leading to an increase in their liability for abuse. Such a potential influence of methylxanthines on dependence phenomena appears particularly relevant to cocaine and nicotine abuse. In addition to this, consumption of methylxanthines has been shown to be associated with the intake of addictive substances even without overtly influencing their abuse liability. This phenomenon appears evident with ethanol, the consumption of which is likely promoted by the methylxanthine caffeine by means of a mechanism involving a reduced self-perception of ethanol intoxication. Interactions between methylxanthines and other psychoactive substances appear more complex, and their relevance to drug dependence is often undetermined. In this regard, a potential facilitatory influence of methylxanthines on the addictive effects of drugs such as amphetamines, methylphenidate, and cannabis derivatives is postulated, but not conclusively proven. An issue of particular relevance when examining the role played by methylxanthines in dependence phenomena is that adenosine receptors, to which methylxanthines bind with high affinity, can interact with several neurochemical pathways involved in the effects of addictive psychoactive substances. Of paramount interest in this regard is the finding that methylxanthines, and in particular caffeine, may interact with the dopaminergic system, which plays a crucial role in drug-dependence phenomena (Fig. 2).

In conclusion, data in this chapter indicate that caution should be exercised in the combined consumption of methylxanthines and other psychoactive substances. In fact, although consumption of methylxanthines does not seem to be particularly harmful per se, it could nevertheless result in modification of the effects of other psychoactive substances, and this might have implications in terms of both drug dependence and drug-induced toxicity.

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Methylxanthines and Human Health: Epidemiological and Experimental Evidence

Marie-Soleil Beaudoin and Terry E. Graham

Contents

Abstract When considering methylxanthines and human health, it must be recognized that in many countries most caffeine is consumed as coffee. This is further confounded by the fact that coffee contains many bioactive substances in addition to caffeine; it is rich in phenols (quinides, chlorogenic acid, and lactones) and also has diterpenes (fatty acid esters), potassium, niacin, magnesium, and the vitamin B_3 precursor trigonelline. There is a paradox as consumption of either caffeine or caffeinated coffee results in a marked insulin resistance and yet habitual coffee consumption has repeatedly been reported to markedly reduce the risk for type 2 diabetes. There is strong evidence that caffeine reduces insulin sensitivity in

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skeletal muscle and this may be due to a combination of direct antagonism of A_1 receptors and indirectly β-adrenergic stimulation as a result of increased sympathetic activity. Caffeine may also induce reduced hepatic glucose output. With the exception of bone mineral, there is little evidence that caffeine impacts negatively on other health issues. Coffee does not increase the risk of cardiovascular diseases or cancers and there is some evidence suggesting a positive relationship for the former and for some cancers, particularly hepatic cancer.

Keywords Caffeine · Coffee · Insulin resistance · Type 2 diabetes · Adenosine · Cardiovascular disease

Abbreviations

AUC	Area under the curve
cAMP	Cyclic AMP
CGA	Chlorogenic acid
CNS	Central nervous system
CVD	Cardiovascular disease
CYP1A2	Cytochrome P450 1A2
FFA	Free fatty acid
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
ISI	Insulin sensitivity index
OGTT	Oral glucose tolerance test
SNS	Sympathetic nervous system
T2D	Type 2 diabetes

1 Introduction

The topic of this chapter is methylxanthines and human health. However, caffeine is the dominant methylxanthine consumed by humans and, furthermore, humans rarely consume caffeine by itself. The vast majority of caffeine consumption is as coffee. Therefore, in order to address human health practically, we have chosen to review both caffeine and coffee. It is very important to note that coffee is a complex food that is composed of thousands of compounds and that caffeine is only one of at least a hundred that are biologically active (Ranheim and Halvorsen 2005). In addition, much of the laboratory-based research with humans and animal models is with pure caffeine and yet the majority of epidemiology research is based on coffee consumption. One should not transpose the effects of caffeine to those of coffee without strong supporting data. A further challenge in establishing the effects of caffeine on human health is to consider the physiological effects of caffeine and not the pharmacological actions. Humans normally experience systemic plasma concentrations of up to 30–60 μmol/L, while many investigations use levels that are orders of magnitude greater.

The focus of the chapter is on human health and thus we will restrict the review to the ingestion of caffeine or other methylxanthines and coffee rather than other forms of administration. There appears to be an endless supply of novel commercial products that contain caffeine. These are designed for ingestion, skin application, or even to be aspirated. For example, Reissig et al. (2009) reviewed caffeinated energy drinks; they reported that, in the USA, bottles/cans of these items provided from 50 mg to over 500 mg of caffeine and their popularity is reflected by the estimation that sales in 2006 were US $5.4 billion. Nevertheless, coffee remains by far the most common dietary source of caffeine in North America, Europe, and Brazil (Health Canada 2007; Frary et al. 2005; Camargo et al. 1999). In addition, coffee is the main source of caffeine for athletes, used as a way to improve performance (Tunicliffe et al. 2008). On the other hand, although tea generally contains less caffeine than coffee, it constitutes a major source of caffeine in heavy tea-consuming countries such as Japan (Yamada et al. 2009) and the UK (Lundsberg 1998). Similarly, mate is the main contributor to caffeine intake in Argentina (Olmos et al. 2009). Despite the heterogeneity of caffeine dietary sources, health issues have mostly been investigated in relation to pure caffeine or coffee. Consequently, while the current chapter will focus on coffee, caffeine, and health, it will also acknowledge the potential health effects of other caffeine sources, such as tea, when the information is available.

There have been a number of broad reviews on either caffeine or methylxanthine-containing foods and human health. Nawrot et al. (2003) concluded that moderate caffeine consumption (approximately 400 mg/day) was not associated with any adverse effects, including cardiovascular health and cancers. Others (van Dam 2008; Ranheim and Halvorsen 2005) have pointed out that consumption of boiled/unfiltered coffee increases the levels of blood lipids and is a risk for cardiovascular disease (CVD). However, filtered coffee reduces the risk of CVD and type 2 diabetes (T2D). It is commonly proposed that this may be due to the antioxidant, heterocyclic compounds in coffee. Similarly, Engler and Engler (2006) concluded that cocoa and chocolate have beneficial cardiovascular effects owing to their high flavonol content.

One often assumes that ill-health issues of obesity and the associated diseases such as T2D (and the complications of neuropathies, blindness, renal failure, amputations) and CVD are predominantly problems for the developed countries. While obesity is obviously a major problem in countries such as Canada and the USA, it is now a significant issue in countries such as Brazil, Guatemala, Nigeria, India, and China (Misra and Khurana 2008). Given this, it is not surprising that T2D is a global health problem. For example, almost 80% of diabetes deaths occur in low-and middle-income countries (WHO 2010). The World Health Organization estimates that over 4% of the world population will have T2D in two decades. Thus, if caffeine and coffee are nutritional factors associated with this disease, this is important in global health.

For both ethical and economic reasons, it is also important to note that not only is the frequency of occurrence of T2D increasing dramatically, but also that the average age of diagnosis is becoming younger. It is well documented that key risk factors are inactivity and obesity. Lifestyle intervention (physical activity and a healthy diet) is one of the best treatments and is also very important in preventing or delaying T2D. In this chapter the main focus will be on the relationship between caffeine and coffee and carbohydrate homeostasis and the primary health issue will be T2D.

The regulation of insulin and carbohydrate homeostasis is complex, involving many tissues and processes before one superimposes caffeine, a biologically active compound that can affect almost every tissue of the body. It also is a very important question as caffeine is a stable part of the diet of most adults globally. To add to the complexity, the most common source of caffeine is coffee, which contains many bioactive substances in addition to caffeine.

In order to evaluate the potential health impact of methylxanthines, it is fundamental that their impact on each tissue is understood. Methylxanthines are known to directly affect many tissues and this is a major complication for establishing the primary actions of caffeine in the human body. Once any one tissue has responded to caffeine, this response can result in a secondary action that is indirectly associated with the ingestion of caffeine. Figure 1 summarizes this with reference to the potential for caffeine to interfere with the actions of insulin on skeletal muscle. For example, caffeine stimulates the central nervous system (CNS) and the resultant changes in activity in the sympathetic nervous system (SNS) output could result in

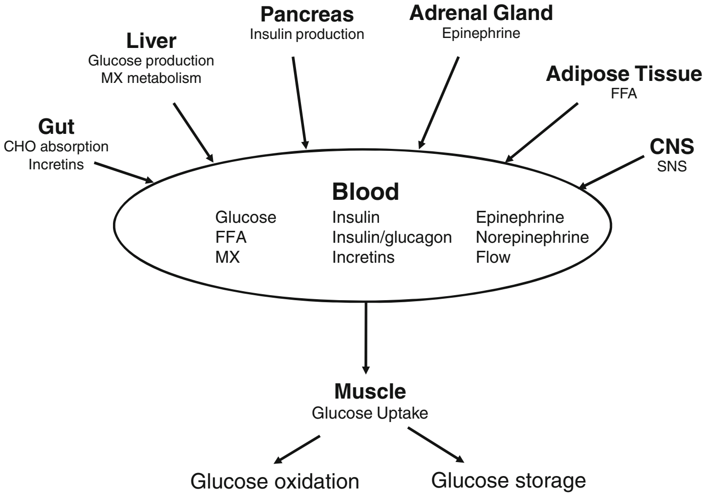

Fig. 1 Summary of the putative primary and secondary effects of caffeine on factors affecting carbohydrate homeostasis in some of the biological systems in the human. Caffeine affects several tissues simultaneously, which complicates the understanding of the physiological effects of caffeine. *CHO* carbohydrate, *CNS* central nervous system, *FFA* free fatty acids, *MX* methylxanthine, *SNS* sympathetic nervous system

further actions from a number of tissues. As will be reviewed briefly herein and has been discussed at length in other chapters, the main action of caffeine in its physiological range appears to be that of an adenosine receptor antagonist. Thus, any tissue that expresses such receptors could respond to caffeine. However, there are several subtypes of adenosine receptors and they are found in most tissues. This results in a vast array of possible primary and secondary responses. Owing to this complexity and multiple interactions, many scientists restrict their investigations to merely one tissue or cell line and only to caffeine. While this reductionist approach provides many scientific advantages, it dramatically restricts one's ability to understand the physiological effects of caffeine on the human, let alone the effects of coffee ingestion.

2 Caffeine, Coffee, and Carbohydrate Homeostasis

2.1 Acute Ingestion of Caffeine and Carbohydrate Homeostasis

Caffeine and coffee have been extensively studied in regard to their effects on insulin resistance and T2D. As mentioned, caffeine and coffee constitute two distinct metabolic challenges and appear to have different impacts on human health. In the case of insulin resistance, extensive literature supports that caffeine acutely decreases insulin sensitivity. In contrast, a similar body of literature documents that chronic consumption of coffee decreases the risk of T2D.

