Barry B. Halliwell
Henrik E. Poulsen *Editors*

Cigarette Smoke and Oxidative Stress

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With 66 Figures, 18 in Color and 8 Tables

Springer

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Preface

From a public health point of view, there is little doubt that one of the most important preventable causes of disease worldwide is tobacco smoking. It is also clear that tobacco smoke contains a vast number of chemicals with important biological effects in disease processes. The gas phase of tobacco smoke is oxidizing, the tar phase is reducing, and whole smoke is roughly neutral, so its effects on oxidative stress may be an "antioxidant paradox."

From a scientific point of view, we found it of interest to make a comprehensive overview of what we presently know about oxidative stress and tobacco smoke, because smoking is presently the best-known common condition associated with oxidative stress, and it may serve as a model for others. To this end, we have asked distinguished researchers from the public and the private sectors to evaluate the present scientific status in their particular area. Authors were selected purely because of their scientific merits.

We do not claim that all the well-described health hazards associated with cigarette smoking stem from oxidative stress, nor should we. However, we ought to be able to find out, and for some of those health hazards, we can already say. We hope this book will stimulate more research to find answers to the remaining questions.

Barry Halliwell and Henrik E. Poulsen

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Chapter 1

Oxidative Stress

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Barry B. Halliwell and Henrik E. Poulsen

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1.1 What is Oxidative Stress?

Oxygen was discovered by the Swedish scientist Carl Wilhelm Scheele and reported in his thesis *Luft und dem Feuer* (Air and Fire) from Uppsala and Leipzig in 1777. Later it was realized that in higher animals, breathing supplies the cells with oxygen and serves to eliminate the carbon dioxide formed from cellular metabolism. The well-known reaction between oxygen and fuel (e.g., carbon in wood) requires high temperatures. However, it was discovered that special proteins in the cells—enzymes—are able to catalyze this combustion at body temperature. The trick is that the enzymes can bind both oxygen and the substrate and bring them into close proximity so that chemical reaction can occur and the liberated energy can be stored as ATP for use elsewhere and later in the cell.

Oxygen was consequently considered a good thing. However, experience from exposure to high-oxygen concentrations in deep-sea divers and premature babies showed that organ damage could be a result of exposure to too much oxygen. As we learned to measure oxidative damage better, we realized that it happens in vivo even at normal atmospheric O_2 levels.

It is now well established that free radical chemistry occurs in biology, and it is also becoming increasingly clear that free radicals not only function in cellular respiration, as damaging species, but also in the signaling systems within cells. As an example of the acceptance of these phenomena, the journal *Nature Medicine* has "oxidative stress" among its limited number of keywords for paper submission.

1.2 Oxidative Stress

Oxidative stress was initially defined by Sies (1985, 1986) as a serious imbalance between oxidation and antioxidants, "a disturbance in the prooxidant–antioxidant balance in favor of the former, leading to potential damage." The definition seems simple; however, it builds on definitions about oxidation, antioxidants, and balance.

The definition of oxidation seems also simple: loss of electrons by a species, gain of oxygen, or loss of hydrogen. However, if something is oxidized, something else must be reduced. The effect depends on the context. As put forward by Buettner (1993), there is a pecking order of oxidants. In biology, substances very high in the pecking order (e.g., the hydroxyl radical) will almost always be an oxidant; other substances (e.g., NO· or H_2O_2, can act as oxidants or reductants, depending on whether they react with substances lower or higher in the pecking order.

An antioxidant is more difficult to define. A popular (but not comprehensive) definition was put forward by Halliwell: An antioxidant is any substance that, when present at low concentrations as compared with those of an oxidizable substrate, significantly delays or prevents the oxidation of that substrate. (Its shortcomings are discussed in an upcoming book, *Free Radicals in Biology and Medicine*, 4th ed., Halliwell and Gutteridge, 2006.) As argued above, the chemical terms are oxidation and reduction, and an antioxidant is clearly different from a reducing agent. A reducing agent may even be a prooxidant if it reduces oxygen to free radicals or converts transition metal ions to lower oxidation states that react more readily with peroxides. Many biological reducing agents are Janus-faced: They can be anti- or prooxidants, depending on the levels of O_2 and transition metal ions around.

Balance or imbalance is poorly defined. Generally, we think of our environment as

an oxidative environment, and this presumably is true for outer surfaces of the body. However, our knowledge is limited regarding intracellular conditions. They are generally reducing, but with some subcellular variations (e.g., the endoplasmic reticulum is more oxidized than the mitochondria). Even within the cytosol there might be considerable differences between locations close to the cell membrane and close to the nuclear membrane. It could be that there is a balance between oxidants and antioxidants, but it seems rather unlikely. The cell must rapidly and transiently modulate its redox state to send signals.

1.3 Measurement of Oxidative Stress

The term oxidative stress rests on definitions that are not always sufficiently clear; consequently, oxidative stress is a somewhat vague term, as is oxidative damage. Halliwell and Whiteman (2004) have defined the latter as "the biomolecular damage that can be caused by direct attack of reactive species during oxidative stress."

In very simple noncompartmentalized systems, e.g., an in vitro system with a limited number of oxidants and targets for oxidation, it is often self-evident how oxidative stress is defined and measured: by simply measuring antioxidants, free radicals, and other reactive species (RS) and doing a balance sheet. Care must be taken: What is seen depends on what is measured. RS can be measured directly (e.g., by electron spin resonance or various trapping methods), or indirectly by examining end products of their reaction with biomolecules (oxidative damage).

More-complicated systems need much more careful approaches. A cell is compartmentalized with many different molecular targets for oxidation. Lipids in the outer cell membrane most probably have quite a different oxidative environment as compared with the inner mitochondrial membranes. Nucleic acids also exist in different compartments, and the oxidative environment is quite different between transcribing and nontranscribing DNA in the nucleus, DNA in the mitochondria, and the different types of RNA. Likewise for protein oxidation, plasma proteins and cellular proteins exist in different compartments and thereby in different oxidative environments.

At the next level of complexity, different organs and different parts of the organs may present quite different conditions. For example, the liver receives a mixture of arterial and venous blood and thereby much lower oxygen concentrations than most other organs, and even within the liver, cells in the first part of the sinusoid live in a quite different oxidative environment than those in the end of the sinusoid.

That the structure and organization is complicated should not make researchers refrain from trying to define the system under investigation. Rather, it should in many cases make us more humble in the interpretation of data obtained in complicated systems, and more careful in defining and understanding the limitations of simple methods used to investigate complicated systems.

1.4 Biomarkers of Oxidative Stress

When considering the effect of increased oxidative stress (or decreased for that matter), the issue of target is mandatory. From a general point of view, lipids, proteins, carbohydrates, and DNA are considered important macromolecules. For measurement of the

oxidation of these molecules, the biomarker approach is most often used. A biomarker of disease is defined as a molecular indicator of a specific biological property, a biochemical feature or facet that can be used to measure the progress of disease or the effects of treatment. The reader should be aware that there are other forms of biomarkers, e.g., biomarkers of exposure.

Such a biomarker should fulfill certain criteria, as given in Table 1.1.

Table 1.1 Criteria for the ideal biomarker of oxidative stress

No.	Criterion
1	It should be predictive of development of the disease or condition under investigation (example: lipid peroxidation in plasma should predict arteriosclerotic events or cardiovascular death).
2	It should reflect biological event(s) that can be related to the pathogenesis of the disease or condition.
3	It should be stable over short periods (weeks, months) in stable individuals.
4	It should produce identical results when the same sample is measured in different laboratories.
5	The sample from which it is measured should be stable on storage.
6	The biomarker should relate to immediate events within short periods or should reflect integration of events over a well-defined period.
7	Preferentially, the biomarker measurement should be noninvasive or measurable in an easily available biological specimen (example: urine, sputum) or in minimally invasively obtainable biological specimen (example blood or plasma).
8	The cost of sample analysis should be low, and it should be possible to perform a large number of analyses within a reasonable time.

Whereas the methods to measure events that are related to oxidative stress—be it oxidation, free radicals, or antioxidants—are numerous, it should be realized that very few, if any, of them fulfill the criteria in Table 1.1, and hence cannot yet be considered biomarkers of oxidative stress. To our knowledge, there are no publications that in a proper scientific way fulfill criterion 1, namely predictive of development of disease. Nonetheless, recent studies with F_2-isoprostanes and 3-nitrotyrosine look promising in this direction.

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Chapter 2

Tobacco Smoke Constituents Affecting Oxidative Stress

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Jan B. Wooten, Salem Chouchane, and Thomas E. McGrath

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2.1 Introduction

Cigarette smoke is a highly complex aerosol composed of several thousand chemical substances distributed between the gas and the particulate phases. A frequently cited estimate for the number of these constituents is ca. 4,700 (Dube and Green 1982). The enormous complexity of cigarette smoke is the result of multiple thermolytic processes that occur in heated tobacco within the confines of the burning cigarette rod. These processes involve distillation, pyrolysis, and combustion, and are influenced by factors including the design of the cigarette (Norman 1999) and the composition of the tobacco (Bokelman and Ryan 1985; Leffingwell 1999). Numerous organic chemical classes are represented in cigarette smoke including saturated and unsaturated hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids, esters, phenols, nitriles, terpenoids, and alkaloids (Baker 1999; Dube and Green 1982; Hoffmann et al. 2001). Whereas the composition of cigarette smoke is complex, certain smoke constituents have received greater analytical scrutiny than have others, either because of their greater relative abundance in smoke (which makes them easy to analyze), their known pharmacological properties (Seeman et al. 2004), and/or because they are believed to be carcinogenic or potentially harmful to smokers (IARC Monographs 1986; USCPSC 1993).

Large numbers of data on the composition of mainstream smoke have been published, and the subject has been reviewed in detail (Baker 1999). The objective of this chapter is to take a more focused look at the chemical constituents in cigarette smoke that relate to oxidative stress. In particular, we examine smoke constituents that are known to (1) increase oxidant burden, (2) decrease antioxidant protection, or (3) result in the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Section 1 provides a brief description of the thermal conditions inside a burning cigarette and some relevant properties of cigarette smoke. Section 2 is an overview of the existing information related to smoke chemistry and oxidative stress. Section 3 explores how certain tobacco leaf constituents affect the delivery of some of the cigarette smoke constituents known to influence oxidative stress. Phenolic compounds originate from the pyrolysis of polyphenols, carbohydrates, and other precursors in tobacco leaves, whereas trace metal ions present initially in the leaves are known to transfer to cigarette smoke. This discussion draws on recent results from our own laboratory and literature reports. Section 4 discusses the important topic of free radicals, ROS, and reactive RNS,

their potential involvement in the toxicity of cigarette smoke in general, and the in vitro cytotoxicity of individual smoke constituents in particular. Special emphasis is given to the role of free radicals and the redox chemistry of phenolic compounds, including some current results.

2.1.1 Pyrolysis and Combustion inside Cigarettes

Before discussing cigarette smoke and oxidative stress, we present some basic principles of cigarette smoke formation and properties that may help to convey the complexity of tobacco smoke. An elaborate description of the fluctuating thermal gradients and vapor environment inside a cigarette during smoking has been given by Baker (1999). The chemical complexity of cigarette smoke is strongly dependent on the heating conditions inside the lit cigarette. To summarize briefly, when a smoker lights and draws on a cigarette, the temperature of the ignited tobacco rises rapidly, and a hot coal forms at the lit end of the cigarette that is the center of combustion (the combustion zone). Peak temperatures inside the coal can exceed 900 °C. The high temperature inside the coal during a puff causes an increase in the viscosity of the air flowing through the coal and a concomitant increase in the resistance to the draw of air through the cigarette. This effect forces air to be drawn primarily from the periphery of the coal at the paper burn line rather than through the center of the coal. The depletion of oxygen due to combustion inside the coal and the flux of air around the coal results in the formation of a region immediately behind the coal that is depleted of oxygen, but where the temperatures remain high enough to promote the thermal decomposition of the unburned tobacco. For this reason, this area behind the coal is known as the pyrolysis/distillation zone. Copious amounts of volatile and semivolatile smoke constituents evolve from this zone. These constituents result in part from the pyrolysis of tobacco and in part from distillation of volatile constituents native to tobacco because of the heat of the encroaching coal.

The smoke constituents drawn through the cigarette rod during a puff and delivered to the smoker are termed mainstream smoke. In the interim period between puffs when no air is being drawn through the cigarette, the coal undergoes smoldering combustion driven primarily by diffusion of oxygen into the coal. The smoke escapes from the periphery of the coal to the surrounding air. This smoke is termed sidestream smoke. Typical coal temperatures during smoldering combustion are less than 800 °C. The different thermal conditions and air flow through and around the coal during smoldering combustion, in comparison to combustion during a puff, causes the sidestream and mainstream smoke composition to differ significantly, the primary difference being the relative abundance of the smoke constituents (Baker 1999).

2.1.2 Cigarette Smoke Properties

The smoke emitted from a lit cigarette is a dense aerosol composed of microscopic droplets, known as the particulate phase, dispersed in a vapor of air and other gases derived from the burning tobacco. The particulate phase of cigarette smoke overall acts as a reducing agent, which may play a role in its toxicity (Church and Pryor 1985; Lakritz et al. 1972; Schmeltz et al. 1977). There are some 10^9–10^{10} particles per cubic centimeter in

fresh mainstream smoke, and the particle size varies from 0.1 to 1.0 μm in diameter. The standard method for separating the particulate fraction of cigarette smoke from the gas-phase constituents is to pass the cigarette smoke through a fiberglass filter called a Cambridge pad (Baker 1999; Dube and Green 1982). This filter has a trapping efficiency of 99% for particles with an aerodynamic diameter larger than 0.1 μm. Thus, the particulate phase of mainstream smoke is defined operationally by the method employed to trap nonvolatile and semivolatile materials.

The total particulate matter (TPM) includes *all* the material collected on the Cambridge pad (Baker 2002). Tar is the term applied to the smoke particulate fraction collected on the Cambridge pad, minus the content of nicotine and water. A small portion of the mainstream smoke constituents are distributed between the gas and particulate phases. These organic substances are described as semivolatile constituents, and they typically have molecular weights in the range of ca. 60–200 (Baker 1999). The gas phase of cigarette smoke is the component that passes unobstructed through the Cambridge filter pad, which includes the gaseous constituents (oxygen, nitrogen, nitric oxide, carbon dioxide, carbon monoxide, etc.) and the volatile and some semivolatile organic constituents.

The TPM collected on a Cambridge filter pad is mostly soluble in either water or organic solvents (at least 95–99%), and thus differs from respirable particulate matter such as carbon black (e.g., diesel exhaust particles and other forms of soot) that is prevalent in the environment. Such materials have been termed poorly soluble particles (PSP) and have recently attracted the interest of researchers because of potential adverse effects related to the generation of ROS. Primarily surface-driven mechanisms have been invoked to explain ROS generation from PSPs and therefore appear to differ from the ROS generation mechanisms of cigarette smoke (Knaapen et al. 2004). ROS generation in cigarette smoke particulate matter is believed to be based on the redox cycling of quinones derived from TPM constituents (Dellinger et al. 2001).

2.2 An Overview of Cigarette Smoke Chemistry and Oxidative Stress

In this section, we discuss a number of chemical classes of smoke constituents that have been documented to affect oxidative stress. Because of the complexity of cigarette smoke, however, it is impossible to be comprehensive, and much remains unknown. We also do not attempt to address the relative importance of the various chemical classes to induce oxidative stress as there are many complex biological interactions and processes involved. Rather, we focus on the chemistry of the smoke constituents, citing the appropriate literature references that make the connection between the smoke constituents and their biological effects. Among the smoke constituents that we include in our overview are organic compounds or metal ions that act as electrophiles, free radicals, reactive anions or metal ions that act as reducing agents (donate an electron), or free radicals or metal ions that act as oxidizing agents (accept an electron).

ROS and RNS are generated when mainstream cigarette smoke interacts with aqueous media or physiological fluids. Some smoke constituents become involved in oxidative stress only after they are chemically modified by metabolic processes in vivo. For example, benzo[*a*]pyrene can be metabolized to its corresponding quinone, which can generate ROS via a redox cycling mechanism (Briede et al. 2004; Winston et al. 1993).

This quinone and related substances that are not initially present in cigarette smoke are not otherwise included in our discussion. Another distinction can be made between oxidants that form by the direct action of cigarette smoke constituents and secondary oxidants that form in response to inflammation resulting from smoking-related oxidative stress. These topics are addressed in other chapters.

2.2.1 Particulate-Phase Constituents

2.2.1.1 Free Radicals

Free radicals were discovered in cigarette smoke and other charred organic materials soon after the development of electron paramagnetic resonance spectroscopy (EPR) (Lyons et al. 1958). However, it was not until 1983 that Prof. William A. Pryor of Louisiana State University employed EPR to associate cigarette tar radicals with hydroquinone and catechol, and to suggest their possible involvement with smoking-related diseases (Pryor et al. 1983a, c). Subsequently, the Pryor research group conducted many studies to characterize the smoke radicals, and in vitro assays were performed, suggesting that cigarette smoke could cause oxidative stress or oxidative damage to essential biological molecules. For example, Church and Pryor (1985) proposed that the excess superoxide that forms in lung tissue in response to exposure to cigarette smoke might be one possible mechanism responsible for the inactivation of α_1-protease inhibitor, a protein associated with the onset of emphysema in deficient individuals. In the same report, the authors noted that cigarette tar incubated with DNA exhibits an EPR signal in the recovered DNA. Later, it was shown that DNA damage could occur by the attack of hydroxyl radicals generated from the bound tar radicals (Pryor 1992; Pryor et al. 1998). Pryor (1992) noted that such molecular damage is not unique to tobacco smoke, but also occurs from smoke from other sources such as diesel fuel and wood.

Extensive studies were initiated by the Pryor group to characterize the cigarette TPM radicals. Organic extracts of cigarette smoke condensate revealed the presence of as many as five different EPR signals (Church and Pryor 1985). Treating the alcoholic extract of TPM with sodium hydroxide in the presence of air gave an EPR spectrum dominated by the characteristic five-line spectrum of the *p*-benzosemiquinone radical, thus revealing an abundant source of radical precursors (Pryor et al. 1983b). Subsequently, the semiquinone radicals were shown to be concentrated in the aqueous extract of cigarette tar (ACT). The EPR spectrum of fresh ACT in air-saturated pH 10 buffer solutions was found to exhibit the intense resonances of the semiquinone radicals of both hydroquinone and catechol. The pattern of resonances in the ACT spectrum was indistinguishable from the combined spectra of pure hydroquinone and catechol allowed to autooxidize in air-saturated solutions at pH 9, showing that the radicals in ACT derive from the hydroquinone and catechol in cigarette smoke.

The Pryor group (Zang et al. 1995) and "Tanigawa et al. (1994)" also demonstrated by EPR spin-trapping experiments that aqueous dimethyl sulfoxide (DMSO) solutions of ACT, buffered at pH 9 and saturated with air, contain superoxide radical anions, one of the ROS involved in oxidative stress. The mechanism proposed for the formation of superoxide in ACT was the autooxidation of the hydroquinone anion (and related anions) in air to give benzosemiquinone radical and superoxide, as shown in Fig. 2.1 (Brunmark and Cadenas 1989; Zang et al. 1995). Spin-trapped adducts of the hydroxyl radical, an-

other important stress-related ROS, were also identified in ACT. The mechanism put forth for the formation of hydroxyl radicals was the catalytic disproportionation of hydrogen peroxide (H_2O_2) by transition metal ions, the well-known Fenton reaction (Cosgrove et al. 1985).

Hydrogen peroxide is a naturally occurring by-product of oxidative stress. It is formed during normal respiration in living organisms by catalytic disproportionation of superoxide radicals by superoxide dismutase (SOD). Another enzyme, catalase, is highly efficient at converting H_2O_2 to "innocuous products, water, and molecular oxygen." If this cellular defense mechanism is overwhelmed, the excess H_2O_2 can undergo disproportionation via the Fenton reaction to form hydroxyl radicals. The hydroxyl radicals derived from H_2O_2 are highly oxidizing species that are well known to cause oxidative damage to essential biomolecules, including DNA (Halliwell and Gutteridge 1999). H_2O_2 has been found in ACT and in aged unbuffered aqueous solutions of catechol, a smoke constituent abundant in both ACT and TPM. The H_2O_2 concentration in smoke condensate has been shown to increase with age, pH, and temperature (Nakayama et al. 1989; Stone et al. 1995). Exogenous H_2O_2 found in cigarette smoke and H_2O_2 that forms by the physiological response to smoke constituents are presumed to be a source of oxidative stress and/or damage in smokers.

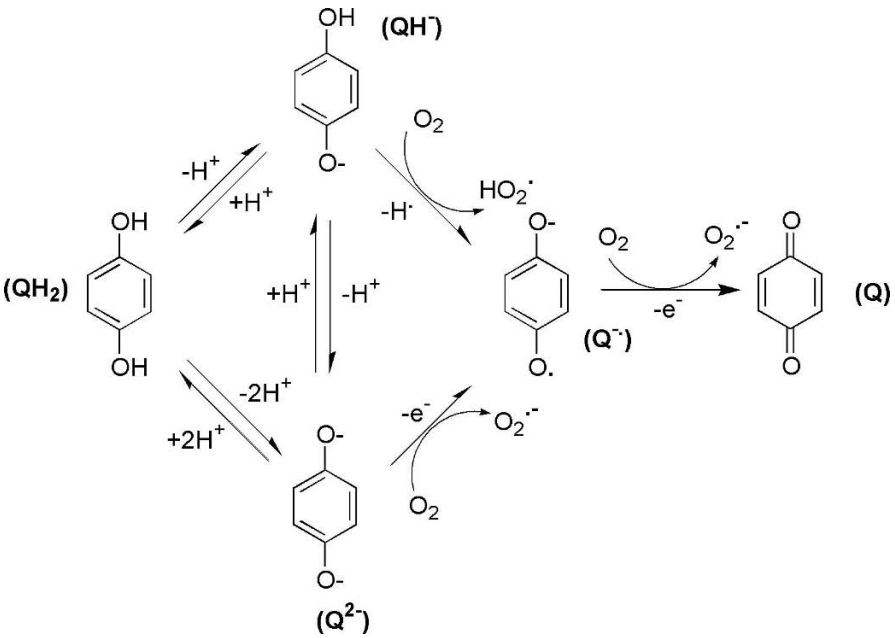

Fig. 2.1 Generation of semiquinone and superoxide radicals by autooxidation of hydroquinone, an abundant dihydroxybenzene found in the particulate phase of cigarette smoke (Zang et al. 1995)

2.2.1.2 Quinones

Quinones are readily formed from cigarette smoke constituents that can undergo autooxidation. Benzoquinone, for example, forms by the autooxidation of hydroquinone in

ACT (Sect. 2.1.1) or by oxidation in vivo in living organisms. The toxicology of quinones has been studied extensively (Bolton et al. 2000; Monks et al. 1992). In general, the toxicity of quinones is believed to occur by two mechanisms, the redox cycling mechanism, which generates excess ROS as byproducts, and the formation of covalent bonds with essential biological molecules (especially molecules containing thiol groups) (Rodriguez et al. 2004; Seung et al. 1998). Both mechanisms can contribute to the onset of oxidative stress. Quinones derived from cigarette smoke constituents undergo redox cycling in living organisms by entering into the NADPH reductase pathway (Bolton et al. 2000; Hirakawa et al. 2002; Squadrito et al. 2001). The reduction of quinones by NADPH or ascorbate regenerates the parent quinols, thereby creating the redox cycle (Roginsky et al. 1999a). Redox cycling of xenobiotic quinones can significantly increase the cellular burden of ROS and deplete their antioxidant defenses.

Whereas redox cycling of quinones is recognized as a significant source of oxidative stress from cigarette smoke, α,β-unsaturated ketones derived from particulate-phase constituents, such as benzoquinone, can also undergo electrophilic substitution in a manner similar to a number of gas-phase constituents such as acrolein (see Sect. 2.2.3). For example, pure benzoquinone in oxygenated aqueous solutions undergoes Michael addition via a semiquinone intermediate to form intensely colored condensation products; the color of the solution changes to a deep purple within minutes of dissolution, an indication of the presence of conjugated Michael addition products. This can occur for various quinones even at physiological pH, depending on the p*K* of the parent dihydroxybenzene (Pedersen 2002) and the redox potentials of the corresponding semiquinone radicals (Roginsky et al. 1999b). Quinones react readily with cellular nucleophiles, especially glutathione (GSH) and other thiols (Lau et al. 1988).

2.2.1.3 Trace Heavy Metals

Tobacco plants transport metal ions from the soil through the roots into the leaves (Lougon-Moulin et al. 2004; Tso 1990). Trace amounts of heavy metals accumulate in the leaves, and they are known to transfer in trace quantities from the cured and processed tobacco to mainstream cigarette smoke. These metals include cadmium, lead, mercury, arsenic, iron, copper, chromium, nickel, and selenium (Hoffmann et al. 2001; IARC Monographs 1986; Smith et al. 1997; Stohs and Bagchi 1995). The most abundant redox-inactive metals in cigarette smoke generally are cadmium, lead, mercury, and arsenic. The yield of these metals in cigarette smoke is influenced by cigarette design, but the yield generally correlates with tar yields. The most abundant redox-active metals in cigarette smoke are copper and iron, with copper being more abundant than iron, ca. 0.19 versus ca. 0.042 μg per cigarette, respectively (Stohs et al. 1997).