Acute administration of alkaloid caffeine impairs glucose homeostasis in healthy (Graham et al. 2001; Dekker et al. 2007; Battram et al. 2006; Keijzers et al. 2002; Norager et al. 2006), obese (Petrie et al. 2004; Lee et al. 2005), and diabetic (Robinson et al. 2004; Lee et al. 2005; Lane et al. 2004, 2008) subjects. In responsive populations, consumption of caffeine 1 h before an oral glucose tolerance test (OGTT) has been consistently shown to increase insulin area under the curve (AUC) by 25–42% (Graham et al. 2001; Robinson et al. 2004; Dekker et al. 2007; Thong and Graham 2002). Despite this exaggerated insulin response, most (Pizziol et al. 1998; Graham et al. 2000; Battram et al. 2006; Robinson et al. 2004; Dekker et al. 2007), but not all (Petrie et al. 2004; Thong and Graham 2002), studies reported exaggerated blood glucose response and elevated glucose AUC. An insulin sensitivity index (ISI) was developed to estimate insulin sensitivity based on the insulin and glucose AUC (Matsuda and DeFronzo 1999). Caffeine has consistently reduced the ISI by 14–25% compared with a placebo (Table 1) (Battram et al. 2006; Petrie et al. 2004; Robinson et al. 2004; Thong and Graham 2002).

In addition, the effects of caffeine on insulin sensitivity have been examined with the euglycemic–hyperinsulinemic clamp technique. In agreement with the studies employing the OGTT method, ingesting 5 mg caffeine per kilogram of body weight prior to the insulin clamp reduced the glucose infusion rate (i.e., created an insulin resistance) by 13–37% compared with a placebo (Fig. 2, Table 2)

Table 1 Summary of contemporary studies that examined the effects of caffeine on glucose and insulin metabolism during an oral carbohydrate challenge in humans

Reference	Subjects	Treatments	Study design	Results
Pizziol et al. (1998)	30 (12 males, 18 females) healthy subjects	CAF (200 mg) or DECAF (50 mL)	DECAF or DECAF + CAF 5 min prior to OGTT (4 h)	DECAF + CAF increased glucose AUC. No significant difference for insulin AUC
Graham et al. (2001)	18 young, fit males	CAF (5 mg/kg BW) or PL	Ingestion of capsule 1 h prior to OGTT (2 h).	CAF increased C-peptide (37%), insulin (60%), and glucose (24%) AUC
Thong and Graham (2002)	7 active males	PL, CAF (5 mg/kg BW), PRO (80 mg), CAF + PRO	Ingestion 90 min prior to OGTT (2 h)	CAF increased insulin (42%) and C-peptide (37%) AUC. No change in glucose AUC. CAF + PRO did not differ from PL
Lane et al. (2004)	14 (11 males, 3 females) T2D, CAF users	CAF (375 mg) or PL	250 mg CAF 1 h prior to 125 mg CAF + boost (75 g CHO). Blood samples drawn from 0 to 180 min	CAF increased glucose AUC (21%) and insulin AUC (48%)
Petrie et al. (2004)	9 obese, healthy males	CAF (5 mg/kg BW) or PL	Ingestion of capsule 1 h prior to OGTT (2 h) before and after 12-week weight-loss program	CAF increased insulin AUC before and after weight loss. No change in glucose AUC
Robinson et al. (2004)	12 males with T2D	CAF (5 mg/kg BW) or PL	Ingestion of capsule 1 h prior to OGTT (3 h)	CAF increased glucose AUC (16%) and insulin AUC (25%). CAF decreased insulin sensitivity by 14%
Norager et al. (2006)	30 (15 males, 15 females) elderly (> 70-year-old) subjects	CAF (6 mg/kg BW) or PL	Cycling at 65% of maximum O_2 uptake 1 h after capsule ingestion	CAF increased insulin resistance (HOMA-IR) by 23% at the end of exercise
Battram et al. (2007b)	14 tetraplegic patients	CAF (4 mg/kg BW) or PL	Ingestion of capsule 1 h prior to 2 h-OGTT	No effects of CAF on glucose, insulin, proinsulin or C-peptide

Dekker et al. (2007)	12 males, noncaffeine users	CAF (5 mg/kg BW) or PL	Habituation. 1 PLA and 3 CAF trials separated by 7 days + consumption of 5 mg/kg CAF every day	CAF increased glucose AUC at day 0 and insulin AUC at day 0 and day 14
Lane et al. (2008)	10 (5 males, 5 females) T2D, CAF users	CAF (500 mg) or PL	250 mg CAF with breakfast (Boost Plus – 90 g CHO) + 250 mg CAF with lunch. Continuous blood glucose monitoring for 72 h	CAF increased average daytime glucose and postprandial glucose responses
Robinson et al. (2009)	27 pregnant women (19 without GDM; 8 with GDM)	CAF (3 mg/kg prepregnancy BW) or PL	Ingestion of capsule 1 h prior to an OGTT (2 h)	CAF increased glucose AUC and C-peptide AUC in GDM women. CAF decreased insulin sensitivity by 18% in GDM women

AUC area under the curve, *BW* body weight, *CAF* caffeine, *CHO* carbohydrate, *DECAF* decaffeinated coffee, *GDM* gestational diabetes mellitus, *HOMA-IR* homeostasis model assessment of insulin resistance, *OGTT* oral glucose tolerance test, *PL* placebo, *PRO* propanolol, *T2D* type 2 diabetes

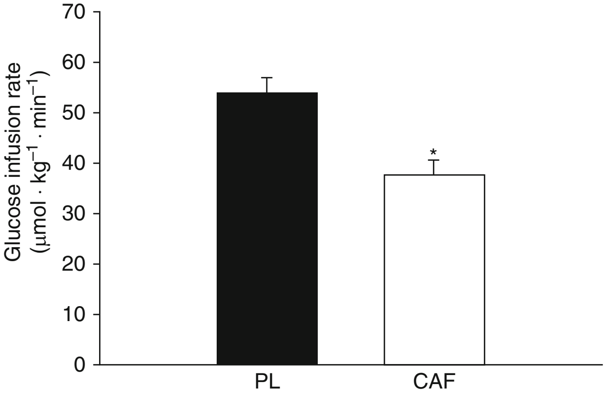

Fig. 2 Caffeine-induced insulin resistance shown during a euglycemic–hyperinsulinemic clamp. Glucose infusion rates for a placebo (*PL*) and caffeine (*CAF*) are shown for seven healthy males. The *asterisk* indicates that the ingestion of caffeine lowered the glucose disposal compared with the placebo. (Reproduced from Thong et al. 2002 with permission)

(Keijzers et al. 2002; Greer et al. 2001; Thong et al. 2002; Battram et al. 2005; Lee et al. 2005; Battram et al. 2007a). As discussed below, this likely results from caffeine antagonism of adenosine receptors in skeletal muscle.

It must be noted that studies using euglycemic–hyperinsulinemic clamps investigate different aspects of metabolism than those using OGTTs. The former is the gold standard to examine peripheral tissues responses to insulin. On the other hand, an OGTT provides valuable data on postprandial metabolism and takes into account other tissues, such as the gut, pancreatic β cells, and the liver. Given that caffeine may affect gut incretins, liver glucose output, β-cell function, as well as adipose tissue and muscle, the information from both of these techniques is essential to obtain a better understanding of the effect of caffeine on carbohydrate metabolism. The consistent finding using either approach that caffeine resulted in a 15–30% reduction in insulin's effectiveness is impressive. It may suggest that the major reason for this immediate effect is the same in both situations: impaired glucose disposal in skeletal muscle.

While all of these investigations examined caffeine and focused on hyperglycemia and applications for T2D, it is noteworthy that de Galan et al. (2002) infused the dimethylxanthine theophylline, used type 1 diabetics, and infused glucose to maintain hypoglycemia rather than euglycemia. They found that this methylxanthine also caused an insulin resistance, but noted that for a person with type 1 diabetes who is hypoglycemic, this would be beneficial. A practical extension of this finding could be to suggest that in a hypoglycemic state, ingesting a beverage such as a cola would be particularly helpful. Not only would this provide a large amount of easily absorbed carbohydrate, but presumably the caffeine would also cause insulin resistance, thus further increasing the low amount of blood glucose.

In summary, the available evidence strongly supports that acute ingestion of caffeine impairs insulin sensitivity and glucose disposal in lean, obese, and diabetic

Table 2 Summary of contemporary studies that examined the effects of caffeine on glucose disposal during a euglycemic–hyperinsulinemic clamp in humans

Reference	Subjects	Treatments	Study design	Results
Greer et al. (2001)	9 sedentary, lean male, non-CAF users	CAF (5 mg/kg BW) or PL	Ingestion of capsule immediately followed by 180-min clamp	CAF decreased GIR by 24% and decreased CHO storage by 35%
Keijzers et al. (2002)	12 healthy subjects (6 females, 6 males)	CAF infusion (3 mg/kg BW) or saline	120-min clamp started 30 min after the start of the CAF infusion	CAF decreased insulin sensitivity by 15%
Thong et al. (2002)	7 recreationally active males	CAF (5 mg/kg BW) or PL	1-leg extension (1 h), rest (2 h), CAF or PL, rest (1 h), 100-min clamp	CAF decreased GIR (30%), decreased leg glucose uptake (51% exercised leg vs. 55% rested leg)
Battram et al. (2005)	12 healthy, young males; CAF users	CAF (5 mg/kg BW), PL and PL + high or low epinephrine	Ingestion of CAF or infusion of epinephrine 30 min prior to 120-min clamp	CAF and high EPI decreased GIR by 34 and 13%, respectively. EPI levels similar between CAF and low EPI
Lee et al. (2005)	Lean ($n = 8$), obese ($n = 7$), and T2D ($n = 8$) males	CAF (5 mg/kg BW) or PL	Ingestion of capsule 30 min prior to 180-min clamp before and after a 13-week aerobic exercise program	CAF decreased insulin sensitivity by 23–37% in all three groups before and after the exercise program
Battram et al. (2007a)	8 healthy males	PL + saline; CAF (5 mg/kg BW) + saline; PL + EPI; CAF + EPI	Ingestion of capsule 30 min prior to 120-min clamp	CAF (26%), EPI (24%), and CAF + EPI (42%) decreased insulin-corrected GIR vs. PL. Trend for CAF + EPI to be different from EPI or CAF ($p < 0.08$)

BW body weight, *CAF* caffeine, *CHO* carbohydrate, *EPI* epinephrine, *GIR* glucose infusion rate, *PL* placebo, *T2D* type 2 diabetes subjects. While data from studies investigating caffeine with either an OGTT or a euglycemic–hyperinsulinemic clamp are very convincing, such studies use a reductionist approach, investigating caffeine in isolation. As noted previously, caffeine is most often consumed in the form of beverages or foods. In North America, 60–75% of caffeine intake comes from coffee (Health Canada 2007). Therefore, investigating the effects of caffeinated coffee on insulin sensitivity and glucose uptake is highly relevant.

2.2 Acute Ingestion of Coffee and Carbohydrate Homeostasis

Similar to caffeine, acute consumption of coffee (note that the term "coffee" will refer to caffeinated coffee) is detrimental to glucose tolerance in healthy subjects. Coffee has been shown to acutely increase glucose AUC compared with decaffeinated coffee in healthy men during an OGTT (Battram et al. 2006; Greenberg et al. 2009; Johnston et al. 2003). In addition, Moisey et al. (2008) showed that, in comparison with decaffeinated coffee, regular coffee increased glucose AUC, insulin AUC, and decreased the ISI when consumed prior to both high and low glycemic index cereal meals (Table 3). Furthermore, this effect was also observed following a second meal (Moisey et al. 2010).

In these studies, coffee significantly impaired glucose disposal, when compared with decaffeinated coffee. One can then ask whether this effect is due to deleterious effects of coffee and/or to possible beneficial effects of decaffeinated coffee. There is limited information in this regard and the results are not consistent. While Battram et al. (2006) found that the glucose AUC during an OGTT was significantly lower following consumption of decaffeinated coffee compared with a placebo, Kacker (2003) did not report differences in insulin or glucose AUCs between decaffeinated coffee and water treatments during an OGTT. Recently, van Dijk et al. (2009) also failed to show an effect of decaffeinated coffee ingested prior to an OGTT and in contrast to all of these investigations, Greenberg et al. (2009) found that decaffeinated coffee resulted in impaired glucose metabolism compared with a placebo. While there is limited information and no consensus regarding decaffeinated coffee, taken collectively, a large number of investigations have revealed that acute ingestion of regular coffee is detrimental to glucose tolerance.

2.3 Chronic Ingestion of Caffeine and Coffee and Carbohydrate Management

Investigation of the effect of coffee consumption on carbohydrate homeostasis highlights a discrepancy between acute and chronic exposure to coffee. On one hand, acute consumption of coffee is detrimental to glucose tolerance. On the other hand, regular consumption of coffee is associated with a reduced risk of T2D.

Table 3 Summary of contemporary studies investigating the acute effects of coffee (caffeinated and decaffeinated) on glucose and insulin metabolisms in humans

Reference	Subjects	Treatments	Study design	Results
Johnston et al. (2003)	9 (4 males, 5 females) lean subjects	COF, DECAF, or water	400-mL drink ingested simultaneously with 25 g glucose. Blood samples were taken for 3 h	Between 0 and 30 min, COF increased glucose and insulin AUC vs. DECAF. COF also increased glucose AUC vs. PL
Battram et al. (2006)	11 healthy, young males	DECAF, COF, caffeine, PL	Ingestion of drink 1 h prior to OGTT	Caffeine increased glucose and insulin AUC. DECAF decreased glucose AUC vs. PL
Moisey et al. (2008)	10 healthy males	COF (5 mg/kg BW) or DECAF	Ingestion of drink 1 h prior to high GI or low GI cereal	COF increased glucose AUC, insulin AUC (NS for high GI), and C-peptide AUC for both low and high GI cereals
van Dijk et al. (2009)	15 overweight males	DECAF (12 g), trigonelline (500 mg), CGA (1 g), or PL	Ingestion 30 min prior to 2 h OGTT	CGA and trigonelline reduced glucose and insulin concentrations 15 min into OGTT No change in AUC
Moisey et al. (2010)	10 healthy males	COF (5 mg/kg BW), DECAF, or water	Ingestion of treatment + high glycemic cereal 3 h prior to a 3-h OGTT	In the OGTT, COF increased insulin AUC by 49 and 57% compared with DECAF and water, respectively COF increased glucose AUC and decreased ISI compared with DECAF and water during the OGTT
Greenberg et al. (2009)	11 healthy young men	COF (6 mg/kg BW), DECAF, caffeine, or water	Ingestion of treatment 1 h prior to OGTT	In the OGTT, DECAF had lower glucose AUC than caffeine The PL had lower insulin AUC than DECAF, COF, and caffeine

AUC area under the curve, *CGA* chlorogenic acid, *COF* caffeinated coffee, *DECAF* decaffeinated coffee, *GI* glycemic index, *ISI* insulin sensitivity index, *NS* nonsignificant, *OGTT* oral glucose tolerance test, *PL* placebo

While habitual intake of caffeine can lead to habituation and dampened responses in parameters such as increased blood pressure in some individuals (Debrah et al. 1995; Lovallo et al. 2004), Dekker et al. (2007) found that caffeine continued to result in an insulin resistance in caffeine-naïve subjects after 14 days of caffeine ingestion. In addition, in the many studies summarized above, most subjects had been regular consumers of coffee for years or decades. While there was a only brief period of withdrawal (1–2 days) prior to testing, they responded to caffeine or coffee consumption with a marked insulin resistance. Thus, any habituation to caffeine appears to be minimal and/or rapidly reversible.

In a meta-analysis of consumption of coffee and T2D, the relative risks for heavy (six or more cups per day) and moderate (four to six cups daily) coffee drinkers were 0.65 and 0.72, respectively, compared with the lowest coffee consumers (zero to two cups daily) (van Dam and Hu 2005). These findings have been replicated across sexes, geographical locations, and obesity levels (reviewed in van Dam 2008; van Dam and Hu 2005; van Dam et al. 2006; Pereira et al. 2006; Salazar-Martinez et al. 2004; Agardh et al. 2004; Tuomilehto et al. 2004), and this relationship is also documented in patients with impaired glucose tolerance at the baseline (Smith et al. 2006). Coffee appears to have dose-dependent protective effects on T2D (Fig. 3). A recent meta-analysis suggested that for every additional cup of coffee consumed daily, the risk of T2D is reduced by 7% (Huxley et al. 2009). An inverse, linear relationship has also been reported between T2D and the consumption of decaffeinated coffee and tea, although fewer studies investigated this association (Huxley et al. 2009). In fact, drinking daily three or four cups of regular coffee, decaffeinated coffee, or tea decreased the risk of T2D by 25, 33, and

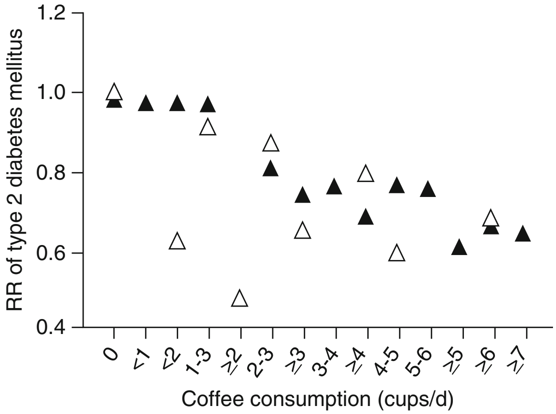

Fig. 3 Summary of the relative risk of type 2 diabetes with habitual coffee consumption. This figure summarizes the data from various epidemiology studies that were summarized by Huxley et al. (2009). The *closed symbols* represent the consumption of caffeinated coffee and the *open symbols* represent the consumption of decaffeinated coffee. Each additional daily cup of coffee consumed reduced the relative risk by 7% and consuming three or four cups of coffee a day resulted in a 25% lower risk compared with not drinking coffee. Similar results were found for decaffeinated coffee, but the number of studies was limited

20%, respectively (Huxley et al. 2009). Interestingly, a small prospective study found that moderate consumption of regular coffee prior to pregnancy, but not decaffeinated coffee, decreased the risk of gestational diabetes by 50% compared with the risk for nonconsumers (Adeney et al. 2007). It is noteworthy that Robinson et al. (2009) found that acute ingestion of caffeine did not cause insulin resistance in pregnant women who did not have gestational diabetes and yet those with this illness responded to caffeine with an insulin resistance (Table 1). In summary, regular coffee drinking is protective against diabetes (T2D and gestational diabetes) in a dose-dependent manner and across ethnic groups and a wide range of drinking habits.

The beneficial effects of coffee consumption are attributed to coffee compounds other than caffeine. Caffeine constitutes only approximately 2% of coffee, and some of the bioactive compounds in coffee are still unknown (Tunicliffe and Shearer 2008). The concentrations of these will vary with the type of bean, the growing conditions, the roasting techniques, and the method of brewing and filtering the coffee. Tse (1991, 1992) isolated an unidentified cholinomimetic compound from coffee and infusion into rodents resulted in a decrease in blood pressure and heart rate. Coffee also contains diterpenes (fatty acid esters), which elevate the level of blood cholesterol, and minerals such as potassium, niacin, and magnesium. Furthermore, coffee is a major dietary source of the vitamin B_3 precursor trigonelline, and is also rich in phenols [quinides, chlorogenic acid (CGA), and lactones]. These compounds are formed during the roasting of the beans and appear to have a variety of biological actions, including being antioxidants.

Oxidative stress occurs in the body when free radicals overcome the protective effects of antioxidants. The resulting cellular damage is associated with several health problems, including diabetes, CVD, and cancer. Fruits and vegetables are typical sources of antioxidants in the diet. However, the volume and frequency of coffee consumption in Western countries make coffee the major source of dietary antioxidants (Tunicliffe and Shearer 2008). The extent of their efficacy in humans in vivo is still debated.

Two classes of compounds have been specifically suggested to be beneficial to insulin sensitivity: CGAs and quinides. In diabetic rats, plant extracts containing mostly 5-caffeoylquinic acid (5-CQA), a common CGA in coffee, lowered plasma glucose levels (Andrade-Cetto and Wiedenfeld 2001). In humans, 1 g CGA and 500 mg trigonelline independently improved glucose and insulin concentrations early during an OGTT. However, this effect was not seen with decaffeinated coffee providing 264 mg CGA and 72 mg trigonelline (van Dijk et al. 2009). The possibility remains that prolonged or high-concentration exposure to these compounds may be necessary before there is a health benefit.

Shearer et al. (2007) provided direct evidence that short-term consumption of decaffeinated coffee had a beneficial result. They fed rats a high-fat diet for 28 days along with decaffeinated coffee, decaffeinated coffee with caffeine added, or water as the available fluid. The decaffeinated coffee treatment created an insulin-sensitive state as an increased glucose infusion rate was required during an insulin clamp compared with decaffeinated coffee with caffeine and water treatments.

The concentration of CGAs in coffee reaches 2.5 mmol/L but very little CGA is detected in the plasma of coffee drinkers because it is hydrolyzed rapidly (Tunicliffe and Shearer 2008). This suggests that advantageous effects of CGA could occur in the gastrointestinal system, before CGA is absorbed and degraded, or could be mediated indirectly, through its main product, caffeic acid (McCarthy 2005).