Many investigations have suggested that metal-induced oxidative stress can be partially responsible for the toxicity of these metals (Ercal et al. 2001). Redox-active metals, such as iron, copper, nickel, and chromium, can undergo redox cycling in oxygenated aqueous solutions, with the concomitant formation of ROS, whereas redox-inactive metals such as lead, cadmium, and mercury can deplete cells of thiol-containing antioxidants and reduce the activity of antioxidant enzymes. Heavy metals can exert other molecular effects such as inhibition of DNA repair and activation of cellular signaling (Bal and Kasprzak 2002; Barchowsky and O'Hara 2003; Kasprzak 2002; Waisberg et al. 2003). Thus, both redox-active and redox-inactive metals can potentially cause an increase in ROS in smokers.

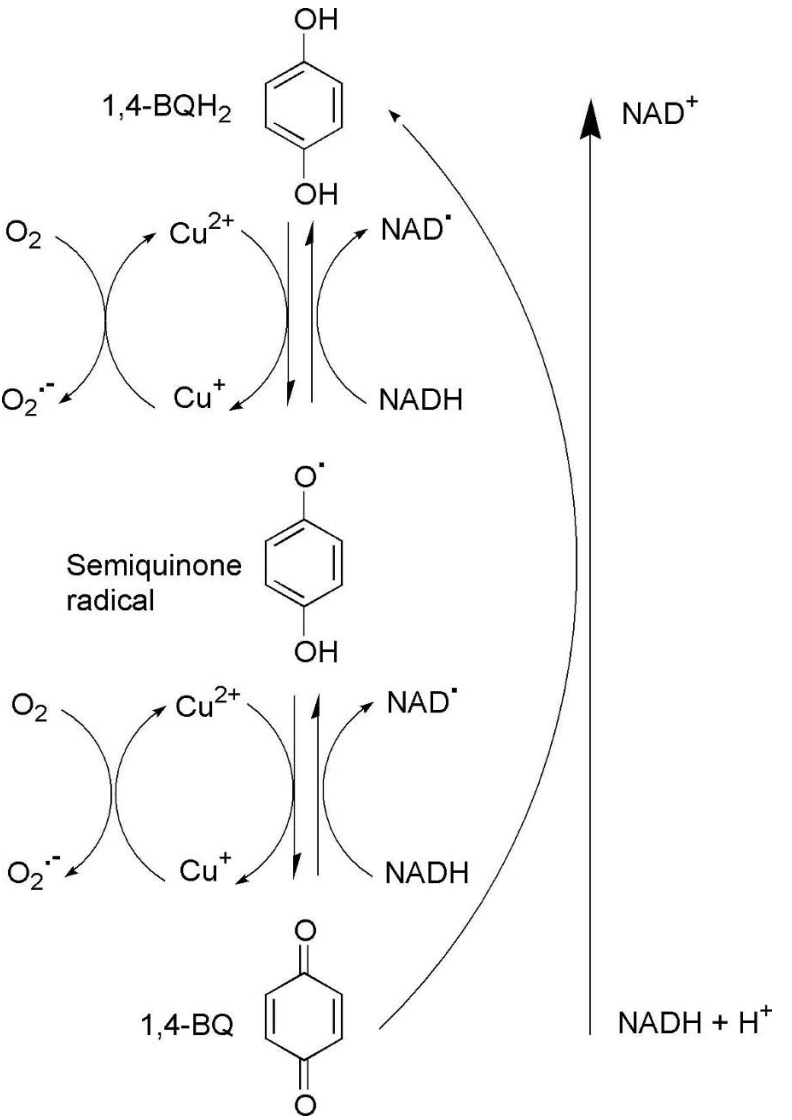

Fig. 2.2 Redox cycling mechanism for the oxidation of quinols to quinones with the formation of reactive oxygen species (Hirakawa et al. 2002)

Transition metals in the tar of cigarette smoke are notable because of their capacity to promote the formation of hydroxyl radicals via the Fenton reaction, both in aqueous extracts of cigarette smoke and in living tissues. In particular, both Fe^{2+} and Cu^{1+} are known to be active in the formation of hydroxyl radicals. These ions can also readily form complexes with many organic molecules, including those that undergo redox cycling (Stohs and Bagchi 1995; Stohs et al. 1997). Cu^{2+} has been shown to oxidize catechol

and hydroquinone to their respective quinones. It can enter into a redox cycle involving hydroquinone in the presence of molecular oxygen, forming semiquinone radicals and generating superoxide radical anions, as shown in Fig. 2.2. In contrast, Fe^{3+} does not significantly enhance the rate of oxidation of hydroquinone (Hirakawa et al. 2002; Li and Trush 1993; Li et al. 1995).

2.2.2 Gas-Phase Constituents

2.2.2.1 Oxidizing Radicals and RNS

Cigarette smoke contains abundant oxidizing agents that are found in the gas–vapor phase (Church and Pryor 1985; Pryor 1992). Even though nitric oxide (NO·) is itself a radical, it is neither particularly reactive nor toxic. NO· combines slowly with molecular oxygen in air (over a period of seconds) to form the toxic oxidant and nitrating agent, NO_2·. According to a mechanism proposed by Pryor et al. (1983b), NO_2· reacts rapidly with other smoke constituents such as isoprene and butadiene to form nitroso-carbon-centered radicals. Carbon-centered radicals are generally highly reactive species. The gas-phase carbon-centered radicals in smoke react instantaneously with molecular oxygen to form peroxyl radicals that react with smoke gas-phase NO· to form alkoxyl radicals and NO_2·, thereby creating a continuous cycle. There are two interesting consequences of the above reaction scheme: (1) the oxidizing radicals in cigarette smoke are formed by reactions between the gas-phase constituents, and not primarily by pyrolysis or combustion reactions in the burning tobacco, and (2) the radicals collected inside an enclosed container of gas-phase smoke increase until the supply of NO· is depleted, persisting for several minutes.

Because the radical species that form from reactions of NO· and other gas-phase smoke constituents are all short-lived, spin-trapping methods must be employed to detect them by EPR spectroscopy, as in the case of the reactive oxygen species. The Pryor group employed the spin trap α-phenyl-*N*-*tert*-butylnitrone (PBN) to detect the oxidizing gas-phase radicals in cigarette smoke. The primary spin adducts found in benzene solutions of PBN bubbled with gas-phase smoke are from alkoxyl radicals, the least reactive, and therefore the longer-lived of the oxidizing radicals. Other researchers developed alternative methods to detect free radicals. For example, Flicker and Green (1998, 2001) developed a chromatographic-based method that is specific for carbon-centered radicals in whole mainstream smoke (including the TPM and the gas phase). The involvement of gas-phase free radicals in oxidative damage is unclear, because it is generally believed that the reactive gas-phase radicals are quenched immediately on contact with surfaces of the respiratory tract (Rahman and MacNee 1996a; 1996b).

NO· itself at physiological concentrations (ca. 0.1–10 n*M*) is relatively unreactive with nonradical molecules (Halliwell and Gutteridge 1999). However, it can react with tyrosyl radical, which is present at the active sites of some enzymes, particularly ribonucleotide reductase (Kwon et al. 1991; Lepoivre et al. 1994). NO· may be converted to a number of more reactive derivatives, known collectively as RNS, such as NO_2·, N_2O_3, and N_2O_4 and $ONOO^-$ (peroxynitrite). DNA damage and nitration of tyrosine in cells exposed to the gas phase of cigarette smoke has been attributed to the action of RNS (Eiserich et al. 1994; Spencer et al. 1995). NO· is reported to enhance the toxicity of phenolic compounds by oxidation to their respective quinones (Urios et al. 2003).

2.2.2.2 Peroxynitrite

Peroxynitrite is an RNS that forms from the reaction of NO· and superoxide. Peroxynitrite is not itself a free radical, being derived from two free radicals, but it is a powerful oxidant that has been shown to induce damage to essential biomolecules in physiological media (Denicola and Radi 2005; Halliwell and Gutterridge 1999). Simultaneous generation of NO· and superoxide favors the production of peroxynitrite anion (Beckman et al. 1990). This peroxynitrite-forming reaction has since been shown to be diffusion controlled ($k_{obs} = 6.7\times10^9\ M^{-1}\ s^{-1}$), indicating that competition between NO· and SOD for superoxide is feasible (Huie and Padmaja 1993), and most of the toxicity of superoxide has been attributed to the formation of peroxynitrite (Koppenol 1998).

It is generally believed that NO· in cigarette smoke reacts with superoxide derived from the reducing constituents in the particulate phase of cigarette smoke, i.e., dihydroxybenzenes such as hydroquinone and catechol, to form peroxynitrite (Müller et al. 1997). Based on kinetic and other considerations, Squadrito and Pryor (1998) proposed that peroxynitrite readily forms in vivo, combining rapidly with abundant intracellular carbon dioxide to form metastable nitrating, nitrosating, and oxidizing intermediates. Apart from carbon dioxide, peroxynitrite is believed to react rapidly only with molecules localized in the cellular vicinity of its formation. Peroxynitrite can react with and inactivate essential proteins including hemoglobin, myeloperoxidase, GSH peroxidase, and others. Because peroxynitrite is short-lived in living tissues and difficult to measure directly, the detection of 3-nitrotyrosine (the nitration product of tyrosine by peroxynitrite) is usually taken as evidence of its existence in vivo (Eiserich et al. 1994; Reiter et al. 2000).

Peroxynitrite has been identified as an oxidative stress-inducing compound of aqueous cigarette smoke fractions (Müller and Gebel 1994, 1998; Müller et al. 1997). After depletion of intracellular GSH content by electrophilic aldehydes, peroxynitrite interferes with specific target molecules, resulting in the activation of stress-related signal transduction and gene expression in cigarette smoke-treated cells in vitro (Müller and Gebel 1994). Furthermore, gene expression profiling in respiratory tract tissues obtained from cigarette smoke-exposed rats revealed a pronounced activation of stress response via upregulation of oxidative stress-related genes, many of which counteract cigarette smoke-induced peroxynitrite stress (Bosio et al. 2002), although other nitration reactions can occur.

2.2.2.3 Glutathione Depleting Substances

Glutathione is abundant in cytoplasm, nuclei, and mitochondria and is the major water-soluble antioxidant in these cell compartments at millimolar concentrations (Ault and Lawrence 2003). High levels of GSH are found in the extracellular lung lining fluid (about 100 μmol/l), but not in blood plasma, where concentrations are very low (<1 μmol/l). Among the intracellular nonprotein thiols such as cysteine, homocysteine, α-lipoic acid, and coenzyme A, GSH accounts for more than 90% of the total thiols. GSH and other thiols react more easily with α,β-unsaturated aldehydes at the β-carbon than at the carbonyl carbon (Meacher and Menzel 1999). Both α,β-unsaturated and saturated aldehydes are direct-acting chemicals, i.e., they require no metabolic activation. The

yields of acrolein and crotonaldehyde, two α,β-unsaturated aldehydes in cigarette mainstream smoke, range from 5 to 60 and <1 to 25 μg per cigarette, respectively (Counts et al. 2004).

Modifications of intracellular GSH by electrophiles in the gas phase of cigarette smoke were first reported decades ago (Gaisch and Nyffeler 1976; Leuchtenberger et al. 1974, 1976). As shown in Fig. 2.3, electrophilic cigarette smoke constituents react with thiol-containing proteins. Eiserich (1995) reported that the concentration of protein sulfhydryl groups in blood plasma is about 500 μ*M*. After exposure to cigarette smoke, the concentration of protein sulfhydryl groups was reduced by ca. 60%. Reddy et al. (2002) investigated this effect in more detail. Solutions of GSH in phosphate buffer exposed to gas-phase cigarette smoke resulted in a significant depletion of GSH, attributed primarily to reaction with acrolein, and a concomitant appearance of oxidized GSH (GSSG). The amount of GSSG formed, however, accounted for only 25% of the GSH depletion. NO·, which is abundant in the cigarette smoke gas phase, can react with GSH to form *S*-nitroso-GSH (GSNO), but Reddy et al. (2002) found that only ca. 1% of the overall reduction in GSH could be attributed to GSNO formation. A more recent investigation by Cahours et al. (2004), using an alternative assay, showed similar amounts of GSSG and GS-aldehyde formation, but the relative percentage of GSNO accounted for more than 30% of the overall GSH depletion.

Hagedorn et al. (2003) developed a GSH depletion assay for gas-phase, particulate-phase, and whole mainstream cigarette smoke. GSH consumption was reported to be two and three times higher for particulate phase and whole smoke, respectively, in comparison to gas-phase smoke. The assay showed that the depletion of GSH in solutions of GSH treated with gas-phase cigarette smoke correlates well with the cytotoxicity of the gas phase, as determined by the neutral red uptake (NRU) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)–tetrazolium assays.

The conjugation of cigarette smoke electrophiles with GSH can proceed spontaneously or by catalysis by GSH *S*-transferases. GSH *S*-conjugates are catabolized to their

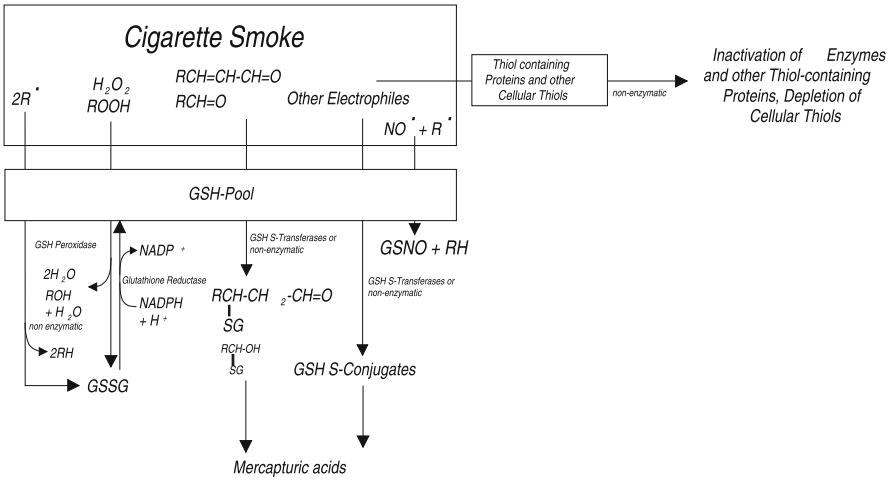

Fig. 2.3 Cigarette smoke-induced depletion of cellular thiols

corresponding mercapturic acids, which are subsequently excreted into the urine. 3-Hydroxypropylmercapturic acid (3-HPMA) is a urinary metabolite of acrolein and can be used as biomarker of cigarette smoke exposure (Mascher et al. 2001). 3-HMPA excretion in smokers as compared with nonsmokers is about three to four times higher (Martin and Tricker 2004).

2.3 Tobacco Leaf Constituents Affecting Smoke Chemistry and Toxicity

2.3.1 Phenolic Compounds

Phenolic compounds are an important class of chemicals that form during the thermal decomposition of biomass (Achladas 1991; Amen-Chen et al. 1997) and tobacco (Schlotzhauer and Chortyk 1987). Their formation, identification and quantification in cigarette smoke has been extensively studied and reviewed (Arrendale et al. 1984; Brunneman et al. 1976; Chen and Moldoveanu 2003; Counts et al. 2005; Crouse et al. 1963; Forehand et al. 2000; Klus and Kuhn 1982; Risner and Cash 1990; Yang and Wender 1962). The most abundant phenolic constituents in tobacco smoke are phenol, dihydroxybenzenes, and their methyl-substituted derivatives. Hydroquinone, catechol, and their methyl-substituted derivatives have been shown by us (see Sect. 4.3) and others (Smith et al. 2002a) to be highly cytotoxic.

Most of the research on phenolic compounds has focused on the formation of catechol and phenol from tobacco, tobacco extracts, and selected tobacco constituents such as polyphenols and lignin (Patterson et al. 1976; Sakuma et al. 1982; Schlotzhauer and Chortyk 1981, 1987; Schlotzhauer et al. 1967, 1982, 1992; Sharma et al. 2000; Spears et al. 1965; Zane and Wender 1963). Despite numerous papers addressing the formation of phenolic compounds from tobacco, there are few data available on their temperature of formation or the contribution of specific tobacco constituents to the yield of phenolic compounds in cigarette smoke TPM (Carmella et al. 1984; Schlotzhauer and Chortyk 1981; Schlotzhauer et al. 1969; Torikaiu et al. 2005). Such information is essential to understand the apportionment of phenolic compounds from tobacco leaf constituents and to develop strategies to reduce the yield of these cytotoxic agents.

We have systematically studied the formation of phenolic compounds from heated tobacco and tobacco leaf constituents in our laboratory. Pyrolysis experiments were carried out in a tube furnace in the heating range from 250 to 600 °C (McGrath et al. 2003). The effect of pyrolysis temperature and water extraction on the formation of phenolic compounds was investigated. Smoking experiments were carried out under Federal Trade Commission smoking conditions (Federal Register 1967, 1980), using cigarettes made from the three individual types of tobacco found in typical American blend cigarettes: bright, burley, and oriental tobaccos. Two reference cigarettes, 2R4F (Chen and Moldoveanu 2003) and IM17 (an industry monitor), containing a representative blend of these tobaccos, were also studied. The 2R4F and IM17 cigarettes have the same blend composition, but the 2R4F cigarette has ventilation holes in the filter tip, whereas the IM17 cigarette does not.

2.3.1.1 Phenolic Compound Yields in TPM

The chemical structures of the ten phenolic compounds in our study (hydroquinone, catechol, resorcinol, 3-methyl catechol, 4-methyl catechol, guaiacol, phenol, *o*-, *m*-, and *p*-cresol) are shown in Fig. 2.4. Quantitative yields of phenolic compounds from smoking and pyrolysis experiments were determined by gas chromatography mass spectrometry (GC/MS) and high-performance liquid chromatography (HPLC). The yield of phenolic compounds was calculated using a calibration curve obtained from the analysis of standard solutions and the yields are reported as the average of three independent measurements. The yields of phenolic compounds in the TPM from the five cigarettes are shown in Fig. 2.5, expressed as micrograms of phenol per milligram of TPM. The TPM yields per cigarette were 7.3±0.7, 14.2±0.2, 16.3±1.0, 8.6±1.3, and 9±1.2 mg, respectively, for the 2R4F, IM17, bright, burley and oriental cigarettes.

Of the ten phenolic compounds measured, hydroquinone and catechol are the most abundant in the TPM of all five cigarettes. The two reference cigarettes gave relatively similar yields of all phenols. Except for the yields of catechol, phenol, and 4-methylcatechol, the yields of phenolic compounds from all three single-component blend cigarettes were quite similar. The 100% bright cigarette gave the highest yield of hydroquinone and 4-methylcatechol. The 100% burley cigarette yielded approximately 47% less catechol as compared with the bright and oriental cigarettes. The trend in the yields of hydroquinone and catechol obtained from the three single-component blend cigarettes followed the order: bright →oriental → burley.

Tobacco polyphenols such as chlorogenic acid and rutin have previously been shown to be precursors of phenolic compounds in cigarette smoke (Carmella et al. 1984; Sakuma et al. 1982; Schlotzhauer et al. 1967, 1982; Sharma et al. 2000; Zane and Wender 1963). The polyphenol content of cigarette tobacco filler ranges from ca. 2.2 mg per cigarette for 100% burley tobacco to ca. 14.22 mg per cigarette for 100% bright tobacco (Table 2.1). Comparison of the polyphenol content of the tobacco filler and the yield of phenolic compounds in the TPM (Fig. 2.5) reveals that the yield of phenolic compounds in the TPM is not directly proportional to the polyphenol content of the tobacco filler. Thus, other tobacco constituents in addition to the polyphenols must contribute to the overall yield of phenolic compounds in cigarette smoke. (see Section 2.3.1.4)

Table 2.1 Polyphenol contents (milligrams per cigarette) for tobacco filler from 2R4F, IM17, and single-tobacco component cigarettes

Cigarette type	Chlorogenic acid	Rutin	Scopoletin	Quinic acid	Caffeic acid	Gentisic acid
2R4F	5.1	1.8	0.01	1.2	0.06	0.01
IM17	6.7	2.2	0.06	1.8	0.13	0.01
Bright	9.7	2.3	0.13	1.9	0.19	0.02
Burley	0.4	0.4	<0.01	1.4	<0.01	<0.01
Oriental	9.0	2.1	<0.01	1.2	0.15	0.01

Polyphenol contents determined from an acetone/water extraction of respective tobacco fillers and quantified by liquid chromatography/mass spectrometry/mass spectrometry

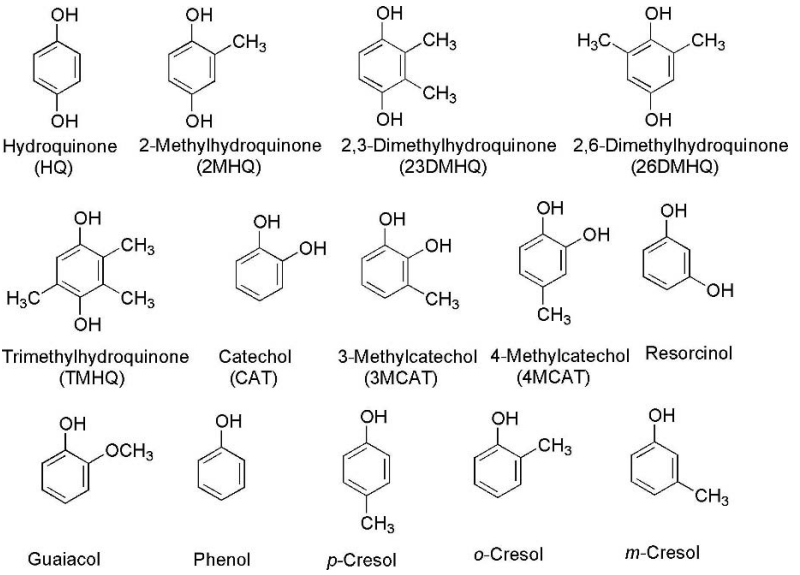

Fig. 2.4 Chemical structures of phenolic compounds found in the total particulate matter (TPM) of mainstream tobacco smoke

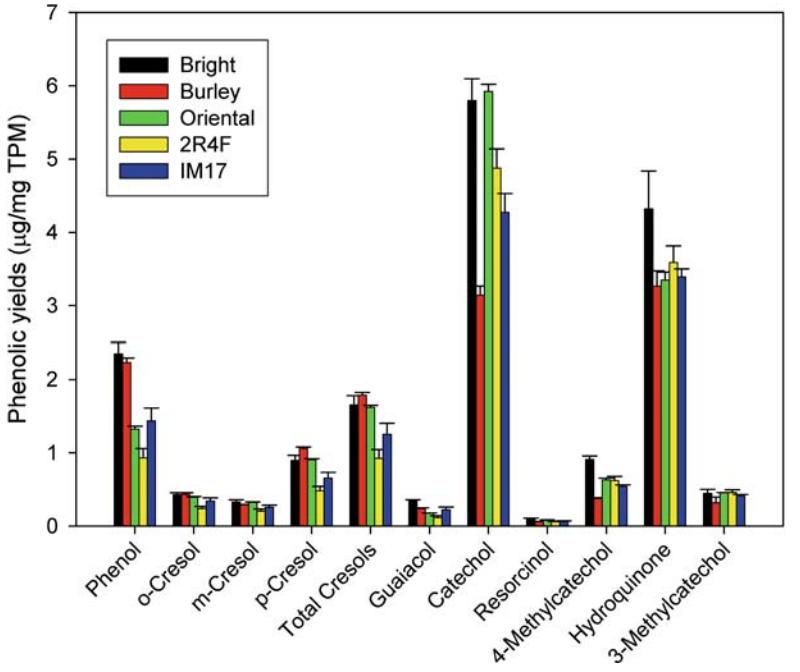

Fig. 2.5 Yields of phenolic compounds in the total particulate matter (*TPM*) of mainstream smoke from several cigarettes smoked under Federal Trade Commission conditions. The cigarettes were three single-component cigarettes containing bright, burley, or oriental tobacco and two reference cigarettes containing the typical American blend of tobaccos (2R4F and IM17)

2.3.1.2 Effect of Temperature on Phenolic Compound Yields

We also investigated the effect of pyrolysis temperature on the formation of phenolic compounds from heated bright tobacco lamina. Samples were first heated at 350 °C for 10 min under helium and the smoke condensate collected and analyzed. The precharred tobacco sample was then heated to 600 °C and held at this temperature for a total of 10 min under helium. The phenolic compounds found in the low-temperature TPM ([LT-TPM] 25–350 °C) and the high-temperature TPM ([HT-TPM] 350–600 °C) were characterized using GC/MS. The constituents of the LT-TPM fraction have previously been described (McGrath et al. 2005). Nicotine is the dominant constituent of the LT-TPM. Furans, furanones, phenols, pyranones, benzenediols, indoles, pyridines, fatty acids, vitamin E, and long-chain hydrocarbons are also present. The HT-TPM fraction was dominated by phenol, mono-, di-, and trimethyl phenols. Indole and methyl indole were also major products, followed by methyl pyridines, substituted pyrroles, methylpyridoindole, stigmasterol, and cholesterol acetates. It is interesting to note that approximately 86% of the total amount of TPM collected by this two-step process forms over the 25–350 °C temperature region.