Quinides, another potentially beneficial class of compounds, are formed from CGAs during the roasting process of coffee beans and are thus specific to coffee. A euglycemic–hyperinsulemic clamp on rats simultaneously with the infusion of decaffeinated coffee extract, synthetic quinide, or saline showed that the synthetic quinide treatment increased the glucose infusion rate (Shearer et al. 2003). While these data strongly support the potential positive impact of decaffeinated coffee and particularly that of CGAs and/or quinides on glucose homeostasis, further research is necessary to confirm their efficacy in humans and their mechanistic bases. Epidemiological evidence documents that drinking coffee can reduce considerably the risk of T2D. This effect is attributed to coffee compounds other than caffeine. However, most products in coffee are still unknown; thus, it is difficult to attribute the positive effects of coffee to one particular class of compounds. It is important to point out that few of the epidemiology studies controlled for the type of coffee consumed. The methods of coffee preparation (e.g., espresso, boiled, filtered, decaffeination) influence the composition of coffee (Ranheim and Halvorsen 2005) and it is unknown how those varied types of coffee modulate metabolic responses.

3 Target Tissues and Actions of Caffeine

Adenosine receptors are ubiquitous, occurring throughout the nervous system, and in the vascular endothelium, heart, liver, adipose tissues, and muscle (Reppert and Weaver 1991; Dixon et al. 1996; Fredholm et al. 1999). Thus, the actions that result from caffeine are dependent on which type of adenosine receptors it blocks and in which tissue the receptors are located. It is unlikely that any one tissue is “dominating” the response, but rather it likely results from a combination of the actions of caffeine on various tissues. With regard to insulin resistance, it appears that the major tissue affected is skeletal muscle. Pertinent to this discussion, the effects of caffeine on muscle could be, in part, secondary to the initial effects on other tissues (Fig. 1). To gain a better understanding for how caffeine can mediate its multiple effects and influence one’s health, it is important to examine the actions of caffeine on various tissues. Two to three cups/mugs of coffee can result in plasma caffeine levels of 20–40 μmol/L (as will ingestion of approximately 5 mg/kg of caffeine) and this is accompanied with lower levels of the dimethylxanthines. Of the latter, paraxanthine is the most abundant, usually reaching 5–8 μmol/L (McLean and Graham 2002). Caffeine has a half-life of approximately 4–6 h (McLean and Graham 2002) and, at biological concentrations (5–50 μmol/L), it is an antagonist to adenosine receptors. This is addressed in detail in other chapters of this book. The receptors are associated with intracellular pathways that influence cyclic AMP (cAMP) production, phospholipase C, and mitogen-activated protein kinases (Schulte and Fredholm 2003). The receptors have several isoforms (A_1, A_{2a}, A_{2b}, and A_3) and caffeine is believed to antagonize all except the A_3 form (Daly and Fredholm 2004). Thus, the action of caffeine on a given tissue depends on its complement of receptor isoforms. The A_1 and A_2 adenosine receptors have opposite actions, with the former associated with G_i protein input onto adenylate cyclase, decreasing the level of intracellular cAMP, while the latter is associated with G_s protein and increases in the level of cAMP. The A_1 receptor is also associated with $G_{\beta\gamma}$ subunits of the heterotrimeric G protein that can have actions separate from those of the G_α subunit that mediates the inhibition of adenylate cyclase. This $G_{\beta\gamma}$ subunit affects calcium release, potassium channels, and voltage-sensitive calcium channels. With such diverse potential effects and a vast number of tissues expressing adenosine receptors, even the primary responses in a person who consumes caffeine are complex and when the secondary events are added to this it becomes an enormous challenge. This section will attempt to address these interactions as they apply to lipid and carbohydrate metabolism in muscle. The distribution of the subtypes of adenosine receptors will not be addressed as this has been addressed elsewhere in the text. It will only be noted in tissues that apply directly to the main topic of lipid and carbohydrate management.

3.1 Skeletal Muscle

Skeletal muscle is the dominant tissue for glucose disposal as it is insulin-sensitive and represents 35–40% of the body mass. Whole-body insulin resistance is commonly associated with limitations in this tissue. Thong et al. (2002) found that caffeine ingestion resulted in a 30% decrease in whole-body glucose disposal (Fig. 2) and this was associated with a 50–55% decrease in leg glucose uptake. This was found for the leg after exercise (when glucose uptake is normally enhanced) as well as when there was no exercise (Fig. 4). Thus, one of caffeine's dominant actions is to impair blood glucose disposal, particularly in skeletal muscle. The mechanisms by which this occurs have been difficult to establish.

Various studies (Vergauwen et al. 1994, 1997; Challis et al. 1984; Han et al. 1998) have demonstrated that functionally muscle has A_1 receptors and that caffeine's effects on rodent skeletal muscle are via the antagonism of A_1 receptors and subsequent elevation of the level of cAMP. However, until recently, only A_2 receptors had been demonstrated (Lynge and Hellsten 2000). Thong et al. (2007) have now shown that indeed A_1 receptors do physically exist in the plasma membrane of rat soleus muscle and, furthermore, A_1 antagonism in vitro resulted in a 30% decrease in glucose uptake. They have also demonstrated that human muscle experiences an increase in the level of cAMP (Thong et al. 2002). In addition, Cheng et al. (2000) demonstrated that streptozotocin-induced (i.e., insulin-free) diabetic rats experienced a decrease in blood glucose level when treated

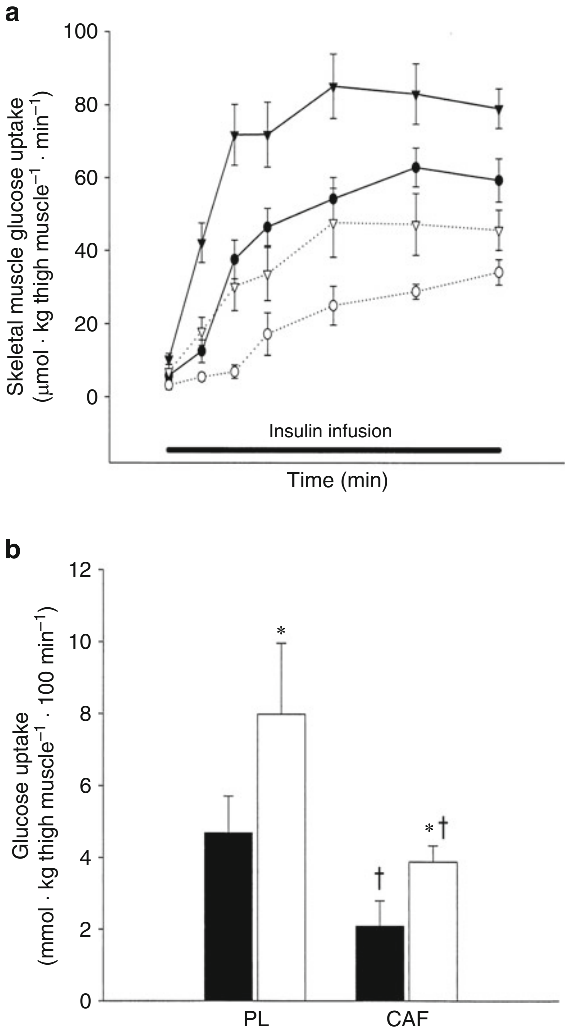

Fig. 4 Caffeine-induced insulin resistance in the leg of human subjects. **a** Summary of the net leg glucose uptake responses during a hyperinsulinemic–euglycemic clamp. The *open symbols* are for the subjects' leg that was not exercised and the *filled symbols* are for the previously exercised leg. The *circles* represent the caffeine-ingestion condition and the *triangles* represent the placebo. **b** Area under the curve data of **a**, with the *filled bars* representing the rested leg and the exercised leg is indicated by the *open bars*. The *asterisk* indicates a significant difference for exercise versus rest and the *cross* reflects a difference in caffeine versus the control. (Reproduced from Thong et al. 2002 with permission)

with dipyridamole to increase endogenous adenosine, an A_1 receptor agonist, while antagonism of A_1 receptors blocked the response. Furthermore, they found that the former treatment enhanced glucose uptake and glycogen synthesis, while A_1 receptor antagonism blocked the uptake of glucose.

There may also be an additional effect of caffeine on muscle carbohydrate metabolism. Several studies (Greer et al. 2001; Thong et al. 2002; Lee et al. 2005) noted that when there was a decreased glucose disposal due to caffeine, the whole-body metabolic rate and respiratory exchange ratio were not altered. Thus, the amount of carbohydrate oxidized was not affected and the amount of carbohydrate stored must account for the depressed glucose disposal. Lee et al.

(2005) estimated this to be a 50–65% depression in carbohydrate storage. Thong et al. (2002) were able to make these measures and estimates both for the whole body (Fig. 1) and the leg (Fig. 4). Their data demonstrated that this preferential impairment of storage rather than oxidation occurred in the leg, i.e., muscle.

Caffeine could have direct intracellular effects, as it is also known to cross cell membranes. Caffeine ingestion resulted in an increase in the level of cAMP in both exercised and rested legs and this was accompanied with a decrease in the level of glycogen synthase (17% fractional velocity and 35% I form) (Thong et al. 2002). Rush and Spriet (2001) reported that physiological concentrations of caffeine could directly inhibit glycogen phosphorylase. The findings are preliminary but they do present the possibility that caffeine could have direct actions on metabolic enzymes associated with carbohydrate storage. Thus, while less glucose enters this tissue during caffeine exposure, of that which does enter, it may be that a smaller fraction than normal is directed into glycogen storage.

We are only aware of one investigation that directly examined coffee and skeletal muscle glucose uptake. Shearer et al. (2007) fed rats a high-fat diet for 4 weeks to induce insulin resistance and they also consumed either water, decaffeinated coffee, or decaffeinated coffee plus caffeine during this time. Those consuming decaffeinated coffee had an increased insulin sensitivity of approximately 100%, but when the decaffeinated coffee had caffeine added, this positive effect was not present. They also found that decaffeinated coffee specifically resulted in increased glucose uptake in a variety of skeletal muscles, while this was not seen when caffeine was consumed and, furthermore, this caffeine treatment also depressed glucose uptake by the heart. Thus, while some components of coffee may increase insulin sensitivity in skeletal muscle, caffeine can result in insulin resistance and the latter could be due to antagonizing A_1 receptors. However, there are a variety of other possible actions of caffeine that could also impact on muscle.