The relative yields of phenols produced over the two temperature regions of 25–350 °C and 350–600 °C, expressed as a percentage of the total yields formed at 600 °C, are shown in Fig. 2.6. Hydroquinone (96%), catechol (97%), guaiacol (95%), 3-methylcatechol

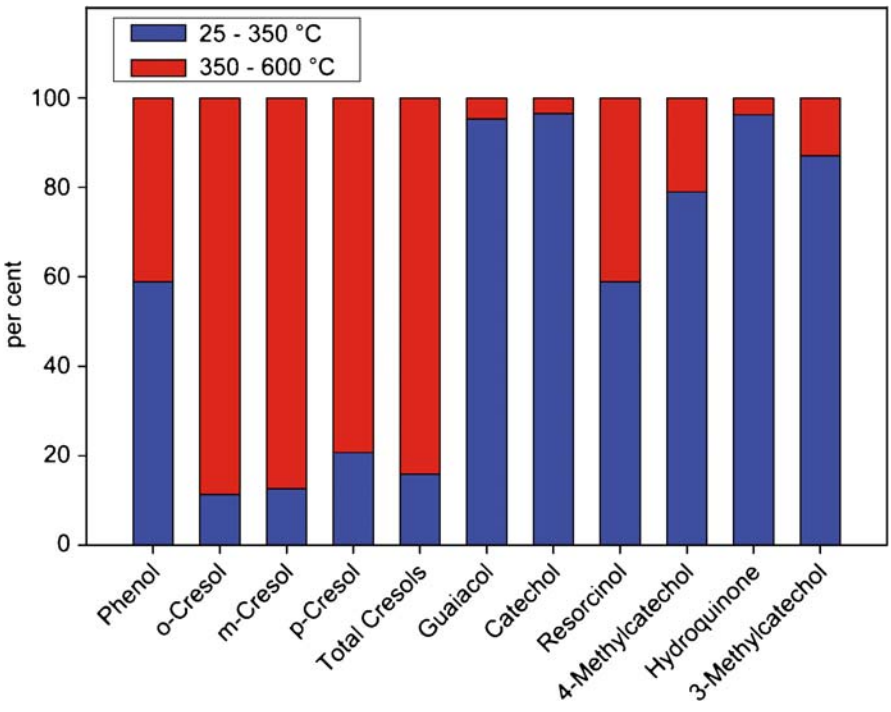

Fig. 2.6 Temperature formation range for phenolic compounds in the total particulate matter (TPM) of bright tobacco heated in a tube furnace under flowing helium for 10 min

(87%) and 4-methylcatechol (79%) are formed predominantly in the LT-TPM fraction, whereas *o*-cresol (89%), *m*-cresol (87%), and *p*-cresol (79%) form predominantly in the HT-TPM fraction. Formation of phenol (59%:41%) and resorcinol (59%:41%) appears to span the two temperature regions. The total yield of the ten phenolic compounds studied accounts for approximately 4% of the total weight of TPM formed.

2.3.1.3 Effect of Water Extraction on Phenolic Compound Yields

To investigate the effect of removing or concentrating potential phenolic precursors from tobacco on the yields of phenolic compounds in cigarette smoke, we extracted samples of bright, burley, and oriental lamina with water. Tobacco polyphenols and brown pigments can be removed and isolated from tobacco by extraction with water, methanol, acetone, and water/methanol solutions (Chortyk et al. 1966; Schlotzhauer and Chortyk 1981; Schlotzhauer et al. 1972, 1969, 1992; Wright et al. 1960, 1964; Zane and Wender 1963). Extraction with water leads to about a 50, 40, and 53% reduction in sample weight of the bright, burley, and oriental lamina, respectively. Water extraction removes the more polar constituents such as inorganic salts, organic salts, polyphenols and alkaloids, while concentrating (on a per-unit weight basis) various carbohydrate, lignin, and lipophilic constituents such as waxes, fatty acids, and high-molecular-weight sterols.

The water-extracted lamina samples were heated at 600 °C under helium, and the yield of phenolic compounds formed were compared with the nonextracted samples. Significant reductions in the yields of hydroquinone (50–60%), catechol (37–41%) and phenol (50–55%) on a per-unit weight basis were observed in the TPM of heated bright and oriental tobacco. A 40% reduction in the yield of hydroquinone was also observed for the TPM of extracted burley tobacco, but, by comparison, there was only a slight reduction in the yield of phenol (21%) and cresols (9%), and there was a significant increase of catechol (85%). When the yields of phenolic compounds from the water-extracted tobacco are normalized to the total amount of material extracted, larger reductions are observed. Decreases of 63–82% for hydroquinone, 53–74% for phenol, and 35–57% for cresols were observed for the burley, oriental, and bright samples, respectively. Whereas the catechol also decreased to around 71% for both bright and oriental tobaccos, the yield of catechol for burley increased slightly by 11%.

Consistent with previous work carried out on the formation of phenol and catechol from extracted tobacco, we found that extraction of tobacco lamina with water removes precursors of hydroquinone, catechol, and phenol from bright and oriental tobacco. Extraction of burley lamina also removes hydroquinone precursors, but significantly concentrates catechol precursors. Because of the longer curing times employed for burley tobacco, precursors to catechol such as chlorogenic acid may be polymerized via enzymatic reactions to water insoluble polymeric precursors (Kameswararo and Gopalachari 1965; Wright et al. 1960, 1964).

2.3.1.4 Phenolic Compound Formation from Tobacco Constituents

To identify possible tobacco precursors of hydroquinone and catechol, we pyrolyzed a number of polyphenolic, carbohydrate, and lignin samples at 600 °C under helium for 10 min and analyzed the collected TPM condensate for hydroquinone, catechol, phenol, and cresols (sum of *o*-, *m*-, and *p*-cresols) by HPLC. The chemical structures of the polyphenols (gentisic, quinic, chlorogenic, and caffeic acids, scopoletin, and rutin) are shown in Fig. 2.7. The yields of hydroquinone, catechol, phenol, and cresols produced from the pyrolysis of these compounds added to bright tobacco under helium gas are shown in Fig. 2.8. The increase in the yield of hydroquinone from the addition of gentisic acid was ca. 6 times higher than that from quinic acid and ca. 17 times higher than from chlorogenic acid on a per-unit weight basis.

The yields of hydroquinone, catechol, phenol, and cresols from the 600 °C pyrolysis of the individual tobacco cell wall constituents are shown in Fig. 2.9. Comparable yields of hydroquinone, catechol, and phenol were formed from cellulose, xylan, glucose, and fructose, with slightly lower amounts of hydroquinone being formed from the pectin sample. The yield of cresols was very similar for cellulose and pectin, with slightly lower yields being formed from glucose and fructose. Although the yield of hydroquinone from the model lignin sample is comparable to the carbohydrates pyrolyzed, the yields of catechol, phenol, and cresols are approximately 9, 11, and 13 times higher, respectively, compared with the cell wall carbohydrates.

Among the 11 tobacco constituents studied, gentisic, quinic, and chlorogenic acids were found to be the most significant precursors of hydroquinone. Caffeic, chlorogenic, and quinic acids are major precursors to catechol, followed by lignin and then the carbohydrates. Lignin yields significantly more catechol compared with the cell wall carbohydrates, (Carmella et al. 1984; Schlotzhauer et al. 1982) but significantly less in comparison to chlorogenic or caffeic acid (ca. 4 and 17 times lower, respectively, on a per-unit weight basis).

To estimate the contribution of each tobacco leaf constituent to the overall yield of the phenolic compounds from the pyrolysis of tobacco, we normalized the yield of phenolic compounds from each of the precursors studied to the amount of each precursor reported in bright tobacco. The estimated level of phenolic compounds from each precursor in bright tobacco leaf lamina is given in Table 2.2. For the normalization step, glucose was used to represent the total reducing sugars, and amounts of cellulose, pectin, hemicellulose, reducing sugars, and lignin were taken from the work of Leffingwell (1999) and Bokelman and Ryan (1985). The amounts of free quinic, caffeic, chlorogenic, and gentisic acids were obtained experimentally from the liquid chromatography mass spectrometry (LC/MS) analysis of an acetone/water extract of bright tobacco.

The carbohydrates and lignin constituents in tobacco were found *not* to be major precursors to hydroquinone. Even though they make up ca. 46% of the weight of tobacco, together they only account for 6–8% of the overall yield of hydroquinone. The polyphenols, which account for less than 2% of the weight of tobacco, account for ca. 17% of the overall hydroquinone yield. Chlorogenic acid and the estimated free quinic acid level account for ca. 11 and 6%, respectively.

From the pyrolysis of the individual tobacco constituents at 600 °C presented in Figs. 2.8 and 2.9, we found that caffeic, chlorogenic, and quinic acids are the major pre-

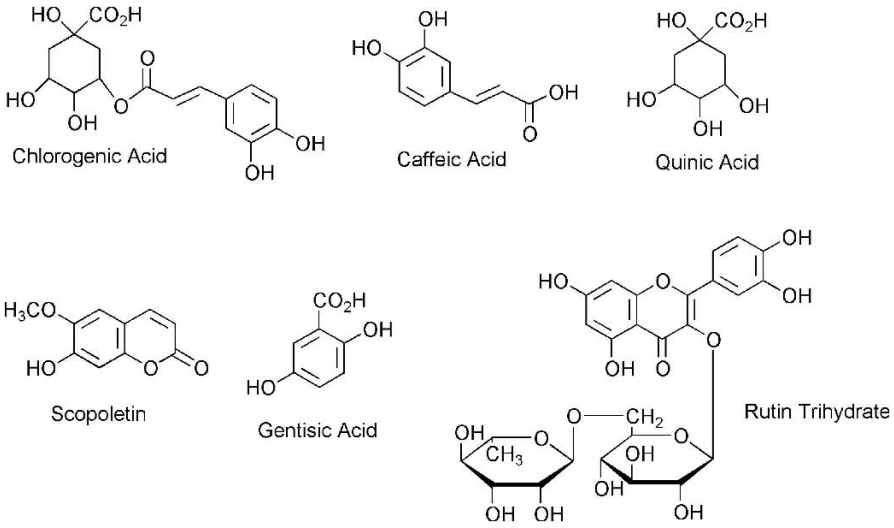

Fig. 2.7 Chemical structures of phenolic precursor compounds found in bright tobacco

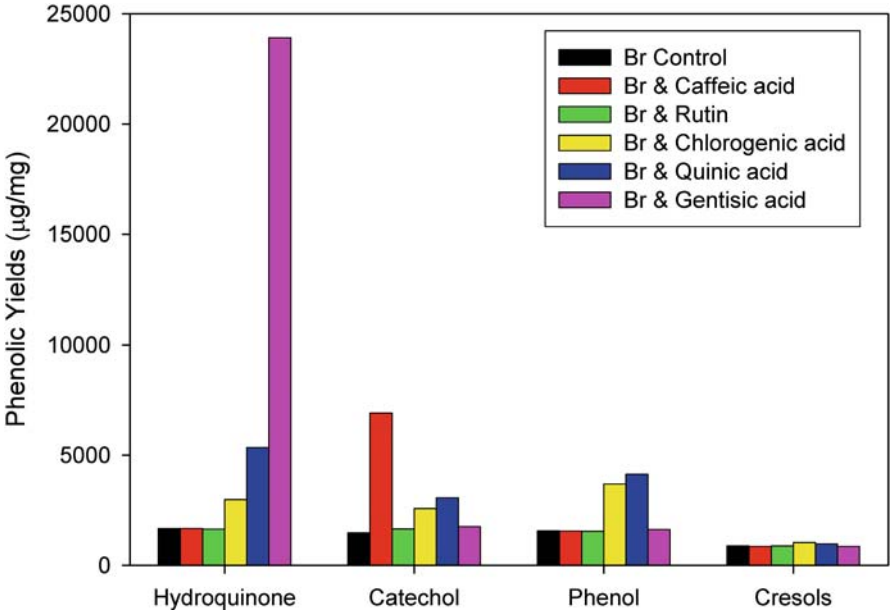

Fig. 2.8 Yield of phenolic compounds from 1 g bright tobacco mixed with 60 mg of the indicated phenolic precursors and heated at 600 °C for 10 min under flowing helium in a tube furnace

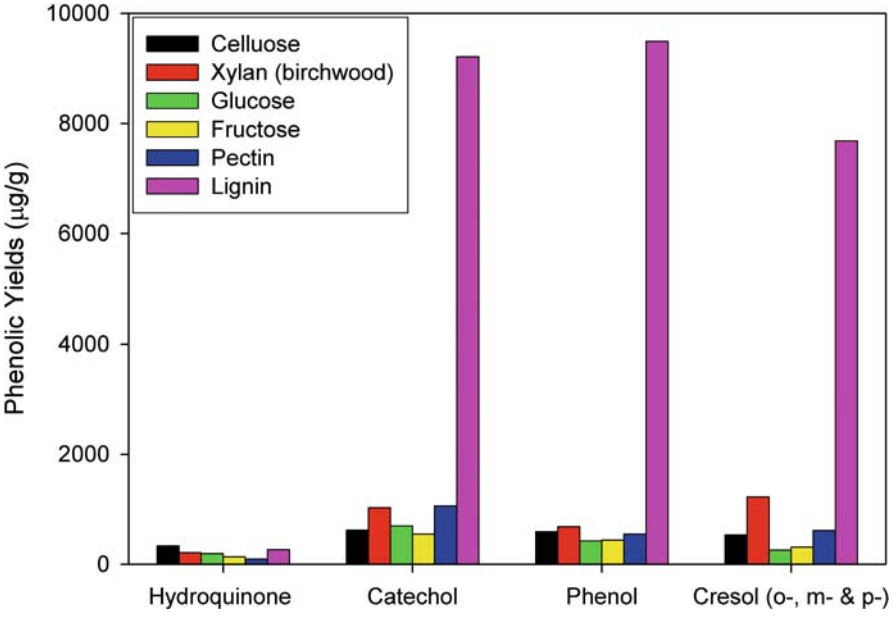

Fig. 2.9 Yield of phenolic compounds from several tobacco leaf constituents heated at 600 °C under flowing helium for 10 min

cursors to catechol, followed by rutin, lignin and, to a lesser extent, the polysaccharides. Upon normalization of the yields, the carbohydrate and lignin together were found to account for approximately one third of the overall catechol yield. Although the carbohydrate content in tobacco is generally approximately five times higher than that of lignin, lignin accounts for 11% of the total yield of catechol, followed by hemicellulose (6.1%), pectin (6.6%), glucose (6%), and cellulose (3.7%). Of the polyphenols, chlorogenic acid (11%), quinic acid (2.7%), caffeic acid (1.2%), and rutin (0.8%) contribute ca. 16% towards the overall yield of catechol. The individual contributions from the pyrolysis for chlorogenic acid, glucose, cellulose, and rutin to the overall yield of catechol reported here are very similar to those previously reported by Carmella et al. (1984).

Based on the assumptions employed for the normalization, we found that ca. 24% of the overall hydroquinone yield and ca. 49% of the overall catechol yield can be accounted for by the 11 tobacco constituents examined. It should be noted that the addition of potassium nitrate and potassium acetate to pure cellulose (1% [w/w] potassium levels) led to a threefold increase in the yields of hydroquinone and catechol. Calcium (in the form of $CaCO_3$) also significantly increases the yield of catechol when added to cellulose. The influence of the two most abundant endogenous inorganic cations (potassium and calcium) can potentially increase the overall contribution of tobacco carbohydrates to catechol yields.

Table 2.2 Estimated source apportionment for hydroquinone and catechol in the TPM of smoke from 100% bright tobacco heated at 600 °C for 10 min under helium

	Wt%	HQ yield[a]	Percentage of total[b]	CAT yield[a]	Percentage of total
Carbohydrates					
Cellulose	10	38	2.4	62	3.7
Glucose	14	34	2.1	100	6.0
Pectin	10	13	0.8	110	6.6
Hemicellulose	10	20	1.2	102	6.1
Lignin	2	5	0.3	184	11.0
Polyphenols					
Chlorogenic acid	0.84	183	11.0	155	10.5
Rutin	0.41	1	0.1	12	0.8
Quinic acid	0.15	92	5.5	39	2.7
Caffeic acid	0.02	0	0.0	18	1.2
Gentisic acid	0.0015	6	0.4	0	0.0

Wt% Estimated weight percentage of each component in bright tobacco lamina (Bokelman and Ryan 1985)

[a]Yields of hydroquinone (*HQ*) and catechol (*CAT*), respectively, for each tobacco constituent normalized to the amount of each constituent found in a methanol/water extract of bright tobacco

[b]Contribution to the total yield

2.3.2 Trace Heavy Metals

The yields reported in the literature of trace metals that transfer from the cigarette tobacco to the mainstream cigarette smoke vary widely. For example, Purkis et al. (2003) reported the yields of several trace metals in the TPM from three different cigarettes (5-, 8-, and 12-mg tar delivery), tested by five independent laboratories under the same smoking regime. For the 8-mg product, the yield (nanograms per cigarette) for cadmium was 20.6–35.3; for lead, 8.8–16.8; for mercury, 0.4–3.5; for arsenic, 1.9–2.2; for chromium, 2–8.7; for nickel, 2–5.3; and for selenium, 0.8–6. Although the Cambridge filter is the most common method of collecting organic compounds from cigarette smoke condensate, it is not suitable for the collection of tar from mainstream cigarette smoke for trace metal analysis because of the trace metal impurities in the pad. Instead, quartz glass filters have been used for collection of inorganic compounds in tobacco smoke, because their background contamination is relatively low. Although cold traps and jet impaction traps have been employed, electrostatic precipitation into quartz tubes has become the preferred technique for collection of cigarette smoke condensate for trace metal analysis. Samples must be handled with meticulous care to avoid potential sources of metal contamination from the laboratory environment (Counts et al. 2004, 2005; Gregg et al. 2004; Roemer et al. 2004). In general, the use of an isolation clean room is required for accurate trace metal analysis.

A variety of analytical techniques has been employed for determining trace metals in mainstream cigarette smoke. In the most recently reported measurements, the two

Table 2.3 Trace metal analysis for 1R4F research cigarettes and three commercial products reported by different labs under different smoking regimes

Reference	Cigarette	Smoking Conditions	Tar (mg/cig.)	Cd (ng/cig.)	Pb (ng/cig.)	Hg (ng/cig.)	As (ng/cig.)	Cr (ng/cig.)	Ni (ng/cig.)	Se (ng/cig.)	Method	No. labs/reps.
Gregg et al. 2004	1R4F	ISO[a]	9	63.2	39.3	4.6	6.1	–[b]	–[b]	–[b]	ICP-MS	2 labs (average)
Torrence et al. 2002	1R4F	FTC[c]	9	64.2±6.3[d]	38.2+/–1.8	–[e]	6.9±0.5	–[e]	–[e]	–[e]	ICP-MS	20 cigarettes
Chang et al. 2002	1R4F	FTC	9	–[e]	–[e]	5±0.4	–[e]	–[e]	–[e]	–[e]	CV-AAS	20 cigarettes
Chen and Moldovea-nu 2003	1R4F	ISO	9.38	55.1	42.5	5.4	12.2	57.7	6.4	34.9	Various	1–4 labs
Counts et al. 2004	1R4F	ISO	9.1±0.4	64.4±2.9	36.7±1.5	5.5±0.5	4.7±0.8	–[b]	–[b]	–[b]	GF/CV-AAS	20 cigarettes/ replicates
Counts et al. 2004, 2005	1R4F	MDPH[f]	19.1±0.8	143.6	69.5	10	11.2	–[b]	–[b]	–[b]	GF/CV-AAS	20 cigarettes/ replicates
	1R4F	HC[g]	26.3±1.4	160.1	89	10.1	11.9	–[b]	–[b]	–[b]	GF/CV-AAS	20 cigarettes/ replicates

Cd cadmium, *Pb* lead, *Hg* mercury, *As* arsenic, *Cr* chromium, *Ni* nickel, *Se* selenium, *mg/cig.* milligrams per cigarette, *ng/cig.* nanograms per cigarette, *labs/reps.* laboratories/replicates, *ISO* International Organization for Standardization, *ICP-MS* inductively coupled plasma mass spectrometry, *FTC* Federal Trade Commission, *CV-AAS* cold-vapor atomic absorption spectrometry, *GF/CV-ASS* graphite-furnace atomic absorption spectrometry, *MDPH* Massachusetts Department of Health, *HC* Health Canada

[a]ISO puffing conditions: 35-ml volume, 60-s interval, 2-s duration

[b]Below detection limit or too low to quantify

[c]FTC puffing conditions; 35-ml volume, 60-s interval, 2-s duration

[d]Mean±SD

[e]not measured

[f]MDPH puffing protocol: 45-ml volume, 30-s interval, 2-s duration, 50% filter ventilation blocking

[g]HC puffing protocol: 55-ml volume, 30-s interval, 2-s duration, 100% filter ventilation blocking

most common techniques were either inductively coupled plasma mass spectrometry (ICP-MS), or graphite-furnace (GF-) or cold-vapor atomic absorption spectrometry (CV-AAS). Because of its higher volatility, mercury is usually measured by cold-vapor atomic absorption spectrometry (Chang et al. 2002; McDaniel et al. 2001).

The yields of several trace metals in the mainstream smoke collected under International Organization for Standardization (ISO) smoking machine conditions from 48 commercial brands of filtered cigarettes were reported by Counts et al. (2004). The tar yield in the various brands ranged from 0.9 to 14.4 mg per cigarette. To a very good approximation, the amount of cadmium, lead, mercury, and arsenic in the smoke condensate was found to be proportional to the yield of tar per cigarette. The overall yield of trace metals showed a dependence on the cigarette brand, design, and the smoking machine conditions employed (Counts et al. 2005). For the 48 brands tested, the range of the average yield per cigarette for each metal was cadmium, 1.6–101.1 ng (48 brands); lead, 13.0–31.4 ng (5 brands); mercury, 1.5–4.7 ng (40 brands); and arsenic, 3.9–5.5 ng (3 brands). For the brands not included in this summary, the levels of these metals in the cigarettes were either below the detection limits of the analytical methods, or too low to quantify. A similar survey of cigarette brands sold in the United Kingdom was made by Gregg et al. (2004). In both studies, some of the variation in trace metal yields between cigarettes was likely because of the variation in the trace metal composition of regional cigarette tobaccos. Other reviews of metal ions in cigarette smoke include (Baker 1999; Hoffmann et al. 2001; IARC Monographs 1986; Smith et al. 1997). In some of these reports, single values are given for the yield of particular metals without indicating the cigarette design, TPM yield, or smoking regimen (see examples cited by Baker [1999] and Stohs et al. [1977]). Such reports are not very meaningful in light of the large range in tar yields reported for different commercial products.

The remaining toxic elements that have been assayed in commercial cigarette products are nickel, chromium, and selenium. The yields of these elements in the most recent studies with commercial filtered cigarettes are either below the detection limit of the methods or too low to quantify (<2 ng per cigarette), over a range of cigarette tar yields. (See, for example, the results of Counts et al. [2004, 2005], Gregg et al. [2004], and the summary for 1R4F research cigarettes given in Table 2.3.) In a systematic comparison of the analyses of 1R4F research cigarettes by four different laboratories, Chen and Moldoveanu (2003) reported high values of 57.7, 6.4, and 34.9 ng per cigarette for chromium, nickel, and selenium, respectively (Table 2.3). The authors noted that different analytical limits of quantification between laboratories contributed to uncertainties in several reported yields. Other potential contributors to these analytical variations are the laboratory environmental or apparatus contaminates addressed by Torrence et al. (2002). Thus, appropriate analysis precautions appear necessary to ensure that accurate data are available for health, regulatory, and tobacco science groups.

2.4 Free Radicals, ROS, and RNS

Cigarette smoke contains a large amount of free radicals (Pryor et al. 1983a, c) and constituents that readily produce free radicals (Cosgrove et al. 1985; Pryor et al. 1983a). The free radicals in cigarette smoke can be classified into two categories: (1) free radicals that form during the burning of tobacco and the smoking process and (2) free radicals that are *not* initially present in the smoke, but are generated either when the gas phase or the

TPM constituents are oxidized in the smoke aerosol, or when they dissolve in oxygenated aqueous solutions or biological media. The first category includes the radicals in the TPM and the gas phase, whereas the second category includes semiquinone radicals, ROS, and RNS.

2.4.1 Particulate-Phase Free Radicals

The TPM free radicals from cigarette smoke are known to be stable and last for an indefinitely long period of time (Chouchane et al. 2005; Pryor et al. 1983a, c, 1998; Zang et al. 1995). They can be detected using EPR either directly on the filter used to collect the TPM or in solution by extraction of the TPM. A cellulose filter is employed because the Cambridge pad exhibits a background EPR signal. Figure 2.10 shows the EPR signal of cigarette smoke TPM free radicals detected directly on a cellulose filter (Chouchane et al. 2005). The EPR signal is characterized by a broad singlet with a g-factor equal to 2.0028. The EPR spectrum in Fig. 2.10 is very similar to previously reported spectra (Pryor et al. 1983a; Pryor et al. 1983c). The concentration of these radicals in TPM can be as great as 10^{17} radicals per gram of TPM depending on the tobacco, cigarette type, and the smoking regime. The chemical nature of the radicals in cigarette smoke TPM has never been fully characterized. The accepted view of TPM radicals is that they consist primarily of semiquinone radicals in a polymeric tarry matrix. (Pryor et al. 1983a; Pryor et al. 1983c). Semiquinones undoubtedly account for part of the radicals, but recent findings suggest that the TPM radicals are not simply semiquinone radicals, but can be distinguished as oxygen-centered radicals or carbon-centered radicals (Chouchane et al. 2005).

Using bright tobacco cigarette filler heated in a tube furnace under helium atmosphere, we observed that the free radical yield in TPM increases with the heating temperature, as shown in Fig. 2.11. Maskos et al. (2005) made similar observations and showed that the TPM radicals from bright tobacco filler heated at 200–400 °C exhibit g-factors that vary from 2.0039 to 2.0050, characteristic of oxygen-centered radicals, whereas radicals from tobacco heated at ca. 600 °C exhibit a g-factor equal to 2.0028, characteristic of carbon-centered radicals. Experiments in our laboratory showed that TPM radicals from both cigarette smoke and pyrolyzed tobacco undergo an aging process when exposed to air, exhibiting an increase in the intensity of the EPR signals and a shift in the g-factors after the TPM is aged for more than 24 h, as shown in Fig. 2.12. The results of Maskos et al. (2005) and our own suggest that a significant fraction of the cigarette smoke radicals trapped on the filter is initially carbon-centered radicals that convert to oxygen-centered radicals on exposure to molecular oxygen in air.

In experiments utilizing a smoking machine, we measured the yield of free radicals in the TPM from several cigarettes containing different amounts of polyphenolic compounds in the tobacco filler, as shown in Fig. 2.13 (Chouchane et al. 2005). The cigarettes used in our study were the same cigarettes employed in the phenolic compound analysis (see Sect. 2.3.1). We found that the yield of free radicals generated in the TPM of the smoke from these cigarettes was *not* directly related to the total amount of polyphenolic compounds in the tobacco leaf filler. For example, a cigarette containing 100% bright tobacco, which contains a significantly higher amount of polyphenolic compounds in comparison to burley tobacco (Table 2.1), did not generate the highest amount of free radicals in the TPM. However, with the exception of the bright cigarettes, a slight trend was observed between the TPM radicals and the dihydroxybenzenes in the TPM

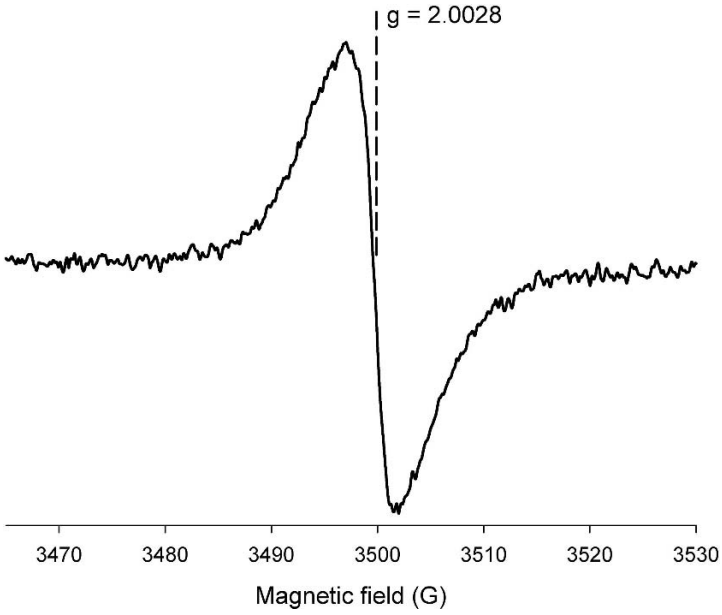

Fig. 2.10 EPR signal of free radicals in the total particulate matter (TPM) of mainstream smoke from a single 2R4F cigarette. The spectrum of the fresh TPM was measured directly on the cellulose collection filter (Chouchane et al. 2005)

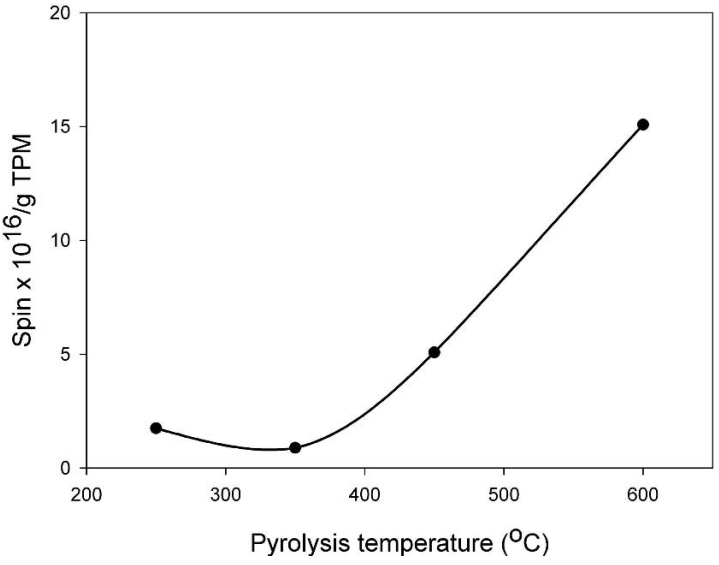

Fig. 2.11 Effect of pyrolysis temperature on the yield of free radicals in the total particulate matter (*TPM*) from bright tobacco. The tobacco was heated for 10 min in a tube furnace at the indicated temperatures under a helium atmosphere

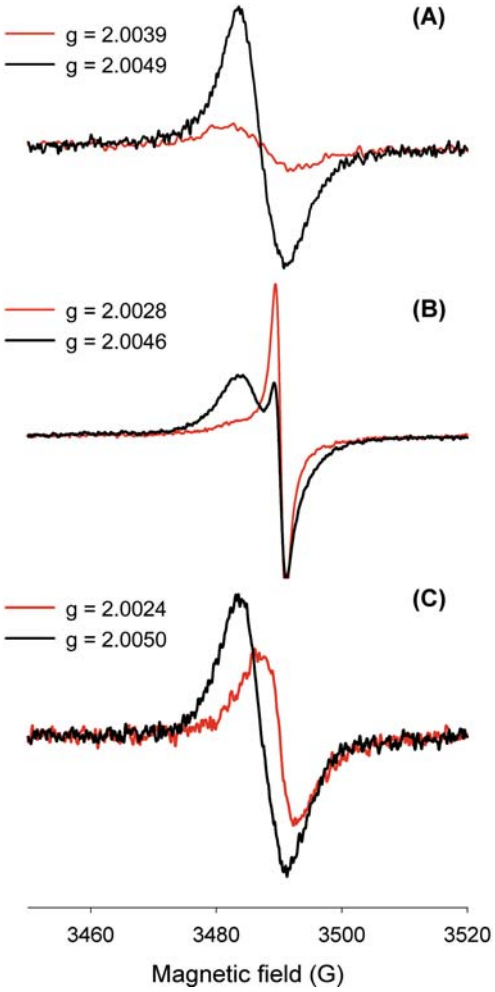

Fig. 2.12 Effect of aging on the free radicals in the total particulate matter (TPM) of smoke from 100% bright tobacco cigarettes or bright tobacco heated under helium in a tube furnace. EPR spectra of free radicals in fresh (·····) and aged (—) TPM for a week from **a** tobacco pyrolyzed in at tube furnace at 450 °C, **b** tobacco pyrolyzed at 600 °C in a tube furnace, and **c** a smoked cigarette

(Fig. 2.14), suggesting that these phenolic compounds do contribute to the formation of the TPM radicals. This result differs, however, from the data previously reported by Blakley et al. (2001) that showed that, there is no relationship between the radicals and the yield of phenolics in the TPM.

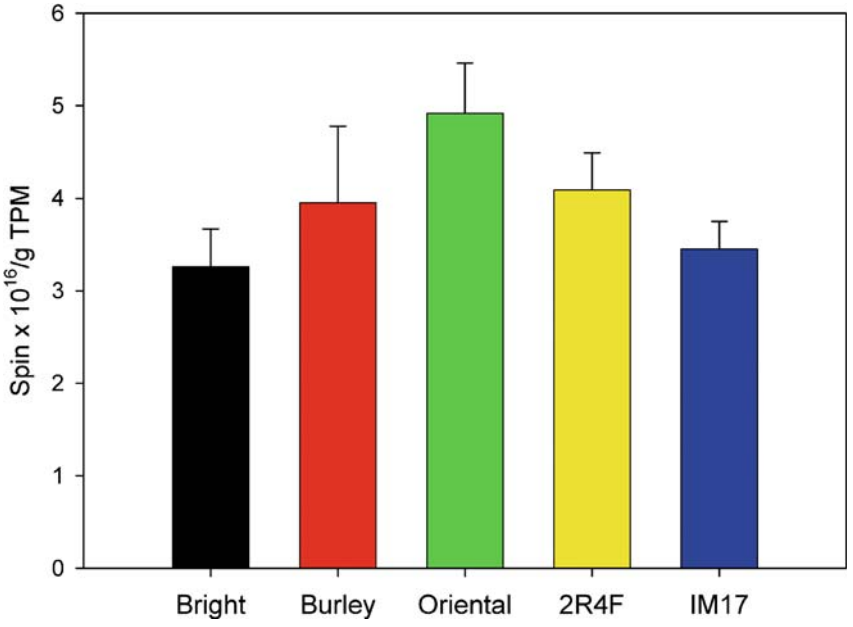

Fig. 2.13 Yield of free radicals in fresh total particulate matter (*TPM*) of mainstream smoke of different cigarettes (Chouchane et al. 2005)

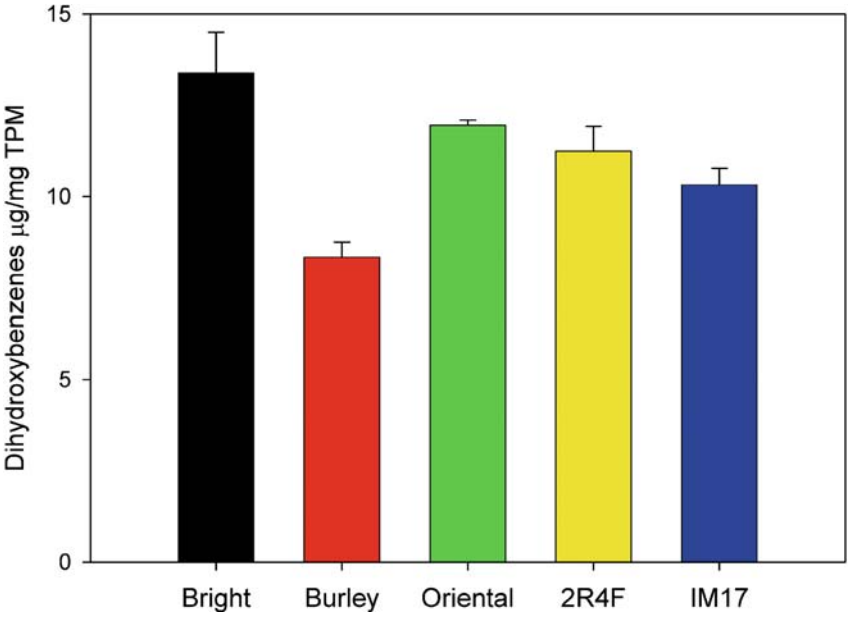

Fig. 2.14 Yield of dihydroxybenzenes in the total particulate matter (*TPM*) of mainstream from different cigarette smoke (Chouchane et al. 2005)

2.4.2 Gas-Phase Free Radicals

The radicals in the gas–vapor phase of cigarette smoke are oxidizing, and they are generally more reactive than are the TPM radicals. EPR spin-trapping techniques are usually used to detect the free radicals in the gas phase of cigarette smoke, typically using the spin trap PBN in benzene solution (Bluhm et al. 1971). Figure 2.15 shows the resulting EPR signal of the gas phase of cigarette smoke, separated from the TPM by a Cambridge filter, and bubbled into a benzene solution of 100 m*M* PBN (Chouchane et al. 2005). The measured hyperfine coupling constants of the radical adducts (confirmed by spectral simulation) are $a_N = 13.9$ G and $a_H = 2.0$ G, and the g-factor is 2.00565. These parameters are in agreement with previously reported hyperfine coupling constants for the PBN adducts of alkoxyl radicals (Pryor et al. 1983a, c). Quantification of the gas-phase radicals from different cigarettes shows that the yield of radicals per cigarette falls in the order: 2R4F > IM17 > burley > oriental > bright (Fig. 2.16) (Chouchane et al. 2005).

NO· is known to be the predominant precursor of other cigarette smoke gas-phase radicals (Sect. 2.2.1). Nitrate, amino acids, ammonium salts, and other nitrogen-containing compounds can potentially produce NO· in the gas phase of cigarette smoke by thermolytic decomposition. Notably, however, gas-phase free radicals can also be produced by heating tobacco leaf constituents that do not contain nitrogen, e.g., cellulose (Pryor et al. 1983a). Im et al. (2003) showed that the evolution of NO· from heated tobacco occurs in two distinct temperature regimes from primarily two sources. Between 275 and 375 °C, under pyrolytic or combustion conditions, the source of NO· was attributed to the decomposition of nitrate. At a higher temperature range (425–525 °C), NO· is produced only in an oxidative environment. In this thermal regime, the NO· was attributed to the oxidation of the char, which contains nitrogen originating from the decomposition of amino acids and protein at lower temperatures.

2.4.3 Cytotoxicity of TPM Constituents

The TPM of cigarette smoke is a very complex mixture that contains numerous substituted phenolic compounds (Smith et al. 2002a). Among these compounds, the dihydroxybenzenes are notable because they can act either as prooxidants or antioxidants. In this section, we present evidence for their possible involvement in the cytotoxicity of cigarette smoke TPM. As discussed in Sect. 3.1.1, the most abundant phenolic compounds found in the TPM from blended or single tobacco cigarettes are phenol, dihydroxybenzenes, and their methyl-substituted derivatives, with hydroquinone and catechol exhibiting the highest yields (see Fig. 2.5). Hydroquinone and catechol are abundant in the smoke of commercial cigarettes (Baker 1999; Counts et al. 2004; Roemer et al. 2004). They are known to generate semiquinone and superoxide radicals via the redox cycling mechanism in aqueous solutions (Samuni et al. 2003; Squadrito et al. 2001), and they have been shown to induce damage in physiological systems (DeCaprio 1999; Deisinger et al. 1996; do Céu Silva et al. 2003; McCue et al. 2003).

We have measured the in vitro cytotoxicity of hydroquinone, catechol, and their methyl-substituted derivatives. The results, given in Table 2.4 (Chouchane et al. 2004), are reported in terms of the 50% effective concentration (EC_{50}), the effective concentration required to kill 50% of mouse embryo BALB/c 3T3 cells in the NRU assay. We found that the methyl-substituted dihydroxybenzenes exhibited a higher cytotoxicity

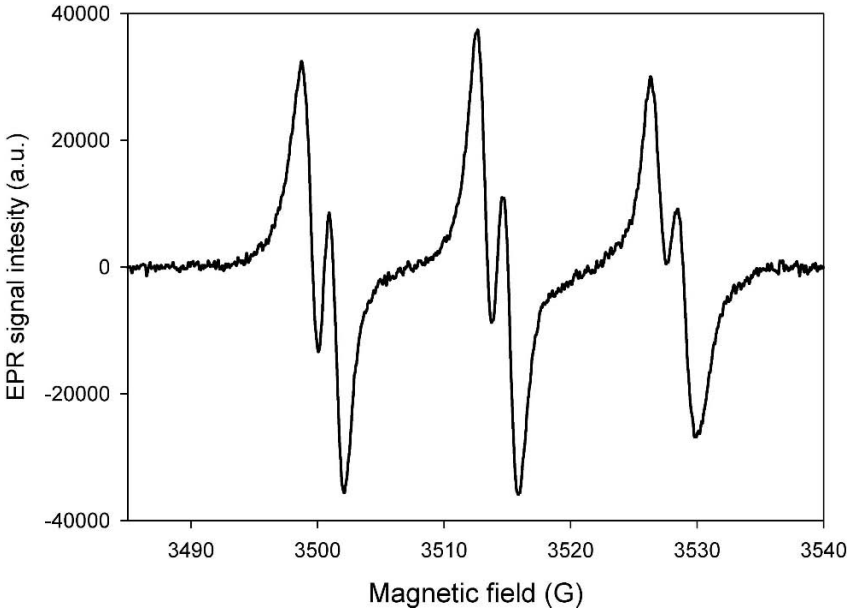

Fig. 2.15 EPR signal of spin adducts of radicals in the gas phase of cigarette smoke. The gas phase was separated from the total particulate matter (TPM) using a Cambridge pad and bubbled into a solution of benzene containing a 100-m*M* α-phenyl-*N*-*tert*-butylnitrone (PBN) spin trap. The sample was degassed and analyzed by EPR spectroscopy (Chouchane et al. 2005)

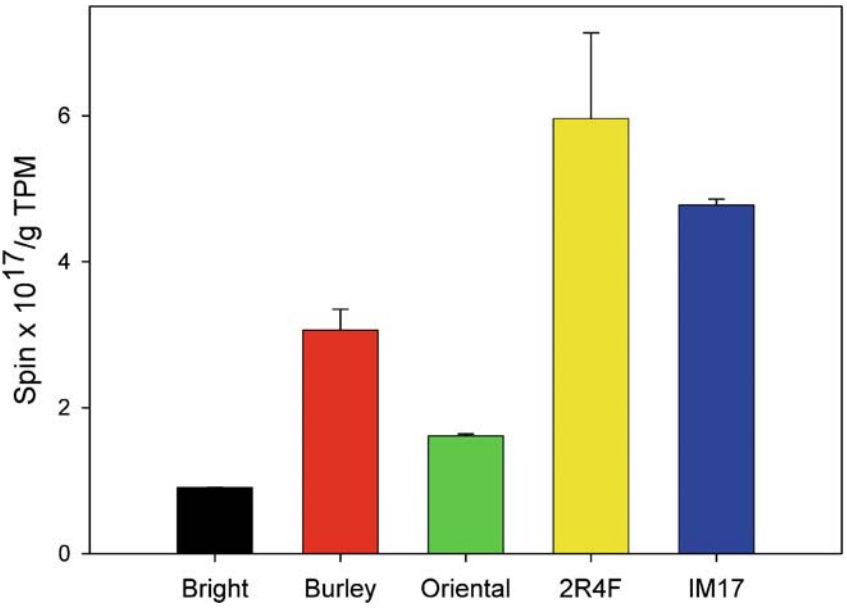

Fig. 2.16 Yield of free radicals found in the gas phase of mainstream smoke from different cigarettes (Chouchane et al. 2005)

than did their unsubstituted parent compounds. Similar results were reported for substituted phenols by Moridani et al. (2003), who attributed the difference in cytotoxicity to their higher lipophilic character and their lower redox potentials. The dihydroxybenzenes shown in Fig. 2.4 are present in the TPM of cigarette smoke, together with many other substances in a complex matrix. The EC_{50} values of the pure compounds, however, do not necessarily reflect the cytotoxicity of such a mixture. The cytotoxicity of the matrix depends on a number of factors such as the structure, concentration, and redox potentials of the individual dihydroxybenzenes. Moreover, molecular interactions between the dihydroxybenzenes (and their oxidation products) and other TPM constituents can affect the cytotoxicity of a mixture of TPM constituents.

Table 2.4 Yield of dihydroxybenzenes in the total particulate matter from 2R4F cigarettes and their specific in vitro cytotoxicity expressed as the 50% effective concentration (EC_{50}) determined by the neutral red uptake assay (Chouchane et al. 2004)

Dihydroxybenzenes	EC_{50} (m*M*)	Yield (μg/cig.)
Hydroquinone	0.021±0.004	39.3±1.26[a]
2-Methylhydroquinone	0.011±0.001	4.02±0.03[b]
2,3-Dimethylhydroquinone	0.015±0.005	1.37±0.07[b]
2,6-Dimethylhydroquinone	0.016±0.002	0.49±0.04[b]
Trimethylhydroquinone	0.026	1.83±0.03[b]
Catechol	0.33	45.3±0.52[c]
3-Methylcatechol	0.036±0.005	5.3
4-Methylcatechol	0.052±0.013	4.4
Total		102.0

EC_{50} 50% effective concentration, *μg/cig.* micrograms per cigarette
[a]Mean±SD, *n*=3
[b]Mean±SD, *n*=4
[c]Mean±SD, *n*=10

When dissolved in the cell culture medium employed in the cytotoxicity assay (e.g., Dubelcco's Modified Eagle's Medium (DMEM), we found that the dihydroxybenzenes generate significant amounts of the corresponding semiquinone radicals, as represented by their EPR spectra shown in Fig. 2.17. However, the yield of semiquinone radicals depends on the structure of each dihydroxybenzene and its redox potential. Standard one-electron reduction potentials for the redox couple $Q/Q^{-}\cdot$ at 25 °C and pH 7.0 have previously been reported for several dihydroxybenzenes found in TPM including hydroquinone, 78 mV; methylhydroquinone, 23 mV; 2,3-dimethylhydroquinone, –74 mV; 2,6-dimethylhydroquinone, –80 mV; and trimethylhydroquinone, –165 mV (Wardman 1989). The reduction potential decreases with methyl substitution. Among the most abundant dihydroxybenzenes in TPM, hydroquinone is the most potent semiquinone radical generator (Fig. 2.18), and it has a high reduction potential. The reduction potentials of the dihydroxybenzenes increase with their capacity to undergo autooxidation and generate semiquinone radicals, as shown in Fig. 2.19. An association between the cytotoxicity of quinones and their reduction potential has been previously proposed. Nemeikaite-Ceniene et al. (2002), for example, observed that the toxicity of natural hydroxyanthraquinones increases at pH 7 with an increase of their reduction potential, pointing to an oxidative stress mechanism.