3.2 *Adrenal Medulla and the Sympathetic Nervous System*

A number of areas of the CNS are affected by caffeine, but of particular interest for this topic is the SNS. As discussed previously, the SNS is stimulated when the CNS is exposed to caffeine. Epinephrine originates from efferent stimulation of the adrenal medulla, but circulating norepinephrine is the result of “spillover” or “washout” from the SNS activity of specific tissues. There are a number of reports that caffeine increases circulating levels of epinephrine, but changes in norepinephrine concentration have been inconsistent (Robertson et al. 1981; Arciero et al. 1995; Van Soeren et al. 1993; Graham and Spriet 1995). We (Graham et al. 2000) have reported an increase in norepinephrine “spillover” from the leg of humans during exercise. These endocrine responses may in turn precipitate some of the metabolic responses associated with caffeine ingestion. Increased SNS activity can directly result in decreased glucose clearance and can also mobilize free fatty acid (FFA) and the latter could result in insulin resistance.

Epinephrine constitutes a potential mechanistic explanation as elevations can result in a decrease in glucose uptake in muscle (Battram et al. 2007a; Howlett et al. 1999). Comparison of people with tetraplegia (Van Soeren et al. 1996; Mohr et al. 1998; Battram et al. 2007b) and able-bodied individuals provides insight into one of the actions of caffeine. Battram et al. (2007b) found that caffeine consumption followed by an OGTT in people with tetraplegia did not result in any indication of insulin resistance. This may be due to changes associated with the profound muscle atrophy that they experience or may be due to the SNS. Able-bodied individuals respond to caffeine ingestion with an increase of about 100% in the level of circulating epinephrine. However, patients with tetraplegic injuries have a denervated adrenal medulla and very little circulating epinephrine and when they ingest caffeine, there is no increase in the level of epinephrine (Van soeren et al. 1996; Battram et al. 2007b). This strongly suggests that the caffeine–epinephrine response is not a direct action on the medulla but rather is secondary to stimulation of the CNS. This finding also suggests that caffeine-induced insulin resistance could be a product of increased levels of catecholamines. The importance of epinephrine as a mediator of caffeine effects was also supported when simultaneous ingestion of caffeine and propanolol, a β-adrenergic receptor blocker, was shown to counteract the deleterious effects of caffeine alone on glucose disposal (Thong and Graham 2002). Thus, it appears that epinephrine is essential to mediate caffeine effects on glucose tolerance.

Mechanistically, epinephrine activation of β-adrenergic receptors decreases whole-body glucose metabolism by 40%, probably through inhibition of glucose transporter type 4 activity (Bonen et al. 1992; Han and Bonen 1998). This effect is of a magnitude similar to what is reported with caffeine alone (Tables 1, 2). However, epinephrine levels ranging from 2 to 4 nmol/L are necessary to generate this magnitude of a decrease in glucose disposal, while ingestion of 5 mg of caffeine per kilogram of body weight only increases the plasma epinephrine concentration to 0.3–0.6 nmol/L (Graham et al. 1998; Thong and Graham 2002). Owing to this difference in plasma concentrations, it is unlikely that epinephrine alone mediates the effects of caffeine on glucose disposal. In addition, Battram et al. (2005, 2007a) reported that the glucose infusion rate during a euglycemic–hyperinsulinemic clamp was reduced by 26 and 24% with caffeine and epinephrine infusions, respectively. This similar impairment was achieved even though the epinephrine concentration in the caffeine trial was 50% less than that in the epinephrine trial. Most importantly, they found that simultaneous ingestion of caffeine and infusion of epinephrine resulted in a far greater impact on glucose disposal (42%), despite lower epinephrine levels in this treatment compared with that of epinephrine infusion alone. In addition, as mentioned previously, Thong et al. (2007) showed that A_1 antagonism resulted in a 30% decrease in glucose uptake in skeletal muscle in vitro and there was no epinephrine present in the medium. Taken together, these data suggest that epinephrine is likely an important factor, but not sufficient, to entirely explain the caffeine effect. There is also clear evidence that antagonism of A_1 receptors in muscle is very important.

3.3 Adipose Tissue

Intravenous infusion of a triglyceride emulsion plus heparin for at least 3 h decreases glucose disposal during a euglycemic–hyperinsulinemic clamp (Boden et al. 2001; Belfort and Mandarino 2005; Dresner et al. 1999; Kruszynska et al. 2002; Itani et al. 2002; Homko et al. 2003). It is well known (Battram et al. 2006; Petrie et al. 2004; Thong et al. 2002; Dekker et al. 2007) that caffeine results in an elevation of FFA and thus this in turn could also induce insulin resistance. Catecholamines can directly increase the level of plasma FFA and it is not clear whether or not the FFA mobilization is due to a direct action of caffeine on adipocytes or is due to the increase in SNS activity described earlier. Adipocytes express adenosine A_1 receptors and these are tonically active, inhibiting adenylate cyclase and reducing both the level of cAMP and lipolysis (Liang et al. 2002). Adipocytes also have adrenergic β_2 receptors and thus they could mediate the lipolysis. Investigations with different subject populations have assisted in establishing critical aspects of the hierarchy of metabolic events associated with caffeine. When people with tetraplegia ingest caffeine, they experience no increase in the levels of epinephrine as noted earlier and yet there is a large increase in circulating FFA concentration that is comparable with that observed in able-bodied subjects (Van soeren et al. 1996; Battram et al. 2007b). This clearly demonstrates that caffeine directly enhances lipolysis independent of the SNS. Similarly, Johansson et al. (2007) studied rodent adipocytes with the A_1 receptor knocked out and concluded that this receptor was indeed responsible for the antilipolytic actions of caffeine.

Regardless of the mechanism of FFA mobilization, there are at least two reasons to believe that the rise in FFA concentration is not critical for the skeletal muscle insulin resistance. First, Greer et al. (2001) performed hyperinsulinemic–euglycemic clamps immediately after subjects had ingested caffeine. They observed a marked decrease in glucose disposal (Table 2) and yet the circulating FFA levels decreased rapidly and markedly from the onset of the hyperinsulinemia. In addition, as noted above, Battram et al. (2007b) found that caffeine followed by an OGTT did not result in insulin resistance in people with tetraplegia and yet there was a normal increase in FFA concentration. Thus, it appears that the mechanisms that influence skeletal muscle insulin sensitivity are (1) caffeine-induced stimulation of the SNS and increased circulation of epinephrine and (2) direct caffeine antagonism of A_1 receptors on the muscle tissue.

3.4 Liver

With regard to the topic of caffeine and carbohydrate homeostasis, most of the research focus has been on skeletal muscle, but the liver could be involved both in the regulation of the circulating levels of caffeine and also in the regulation of blood glucose concentrations. In addition, this tissue experiences concentrations of caffeine and glucose that far exceed those of other tissues. Once the caffeine or glucose is absorbed, it enters the portal circulation and is delivered to the liver via the portal artery. The liver can take up the glucose to store it as glycogen and can also liberate glucose into the systemic circulation. In addition, the hepatic P450 system metabolizes the caffeine to dimethylxanthines. These dimethylxanthines plus caffeine that were not catabolized in the first pass then enter the vena cava and their concentrations are diluted as they circulate systemically. Similarly, glucose that is not cleared, as well as any that is released from the liver owing to either glycogenolysis or gluconeogenesis, will enter the systemic circulation. Thus, the liver is exposed to a far higher concentration of caffeine than any other tissue of the body and also is fundamental to the dynamic regulation of blood glucose.

Rarely has the liver been investigated in vivo as its portal circulation and complex metabolism present methodological challenges. In dogs, adenosine infusion has been shown to increase hepatic glucose output and to inhibit the suppressive effects of insulin (McLane et al. 1990). Pencek et al. (2004) infused caffeine and glucose into the portal vein of conscious dogs during a hyperinsulinemic–hyperglycemic clamp. When the hepatic portal artery, portal vein, and a systemic (hepatic) artery were simultaneous sampled, the respective concentrations were 101, 83, and 35 μmol/L, illustrating the differences in caffeine levels throughout the circulation. The hepatocytes experienced threefold greater caffeine concentration compared with other tissues. In this study no differences were found in the levels of arterial glucagon, cortisol, and norepinephrine, but caffeine did induce a very modest increase in the level of epinephrine. Nevertheless, caffeine resulted in an approximately 100% increased net hepatic glucose uptake. Despite this, whole-body glucose level did not improve, implying that there was a decreased peripheral glucose uptake. Thus, the hepatic response to caffeine did not contribute to the exaggerated blood glucose response associated with caffeine and carbohydrate ingestion, but rather could minimize it.

The few studies of either adenosine or caffeine on hepatic carbohydrate metabolism support that the A_1 receptor enhances glucose release and caffeine reduces hepatic glucose output, but the metabolic factors involved have not been established. Buxton et al. (1987) reported that adenosine stimulated glucose release from isolated rat liver; they also found that adenosine stimulated glycogen phosphorylase activity in isolated hepatocytes. However, when Pencek et al. (2004) infused caffeine and glucose into the portal vein of conscious dogs, they found that the ratio of hepatic glycogen synthase to phosphorylase activity did not change. However, the level of hepatic glucose 6-phosphatase increased and lactate production increased by 40%. This may account for the observation (Graham et al. 2000) that caffeine consumption by human subjects resulted in elevated arterial lactate levels both at rest and during exercise despite no change in leg lactate production. We are not aware of any investigations of liver carbohydrate metabolism and coffee; however, both CGA (Arion et al. 1997) and a derivative (Herling et al. 1999) have been found to inhibit hepatic glucose 6-phosphatase, a key reaction in the production of glucose by the liver. Furthermore, Herling et al. (1999) also demonstrated a dose-dependent reduction in blood glucose level in rats exposed to a synthetic derivative of CGA. As mentioned previously, infusing rats with a synthetic quinide during a euglycemic–hyperinsulinemic clamp increased the glucose infusion rate by approximately 40% (Shearer et al. 2003). There was no change in the glucose uptake of a variety of skeletal muscles or the heart and they proposed that this positive impact on whole-body insulin sensitivity was due to a reduced net hepatic glucose output. This presents the possibility that caffeine and other components of coffee could all reduce hepatic glucose release.

3.5 Gastrointestinal System

While skeletal muscle, the pancreas, and the liver have been recognized as essential tissues in modulating insulin responses, intestinal-derived factors are now believed to play an important role in carbohydrate homeostasis. Two peptides, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), can enhance glucose-induced insulin secretion of β cells. These incretin hormones are secreted from the gastrointestinal tract and their resulting actions are mediated through G-protein-coupled receptors in a variety of tissues, most importantly on the pancreas. In humans, incretin actions on pancreatic β cells are thought to account for approximately 50–70% of the total postprandial insulin secretion (Baggio and Drucker 2007).

Both dietary carbohydrates and lipids stimulate GLP-1 and GIP secretion probably through direct interaction between nutrients and cells. Potential mechanisms of incretins' insulinotropic effects include activation of insulin gene transcription, enhanced messenger RNA stability, and improved insulin bioavailability (Baggio and Drucker 2007). In addition to well-documented effects on the pancreas, extra-pancreatic incretin actions have also been described. For instance, Sandhu et al. (1999) described that infusion of GLP-1 during a hyperinsulinemic clamp in depancreatized dogs increased the glucose infusion rate and whole-body glucose utilization. This insulin-sensitivity effect was absent during a clamp with lower (fasting) levels of insulin. The main tissue involved in glucose disposal is skeletal muscle, and they found evidence of GLP-1 receptor messenger RNA in skeletal muscle. This contrasts with other studies that did not find the presence of GLP-1 receptors in muscle in humans and rats (Bullock et al. 1996; Wei and Mojsov 1996). This could suggest a species-specific expression of GLP-1 receptors and actions; therefore, additional studies are necessary before this finding can be extended to humans.

These hormones are currently the focus of a great deal of pharmacological investigation regarding T2D. Intravenous injection of GLP-1 in fasted, T2D patients increased the level of C-peptide concurrently with the level of insulin, suggesting that at least some of the effects of incretin mimetics occur at the pancreas level, potentiating insulin secretion after a meal (Meier et al. 2004). In addition, with long-term treatment, GLP-1 mimetics and dipeptidyl peptidase-4 inhibitors can reduce the level of glycosylated hemoglobin (HbA_{1c}) by 0.8–1.75% (Drucker and Nauck 2006).

To the best of our knowledge, the impact of caffeine on the secretion of incretins has not been investigated. However, Johnston et al. (2003) examined the effects of ingesting coffee or water concurrent with 25 g dextrose. They showed that coffee consumption resulted in a blunted GIP response and there was a trend for decaffeinated coffee to induce a lower response than that of regular coffee. However, these findings are controversial as recently Greenberg et al. (2009) failed to find any effect of either decaffeinated coffee or regular coffee on GIP responses for the first 2 h of an OGTT challenge.

Johnston et al. (2003) suggested that antioxidants present in coffee, especially CGA, could act to delay glucose absorption, hence explaining the blunted GIP secretion with coffee. It is not known if caffeine itself could have an impact on carbohydrate homeostasis prior to its absorption from the gastrointestinal system. The effects reported for caffeine on the gastrointestinal system have been inconsistent regarding responses such as gastric secretion, gastroesophageal reflux, and jejunal secretions. Van Nieuwenhoven et al. (2000) conducted a comprehensive investigation of gastrointestinal function and caffeine. However, it was a complicated study as the caffeine was ingested with a carbohydrate–electrolyte solution and after a rest period, 90 min of steady-state exercise was performed. They found that the caffeine ingestion did not affect gastric emptying, orocecal transit time, gastroesophageal reflux, gastric pH, or gastrointestinal transit time. While intestinal permeability was unaffected, intestinal glucose uptake was greater following the caffeine–carbohydrate–electrolyte ingestion as compared with carbohydrate–electrolyte consumption alone. The authors speculated that caffeine stimulated the sodium–glucose-linked transporter that is associated with glucose uptake by the jejunum. The authors also pointed out that the lack of detrimental effects of caffeine could be due to the low dose (120 mg). Yeo et al. (2005) reported that coingesting caffeine and a glucose solution during prolonged exercise resulted in the greatest increase in carbohydrate oxidation and this was due to increased exogenous carbohydrate oxidation. However, this is in contrast to previous reports in which it was stated that caffeine did not influence carbohydrate absorption or oxidation during exercise (Jacobson et al. 2001; Sasaki et al. 1987).

Incretin effects on carbohydrate homeostasis likely include both pancreatic and extrapancreatic responses. It is unknown if caffeine or coffee affects the incretin response. This is an area of science that is in its infancy regarding the incretin impact both on the pancreatic β cells and on other tissues, especially skeletal muscle. Given the emphasis that is placed on altered incretin responses in the T2D state, the putative roles of coffee and caffeine need to be explored in detail. While caffeine may have effects on gastrointestinal glucose absorption, the understanding is far from complete at this time. The possibility that aspects of coffee may blunt some of the incretin response needs to be investigated further. It is not clear how this would be associated with the positive effect of chronic consumption of coffee on the risk for T2D. It would also seem highly unlikely that increased absorption would result in the increased glucose AUC and insulin resistance, but it could be a contributing factor.

3.6 *Pancreatic β Cells*

With regard to pancreatic β cells, caffeine could also have a direct effect on insulin secretion. The β cells have A_1 receptors that inhibit insulin secretion (Hilliare-Buys et al. 1989; Töpfer et al. 2008) and exposure to caffeine can increase insulin release (Töpfer et al. 2008). In studies in which caffeine and OGTT challenges are given to humans, normally the caffeine is ingested 1 h prior to the OGTT. The reports summarized in Table 1 did not observe an increase in the level of either insulin or C-peptide prior to the OGTT. Furthermore, if the caffeine did promote insulin secretion, this should lower the blood glucose level, but this was not observed either prior to or during the OGTT. Finally, during insulin clamp studies the insulin secretion would not be a factor. Thus, it appears that a caffeine-induced insulin secretion is not a factor in carbohydrate management following a single ingestion of caffeine. In contrast, Wu et al. (2005) found that habitual consumption of coffee was inversely associated with fasting concentrations of C-peptide. This result was found for both decaffeinated and caffeinated coffee, but not for tea, suggesting that bioactive factors in coffee, other than caffeine, accounted for this change. It is noteworthy that the C-peptide reduction was greater for obese (27%) or overweight (20%) women compared with normal-weight women (11%).

Figure 1 summarizes the known effects of caffeine and caffeinated coffee that may acutely affect carbohydrate homeostasis. The major site of the insulin resistance is clearly skeletal muscle and it appears that the metabolic alteration is initially the inhibition of uptake of glucose and subsequently its storage, while carbohydrate oxidation is unaffected. An increase in SNS activity or at least the presence of a normal concentration of epinephrine appears to be important, but role these play has not been established. In addition, there may be an enhanced gastrointestinal absorption of glucose and there appears to be a decreased net hepatic glucose output. So little is known regarding the habitual effects of the noncaffeine components of coffee on these tissues and mechanisms to allow us to speculate regarding how coffee consumption decreases the risk for T2D. Van Dam et al. (2006) proposed that since the relative risk for T2D is similar in obese and nonobese coffee drinkers, this must mean that the absolute reduction in risk is greater for the obese coffee drinkers and they may particularly benefit from coffee!

4 Caffeine, Coffee, and Cardiovascular Disease: Epidemiology and Mechanisms

The association of caffeine and CVD is reviewed in depth in Riksen et al. (2010). Once caffeine enters the systemic circulation, every tissue of the body is exposed to this substance. It is well known that caffeine and coffee ingestion result in a modest increase in vascular resistance and thus an increase in blood pressure, particularly the diastolic pressure (Jee et al. 1999; Smits et al. 1983, 1991; Quinlan et al. 2000). Smits et al. (1991) also showed that a caffeine challenge as little as 1 mg/kg (plasma concentration of 3.1 μmol/L) was enough to increase the pressure and higher levels of caffeine did not result in a larger response. Furthermore, caffeine almost completely attenuated the hemodynamic responses to the adenosine transport antagonist dipyridamole, suggesting that indeed caffeine is acting as an adenosine receptor antagonist. Similarly, Quinlan et al. (2000) confirmed that plasma caffeine concentrations of 5–10 μmol/L caused an increase in blood pressure, but the response was quite modest. Graham et al. (2000) reported that caffeine ingestion resulted in a 5 mm Hg increase in mean blood pressure and a 21% increase in leg vascular resistance but no difference in leg blood flow or oxygen uptake. While these effects could also be the result of increased levels of epinephrine (see the discussion earlier), Smits et al. (1990, 1991) demonstrated that the hemodynamic effects of caffeine were independent of a catecholamine effect.

Martin et al. (2006a) reported that approximately half of their healthy subjects demonstrated a blunted response to a forearm vascular conductance test and an adenosine infusion. The exercise hyperemia was the same in both groups but when nitric oxide production was inhibited, this affected only the “responders.” They concluded that those with a blunted response to adenosine also had less contribution from nitric oxide. When the methylxanthine derivative aminophylline was employed, the response to adenosine was markedly blunted, but only in the adenosine “responders” (Martin et al. 2006b). This suggests that there could be caffeine “responders” and “nonresponders”. However, this does not appear to be due to a CYP1A2*1F polymorphism as the differences were abolished when the subjects were infused with dipyridamole (Martin et al. 2007). The scientists proposed that the nonresponders could have a greater activity in the adenosine transporter and thus less endogenous adenosine available for the receptors.

Caffeinated coffee consumption has also been extensively investigated in relation to CVD risk. Early epidemiology studies reported increased risk of CVD in regular coffee drinkers (reviewed in Higdon and Frei 2006; Ranheim and Halvorsen 2005; van Dam 2008). This was based on the knowledge that acute consumption of coffee leads to a small increase in blood pressure and may be associated with hypertension in the long term (Jee et al. 1999). Despite these early suggestions, recent evidence does not support a role of coffee consumption in CVD prevalence. It is possible that the early studies did not adequately control for unhealthy behaviors associated with coffee drinking (e.g., cigarette smoking, physical inactivity, and alcohol consumption), therefore mistakenly associating increased CVD risk with coffee consumption.

In recent prospective studies (summarized in Table 4), high coffee consumption (four or more cups daily) did not increase the risk of heart-failure hospitalization/mortality (Ahmed et al. 2009), coronary heart disease (Kleemola et al. 2000), or cardiovascular mortality (Mineharu et al. 2009). Furthermore, coffee consumption was not associated with elevated CVD risk in postmyocardial infarction patients (Silletta et al. 2007; Mukamal et al. 2009). Similarly, in women, no significant relationships were found between the risks of stroke (Lopez-Garcia et al. 2009), myocardial infarction (Rosner et al. 2006), cardiovascular mortality (Mineharu et al. 2009), and moderate-to-heavy coffee consumption (four or five cups daily).