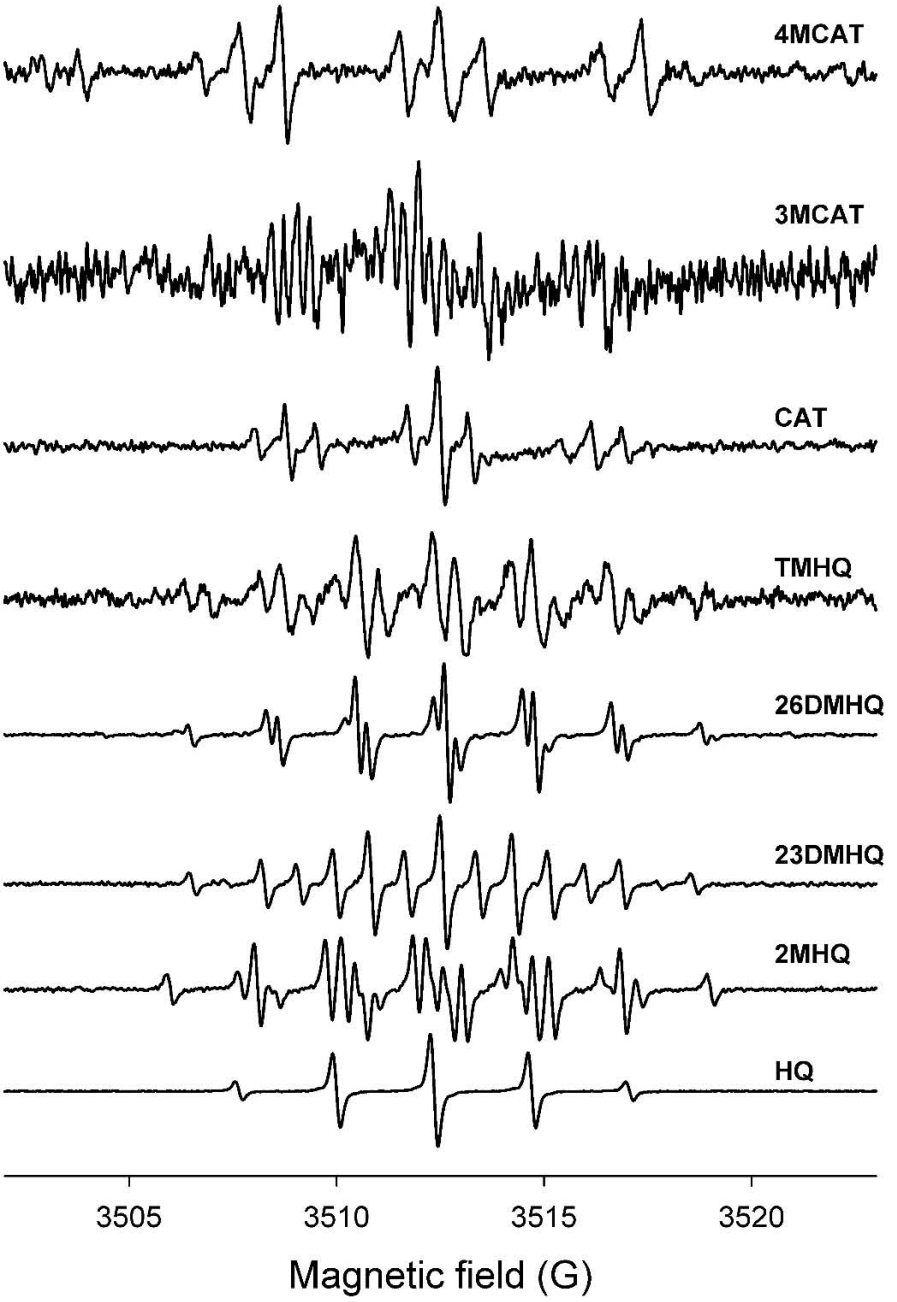

Fig. 2.17 EPR spectra of semiquinone radicals observed in 1-m*M* solutions of dihydroxybenzenes in DMEM (Chouchane et al. 2004)

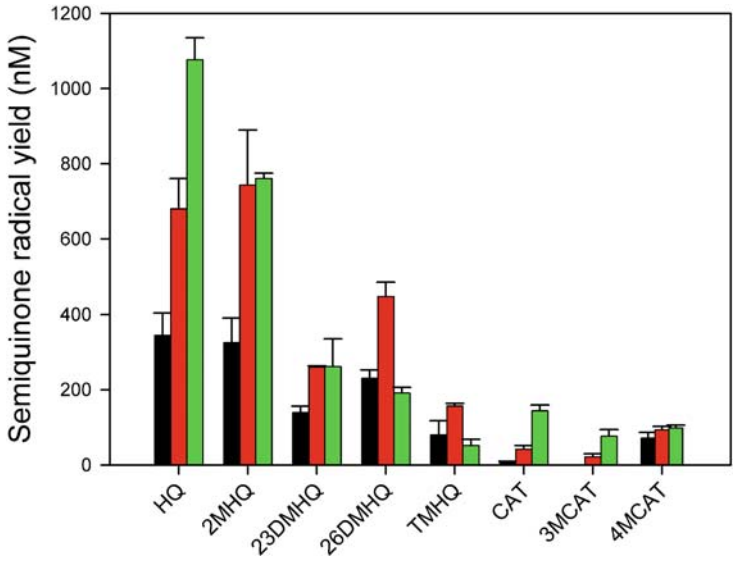

Fig. 2.18 Yield of semiquinone radicals obtained when 1-m*M* dihydroxybenzenes were dissolved in cell culture medium DMEM (Chouchane et al. 2004)

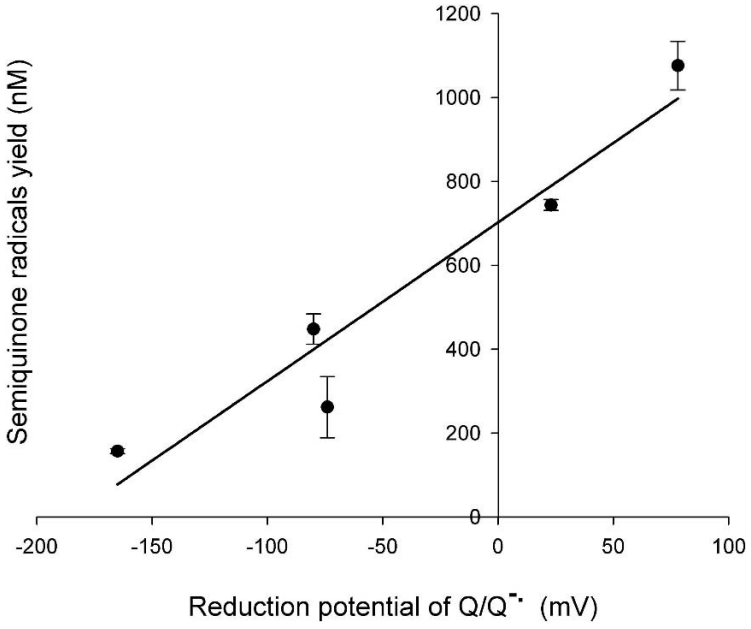

Fig. 2.19 Yield of semiquinone radicals formed in 1-m*M* solutions of dihydroxybenzenes dissolved in DMEM versus their respective reduction potentials to the semiquinone radical ($Q/Q^{-\cdot}$) (Chouchane et al. 2004)

2.4.4 ROS and RNS in Aqueous Solutions of Cigarette Smoke

The examination of the chemistry of cigarette smoke-bubbled aqueous solutions is important on two counts. First, immediately after a smoker takes a puff, smoke enters an environment of high humidity, and the smoke deposits in the epithelial lining fluid (ELF) of the respiratory tract. Second, reactive species can form in aqueous solutions of cigarette smoke that are not generated inside the burning cigarette, but rather form in solution by reaction between smoke constituents, or by reactions of smoke constituents with dissolved oxygen.

2.4.4.1 Superoxide and Hydroxyl Radicals

Several cigarette smoke constituents have been suggested to be responsible for the generation of reactive oxygen species. Among these constituents, dihydroxybenzenes are good candidates. Oxidation of hydroquinone (QH_2) by molecular oxygen in aqueous solution generates the semiquinone ($Q^{-}\cdot$) and superoxide radicals following reaction 1:

$$QH_2 + O_2 \rightarrow Q^{-}\bullet + O_2^{-}\bullet + 2H^{+} \quad (1)$$

Spontaneous disproportionation of the superoxide radical anion, or catalytic disproportionation in vivo by SOD, generates H_2O_2 (reaction 2). In the presence of transition metal ions, H_2O_2 can undergo disproportionation to generate hydroxyl radical, a powerful oxidant (reaction 3).

$$O_2^{-}\bullet + 2H^{+} \rightarrow H_2O_2 \quad (2)$$

$$H_2O_2 + Fe^{2+} \rightarrow \bullet OH + OH^{-} + Fe^{3+} \quad (3)$$

Superoxide and hydroxyl radicals have been shown to form in aqueous extracts of cigarette smoke (Pryor et al. 1998; Zang et al. 1995). It was also shown that aqueous extracts of TPM produce hydroxyl radicals that can be spin-trapped with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). The hydroxyl radicals arise from the metal mediated decomposition of H_2O_2, as shown above (Cosgrove et al. 1985; Pryor 1992).

The DMPO–superoxide radical adduct is unstable, with a half-life of 80 s at pH 6 and 35 s at pH 8. The superoxide spin adduct slowly decays to form the DMPO–hydroxyl radical adduct (Buettner and Oberley 1978; Finkelstein et al. 1979, 1980, 1982). The Pryor group observed superoxide radicals in ACT in a high-pH solution, but not hydroxyl radicals. In the same study, superoxide radicals were detected in ACT by employing a much higher concentration of DMPO (ca. 1 *M*) (Zang et al. 1995). With the development of new spin-trap molecules for reactive oxygen species, particularly superoxide radical, it is possible to overcome some of the problems encountered with the use of DMPO. 5-Diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO), for example, can trap both superoxide and hydroxyl radicals. The DEPMPO–superoxide radical adduct has a half-life of 13 min (Frejaville et al. 1995), allowing its detection by EPR within a shorter period in comparison with the DMPO adduct. As shown in

Fig. 2.20, when whole cigarette smoke is passed through a phosphate buffered solution pH 7.4 containing DEPMPO (100 m*M*), both superoxide radical and hydroxyl radical are trapped. Under the same experimental conditions using DMPO, only superoxide radicals are trapped.

If superoxide is involved in the adverse effects of cigarette smoke, a strategy aimed at the elimination of excess superoxide might minimizes these effects. For example, acute exposure to cigarette smoke is known to induce the infiltration of neutrophils into the airways in guinea pigs, a phenomenon associated with a defensive response to oxidative stress. This response is manifest by activation of the nuclear factor-kappaB (NF-κB) transcription factor and increased expression of interleukin 8 (IL-8) mRNA (Nishikawa et al. 1999). Prior treatment of the guinea pigs with SOD, to reduce the accumulation of superoxide, inhibited neutrophil accumulation and reduced both NF-κB activation and IL-8 mRNA expression. Another example was reported by (Smith et al. 2002b): Intratracheal instillation of a SOD-mimetic (AEOL 10150, a manganese porphyrin) into the airways of rats was shown to provide a marked protective effect against cigarette smoke-induced inflammation and damage.

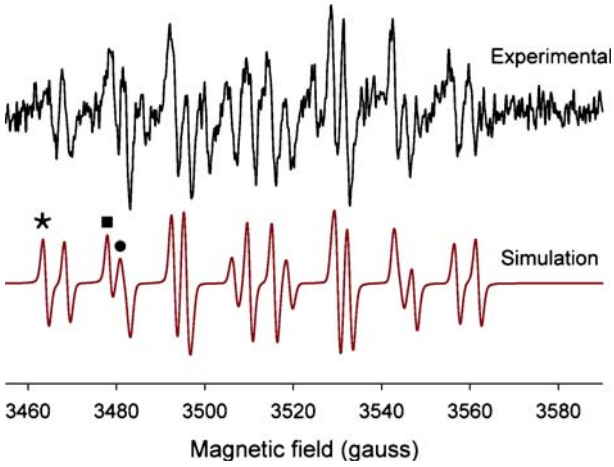

Fig. 2.20 EPR signal of spin adducts of superoxide radicals and hydroxyl radicals trapped in a phosphate buffer pH 7.4 containing 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) 100 m*M* bubbled with whole mainstream smoke. ▪ DEPMPO-superoxide radical adduct, • DEPMPO-hydroxyl radical adduct, * DEPMPO-carboxyl radical adduct

2.4.4.2 Hydrogen Peroxide

There are relatively few reports of H_2O_2 measurements in cigarette smoke under any conditions. One difficulty in analyzing for H_2O_2 is its transient nature, and the established methods of analysis are slow and laborious. Both a polarographic method developed for the analysis of H_2O_2 in TPM (Nakayama et al. 1989) and a previously reported colorimetric method developed to analyze whole smoke (Nakayama et al. 1984) require

the removal of the organic phase by chromatography or solvent extraction as a preliminary step in the analysis. Another approach to measure H_2O_2 in cigarette tar is to use an oxygen electrode, but the oxygen electrode is not specific to H_2O_2, and the addition of catalase to the analyte solutions is required to confirm that the oxygen adsorbed species is because of H_2O_2 (Stone et al. 1995).

By application of the polarographic method, Nakayama et al. (1989) found that the aqueous extracts of cigarette tar produce H_2O_2 for prolonged periods up to 24 h and longer. The dihydroxybenzenes in tar, i.e., hydroquinone, catechol, etc., provide an ample reservoir for the formation of semiquinone radicals via autooxidation. The semiquinone radicals generate superoxide radical anions in oxygenated solutions for extended periods, producing H_2O_2 by dismutation. Other pathways of H_2O_2 formation may exist, but they have not been clearly delineated as the semiquinone pathway. The primary source of H_2O_2 in cigarette tar is believed to be superoxide formed by the reduction of atmospheric oxygen by semiquinone radicals in the tar (Tanigawa et al. 1994).

In a series of experiments by Nakayama et al. (1989) on experimental and commercial cigarettes, extracts of TPM in phosphate buffer were bubbled with oxygen for 1 min and incubated in the dark. The yield of H_2O_2 after 4 h ranged from 37 to 123 μ*M* per cigarette. The study included both filtered (with and without ventilation) and nonfiltered cigarettes, and the yield of H_2O_2 varied depending on the cigarette design. In general, nonfiltered cigarettes yielded higher amounts of H_2O_2 than do filtered cigarettes. The results were not normalized to the amount of TPM delivered, but the authors showed that the overall yield of H_2O_2 in most cigarettes is proportional to the yield of TPM

Recently, a fluorometric method was developed that detects the oxidized form of Amplex Red, a fluorescent dye, in the presence of horseradish peroxidase and H_2O_2 (Yan et al. 2005). This method has several advantages over the polarographic method: (1) it only requires a 2-min incubation time and is therefore faster than are previous methods, (2) the H_2O_2 does not have to be separated from the tar matrix, (3) it can be applied to small samples, and (4) the method is readily adapted for automation. Applying their method, Yan et al. (2005) bubbled five puffs of whole smoke from 1R4F or 2R4F research cigarettes into phosphate buffered saline solution (PBS) containing the fluorescent dye, using a smoking machine. Concentrations of 3–8 micromolar H_2O_2 were found in the whole smoke bubbled samples, while there was negligible H_2O_2 formation from gas-phase smoke bubbled samples. Others have developed electrochemical means of detecting hydrogen peroxide. H_2O_2 is an electrochemically active species that will disproportionate at the surface of a metallic electrode. For example, an amperometric detection principle similar to the oxygen detection using the Clark electrode has been utilized. Hydrogen peroxide is selectively detected by an electrode after passing through a H_2O_2 permeable membrane. This method can compliment the flurometric approach with direct quantitative measurement in biological samples in the low n*M* range.

2.4.4.3 NO· and Peroxynitrite

NO· and peroxynitrites are the major reactive nitrogen species derived from cigarette smoke. The yields of NO· from 49 commercial cigarettes smoked on a smoking machine under ISO conditions were reported to be 78–487 μg per cigarette (Counts et al. 2005). As described in Sect. 2.2.1, NO· forms in the cigarette gas phase from the burning of different tobacco constituents. Im et al. (2003) showed that NO· from heated tobacco is

produced in two distinct temperature ranges, a low-temperature range (275–375 °C) in an oxygen-free atmosphere, and a high-temperature range (425–525 °C) that requires an oxygen-containing atmosphere. Nitrates were determined to be the source of NO· formation in the low-temperature ranges, and amino acids and proteins were suggested to be the sources of NO· at the higher-temperature ranges. The individual contribution of these precursors to the overall yield of NO· in cigarette smoke has yet to be determined.

Peroxynitrite forms by the reaction of NO· and superoxide radicals. In smoke-bubbled aqueous solutions, smoke constituents that can reduce molecular oxygen to superoxide, e.g., hydroquinone, continuously generate superoxide, which reacts rapidly with NO· from the gas phase to give peroxynitrite. Alkyl peroxynitrites can also form by reaction of NO· and peroxyl radicals (Halliwell and Gutteridge 1999), which are presumed to form in the gas phase of cigarette smoke (Pryor et al. 1983a, 1984, 1985). Peroxynitrite in cigarette smoke extract has been shown to react with and inactivate the α_1-proteinase inhibitor (Moreno and Pryor 1992). Peroxynitrite has also been identified as an oxidative stress-inducing constituent of aqueous cigarette smoke fractions. After depletion of intracellular GSH by electrophilic aldehydes, peroxynitrite interferes with specific target molecules, resulting in the activation of stress signal transduction and stress gene expression in cigarette smoke-treated cells in vitro (Müller and Gebel 1994, 1998; Müller et al. 1997).

2.5 Summary

Cigarette smoke has considerable potential for inducing oxidative modifications and depletion of antioxidants. In cigarette smoke-exposed aqueous solutions, ROS and RNS form and subsequently act as potent oxidants. Oxidative damage to lipids, proteins, and DNA by cigarette smoke-derived ROS and RNS has been extensively demonstrated both in vitro and in vivo. In many cases, the initially generated reactive intermediates convert cellular constituents into second-generation reactive intermediates (e.g., acrolein, 4-hydroxynonenal) capable of inducing further cytotoxic and genotoxic damage. When free radicals react with nonradicals (e.g., lipids), new radicals can form that may result in a chain reaction of free radicals. Thus, relatively short-lived free radicals may propagate their damaging effects beyond the limits set by their short half-lives and limited diffusion times. ROS and RNS activate numerous redox sensitive signaling pathways that modulate cellular responses, such as inflammation, which may itself result in the formation of endogenous oxidative species. Therefore, the oxidative damage resultant from cigarette smoke exposure is complex and likely mediated by both the oxidative potential of cigarette smoke and indirect biological responses.

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Chapter 3

Oxidative Modifications of Proteins and Lipids by Cigarette Smoke (CS). A Central Role for Unsaturated Aldehydes in CS-Mediated Airway Inflammation

3

Albert van der Vliet

Contents

3.1 Introduction

Voluntary and involuntary cigarette smoking remains a major health concern, smoking not only being the main cause of lung cancer, but also being a major causative factor in cardiovascular diseases and chronic lung inflammatory diseases such as emphysema and asthma (Eisner et al. 1998; Schwartz et al. 2000). Although tobacco smoking is a preventable habit, exposure to tobacco smoke may have more long-lasting effects, and its consequences may be manifest long after exposure. In this regard, several clinical and epidemiological studies have indicated that prenatal tobacco smoke exposure or exposures early in life may have a significant affect on lung function, susceptibility to respiratory infection, and asthma risk (DiFranza et al. 2004; Gilliland et al. 2002; Kabesch et al. 2004). Hence, despite necessary efforts to reduce the smoking habit, the more delayed health effects of involuntary smoking can be expected to be a major and growing health issue over the next few decades. Thus, despite continued success in reducing smoking habits in most Western countries, continued research is needed to understand further the mechanistic basis underlying both acute and more chronic and protracted health effects. In particular, the long-term effects of pre- or postnatal smoke exposure require additional investigation, which may lead to improved diagnosis and management of tobacco-related health effects later in life. Determining how tobacco smoke contributes to disease and what components of tobacco smoke are primarily responsible for the adverse health effects of smoking will lead to improved mechanistic understanding of tobacco-related diseases and perhaps open new avenues for therapeutic management of patients with tobacco-related disease.

Although cigarette smoke contains thousands of toxic chemicals including many carcinogens, a wealth of evidence supports the notion that a major part of the toxicity associated with cigarette smoking is related to oxidative stress, caused by reactive oxidants and radical species in tobacco smoke itself or by secondary oxidative events that are initiated by inflammatory–immune processes that are activated by smoke exposure. It is the main purpose of the present chapter to briefly overview the oxidant chemistry of tobacco smoke and to outline the major oxidative modifications in biological targets that are induced by tobacco smoke, with special focus on interactions with biological lipids and proteins. As will be clarified in the next several sections, a large body of evidence supports the notion that a major part of the "oxidative stress" that is induced by cigarette smoke is because of the presence of unsaturated aldehyde species, rather than free radicals, within cigarette smoke. In particular, α,β-unsaturated aldehydes such as acrolein and crotonaldehyde are abundant components of cigarette smoke, and possess high reactivity toward many biological targets. Because similar aldehydes are also produced during the oxidation of biological lipids, e.g., as a result of activated inflammatory–immune processes or initiated by cigarette smoke-derived radicals, this class of molecules may contribute importantly to many of the cellular effects of cigarette smoke. For this reason, the majority of this chapter will be devoted to the biochemistry and cellular effects of this class of molecules as well as cellular defenses against these agents. Finally, some recent efforts and strategies to identify critical cellular targets for these aldehydes will be discussed, as these approaches will be instrumental in future studies aimed at determining the precise role of these agents in cellular and biological effects of cigarette smoke.

3.2 Cigarette Smoking and Airway Inflammation

3.2.1 Evidence from Epidemiology and Animal Studies

In addition to being a major cause of lung cancer (which will be discussed in other chapters in this volume, and hence not further discussed here), cigarette smoking is known to cause activation of inflammatory processes within the central airways, peripheral airways, and the lung parenchyma, even in smokers with normal lung function. Moreover, tobacco smoke exposure also results in increased or altered inflammation in patients with chronic obstructive pulmonary disease (COPD) and is a well-known contributing factor in emphysema or other forms of COPD (Rahman and MacNee 1996; Saetta 1999). Similarly, cigarette smoking is also known to contribute to allergic airway diseases such as asthma, and appears to contribute to increased inflammation with neutrophilia, which is generally associated with increased disease severity (Chalmers et al. 2001; Floreani and Rennard 1999; Rahman and MacNee 1996; Saetta 1999). The ability of environmental tobacco smoke (ETS) to promote airway inflammation has been investigated in several animal studies (Witschi et al. 1997), and ETS exposure was generally found to augment inflammatory responses that were initiated by other stimuli (other environmental pollutants or pathogens), illustrating the ability of tobacco smoke to alter inflammatory–immune processes. An example of such studies is shown in Fig. 3.1, which illustrates the occurrence of airway neutrophilia in mice on exposure to lipopolysaccharide (LPS) from *Escherichia coli*, a response that was augmented when mice were simultaneously exposed to ETS. As shown, ETS exposure markedly augmented the airway neutrophilia as well as epithelial injury (as determined by protein leak, not shown), when combined with LPS challenge, although such ETS exposure alone did not cause significant neutrophilic inflammation. These findings indicate that tobacco smoke can alter ongoing inflammatory–immune processes, which may be a contributing factor in the airway inflammation and decreased lung function in subjects with asthma or COPD.

Clearly, many factors are involved in the contributing effects of tobacco smoking to chronic airway disease, and it is well appreciated that genetic factors contribute importantly to susceptibility to allergic airway diseases such as asthma (Wills-Karp and Ewart 2004) and to the incidence of COPD in smokers, because only 15% of smokers develop COPD (Saetta 1999). Hence, the contributing effects of cigarette smoking to these disorders most likely result from a combination of the various chemical effects of smoke exposure and genetic variability in inflammatory–immune regulation and/or susceptibility to such tobacco smoke-mediated effects. Despite such complexities, it has become increasingly appreciated that the presence of elevated oxidative stress is one common denominator in the relationship between smoking and lung inflammation, asthma, and COPD (Rahman and MacNee 1996). Such oxidative stress is the result of the many reactive oxidizing components within tobacco smoke components as well as the activation of inflammatory–immune processes, and the relative contribution of these events may be difficult to dissect. Furthermore, as outlined above, cigarette smoking contributes importantly to chronic inflammation, and efforts to outline the molecular mechanisms involved in such tobacco smoke-induced inflammation have pointed to various changes in cell function, including apoptotic or necrotic cell death, altered repair following injury, and alterations in pro- or anti-inflammatory cytokine production, by either epithelial cells or by resident or recruited inflammatory–immune cells. In addition, cigarette smoke exposure is known to affect phagocytic clearance of inhaled pathogens or of

apoptotic or necrotic neutrophils, and can thereby contribute to more chronic inflammation and impaired host defense. For example, it was recently demonstrated in mice that cigarette smoke exposure suppresses the ability to clear *Pseudomonas aeruginosa* infections, which results in augmented inflammation and clinical presentation (Drannik et al. 2004). As will be discussed in the following sections, many such cellular effects appear to be associated with oxidative stress, which may form the basis of the molecular mechanisms by which cigarette smoke causes such alterations.

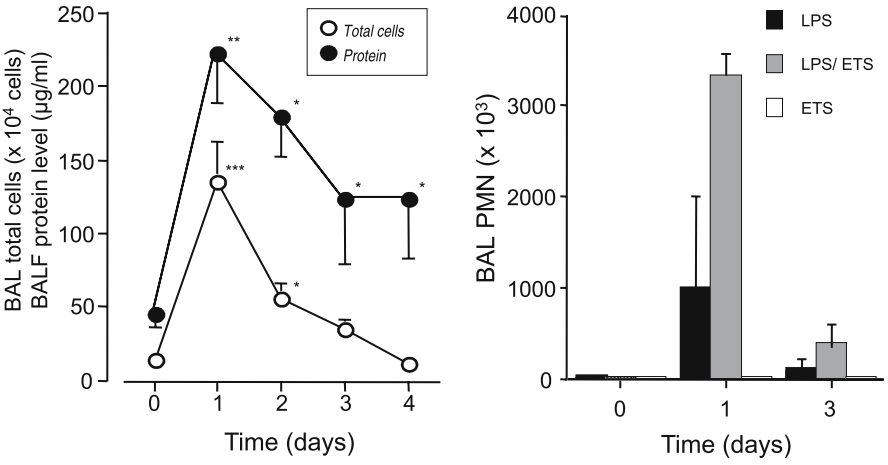

Fig. 3.1 Exposure to environmental tobacco smoke (*ETS*) promotes lipopolysaccharide (*LPS*)-induced airways neutrophilia in mice. **a** Intranasal LPS instillation induces transient airways neutrophilia. LPS (serotype 055:B5; 300 µg/kg) was instilled intranasally into C57Bl6 mice, and lungs were lavaged after 1, 2, 3, or 4 days for total cell count and protein analysis. **b** Effect of ETS on LPS induced airway inflammation. Mice were challenged with LPS as in **a**, and subsequently exposed to ETS for 6 h/day (at the Institute for Toxicology and Environmental Health at the University of California, Davis) starting 18 h after LPS challenge. ETS exposure was monitored and kept at 160 ppm CO, 10 mg/m^3 nicotine, and 50 mg/m^3 total particulate matter, and acrolein concentrations were measured using 2,4-dinitrophenylhydrazine-coated cartridges (SKC, Eighty Four, PA), and found to be 2.1 mg/m^3 (0.9 ppm). Lung lavage was performed immediately after ETS exposure on days 1 and 3 for analysis of total cell count or protein levels.

3.2.2 The Chemical Composition of Cigarette Smoke—Particulate Phase versus Gas Phase

Perhaps the main reason why our understanding of tobacco-related disease is still rather limited is the extremely complex chemical composition of cigarette smoke, which varies depending on smoking habits, the type of tobacco used, and differences between mainstream and sidestream smoke. Moreover, because many components in cigarette smoke (CS) are short-lived and undergo complex chemical reactions, the chemical composition changes substantially during aging within the ambient environment. Physically, CS can be divided into a particulate tar phase (approximately 10^{12} particles per cigarette) and a gas (or vapor) phase, which comprises all components that can pass through a glass-fiber filter that removes >99% of particles greater than 0.1 µm in diameter (Church and

Pryor 1985; see also Chapter 2). The tar phase of CS contains primarily hydrophilic and nonvolatile components, including water, alkaloids (nicotine), phenols, and traces of various aromatic hydrocarbons, nitrogenous aromatics, nitrosamines, etc. The tar phase can be collected on a filter that traps particulate matter or by low-temperature condensation to produce cigarette smoke condensate (CSC). Studies using electron spin resonance (ESR) have demonstrated that the tar phase contains several relatively stable radicals that can form an active redox system capable of reducing O_2 to generate superoxide ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2), and other reactive oxygen species (Church and Pryor 1985; Zang et al. 1995). The gas phase of CS contains high concentrations of inorganic gases (O_2, N_2, CO, NO, HCN, H_2S, NH_3) as well as various alkanes, alkenes, alcohols, and saturated and unsaturated aldehydes, as volatile products of organic combustion. Usually, the lower homologs of these different classes of products predominate in abundance. In addition, gas-phase CS represents a source of organic and oxygen free radicals, which are most likely generated by autooxidation of the high levels of nitric oxide (NO·) within CS to the much more oxidizing nitrogen dioxide ($NO_2\cdot$) (Cueto and Pryor 1994; Norman and Keith 1965). Hence, both CS tar-phase components as well as the gas phase may be an important source of oxidative stress associated with smoke exposure.

It is clear from the above that CS contains many oxidizing components, in either the gas phase or the particulate (tar) phase. Elucidating the contribution of such oxidizing components within CS to its various biological or cellular effects has been significantly complicated by the various experimental approaches that have been used to study CS-related effects. Whereas some investigators use fresh whole or filtered CS in their studies, it has been a more common practice to expose cultured cells to extracts of CS, obtained after bubbling smoke through an aqueous solution, or to CSC (see above). Such approaches have obvious practical advantages, as they allow more accurate dose-response studies and increase reproducibility. In addition, these approaches could also be rationalized as reflecting the CS components that may dissolve or condense at the respiratory tract surface (i.e., in lung lining fluids) and are capable of directly targeting airway epithelial cells. A main problem with such approaches, however, is the fact that they are poorly standardized, which has made comparisons between different studies using CS extracts or CSC difficult. In addition, and perhaps more importantly, many volatile CS components are lost during the collection of CS extracts and/or CSC, leading to underappreciation of the potential role(s) of such volatile components in the cellular effects of CS. Even though many of the carcinogenic components of CS (e.g., those causing DNA nicking and mutations) may be successfully collected in CSC, it has been appreciated for many years that volatile components within CS may be primarily responsible for CS-induced alterations in lung cell function (Leuchtenberger et al. 1974). Overall, it should be stressed that any model of exposing cultured cells or laboratory animals to CS is associated with some trade-offs (Rennard 2004). However, a growing number of studies demonstrated the important contributions of volatile constituents of CS in cellular dysfunction or oxidative stress, some of which may be successfully collected when fresh CS extracts are prepared (even though a significant fraction is most likely lost). As will be outlined in the next several sections, gas-phase CS components (such as the α,β-unsaturated aldehydes acrolein and crotonaldehyde) play an important role in the oxidative stress that is induced by cigarette smoke exposure. Hence, the remainder of this chapter will be primarily centered on the role of gas-phase constituents (with special emphasis on unsaturated aldehydes) in CS toxicity, although it is important not to trivialize the contribution of other CS constituents in the carcinogenic or mutagenic properties of CS.

3.2.3 The Oxidative Capacity of CS—Oxidation of Proteins and Lipids

The respiratory tract surface and the epithelial lining fluids represent the first biological targets of inhaled CS. Therefore, in an attempt to characterize the oxidative modifications that may occur within lung surface fluids as well as the local antioxidant defenses within such fluids, several studies have been performed in which blood plasma was exposed to CS, as a model biological fluid with comparable composition. These studies have indicated that exposure of gas-phase (filtered) CS readily depletes plasma levels of antioxidant micronutrients (primarily ascorbate) and that products of lipid peroxidation (lipid hydroperoxides, conjugated dienes) can be detected as soon as ascorbate pools are depleted (Frei 1991). Supplementation of ascorbate was furthermore capable of delaying lipid oxidation by CS, indicating the involvement of oxidizing free radicals. Although many CS-derived radicals could potentially be responsible for the oxidation of ascorbate and initiation of lipid oxidation, subsequent studies have illustrated that the major factor involved in such oxidation is the formation of $NO_2\cdot$ by autooxidation of NO·, based on similar rates of ascorbate depletion by comparable concentrations of NO·, and a critical dependence on the presence of molecular O_2 (Eiserich et al. 1997). Indeed, several studies indicated that $NO_2\cdot$ is a strong oxidant capable of initiating lipid oxidation (Eiserich et al. 1997). The associations between CS, lipid oxidation, and antioxidant (ascorbate) status have also been confirmed in measurements in smokers, which are found to contain elevated plasma levels of stable lipid oxidation products such as F_2-isoprostanes (Morrow et al. 1995) as well as reduced plasma ascorbate levels (Lykkesfeldt et al. 2000), compared with nonsmoking subjects. Moreover, supplementation with ascorbate appeared to decrease the plasma levels of F_2-isoprostanes in passive smokers (Dietrich et al. 2003).

Using similar studies with isolated blood plasma or with buffered solutions of purified proteins, exposure to CS was also found to cause oxidative modifications in proteins, as illustrated by decreases in protein reduced thiol (-SH) levels, and increased markers of protein oxidation, including protein carbonyls and the tyrosine oxidation products dityrosine and 3-nitrotyrosine (Eiserich et al. 1994; Reznick et al. 1992). The formation of tyrosine oxidation and nitration products by CS most likely involves the intermediate formation of $NO_2\cdot$ (Eiserich et al. 1994), and could be prevented by supplementation with ascorbate. In contrast, thiol depletion and protein carbonyl formation were not altered by supplementation with ascorbate or metal chelators (in an attempt to prevent radical-mediated oxidation), illustrating that these modifications are not because of radical-mediated oxidation, but rather caused by nonradical constituents in CS. As illustrated in Table 3.1, gas-phase CS contains high concentrations of reactive nonradical components, including unsaturated aldehydes such as acrolein and crotonaldehyde, that could conceivably react with nucleophilic targets such as thiols. Indeed, as will be discussed in more detail in subsequent sections, these aldehydes readily react with nucleophilic amino acid residues (including cysteine thiols) by Michael addition, resulting in protein carbonyl adducts, and comparative studies indicated that the depletion of protein-thiol and the formation of protein carbonyls by CS could be accounted for by the major reactive aldehydes within CS (Eiserich et al. 1995; Reznick et al. 1992).

Despite these rather convincing studies, some controversies still remain. First, although protein carbonyl adduct formation by gas-phase CS may be largely independent of radical chemistry and is caused by alkylation by unsaturated aldehydes (Reznick et

Table 3.1 Major Components of Gas-Phase Cigarette Smoke

Component	μmol/cigarette
Inorganic gases	
Carbon dioxide (CO_2)	1,500
Carbon monoxide (CO)	570
Nitric oxide (NO·)	12
Hydrogen cyanide (HCN)	10
Ammonia (NH_3)	6
Nitrogen dioxide (NO_2·)	1
Alkanes, alkenes, etc.	
Methane	50
Isoprene	6
Methanol	6
Acetonitrile	3
Toluene	0.9
Aldehydes	
Acetaldehyde	20
Formaldehyde	2
Acetone	6
Acrolein	0.8
Crotonaldehyde	0.2
Organic radicals	
Alkyl, alkoxyl, peroxyl	0.02

Data from Eiserich et al. (1997) and Norman (1977) and references therein

al. 1992), more recent studies have also described protein carbonyl formation by CS tar-phase components that was preventable by ascorbate (Panda et al. 2001), pointing to differences in oxidative mechanisms between gas-phase and tar-phase CS. Secondly, the relative importance of oxidizing free radicals within gas-phase CS has been questioned, based on a lack of success in detecting oxidizing radicals within fresh CS using ESR (Maranzana and Mehlhorn 1998). Furthermore, studies with erythrocytes demonstrated that exposure to gas-phase CS depleted cell glutahione (GSH) levels more rapidly than ascorbate, and that depletion of GSH diminished the ability of erythrocytes to recycle oxidized ascorbate (Maranzana and Mehlhorn 1998). Thus, direct thiol modifications by CS may be kinetically favored and therefore more important than are free radical-mediated oxidations. This notion is supported by studies towards oxidation of plasma constituents: Whereas CS-mediated oxidation of ascorbate or tyrosine occurred rather slowly over several minutes, as they required NO_2· formation by autooxidative reactions (Cueto and Pryor 1994; Eiserich et al. 1997; Eiserich et al. 1994), thiol depletion by unsaturated aldehydes in CS occurs much more rapidly (Eiserich et al. 1995; Esterbauer et al. 1991).

The chemical modifications of GSH by gas-phase CS were recently investigated using high-performance liquid chromatography (HPLC) and mass spectrometry (MS) approaches, which confirmed that (radical-mediated) oxidation to oxidized GSH (GSSG) occurred to a relatively minor extent, whereas the majority of GSH depletion could be accounted for by alkylation by the major CS-derived unsaturated aldehydes, acrolein and crotonaldehyde (Reddy et al. 2002). In fact, about 50% of the GSH that was depleted by CS could be accounted for its addition to one single aldehyde, acrolein (Reddy et al. 2002). Studies in which various isolated cells were exposed to CS are consistent with this: CS-mediated depletion of GSH occurs largely by alkylation and not by oxidation to GGSG or mixed disulfides (Rahman and MacNee 1996; Reddy et al. 2002), and formation of protein carbonyls is largely the result of Michael addition of aldehydes (Nguyen et al. 2001). Increased carbonyl levels have also been detected in various circulating proteins in smokers as compared with nonsmokers (Lee et al. 1998; Pignatelli et al. 2001), possibly as a result of similar Michael addition. However, markers of free radical-mediated protein oxidation, such as 3-nitrotyrosine, were also found to be increased in smokers (Petruzzelli et al. 1997), illustrating that radical-mediated processes also contribute to increased protein oxidation in smokers. Determination of chemical mechanisms involved in such protein modifications in vivo is more difficult, because they may have been due to indirect oxidative events caused by increased activation of inflammatory processes, rather than direct CS-mediated oxidation. Similarly, the increased presence of lipid oxidation products in smokers (Dietrich et al. 2003; Morrow et al. 1995) may be because of both direct CS-mediated effects and by oxidative processes because of activation of inflammatory/immune cells (Zhang et al. 2002a, b). Conversely, the process of lipid oxidation results in the formation of a range of α,β-unsaturated aldehydes that can alkylate thiols and modify proteins in a manner similar to CS-derived aldehydes. Figure 3.2 illustrates the structural similarities between acrolein and crotonaldehyde (major α,β-unsaturated aldehydes in CS) and malondialdehyde and 4-hydroxy-nonenal (major products of lipid peroxidation). Importantly, acrolein and crotonaldehyde have recently been identified as additional products of lipid oxidation (Uchida 1999).

In summary, alkylations of biological thiols (GSH, protein cysteine residues) by aldehydes appear to be major "oxidative" effects of gas-phase CS. Similarly, as will be discussed in the next paragraph, cellular effects of CS appear to be related to alterations in cellular redox status because of the action of CS-derived unsaturated aldehydes rather than free radicals, most likely because these aldehydes are readily diffusible and capable of entering cells and reacting with many cell targets. Therefore, the biological effects of CS are expected to be because of modifications of critical cell components by unsaturated aldehydes, and by alterations in cellular defenses against these aldehydes, which will be the subject of subsequent sections.

3.3 Cellular Effects of CS

3.3.1 CS and Cellular Redox Changes—Seminal Studies

In seminal observations over 30 years ago, Green (Green 1968) and Leuchtenberger et al. (Leuchtenberger et al. 1974) discovered that adverse effects of CS on alveolar macrophage function or on lung cell alterations could be prevented by reducing agents such as GSH or cysteine, and that volatile CS constituents were primarily responsible for such

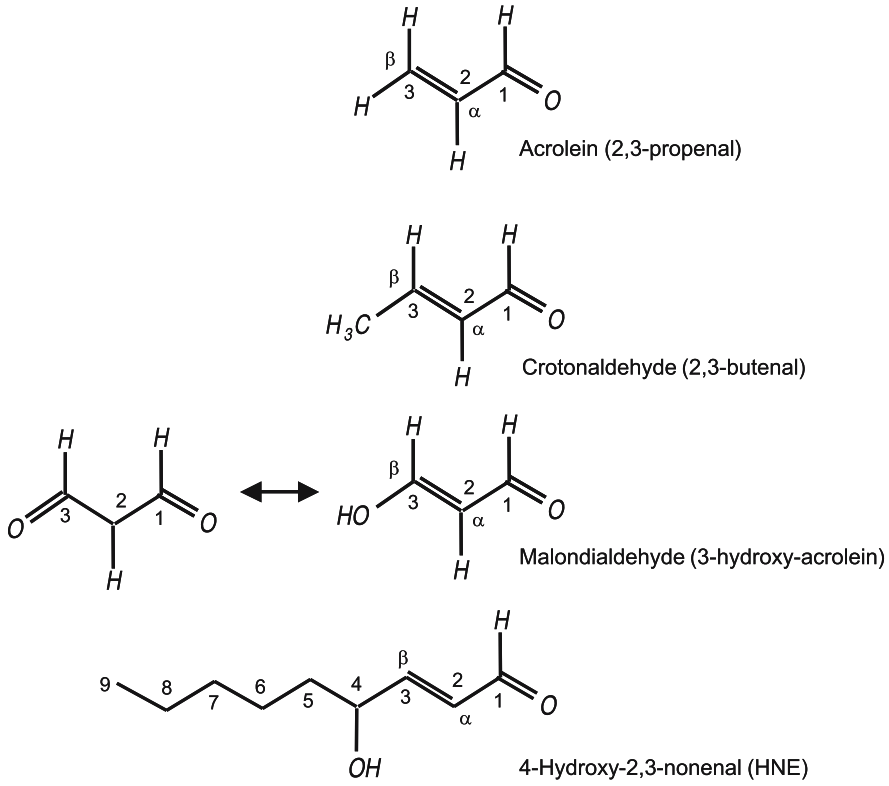

Fig. 3.2 Structures of α,β-unsaturated aldehydes that are present in cigarette smoke (CS) or are formed during lipid peroxidation

effects. In subsequent years, many studies have reported that CS is capable of inducing growth arrest or apoptotic or necrotic cell death (Carnevali et al. 2003; Hoshino et al. 2001; Kim et al. 2004; Rahman and MacNee 1999; Tsuji et al. 2004; Wickenden et al. 2003), or impairing epithelial or phagocytic cell function (Kirkham et al. 2004; Martey et al. 2004; Mio et al. 1997; Nguyen et al. 2001; Wang et al. 2001). In most of these cases, CS effects were related to depletion of cellular GSH and could be countered by reducing agents such as GSH or *N*-acetylcysteine, leading to the general conclusion that oxidative stress generated by CS exposure is responsible for these effects. Several more recent studies have addressed the ability of CS to alter gene expression, in cultured airway epithelial cells or in lung biopsies from current or ex-smokers, and such studies have revealed reversible upregulation of genes that are involved in oxidative stress, GSH metabolism, xenobiotic metabolism, as well as more persistent changes in several putative oncogenes or tumor suppressor genes (Hackett et al. 2003; Spira et al. 2004; Yoneda et al. 2001). Changes in drug metabolizing and antioxidant genes largely involved genes involved in GSH metabolism (GSH peroxidases, glutamate–cysteine ligase, GSSG reductase, GSH *S*-transferases), redox balance (alcohol dehydrogenases, aldo-keto reductases, thioredoxin-related genes) or pentose phosphate cycle, suggesting that many of the cellular ef-

fects may be related to alterations in cellular redox status or changes in GSH-dependent drug metabolism (Hackett et al. 2003).

3.3.2 Effects on Cell Signaling Pathways and Gene Expression—a Role for α,β-Unsaturated Aldehydes

As discussed in the previous section, the major thiol reactive agents within gas-phase CS are α,β-unsaturated aldehydes, of which acrolein and crotonaldehyde are the main members. Hence, these aldehydes can be expected to contribute greatly to the cellular effects of CS, either by altering cellular redox balance by depleting GSH pools or by direct reaction with redox-sensitive targets in proteins or DNA. Indeed, an increasing number of studies have addressed the cellular effects of such aldehydes (as well as similar aldehydes that are generated during lipid oxidation, such as 4-hydroxynonenal) (Esterbauer et al. 1991; Kehrer and Biswal 2000; Leonarduzzi et al. 2000; Li and Holian 1998), and several such studies have documented that these aldehydes can largely mimic the various cellular effects of CS (Hoshino et al. 2001; Mio et al. 1997; Nguyen et al. 2001; Wang et al. 2001). CSCs or extracts have been found to be capable of activating stress responses in cells, resulting in the activation of extracellular regulated kinase-1/2 (ERK) or p38 mitogen-activated protein kinase (MAPK), and of the transcription factor nuclear factor-kappaB (NF-κB), and these responses are associated with induction of stress response genes (*fos*, heme oxygenase) and inflammatory genes such as interleukin (IL)-8, cyclooxygenase-2 or matrix metalloproteinase-1 (Anto et al. 2002; Martey et al. 2004; Mercer et al. 2004; Muller and Gebel 1998). Again, these effects were found to be largely related to GSH depletion and could in part be attributed to CS-derived aldehydes (Finkelstein et al. 2001; Kehrer and Biswal 2000; Mio et al. 1997; Muller and Gebel 1998). In addition to depleting cellular GSH, acrolein readily inactivates thioredoxin and thioredoxin reductase (Park et al. 2005; Yang et al. 2004), thus potentially affecting cell signaling pathways that are controlled by this redox system. We have demonstrated that CS and acrolein are able to induce tyrosine phosphorylation and p38 MAPK activation in neutrophils, and that p38 MAPK activation is related to functional outcomes such as IL-8 production (Finkelstein et al. 2001). It is important to note that acrolein does not always fully replicate the effects of CS, consistent with some studies that suggest that CS aldehydes may synergize with other CS components (including oxidants) to induce their overall effects (Muller and Gebel 1998). Despite rather consistent reports on the ability of CS to activate MAP kinases, the effects on NF-κB are more controversial, because some studies also demonstrated inhibition of (cytokine-mediated) NF-κB activation by CS (Gebel and Muller 2001; Moodie et al. 2004). These apparent discrepancies may be because of variations between CS preparations used, which may have resulted in variable outcomes because of opposing effects of different CS components. Indeed, whereas certain CS components within CS extracts may activate NF-κB, the volatile component acrolein was found to inhibit NF-κB activation, either by upregulation of the inhibitor IκB or by direct chemical modifications of redox-sensitive proteins involved in NF-κB activation (Kehrer and Biswal 2000; Valacchi et al. 2005) (see next paragraph).

Studies with cultured cells or lung biopsies from smokers have shown that CS exposure induces many changes in gene expression, largely involving those encoding oxidative stress-responsive and phase II drug-metabolizing enzymes (Gebel et al. 2004; Hackett et al. 2003; Spira et al. 2004), which are all (at least in part) transcriptionally

regulated by the nuclear factor Nrf2 (NF-E2-related factor) (Gebel et al. 2004). Indeed, acrolein and other unsaturated aldehydes are known activators of Nrf2, presumably by direct chemical modification of the Kelch-like ECH-associating protein 1 (Keap1) sensor, which represses Nrf2 activity (Itoh et al. 2004; Kwak et al. 2003; Levonen et al. 2004; Tirumalai et al. 2002; Wakabayashi et al. 2004). Recently, genetic ablation of Nrf2 in mice was found to enhance susceptibility to cigarette smoke-induced emphysema (Rangasamy et al. 2004), consistent with an important role for oxidative stress, and perhaps aldehyde stress, in the development of this CS-related disorder. One specific consequence of CS exposure to airway epithelial cells is the overproduction of mucin through activation of epidermal growth factor receptors (EGFR) (Shao et al. 2004; Takeyama et al. 2001), and this may contribute to the prominent feature of goblet cell hyperplasia and mucus hypersecretion that is seen in COPD. More-recent studies have suggested that CS-derived aldehydes may be involved in this response, as acrolein has been shown to be able to activate EGFR and to induce expression of mucin genes (Borchers et al. 1999; Deshmukh et al. 2005; Takeuchi et al. 2001). Moreover, such acrolein-induced effects appear to occur in the absence of major cellular redox changes, and appear to be mediated largely by cell surface events such as the activation of cellular metalloproteinases (Deshmukh et al. 2005; Shao et al. 2004). Collectively, these various observations clearly point to an important role for unsaturated aldehydes in overall cellular effects by CS that are thought to contribute to the etiology of CS-related diseases. Therefore, the remainder of this chapter will focus specifically on the biochemistry of these aldehydes.

3.4 Unsaturated Aldehydes and Mediators of CS Effects

3.4.1 Biological Sources—Environmental Pollutants or Products of Lipid Oxidation?

Although the previous section strongly supports a contributing role for unsaturated aldehydes in many of the cellular effects of CS, it is difficult to elucidate their role in vivo without better knowledge regarding the actual doses or concentrations of these aldehydes that reach target cells. Indeed, studies in vitro have shown a wide range of cellular effects of these aldehydes, depending on concentrations that were used, but it is unclear which experimental conditions most accurately resemble in vivo conditions of aldehyde exposure or production. For instance, whereas concentrations of acrolein in mainstream or environmental tobacco smoke can be measured relatively easily, it is much more difficult to determine concentrations that will be encountered within the airways of smokers or of subjects exposed to environmental tobacco smoke. Such determinations are compromised by complications in sampling of specific lung areas, and the reactivity of acrolein with various biological targets, that would result in dramatically underestimating the overall acrolein burden in the lung when based on measurements of unreacted acrolein. Some recent studies documented concentrations of major CS aldehydes (acetaldehyde, acrolein, etc.) in saliva specimens or in exhaled breath condensates (EBC) from nonsmokers and smokers, using chemical derivatization and capillary electrophoresis and/or LC-MS procedures (Andreoli et al. 2003; Annovazzi et al. 2004). These recent findings suggest that acrolein, the main reactive unsaturated aldehyde, can be found in micromolar concentrations in saliva and nanomolar levels in EBC (which, based on dilution associated with EBC collection, corresponds to micromolar levels in

lung surface fluids), and were typically several-fold higher in smokers (Andreoli et al. 2003; Annovazzi et al. 2004). As will be discussed in more detail in the next section, such concentrations of these aldehydes can profoundly affect cell function. More intriguingly, increases in acrolein within EBC of smokers were associated with similar increases in 4-hydroxynonenal and malondialdehyde, well-known end products of lipid oxidation (see Fig. 3.2). Because acrolein and crotonaldehyde are not only major components of CS, but can also be produced during oxidation of various lipids (Uchida 1999), these measurements in EBC suggest that the increased acrolein in smokers may originate from increased lipid peroxidation rather than reflect inhaled acrolein from CS itself. Indeed, recent analysis of various lipid oxidation products in exhaled breath condensates and induced sputum from subjects with COPD or asthma revealed comparable increases in lipid oxidation products including acrolein (Corradi et al. 2004). Because the majority of subjects in this latter study were nonsmokers or ex-smokers, the increased acrolein most likely originated from lipid oxidation rather than inhalation of CS.

The above considerations point to another level of complexity with regard to CS-related disease. First, it is apparent that the biological effects of CS may be mediated not only by the abundant reactive aldehydes within CS, but also by the same aldehydes that are generated on lipid peroxidation, because of the activation of inflammatory–immune processes. Moreover, oxidation of unsaturated fatty acids results in the formation of a variety of similar unsaturated aldehydes, of which 4-hydroxynonenal has received the most attention as a major lipid oxidation product with potential functions as a second messenger molecule (Petersen and Doorn 2004). The similarities in reactivity between the various α,β-unsaturated aldehydes suggest that their cellular effects may in fact represent more general responses to a larger family of aldehydes, although their cellular effects may differ and be more specific because of differences in carbon chain length and other functional groups, that may contribute to specificity in target interactions. For reasons of simplicity, and because it represents the major and most reactive α,β-unsaturated aldehyde in CS, the following paragraphs will deal primarily with acrolein, although many aspects may also apply to other α,β-unsaturated aldehyde family members.