Table 4 Summary of contemporary studies examining the effects of long-term moderate and heavy coffee consumption on cardiovascular disease risk and mortality

Reference	Study design	Subjects	Dependent variable	Results	Conclusions
Jee et al. (1999)	Meta-analysis	11 trials; 522 subjects; mean duration: 56 days; mean coffee consumption: 5 cups/day	Systolic and diastolic BP	Systolic BP increased by 2.4 mm Hg Diastolic BP increased by 1.2 mm Hg	Consumption of coffee is associated with increased BP
Kleemola et al. (2000)	Cohort study; 10-year FU	20,179 Finnish men and women; age 30–59	Fatal and nonfatal CHD; total mortality	Adjusted RR for CHD events and mortality did not differ between low (1–3 cups/day) and high ($\geq$7 cups/day) coffee consumers, in both men and women	Coffee consumption does not increase the risk of CHD or death
Hammar et al. (2003)	Case-control study	Swedish men and women; age 45–70	First nonfatal MI	Increased incidence of MI when consuming boiled coffee vs. filtered coffee: Men RR: 1.41 Women RR: 1.63	Consumption of boiled coffee increased incidence of first nonfatal MI
Bidel et al. (2006)	Cohort study; 20.8-year FU	3,837 Finnish patients with T2D, age: 25–74	CVD, CHD, and stroke mortality	HR for total mortality: 0–2 cups/day: 1.00 3–4 cups/day: 0.77 5–6 cups/day: 0.68 $\geq$7 cups/day: 0.70 Same trend for CVD and CHD mortality	Coffee drinking in T2D patients is associated with reduced total, CVD, and CHD mortality
Rosner et al. (2006)	Cohort study; 5-year FU	32,650 Swedish women; age 40–74	MI events	RR for MI compared with 0–4 cups coffee/week: 5–7 cups/week: 0.84 2–3 cups/day: 0.65 4–5 cups/day: 0.64 $\geq$6 cups/day: 0.65	Coffee consumption does not increase MI risk. Drinking $\geq$5 cups/week decreased MI risk nonsignificantly
Silletta et al. (2007)	Cohort study; 42-month FU	11,231 Italian patients with recent MI	Fatal and nonfatal CVD events	RR for CVD event compared with noncoffee consumers: <2 cups/day: 1.02	No association between moderate coffee consumption and CVD events in post-MI patients

(*continued*)

Table 4 (continued)

Reference	Study design	Subjects	Dependent variable	Results	Conclusions
				2–4 cups/day: 0.91 >4 cups/day: 0.88	
Ahmed et al. (2009)	Cohort study; 9-year FU	37,315 healthy men at the baseline	Heart failure events or mortality	RR compared with ≤1 cup coffee/day: 2 cups/day: 0.87 3 cups/day: 0.89 4 cups/day: 0.89 ≥5 cups/day: 0.89	High coffee consumption does not increase risk of heart failure
Lopez-Garcia et al. (2009)	Cohort study; 24-year FU	83,076 healthy women at the baseline	Stroke events	RR compared with ≤1 cup coffee/month: 1 cup/month to 4 cups/week: 0.98 5–7 cups/week: 0.88 2–3 cups/day: 0.81 ≥4 cups/day: 0.80	Long-term coffee consumption may modestly reduce risk of stroke in women
Mukamal et al. (2009)	Cohort study; 7–9-year FU	1,369 Swedish patients with first acute MI	Mortality after first acute MI	HR compared with <1 cup coffee/day: 1 to <3 cups/day: 0.68 3 to <5 cups/day: 0.56 5 to <7 cups/day: 0.52 ≥7 cups/day: 0.58	Coffee consumption at the time of hospitalization was inversely related to mortality after the first MI
Zhang et al. (2009)	Cohort study; 24-year FU	7,170 women with T2D, but free of CVD or cancer at the baseline	CVD and total mortality	Compared with noncoffee consumers, RR for CVD: ≥4 cups CC/day: 0.76 ≥2 cups DECAF/day: 0.96 Same trend for total mortality	CC and DECAF consumption was not associated with increased CVD or all-cause mortality

In all studies, relative risks and hazard risks were adjusted for possible confounders. None of the studies supported that long-term coffee consumption (except for consumption of boiled coffee) increase cardiovascular disease events. Some studies (Bidel et al. 2006; Rosner et al. 2006; Lopez-Garcia et al. 2009; Mukamal et al. 2009) event suggested protective effects of coffee. Except in the Zhang et al. (2009) paper, no distinctions were made between caffeinated and decaffeinated coffee. *BP* blood pressure, *CC* caffeinated coffee, *CHD* coronary heart disease, *CVD* cardiovascular disease, *DECAF* decaffeinated coffee, *FU* follow-up, *HR* hazard ratio, *MI* myocardial infarction, *RR* relative risk, *T2D* type 2 diabetes mellitus

Some authors have even suggested that coffee could act protectively against CVD (Lopez-Garcia et al. 2008, 2009; Rosner et al. 2006), but more research is needed to confirm this hypothesis. In summary, it does not appear that coffee is detrimental to CVD in men and women, and there is a possibility that coffee may protect against CVD (Lopez-Garcia et al. 2008).

T2D increases the risk of CVD by up to threefold (Stamler et al. 1993), and thus nutritional interventions that alter this risk are very important. The impact of coffee consumption on CVD prevalence in patients with T2D has been investigated. Zhang et al. (2009) reported that neither caffeinated nor decaffeinated coffee increased the risk for total or CVD mortality in diabetic women consuming four or more cups of coffee per day (relative risk for coffee 0.80 and for decaffeinated coffee 0.76). Moreover, neither caffeinated nor decaffeinated coffee increased blood lipid levels, while only decaffeinated coffee (two or more cups daily) reduced glycosylated hemoglobin levels. Another prospective study (mean follow-up 20.8 years) documented that coffee consumption in Finnish diabetic men significantly reduced the relative risk of total mortality, CVD, and coronary heart disease (respective relative risks for five or six cups of coffee per day of 0.68, 0.70, and 0.70) (Bidel et al. 2006).

The possible negative relationship between coffee consumption and CVD risk would likely be mediated through antioxidants present in coffee. As discussed previously, CGAs and quinides are the main candidates with antioxidative properties in coffee. Reduction of inflammation and endothelial dysfunction due to antioxidants in coffee may partly explain the benefits of coffee. Nevertheless, the association between long-term coffee consumption and CVD is still equivocal. Several confounders may explain the discrepancy in the data presented. First, the difference between the effects of caffeinated coffee and decaffeinated coffee has not been investigated. For instance, these beverages have differential effects on hypertension, a mechanism that could be involved in a potential coffee-induced increase in CVD risk. Indeed, acute ingestion of alkaloid caffeine (200–250 mg) can increase systolic and diastolic blood pressure by approximately 2.4 and 1.2 mm Hg, respectively (Jee et al. 1999). The ingestion of caffeinated coffee induces a similar, but lessened, effect, while decaffeinated coffee does not increase blood pressure (Ricksen et al. 2009). Therefore, the hypertensive effects of coffee are attributed to caffeine. Furthermore, only partial habituation to the pressor action of coffee occurs, so even regular coffee drinkers experience the acute hypertensive effects of caffeine. On the basis of this knowledge, the lack of control for caffeine content in coffee may have introduced error and variation in previous investigations. Given the increased risk of CVD in T2D patients and the potential for regular coffee ingestion to reduce the risk for T2D, it becomes even more important to resolve the association between coffee consumption and CVD.

Recent studies have also documented that genetic polymorphisms may modify the association between coffee consumption and CVD risks. However, it should be noted that the data are particular to caffeine rather than to coffee in a general sense. For instance, intake of coffee was only associated with myocardial infarction risk in a subpopulation carrying the CYP1A2*1F allele (Cornelis et al. 2006). CYP1A2 is the gene encoding for cytochrome P450 1A2, a key liver enzyme that is responsible for metabolizing caffeine. Individuals carrying the CYP1A2*1F allele are slow metabolizers of caffeine, compared with individuals who are homozygous for the more prevalent CYP1A2*1A allele. Hypothetically, slower metabolism of caffeine may increase plasma caffeine concentration and exacerbate a possible detrimental effect on myocardial function. However, this study should be considered as preliminary, as its conclusions have been questioned (Ingelman-Sundberg et al. 2006). Nevertheless, it is likely that a number of polymorphisms play roles in the responses to caffeine and other coffee components, and it will require an extensive body of work in order to resolve these components. For instance, the same CYP1A2*1F polymorphism could exacerbate the hypertensive effects of coffee, whereas this effect was nonsignificant in the homozygous CYP1A2*1A subsample (Palatini et al. 2009). Moreover, individuals homozygous for the AA genotype in the CYP1A2 gene did not benefit from the protective effect of coffee on breast cancer (Kotsopoulos et al. 2007) and were more sensitive to its deleterious effects on ovarian cancer (Goodman et al. 2003). The importance of genotype in the strength of the association between caffeine, coffee, and health outcomes is a very new field of research. This present knowledge is very preliminary and undoubtedly represents a very small fraction of the gene–environment interactions that pertain to caffeine and/or coffee. Even within the one situation of the impact of caffeine on insulin sensitivity, there are many factors where a transporter, a receptor, or a second messenger may be affected by a single nucleotide polymorphism. Therefore, the inevitable genetic variability in a population may explain some of the variation and discrepancy in the epidemiology studies presented here.

The method of brewing is also known to modulate the composition of coffee. Cafestol and kahweol are two compounds present in coffee that are known to increase total and low-density lipoprotein cholesterol, two important risk factors for CVD (Higdon and Frei 2006). However, these two molecules are trapped in filter paper during coffee preparation, in such way that filter coffee contains up to 80–100-fold less cafestol and kahweol compared with boiled coffee (Jee et al. 2001). Consequently, different types of coffee brewing techniques may have influenced the data presented and this is a clear illustration of an effect of coffee that is independent of caffeine. Total cholesterol, low-density lipoprotein cholesterol, and apolipoprotein B levels increased in subjects randomized to consumed boiled coffee for 79 days compared with participants who did not drink coffee or who consumed filtered coffee (Van Dusseldorp et al. 1991). In accordance with its effects on serum lipids, the consumption of boiled coffee was associated with increased incidence of myocardial infarction compared with the consumption of filtered coffee in both Swedish males and females (Hammar et al. 2003). In addition, the association between the elevation in serum lipids and coffee was stronger in hyperlipidemic patients (Jee et al. 2001). Taken together, these data suggest that the risk of dyslipidemia following coffee consumption may be exacerbated by drinking boiled coffee and in at-risk populations.

Coffee can also increase the blood content of homocysteine, an amino acid that is associated with cardiovascular risk (Antoniade et al. 2009). Coffee had a dose-dependent positive relationship with homocysteine content in several clinical trials (Nygard et al. 1997; reviewed in Higdon and Frei 2006) and this effect has been attributed, at least in part, to caffeine and CGA (reviewed in Ranheim and Halvorsen 2005). It is unknown how brewing techniques and/or genetic polymorphisms may influence the coffee–homocysteine relationship.

In summary, the health effects of coffee on CVD are still equivocal, although recent studies have pointed toward a neutral and/or a positive effect of long-term coffee consumption for most individuals. On one side, there is evidence that at least one type of coffee (boiled) can be a risk factor for CVD and that certain individuals could be more sensitive to the effects of caffeine. In contrast, there is a possible protective effect of coffee on CVD due to the presence of antioxidants, namely, CGAs and quinides, in coffee.

5 Coffee, Caffeine, and Other Health Effects

The health effects of coffee and caffeine have been extensively studied regarding CVD and T2D. Given the ubiquitous nature of the adenosine receptors and the large number of bioactive compounds in coffee and its effects on numerous tissues, coffee and caffeine are likely to modulate other health outcomes. Here, we will review the available information about the impact of caffeine or coffee consumption on weight control, different forms of cancer, osteoporosis, and cognitive diseases.

There are many weight-loss-oriented commercial products that contain caffeine. Caffeine ingestion can stimulate epinephrine secretion and can mobilize FFAs. This would appear to lend support to the concept that caffeine would also increase metabolism and hence promote weight loss. Fundamental to this topic, any weight-loss supplement must either decrease energy intake (i.e., decrease appetite) or increase energy expenditure. To accomplish the latter, there must either be an increased demand for ATP or a decrease in the efficiency of the production of ATP (i.e., the oxygen cost of producing ATP must become greater perhaps owing to uncoupling proteins in mitochondria). Greenberg et al. (2005) reported that the negative association of coffee consumption and reduced risk for T2D was also associated with weight loss and that this could account for some of the benefit of coffee consumption. However, a direct association with coffee or caffeine and weight control has proven to be elusive. While an increase in energy output of merely a few kilocalories per day can result in a large difference in body weight over years, it is extremely difficult to measure accurately in a short-term experiment. The necessary long-term, longitudinal investigations have not been conducted.

There are a number of accounts that acute caffeine or coffee ingestion can increase the resting or diet-induced thermogenesis by approximately 10% (Acheson et al. 1980; Dulloo et al. 1989; Arciero et al. 1995; Bracco et al. 1995; Astrup et al. 1990; Jung et al. 1981). This would be a significant contribution to the energy balance if it was maintained over decades of caffeine/coffee ingestion. It is interesting to note that comparisons of lean and obese or postobese subjects consistently report that the effect is less in obese and postobese individuals (Acheson et al. 1980; Bracco et al. 1995; Jung et al. 1981). Acheson et al. (2004) found that a large (10 mg/kg) dose of caffeine increased the metabolic rate by 13%, doubled the turnover of lipids, and modestly increased FFA oxidation. However, Bracco et al. (1995) noted that 24 h after caffeine ingestion, the response was blunted. Lopez-Garcia et al. (2006) conducted an epidemiological, prospective study of body weight and caffeine intake of almost 65,000 people for 12 years. While caffeine intake was related to weight loss, the effect was very modest, less than 0.5 kg during the 12 years. There is very little evidence that coffee or caffeine can promote significant changes in body weight.

Recently, Thom (2007) evaluated a new product (Coffee Slender) that contains a green coffee bean extract that is rich in CGAs. The preliminary assessment demonstrated that a single ingestion of the product resulted in a modest decreased blood glucose response to a carbohydrate challenge. More notably, ingestion of the product for 12 weeks resulted in a 5.4-kg weight loss. It was speculated that the CGAs inhibited that absorption of glucose, but no data were provided to support this hypothesis and the findings should be considered preliminary.

The strongest beneficial association between coffee and cancer is perhaps that for liver cancer. Several cohort, case-control, and meta-analysis investigations documented a decreased risk of hepatocellular carcinoma with coffee consumption (Tanaka et al. 2007; Bravi et al. 2007; Montella et al. 2007; Shimazu et al. 2005; Hu et al. 2008; Inoue et al. 2005, 2009; Kurozawa et al. 2005; Gallus et al. 2002a). This negative, dose-dependent association is evident even in low consumers (one cup of coffee daily), but was not reported with tea or decaffeinated coffee in one study (Montella et al. 2007). The underlying mechanisms for this strong association are still unknown, but the beneficial impacts of coffee on liver function have also been documented for liver enzyme function (Ruhl and Everhart 2005a; Honjo et al. 2001) and chronic liver disease (Ruhl and Everhart 2005b; Gallus et al. 2002b; Freedman et al. 2009), suggesting that coffee acts on the whole spectrum of liver function. As with CVD and T2D, one cannot dismiss the effects of antioxidants as a plausible mechanism. Moreover, owing to the lack of differentiation between caffeinated and decaffeinated coffee (except in one study) in the previously reviewed studies, it is not possible to say whether the protective effects are related to the caffeine content of coffee.

There have been some suggestions that coffee consumption may decrease the risk of other diseases, such as colorectal, breast, lung, and bladder cancers. The research did not find a strong association between long-term coffee consumption and any of those types of cancer (La Vecchia and Tavani 2007). The relative risk for colorectal cancer was between 0.91 and 1.0 in high-coffee versus low-coffee consumers (not significant) (Je et al. 2009; Naganuma et al. 2007; Larsson et al. 2006; Michels et al. 2005). Similarly, most large-scales studies (Ishitani et al. 2008; Ganmaa et al. 2008), but not all (Baker and Beehler 2006), did not report significant relationships between coffee and breast cancer in the general public. However, coffee may be protective in specific populations, such as postmenopausal women (Ganmaa et al. 2008) or women who carry high-risk mutations BRCA1 and BRCA2

(Nkondjock et al. 2006). Similar equivocal results have been reported for lung (Kabagambe and Wellons 2009) and bladder (Zeegers et al. 2001; Villanueva et al. 2009) cancers, but the strong association between coffee drinking and smoking, a major risk factor for cancer, complicates data interpretation. After adjustments for smoking, the associations between coffee consumption and lung and bladder cancers were much less pronounced (Villanueva et al. 2009; Pelluchi and La Vecchia 2009; Kabagambe and Wellons 2009). In summary, there are sparse data to support a protective effect of coffee on cancer risks (except for liver cancer), but the current data clearly show that coffee is not an independent risk factor for cancer.

Coffee has also been suggested to be protective against some cognitive diseases (for a detailed discussion see Sawynok 2010). The strongest evidence is related to Parkinson's disease. Consumption of caffeinated coffee (Hu et al. 2007; Tan et al. 2003; Ascherio et al. 2001, 2003; Hernan et al. 2002), tea (Hu et al. 2007; Tan et al. 2003; Ascherio et al. 2001), but not decaffeinated coffee (Ascherio et al. 2001), was linked to a dose-dependent reduction in the incidence of Parkinson's disease. The hazard ratios for the consumption of five or more cups of coffee or three or more cups of tea daily were 0.40 and 0.41, respectively (Hu et al. 2007). In addition, the association between caffeine and Parkinson's disease may be modified by the use of hormone replacement therapy in postmenopausal women. In these women, caffeine intake increased Parkinson's disease risk, whereas in women who did not use hormone replacement therapy, caffeine was protective (Ascherio et al. 2003). Similar caffeine-induced beneficial effects have been suggested for Alzheimer's disease, but the evidence in humans is very sparse. Coffee consumption was inversely associated with Alzheimer's disease in preliminary investigations (Barranco Quintana et al. 2007; Maia and de Mendonca 2002), but more research is needed to confirm this link. De Felice et al. (2009) investigated the role of insulin in Alzheimer's disease with highly differentiated, cultured, hippocampal nerve cells. It is interesting to note that insulin completely prevented the development of synapse abnormalities that are characteristic of Alzheimer's disease and furthermore, this protection was potentiated by rosiglitazone, an insulin-sensitizing drug for T2D treatment. (This has led to the lay press referring to Alzheimer's disease as type 3 diabetes.) While it is quite speculative, given that habitual heavy intake of coffee leads to a decreased risk of T2D, it could be that a positive association between coffee intake and Alzheimer's disease could be associated with increased insulin sensitivity.

Despite strong epidemiological evidence highlighting the benefits of coffee in many health aspects, coffee is nonetheless associated with some negative health outcomes. Most investigations (Harris and Dawson-Hugues 1994; Rapuri et al. 2001; Hernandez-Avila et al. 1991; Kiel et al. 1990; Hallström et al. 2006), but not all (Lloyd et al. 2000), reported that high caffeine consumption (250–450 mg) is associated with increased risk of low bone mineral density, osteoporosis, and osteoporotic fractures in middle-aged women. This situation may be exacerbated in women with low calcium intake (Harris and Dawson-Hugues 1994; Hallström et al. 2006), in lean ($BMI < 25.1$) subjects (Korpelainen et al. 2003), and in women who carry a polymorphism in the vitamin D receptor gene (Rapuri et al. 2001).

Despite its wide acceptance and consumption, caffeine remains a drug, and, as such, may be associated with adverse effects related to abuse, dependence, and with drawal. In some individuals, caffeine at doses that can be found in several cups of coffee can be associated with harmful side effects, such as headache, tachycardia, tremor, insomnia, nausea, and diarrhea (Higdon and Frei 2006). In addition, caffeine withdrawal has been clearly characterized in patients who abstain from doses as low as 100 mg caffeine daily. The onset of caffeine withdrawal occurs 12–24 h after abstinence and can last up to 9 days. The most common symptoms include headache, fatigue, decreased alertness, drowsiness, depressed mood, irritability, and difficulty concentrating (Juliano and Griffiths 2004).

Consumption of caffeine and caffeinated coffee has been associated with several health outcomes. It appears that coffee may be protective not only against T2D and CVD, but also against liver cancer, Parkinson's disease, and Alzheimer's disease. On the other hand, coffee consumption is associated with adverse effects for osteoporosis and caffeine-withdrawal symptoms. Finally, coffee's relationship with several other conditions, such as bladder, colorectal, breast, and lung cancers, is still equivocal and deserves further attention. With the exception of T2D, seldom has the difference between coffee and caffeine been addressed. In any case, it is well recognized that caffeine and coffee are both bioactive substances that have extended effects on several tissues, organs, systems, and ultimately on human health.

6 Summary

The topic of methylxanthines and human health is founded by coffee, the major food source of caffeine, having many bioactive compounds, some of which have antioxidant functions. There is a paradox as consumption of either caffeine or caffeinated coffee results in a marked insulin resistance and yet habitual coffee consumption repeatedly been reported to reduce the risk for T2D. There is strong evidence that caffeine reduces insulin sensitivity in skeletal muscle and this may be due to a combination of direct antagonism of A_1 receptors and β-adrenergic stimulation due to increased sympathetic activity. Caffeine may also induce reduced hepatic glucose output. With the exception of bone mineral, there is little evidence that caffeine impacts negatively on other health issues. Coffee does not increase the risk of CVDs or cancers and there is some evidence suggesting a positive relationship for the former and for some cancers, particularly hepatic cancer. There is limited evidence that caffeine or coffee is effective in promoting weight loss, but there is some evidence that coffee may have a positive effect on neurodegenerative diseases.

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