3.4.2 Cellular Defenses against α,β-Unsaturated Aldehydes

As discussed in the previous sections, α,β-unsaturated aldehydes are among the main components within CS that may be responsible for its effects on cellular function and are also produced during lipid oxidation, a process that is commonly observed in smokers and may involve reactive components of CS itself, as well as the activation of oxidative pathways as a result of inflammatory–immune processes. For these reasons, many studies have addressed the ability of members of this aldehyde family to activate cell signaling pathways, alter gene expression, and/or cell function (Finkelstein et al. 2001; Kehrer and Biswal 2000; Li and Holian 1998; Petersen and Doorn 2004). The α,β-unsaturated aldehydes comprise a family of compounds that are reactive towards many classes of biomolecules, and both the aldehyde group as well as the unsaturated bond, which gives these compounds their strong electrophilic character, are involved in their reactivity. Hence, various metabolic pathways exist to minimize cell exposure to such aldehydes, and/or their unwanted reaction with critical cell targets (Petersen and Doorn 2004). Figure 3.3 illustrates the several metabolic pathways involved in detoxification of α,β-unsaturated aldehydes such as acrolein. A first class of metabolic enzymes that detoxify alde-

hydes are the aldehyde dehydrogenases (ALDH), a large family of enzymes that catalyze the pyridine nucleotide-dependent oxidation of aldehydes to the corresponding acids (Sladek 2003). Conversely, aldehydes can also be detoxified by enzymatic reduction of the aldehyde to the corresponding alcohol, by members of the aldo-keto reductase family (AKR), aldose reductase (AR) and/or alcohol dehydrogenase (ADH) (Niknahad et al. 2003; Sanli et al. 2003; Srivastava et al. 1999). Intriguingly, whereas short-chain aldehydes such as acrolein are relatively poor substrates for reduction by AR, its adduct with GSH, formed by spontaneous or glutathione *S*-transferase (GST) catalyzed conjugation with GSH (see below), is reduced with much higher efficiency by AR (Srivastava et al. 1999), and induction of AR activity has been found to enhance cellular resistance to acrolein (Keightley et al. 2004).

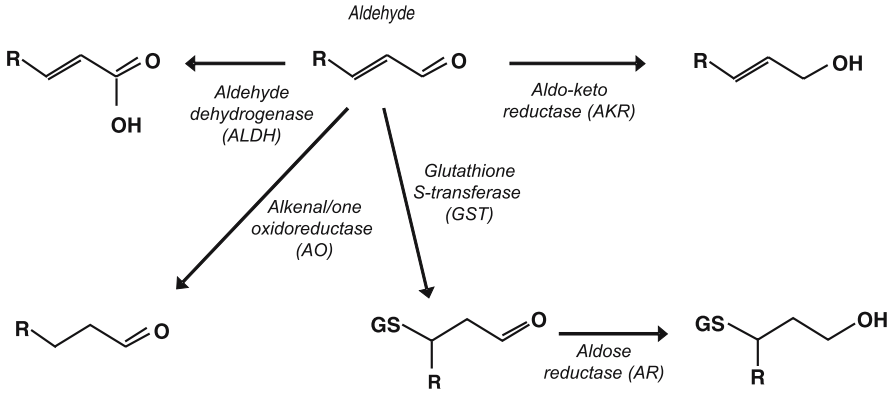

Fig. 3.3 Schematic representation of metabolic pathways in cellular detoxification of α,β-unsaturated aldehydes

Because the unsaturated bond is considered the most reactive moiety within α,β-unsaturated aldehydes, the main detoxification route presumably involves their reduction or conjugation with GSH, the latter catalyzed by a family of GSH *S*-transferases (Berhane et al. 1994; Hubatsch et al. 1998; Pal et al. 2000). Whereas GST P1-1 is most effective against propenals, including acrolein, GST A1-1 and GST M1-1 are more active against hydroxyalkenals, which are produced during lipid peroxidation (Berhane et al. 1994; Pal et al. 2000). GST A4-4, a member of the alpha class GST family, was found to have particularly high activity against 4-hydroxynonenal and other similar lipid oxidation products (Hubatsch et al. 1998). Inhibition of GST activity increases acrolein toxicity, whereas GST induction can protect cells from acrolein toxicity, illustrating the significance of GST-mediated aldehyde detoxification (Cao et al. 2003; He et al. 1998). Recently, an additional enzyme was identified in the detoxification of α,β-unsaturated aldehydes, known as NAD(P)H-dependent alkenal/one oxidoreductase (Dick and Kensler 2004; Dick et al. 2001). This enzyme was initially discovered as a dithiolthione-inducible gene (*DIG-1*), a homolog of an inactivator of leukotriene B_4, and a recently identified enzyme in pig lung that reduces the 13,14 double bond of 15-oxoprostaglandins was found to be virtually identical (Dick et al. 2001; Ensor et al. 1998). Kinetic analysis revealed that this alkenal/one oxidoreductase (AO) may contribute importantly to the detoxification of α,β-unsaturated aldehydes such as acrolein and hydroxynonenal, and that over-

expression of AO reduces hydroxynonenal-induced cell toxicity and Michael addition to proteins (Dick et al. 2001).

Although GSTs appear to be most important in detoxifying unsaturated aldehydes, the significance of aldehyde reduction by AR (Keightley et al. 2004) or elimination of their GSH adducts by multidrug resistance protein (Renes et al. 2000), indicates that addition of GSH to these aldehydes does not fully eliminate their toxicity (Ramu et al. 1996). The importance of GSTs in tobacco-related diseases has been illustrated by several reports of increased disease incidence or decreased lung function associated with genetic GST variants (He et al. 2004). Several GST polymorphisms have been identified, including a *GST M1* null allele, which results in a complete lack of GST M1 activity. A similar null allele also exists for *GST T1*. Several studies have indicated that *GST M1*-null or *GST T1*-null children have increased risk for asthma in relation to environmental tobacco smoke or maternal smoking (Gilliland et al. 2002; Kabesch et al. 2004). Moreover, the *GST M1*-null genotype has also been associated with emphysema (Harrison et al. 1997). Finally, a polymorphism in *GST P1* leads to a 105Ile/Val substitution, which affects catalytic activity against substrates including acrolein (Pal et al. 2000), and has been associated with chronic obstructive pulmonary disease in a Japanese population (Ishii et al. 1999). Collectively, these associations with genetic GST variants may further underscore the potential contribution of unsaturated aldehydes to CS-related disorders.

3.4.3 Effects on Inflammatory–Immune Regulation—Recent Studies

Although many CS constituents are capable of reaching the circulation and thereby contribute to more systemic effects, inhaled (environmental) tobacco smoke will primarily interact with cells at the respiratory surface, which include largely airway and alveolar epithelial cells, as well as alveolar macrophages and extravasated inflammatory–immune cells such as neutrophils, during active episodes of acute or chronic inflammation. Based on geometric considerations, the airway epithelium is the major target for inhaled pollutants such as CS, as it covers the entire surface area of the lung, which in adults resembles the size of a tennis court. Recent studies have clearly established that the airway epithelium may be a main effector in mounting inflammatory responses to inhaled pathogens or allergens (Poynter et al. 2003; Sadikot et al. 2003). Similarly, the epithelium is also thought to be involved in the proinflammatory effects of CS exposure, as reflected by increased neutrophilia and enhanced production of the major neutrophil chemoattractant IL-8, a member of the CXC chemokine family (Chalmers et al. 2001; Mio et al. 1997). Indeed, exposure of cultured respiratory tract epithelial cells to CS extracts was found to cause increased production of IL-8, and increased IL-8 production was also observed on epithelial cell exposure to major aldehyde components in CS, acetaldehyde and acrolein (Mio et al. 1997). Epithelial IL-8 expression is regulated by both transcriptional and posttranscriptional mechanisms (Li et al. 2002), involving the activation of MAP kinases and the transcription factor NF-κB, a main regulator of epithelial cytokine and chemokine expression (Poynter et al. 2003). Recent studies have, however, also shown that α,β-unsaturated aldehydes are capable of inhibiting NF-κB activation, and we recently demonstrated that acrolein at low-micromolar concentrations can inhibit epithelial IL-8 expression by inhibiting NF-κB activation (Valacchi et al. 2005). Similarly, acrolein was also found to inhibit NF-κB activation in macrophages (Li et al. 1999). In

this regard, the action of acrolein on NF-κB signaling is analogous to those of structurally analogous α,β-unsaturated carbonyl compounds, such as 4-hydroxynonenal or cyclopentenone prostaglandins (Ji et al. 2001b; Valacchi et al. 2005).

Although the involvement of unsaturated aldehydes on CS-induced proinflammatory signaling is not straightforward, several lines of evidence suggest that they may represent major causative factors in inducing epithelial oxidative injury and apoptotic or necrotic cell death (Hoshino et al. 2001; Rahman and MacNee 1999; Wickenden et al. 2003). Investigations of acrolein-mediated cell death in cultured bronchial epithelial cells revealed the activation of a process reminiscent of apoptosis, as illustrated by DNA fragmentation and phosphatidylserine externalization (Nardini et al. 2002), although this mode of cell death was not accompanied by caspase activation (not shown). Moreover, whereas cell death by acrolein was closely associated with transient GSH depletion, cell supplementation with either ascorbate or α-tocopherol appeared to prevent acrolein-induced cell death and promote restoration of cellular GSH pools, even though initial GSH depletion could not be prevented (Nardini et al. 2002). Accordingly, increases in cellular oxidant production could be observed following acrolein exposure, illustrating secondary oxidative stress that may be responsible for epithelial cell death. Despite the absence of NF-κB activation and proinflammatory cytokine production under these conditions (Valacchi et al. 2005), such acrolein-induced cell death could conceivably release "danger" signals that can activate antigen-presenting cells and thereby contribute to the initiation of immune responses (Gallucci et al. 1999; Shi et al. 2003). Whether acrolein indeed contributes to increase risk of allergic airway diseases, as has been demonstrated in relation to pre- or postnatal CS exposure, still remains to be established.

From the above, it is clear that the proinflammatory effects of tobacco smoke cannot be easily explained by individual activities of bioactive components on individual cell types, as they most likely involve a combination of effects of its many diverse components on various lung cell types. The studies illustrated in Fig. 3.1 indicate that ETS exposure augments neutrophilia induced by other stimuli, even though it did not induce significant inflammation by itself. Such stimulatory effects on preexisting inflammation suggest that inhaled tobacco smoke may act in part by altering the biological properties of extravasated inflammatory cells such as neutrophils. We and others have studied the effects of CS and/or acrolein on various critical neutrophil activities, such as respiratory burst activation, cytokine production, and apoptotic cell death, the latter being a critical factor in the resolution of inflammation (Fadok et al. 1998; Savill 1997). Initial studies of neutrophil respiratory burst activation indicated that CS (and acrolein) dramatically inhibit this important neutrophil activity in host defense (Nguyen et al. 2001), which suggest a causative mechanism by which CS exposure may diminish host defense and contribute to enhanced respiratory childhood infections (DiFranza et al. 2004; Li and Holian 1998). Also, a decreased ability to clear respiratory infection will contribute to increased and more chronic proinflammatory conditions. Further studies in our laboratory have indicated that neutrophil exposure to acrolein results in increased production of IL-8, by a mechanism involving p38 MAPK activation (Finkelstein et al. 2001). Thus, the observed increases in respiratory tract levels of IL-8 in smokers (Chalmers et al. 2001; Mio et al. 1997) may originate (at least in part) from increased production by extravasated neutrophils.

Although much emphasis has been given to cellular mechanisms by which CS may affect processes that result in the activation of inflammation and inflammatory cell recruitment, it is increasingly recognized that the process of inflammation is also critically regulated by mechanisms that control inflammatory cell clearance in order to terminate

inflammatory processes. The overall state of inflammation can, in fact, be envisioned as a balance between activating mechanisms that recruit inflammatory cells and processes that eliminate inflammatory cells. Apoptotic cell death, regulated by constitutively expressed pathways and by pro- and anti-inflammatory cytokines, is a critical step in terminating the inflammatory response and removing inflammatory cells from the inflamed site. The molecular events that are involved in neutrophil apoptosis and clearance mechanisms of apoptotic cells have been actively studied in recent years and have been comprehensively reviewed (Haslett 1999; Lauber et al. 2004; Maderna and Godson 2003). Many studies have attempted to address the involvement of CS components such as aldehydes in apoptotic or necrotic cell death in various cell types (Haynes et al. 2001; Ji et al. 2001a; Li et al. 1997; Nardini et al. 2002), and concluded that acrolein and other unsaturated aldehydes induce apoptotic cell death. Studies with freshly isolated neutrophils, however, showed the surprising finding that exposure to micromolar concentrations of acrolein actually blocks the constitutive apoptotic pathway in neutrophils and appears to extend their lifespan (Finkelstein et al. 2001). Further mechanistic studies indicated that these effects are closely associated with dramatic changes in cellular GSH status, which were found to correspond with decreased activation of caspases-3, -8, and -9, critical mediators of both intrinsic and extrinsic apoptotic pathways (Finkelstein et al. 2005). Preliminary studies indicate that such inhibition of neutrophil apoptosis also results in reduced phagocytosis by monocyte-derived macrophages (Finkelstein and van der Vliet, unpublished observations). The significance of such inhibitory effects on neutrophil apoptosis for overall CS-related inflammation is still unclear, but may be profound based on recent studies indicating anti-inflammatory signals that are produced as a result of phagocytic clearance of apoptotic cells (Huynh et al. 2002). Conversely, failure to clear apoptotic or necrotic neutrophils can be expected to result in increased deposition of toxic granule proteins that are released from disintegrating cells, which would contribute to proinflammatory conditions (Haslett 1999). In addition to altering neutrophil apoptosis and potentially interfering with their removal, CS and acrolein have also been demonstrated to decrease the phagocytic capacity of alveolar macrophages (Green 1968; Kirkham et al. 2004), resulting not only in reduced antimicrobial activity, but also in diminished clearance of apoptotic neutrophils, which would collectively promote inflammation.

In summary, it is evident from the previous sections that there are a number of mechanisms by which CS exposure can contribute to chronic airway inflammation and to increasingly common diseases such as asthma and COPD. Furthermore, many of these proinflammatory events can be accounted for by acrolein (and other similarly reactive aldehydes in CS), which affects cell function either by direct modification of critical cell constituents, or indirectly by alteration of the cellular redox status and increasing cellular oxidative stress. Figure 3.4 illustrates the several major mechanisms by which acrolein can promote inflammatory–immune processes in the lung.

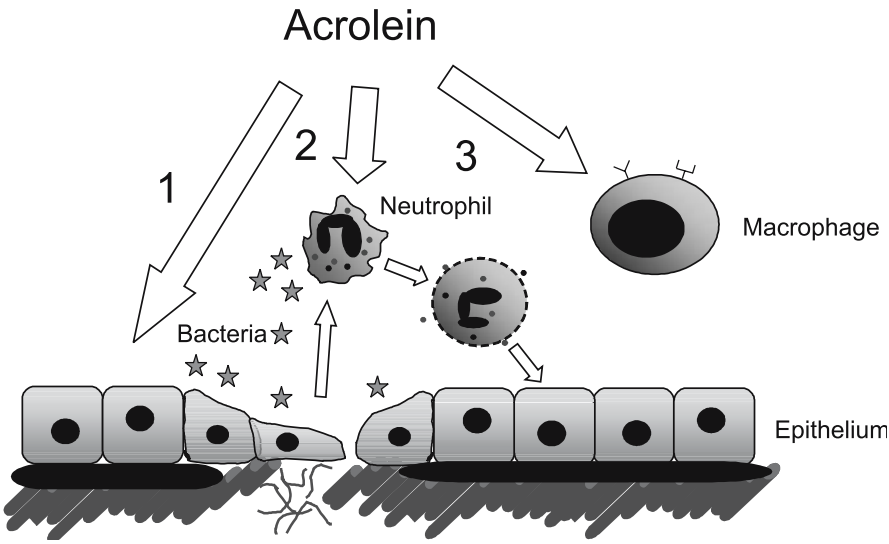

Fig. 3.4 Schematic illustration of the several mechanisms by which acrolein can contribute to airway inflammation. 1. The action of acrolein on airway epithelial cells may cause cell activation and/or injury (Nardini et al. 2002). Such injury, and diminished capacity for repair (Wang et al. 2001), will promote influx of inflammatory cells such as neutrophils. 2. Acrolein can affect neutrophil function by inhibiting oxidative bacterial killing (Nguyen et al. 2001), increasing proinflammatory cytokine production, and interfering with neutrophil apoptosis and promoting necrotic cell death (Finkelstein et al. 2001). Released products from necrotic cells can further promote inflammation by activating or injuring airway epithelial cells. 3. Interaction of acrolein with alveolar macrophages or extracellular proteins results in alterations in macrophage cytokine production (Li and Holian 1998) and in decreased phagocytic activity (Kirkham et al. 2004), further diminishing neutrophil clearance and antimicrobial activity, leading to increased infection and inflammation

3.5 Future Perspectives

3.5.1 Strategies to Detect Biological Targets for Unsaturated Aldehydes

It is evident from the previous paragraphs that acrolein may play a major role in the proinflammatory activities of cigarette smoking and/or exposure to environmental tobacco smoke, and that a variety of cellular and chemical mechanisms may be involved in this. The mechanisms by which acrolein could alter cell function involve either direct interaction with critical cell targets, or indirectly by disturbing cellular homeostasis and redox status (Kehrer and Biswal 2000). Delineating the specific mechanisms by which acrolein affects cell function and what are the specific cellular targets for acrolein (or structurally related aldehydes) has been the subject of considerable recent research effort. In the following, some recent strategies that have been used in these endeavors will be discussed, as well as some limitations and remaining challenges.

In order to clarify further mechanisms by which unsaturated aldehydes affect the cell biology of, e.g., airway epithelial cells, alveolar macrophages, or neutrophils, it is imperative to identify the major cellular targets for these aldehydes and to link modifications of

such targets to functional alterations. Based on the known reactivity of α,β-unsaturated aldehydes, the major modifications in proteins are likely through Michael addition with the main nucleophilic targets, cysteine, histidine, and lysine residues (Fig. 3.5). In this regard, the ability of acrolein (and other aldehydes) to modify cysteine residues has received the most attention, as such residues are often involved in structural or functional protein alterations induced by oxidative or nitrosative events. However, the relative importance of modification of cysteine residues and other amino acid modifications may vary, depending on the protein of interest and the relative abundance and reactivity of these amino acid residues. Several recent studies have implicated reaction with protein cysteine residues by acrolein as a mechanism by which enzyme function is altered. For example, we have recently determined that formation of Michael adducts in cytoplasmic NADPH oxidase subunits may be responsible for the lack of NADPH oxidase activation in neutrophils (Nguyen et al. 2001). Similarly, the central protein kinase that is involved in the activation of NF-κB, I-κB kinase (IKK), has been shown to be subject to redox regulation (Ji et al. 2001b; Reynaert et al. 2004), and was recently identified as a target for alkylation by 4-hydroxynonenal (Ji et al. 2001b), and by acrolein in airway epithelial cells (Valacchi et al. 2005). Although this was not always demonstrated directly, such inactivation most likely occurred by alkylation of the redox-sensitive cysteine residue in the transactivation loop of IKK, providing a direct mechanism involved in the inhibitory effects of unsaturated aldehydes on NF-κB activation. By analogy, preliminary evidence also suggests that acrolein is capable of inactivating caspase-3 (Finkelstein et al. 2001) by alkylation of a cysteine residue that is also subject to redox regulation (Fadeel et al. 1998; Hampton et al. 2002; Mannick et al. 1999).

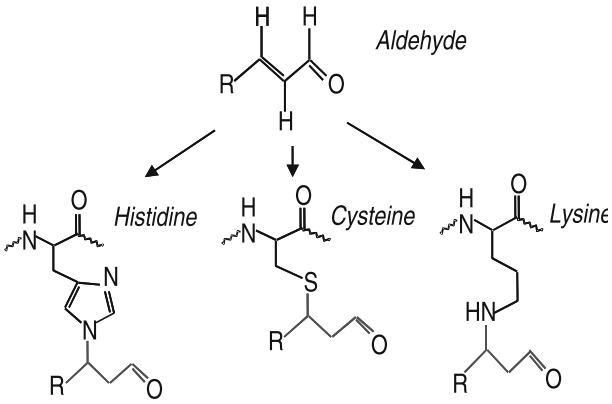

Fig. 3.5 Reaction of α,β-unsaturated aldehydes with protein histidine, lysine, or cysteine residues to form Michael adducts

Despite such examples, identification of the critical protein targets that are modified by acrolein or by other α,β-unsaturated aldehydes is still incomplete, and the exact consequence of such specific modifications to changes in protein interactions or enzymatic function is still largely unknown. One obvious reason for this is the complexity of such chemistry and the diversity of cellular targets. In order to address this issue, advanced proteomic approaches will be needed to identify the exact protein targets, and

the precise modification product(s). Several approaches are currently being used in an attempt to identify direct modifications in protein targets by α,β-unsaturated aldehydes, based largely on their ability to form Michael adducts with several amino acid residues (primarily cysteine, lysine, and histidine residues) (Esterbauer et al. 1991; Parola et al. 1998). Such adducts still contain the aldehyde moiety, which can be employed in strategies to detect them directly. Common approaches to detect protein-bound carbonyl adducts involve derivatization with hydrazides such as dinitrophenylhydrazide (DNPH) or biotin-hydrazide, after which these adducts can be separated using two-dimensional gel electrophoresis approaches, or selectively collected with avidin chromatography for identification by LC-MS or matrix-assisted laser desorption ionization (MALDI)-MS approaches (Conrad et al. 2001; Yoo and Regnier 2004). Although such hydrazide derivatization approaches have been well established and would allow detection of a broad range of Michael adducts of unsaturated aldehydes with various amino acid residues, one major limitation of this approach is that protein carbonyls can also be generated by several other oxidative mechanisms (Levine et al. 1994). Hence, such an approach could yield a high number of false positives, although MS/MS approaches could be used to distinguish alkylation by unsaturated aldehydes and other amino acid oxidation products. Another limitation with such an approach is the fact that the aldehyde moiety is still able to react with biological targets (e.g., Schiff base formation with lysine residues), and such reactions can occur secondary to Michael addition with neighboring amino acid residues, depending on protein structure (Esterbauer et al. 1991; Furuhata et al. 2002).

Alternative approaches to identify protein targets for unsaturated aldehydes have been made possible by the development of specific antibodies against protein adducts of, e.g., acrolein or hydroxynonenal (HNE). In fact, these antibodies have been used in several studies to demonstrate the formation of protein–aldehyde adducts in vivo or to detect adduct formation in specific proteins (Levonen et al. 2004; Parola et al. 1998; Uchida 1999). Although such immunochemical approaches may be more selective, the downside of such approaches is that such antibodies recognize only one specific protein adduct as the main epitope and do not cover the full spectrum of aldehyde reactivity with proteins. For instance, commonly used antibodies to detect protein–HNE adducts specifically recognize a Michael adduct with histidine (Levonen et al. 2004; Parola et al. 1998), and most likely do not detect Michael adducts with other amino acid residues such as cysteine residues. In this regard, such approaches could fail to recognize the potentially important reactions with redox-sensitive cysteine residues in various proteins. Furthermore, aldehyde adducts in which the aldehyde moiety has undergone secondary reactions with other targets (by, e.g., Schiff base formation) would also be left undetected. Similarly, an antibody raised against protein–acrolein adducts (Uchida 1999) is known to recognizes a specific epitope formed by Michael addition to lysine residues, *N*ε-(3-formyl-3,4-dehydropiperidino)-lysine (FDP-lysine), and will similarly not detect Michael adducts with other amino acid residues. Although this antibody has been used successfully to detect protein–acrolein adducts in inflamed tissues (Uchida 1999) and in CS-exposed cells (Nguyen et al. 2001), many potentially important protein modifications (e.g., with cysteine residues) would not be detected.

Whereas endeavors to detect direct cellular targets for unsaturated aldehydes are critical in order to understand the mechanisms by which these agents affect cell function, it has become clear that some redox-sensitive cellular pathways may also be affected indirectly by increased cellular production of oxidants. As such, 4-hydroxynonenal (Uchida et al. 1999), cyclopentenone prostaglandins (Kondo et al. 2001), and acrolein (van der Vliet et al., unpublished observations) have all been shown to rapidly increase cellular

oxidant production, presumably related to changes in mitochondrial function. Moreover, recent proteomic analysis of cysteine-containing proteins revealed cysteine oxidation/modification on a number of cellular proteins following exposure to cyclopentenone prostaglandins, which could be reversed by dithiothreitol, thus reflecting reversible oxidation/thiolation rather than irreversible alkylation (Ishii and Uchida 2004). Thus, although redox-sensitive protein cysteine residues may be susceptible targets for direct modification by unsaturated aldehydes, it is important to follow up analysis of protein cysteine status by additional strategies (reduction or secondary MS approaches) in order to distinguish between direct or indirect modifications. This complexity further illustrates the challenges that can be expected in studies aimed at identifying cellular mechanisms by which unsaturated aldehydes affect cell signaling pathways and function.

3.6 Concluding Remarks

In summary, it is apparent that the spectrum of reactions of α,β-unsaturated aldehydes with biological systems is complex and will not be fully characterized by analysis of one single reaction product in proteins. Hence, successful determination of cellular targets for specific α,β-unsaturated aldehydes will most likely require a combination of various approaches, using both more general derivatization strategies as well as more specific immunochemical approaches. Overall, further analysis by MS/MS techniques will be needed to obtain more-specific identification of aldehyde-induced modifications in target proteins. Although this might be a challenging endeavor, such approaches will be important in identifying the major cellular targets for biologically important aldehydes and may lead to critical discoveries of biological targets that are involved in the proposed roles of α,β-unsaturated aldehydes as potential signaling molecules. The realization that acrolein and other common CS aldehydes are not only products of air pollution, but may also be produced endogenously as potential mediators of lipid oxidation has increased the general scope and significance of such studies toward the biological actions of these aldehydes. Continued efforts to identify cellular mechanisms of acrolein (and related aldehydes) and identification of critical targets will not only be instrumental in further understanding the (bio)chemical mechanisms involved in tobacco-related diseases, but also yield further insights into the more general biochemistry of α,β-unsaturated aldehydes that are receiving increasing attention as more general mediators of conditions associated with oxidative stress and inflammation. As such, important continued research into molecular mechanisms of tobacco-related diseases could not only lead to improved diagnosis and therapeutic management of such cases, but also have the added advantage of having much wider implications for a broader range of pathophysiologic conditions.

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Chapter 4

Cigarette Smoke-Induced Redox Signaling and Gene Expression in In Vitro and In Vivo Models

4

Thomas Müller and Stephan Gebel

Contents

4.1 Introduction

Cigarette smoke (CS) is causally related to cancer development and severe inflammation-related diseases, such as chronic obstructive pulmonary disease (COPD), a syndrome that comprises respiratory disorders, e.g., chronic bronchitis, emphysema, bronchiolitis, and small airway disease (see Chapters 3 and 6), and cardiovascular disease (CVD), including subclinical atherosclerosis, coronary heart disease, and stroke (Peto et al. 1992; US Department of Health and Human Services 1989a, b). Chronic CS inhalation contributes significantly to the risk of these diseases, some of which develop within a few years, whereas other pathophysiologic effects require decades to become clinically detectable. In general, the individual risk is largely dependant on the chronically inhaled dose as well as on the susceptibility of the individual smoker to any of these diseases (reviewed for tobacco-related cancers in Wu et al. 2004). Because CS is a complex mixture estimated to be composed of up to 5,000 different chemicals, it is likely that the development of any of these disorders is not caused by a single constituent, but instead is the result of a complex interaction of a vast variety of CS-derived constituents reacting either directly or indirectly with target molecules in the aqueous, O_2-containing extra- and intracellular milieu of tissues of the respiratory tract and at other sites in the organism. Moreover, each exposure to CS triggers an acute pattern of cellular responses which is superimposed onto the existing responses to any chronic CS exposure. Altogether, these cellular/tissue responses are aimed at inactivating reactive CS constituents *and* adapting to the new microenvironmental conditions. This includes repair as well as synthesis activities; self-destructing, apoptotic mechanisms are activated when the degree of damage is beyond the cell's repair capacity. If, however, the cell is overwhelmed by an attack of damaging principles that is not manageable by cellular defense mechanisms, including the activation of apoptotic pathways, e.g., resulting in unrepaired and therefore inheritable genotoxic damage (reflected by mutational and epigenetic lesions) or cell death by an uncoordinated (necrotic) pathway, the severity of damage generally requires the sustained activation of upstream repair and clearance activities, which may ultimately result in chronic inflammation and disease development.

Generally, in anticipation of stressing conditions, cells (and tissues) are equipped with a whole plethora of means and tools in order to adequately cope with stressors derived from any chemical or physical source, as reflected, for example, by high local extra- and intracellular concentrations of antioxidant molecules such as glutathione (GSH), which are used as a kind of innate "redox buffer," or by redox-sensitive stress-sensing systems, resulting in the activation of signaling pathways followed by an efficient genetic response to combat stress more specifically. In view of this complex network of defense, the intention of this chapter is to review data describing (1) the interference of CS and CS-derived constituents with redox-sensitive and stress-sensing molecules, (2) the potential of CS and CS-derived constituents to activate signaling pathways, and finally, (3) the consequent changes in the gene expression pattern.

4.2 CS-Dependent Redox Effects on Biomolecules Involved in (Oxidative) Stress Sensing and Signal Generation

4.2.1 GSH

One of the most striking features of CS is its strong sulfhydryl (-SH) reactivity. This phenomenon was described more than 40 years ago independently by several groups using different experimental approaches (Fenner and Braven 1968; Green 1968; Lange 1961; Sato et al. 1962; summarized in Stauffer 1974), with the most detailed biological analysis of this effect provided by Leuchtenberger et al. (1974, 1976). For example, it was demonstrated that the in vitro phagocytic activity of isolated rabbit alveolar macrophages was significantly impaired on exposure to CS, yet the effect was completely inhibited in the presence of micromolar amounts of GSH or cysteine in the cell culture medium (Green 1968). Other publications described an inhibitory activity present in CS on specific -SH-dependent enzyme systems (Lange 1961; Sato et al. 1962). The evidence that an essential part of the adverse biological activity of CS might be exerted by its pronounced propensity to react with thiol residues was confirmed and further substantiated by Leuchtenberger et al. (1974, 1976), who demonstrated that this chemical trait of CS could be directly correlated with cytotoxic and carcinogenic effects in hamster lung cell cultures, as indicated by the inhibition of DNA and protein synthesis, atypical proliferation, and malignant cell transformation (Leuchtenberger et al. 1976). These authors also reported that the CS-dependent -SH reactivity was apparently largely attributable to the gas/vapor phase of smoke (Leuchtenberger et al. 1974). Although these data linked the CS-dependent -SH reactivity to CS-related biological effects, they provided no clue as to the underlying mechanism(s). Most of the discussion was related to compounds in CS contributing to the thiol reactivity, with much of the experimental focus on gas-phase components (Stauffer 1974).

There is now strong evidence that chronic exposure to the -SH reactivity effected by CS affects the development of CS-related diseases, especially COPD (for review, see Rahman and MacNee 1999). GSH is viewed as a prime inter- and intracellular target, and genes encoding enzymes involved in the GSH pathway are supposed to play a crucial role in the susceptibility to carcinogens in CS (Yang et al. 2004). As described elsewhere in this book (see Chapter 5), the -SH group containing tripeptide GSH constitutes a first-defense extra- and intracellular antioxidant, which protects cells against endogenous and exogenous oxidative stress (Meister 1991, 1994). The conclusion that this nonprotein thiol is a preferred target of an evidently huge number of different and chemically unrelated CS constituents is documented in numerous publications. These publications report, both in vitro and in vivo, the overall effect of GSH depletion by cigarette whole smoke (Bilimoria and Ecobichon 1992; Müller and Gebel 1998); by various CS fractions, including those derived from both the gas phase and the particulate phase (Müller and Gebel 1998; Rahman et al. 1995); and by single compounds present in CS, e.g., the metal carcinogen cadmium (summarized in Waisberg et al. 2003), and the tobacco-related aldehydes, such as acrolein, formaldehyde, and acetaldehyde (Grafström 1994; Horton et al. 1999; Kehrer and Biswal 2000; Müller and Gebel 1998). -SH-reactive compounds in CS are also represented by the CS particulate phase-associated compound benzoquinone (Abdelmohsen et al. 2003) and by nicotine (Yildiz et al. 1998), and are also indirectly formed from CS-derived precursors in an aqueous O_2-containing environment (Müller et al. 1997). In this context, it is important to note that CS exposure in vitro and

in vivo generally results in a net loss of GSH, as indicated by decreased levels of reduced GSH, whereas the amount of oxidized glutathione (GSSG) does not significantly increase (Müller and Gebel 1994; Park et al. 1998). This observation can be explained by a direct oxidation of GSH by CS-dependent reactive oxygen species (ROS) to yield GSH sulfenic (R-SOH) or GSH sulfinic acid (R-SO_2H); by the reaction of the -SH group with oxides of nitrogen, resulting in the formation of *S*-nitroso-GSH (GSNO); or by a direct covalent glutathiolation of CS-derived constituents as described for the α,β-unsaturated tobacco-related aldehydes acrolein and crotonaldehyde forming a thiohemiacetal or a thioether (Ohno and Ormstad 1985; Reddy et al. 2002). Moreover, additional routes of GSH consumption are represented by the formation of other conjugates of GSH and CS (yet to be identified), which are produced by the broad spectrum of detoxifying GSH *S*-transferases (GSTs) (Sellers and Yang 2002; Wu et al. 2004), GSH peroxidases (GPX) and phospholipid-hydroperoxide GSH peroxidases (PHGPx), which inactivate ROS-dependent peroxides and lipid hydroperoxides, respectively, at the expense of GSH (Thomas et al. 1990). Finally, GSH may be consumed in CS-exposed cells by the substantial formation of GSH conjugates with protein thiols, leading to the formation of protein-mixed disulfides (GSSP) catalyzed by the glutaredoxin system (Fernandes and Holmgren 2004). Because this glutathiolation reaction is readily reversible, it has been postulated as an efficient mechanism for protecting crucial protein -SH residues from irreversible damage (Mallis et al. 2002). Hence, in the recovering cell, functional -SH groups in proteins can easily be recovered from corresponding GSSP conjugates through NADPH-dependent electron transfer also mediated by the glutaredoxin system. In fact, significantly elevated GSSP levels were seen in the blood of smokers in a CS dose-dependent manner, suggesting that GSSP is a sensitive biomarker of oxidative stress (Muscat et al. 2004).

Whereas the effect of CS on extra- and intracellular GSH is well-documented, little information is available on how CS-induced GSH depletion provokes inflammatory processes that can enhance the risk of cancer or COPD. Myriad cellular functions, ranging from enzymatic to organelle mechanisms, are based on functional -SH groups presented by critical protein cysteine residues and by GSH itself (Chakravarthi and Bulleid 2004; Hentze et al. 2002; Molteni et al. 2004; Morito et al. 2003), and which are all protected by high local (millimolar) concentrations of GSH. Accordingly, by compromising cellular GSH homeostasis, cells become increasingly vulnerable to oxidative damage and cell death (D'Alessio et al. 2004), and it can be assumed that it is, among other effects, the sum of CS-dependent cellular lesions induced by -SH modification that contribute to the development of CS-dependent adverse effects. The protective function of GSH becomes clearer when we see that GSH concentrations are significantly elevated in bronchial epithelial cells from smokers (Rusznak et al. 2000) as well as in the extracellular lining fluid of chronic smokers, whereas significantly decreased GSH concentrations are found in the extracellular lining fluid of acute smokers (Barnes 2004; Rahman and MacNee 1999). In this context, it is worth noting that kinetic studies in vivo using rat and guinea pig smoking models revealed that exposure to CS acutely decreased pulmonary and renal GSH levels in a dose-dependent manner, particularly in the rat (Bilimoria and Ecobichon 1992). However, diminished GSH pools were found to be replenished after 3 h of exposure (Bilimoria and Ecobichon 1992), a time frame that was also observed in vitro (Gebel and Müller 2001). The conclusion that CS interferes with -SH functions is corroborated by the finding that *N*-acetyl cysteine (NAC), a precursor of GSH synthesis, has a dramatic chemoprotective effect, both in vitro and in vivo, on CS-induced lesions, such as DNA damage, gene expression, cell survival, and apoptosis (summarized in De Flora et al. 2001).

According to its crucial role as an -SH-residue-protecting agent, the intracellular GSH concentration represents a sensitive stress sensor and, consequently, exhibits signaling potential. This is the overall conclusion from in vitro and in vivo experiments performed in the presence of L-buthionine-[*S*,*R*]-sulfoximine (BSO), a selective inhibitor of γ-glutamylcysteine synthetase (γGST), the rate-limiting enzyme in GSH synthesis (Meister 1995). In fact, a direct effect of lowered GSH levels on stress signal transduction and gene activation has been shown by demonstrating increased DNA-binding activity of the transcription factor activated protein-1 (AP-1) and induced reporter gene transcription (Bergelson et al. 1994) as well as for the expression of the antioxidant gene heme oxygenase-1 (*hmox1*) in cells treated with BSO (Maines 1992 and refs. cited therein). However, most of the data based on BSO treatment show that diminished GSH levels strongly intensify the stress signaling activities of compounds that react directly or indirectly with -SH, such as phorone (Oguro 1996), peroxynitrite (Oh-Hashi et al. 2001), arsenite (Shimizu et al. 1998), and asbestos (Janssen 1995), as well as of CS, both in vitro (Müller and Gebel 1998) and in vivo (Park et al. 1998), whereas, if tested, NAC generally decreased the sensitivity towards these toxic compounds. In addition to activating defense mechanisms as represented by the expression of phase II and antioxidant genes, such as γ*gst*, NAD(P)H:quinone oxidoreductase 1 (*nqo1*) and *hmox1*, stress signals, including those emerging from GSH depletion, also address the expression of inflammation-related genes, such as those encoding growth factors, cytokines, and chemokines, partly via activation of the transcription factor nuclear factor-κB (NF-κB) (Adler et al. 1999; Flohé et al. 1997; Wilhelm et al. 1997) (see below). It can therefore be assumed that in chronic smokers, continually compromised GSH levels per se represent a proinflammatory scenario that may ultimately favor cellular and tissue damage.

4.4.2 Thioredoxin

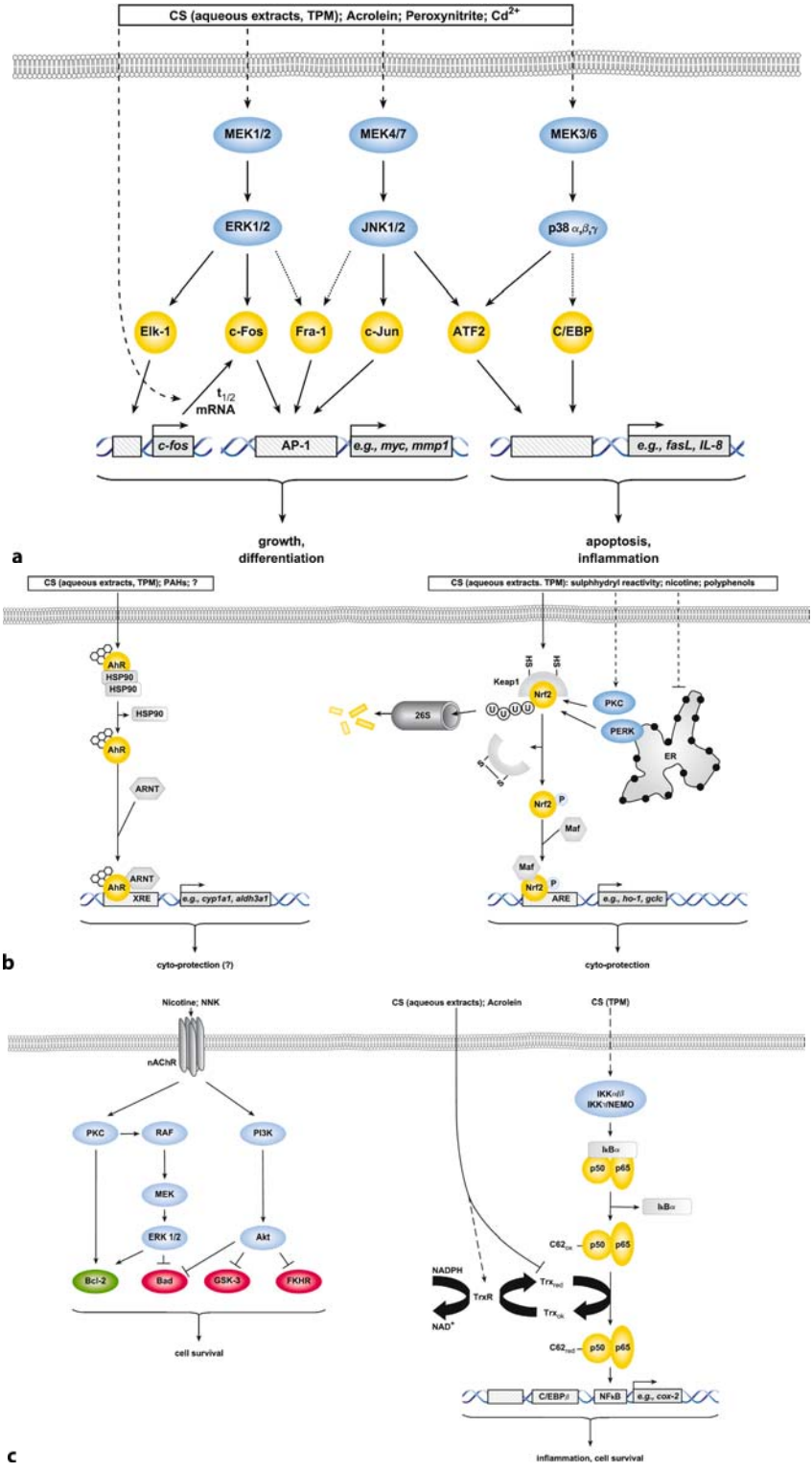
The other major cellular parameter responsible for keeping the cell's redox potential low and consequently the concentration of free -SH residues high is thioredoxin, also known as adult T-cell leukemia-derived factor (for review, see Arnér and Holmgren 2000; Powis and Montfort 2001). Beyond its crucial involvement in ribonucleotide metabolism (Holmgren 1989), another main function of this low-molecular-weight protein (M_r ~12 kDa) is to reduce oxidized -SH residues of functional proteins by means of its dithiol/disulfide (Cys-X-X-Cys) active site, which is fueled by NADPH-released electrons and catalyzed by thioredoxin reductase activity (for review, see Powis and Montfort 2001). In this function, the thioredoxin/thioredoxin reductase system partly overlaps the disulfide reductase activity exerted by the NADPH-dependent GSH/glutaredoxin system, which, however, is uniquely responsible for the protection of critical -SH residues by the formation of GSSP conjugates (Arnér and Holmgren 2000; Aslund and Beckwith 1999; Fernandes and Holmgren 2004). Accordingly, thioredoxin and glutaredoxin, which both belong to the thioredoxin superfamily of disulfide reductases, execute overlapping (i.e., back-up) as well as different disulfide reductase activities, thus further highlighting the biological significance of intracellular reduced thiol residues.

A major important feature of thioredoxin is its ability to interfere with signal transduction pathways by controlling the redox state of certain cysteine residues required by transcription factors to bind to DNA (reviewed in Sun and Oberley 1996). Although the list of transcription factors that rely on the thiol reducing activity of thioredoxin for ef-

ficient DNA binding is still growing, it includes such crucial players as NF-κB (Hayashi et al. 1993; Matthews et al. 1992; Okamoto et al. 1992), as well as p53, AP-1, and hypoxia inducible factor-1α (HIF-1α), the latter three via the redox factor Ref-1 (Gaiddon et al. 1999; Hirota et al. 1997; Lando et al. 2000). According to the fundamental impact on the signaling pathways addressing these transcription factors on the cellular response to changed microenvironmental conditions, and based on its ability to sense alterations of the redox potential, the thioredoxin/thioredoxin reductase system should be a central intracellular stress sensor (Aslund and Beckwith 1999). This hypothesis is supported by data demonstrating that thioredoxin also strongly influences decisions on life and death in the cell, as indicated by its potential to inhibit apoptosis signaling kinase-1 (ASK1) (Fiers et al. 1999; Liu et al. 2000; Saitoh et al. 1998), a mitogen-activated protein kinase (MAPK) that relays signals received via the tumor necrosis factor (TNF) receptor/TNF receptor-associated factor 2 (TRAF2) route to c-Jun N-terminal kinase (JNK) and p38 MAPK. In fact, the strong antiapoptotic effect associated with the overexpression of the thioredoxin/thioredoxin reductase system appears to be instrumental in the development of tumors, as indicated by increased levels of thioredoxin in many human tumors, which is associated with tumor aggressiveness (Powis and Montfort 2001). In mechanistic terms, at least part of the antiapoptotic nature harbored by thioredoxin may be related to its strong NF-κB-activating qualities (Harper et al. 2001).

Although the thioredoxin/thioredoxin reductase system is obviously of fundamental biological significance, there is, in contrast to the numerous effects described for GSH, a surprisingly small number of studies dealing with the effects of CS on thioredoxin or the thioredoxin/thioredoxin reductase system. However, using aqueous extracts of CS, no stimulating effect of this type of CS fraction was detectable on the expression of thioredoxin in in vitro-cultured 3T3 cells as observed by Western blotting (Gebel and Müller 2001). This result was recently confirmed at the transcriptional level for the in vivo situation by two independent rat inhalation studies during which the CS-dependent alteration of the gene expression pattern was studied using DNA microarray technology (Gebel et al. 2004; Gebel et al. in preparation). Although the thioredoxin gene was found to be slightly (~2-fold) induced in the airway epithelium of chronic smokers (Spira et al. 2004b), its low or lack of induction in CS-exposed cells and tissues (beyond a homeostatic [background] expression level) is somewhat surprising because recent data indicate that the thioredoxin gene is controlled by the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) (Kim et al. 2003), which represents a major target for activation by CS, both in vitro and in vivo (see below). Although the expression of thioredoxin is obviously not a major target of CS exposure, there is still strong, though indirect, evidence for the oxidation of its critical cysteine -SH residues by CS. This was deduced from experiments showing, in the first 2–4 h of exposure in CS-treated 3T3 cells, an almost complete failure of thioredoxin to coprecipitate with the p50 subunit of NF-κB, which was paralleled by a significantly decreased efficiency of NF-κB to bind to DNA (Gebel and Müller 2001). Because NF-κB DNA binding is strictly dependent on

Fig. 4.1 Examples of cellular signal transduction pathways triggered by cigarette smoke (*CS*) or CS-related components. Transcription factors are depicted in *yellow*, protein kinases in *blue*, antiapoptotic proteins in *green*, and proapoptotic proteins in *red*. *Hatched boxes* represent transcription factor-binding sites, *dashed lines* represent indirect/unknown mode of action, and *dotted lines* represent transcriptional activation. *P* phosphorylation, *U* ubiquitination. See text for details


CS (aqueous extracts, TPM); Acrolein; Peroxynitrite; Cd2+
MEK1/2
MEK4/7
MEK3/6
ERK1/2
JNK1/2
p38 α,β,γ
Elk-1
c-Fos
Fra-1
c-Jun
ATF2
C/EBP
t1/2 mRNA
c-fos
AP-1
e.g., myc, mmp1
e.g., fasL, IL-8
growth, differentiation
apoptosis, inflammation
a
CS (aqueous extracts, TPM); PAHs; ?
CS (aqueous extracts. TPM); sulphhydryl reactivity; nicotine; polyphenols
AhR
HSP90
HSP90
HSP90
AhR
ARNT
AhR
ARNT
XRE
e.g., cyp1a1, aldh3a1
cyto-protection (?)
Keap1
SH
SH
Nrf2
26S
PKC
PERK
ER
Nrf2
P
Maf
Maf
Nrf2
P
ARE
e.g., ho-1, gclc
cyto-protection
b
Nicotine; NNK
nAChR
CS (aqueous extracts); Acrolein
CS (TPM)
PKC
RAF
PI3K
MEK
ERK 1/2
Akt
Bcl-2
Bad
GSK-3
FKHR
cell survival
IKKα/β/γ
IKKγ/NEMO
IκBα
p50
p65
IκBα
C62ox
p50
p65
NADPH
TrxR
NAD+
Trxred
Trxox
C62red
p50
p65
C/EBPβ
NFκB
e.g., cox-2
inflammation, cell survival
c

the thiol reductase activity of thioredoxin for DNA binding via its p50 subunit (Hayashi et al. 1993; Matthews et al. 1992; Okamoto et al. 1992) (see Fig. 4.1c), these findings indicate that in the early phase of CS exposure, thioredoxin becomes oxidized. In order to render this oxidized, nonfunctional fraction of thioredoxin protein functional again, it appears that CS-exposed cells and tissues transcriptionally activate the thioredoxin reductase gene as evidenced in vitro by RT-PCR analysis (Gebel and Müller 2001) and in vivo by a reproducible 3- to 5-fold induction of this gene, both in the nasal epithelium and in the lungs of CS-exposed rats (Gebel et al., in preparation). This observation is in accordance with results showing a ~4-fold activation of the thioredoxin reductase gene in the lungs of mice exposed for 3 h to CS (Rangasamy et al. 2004), and with a more than 2-fold induction in the airway epithelium of healthy (chronic) smokers (Hackett et al. 2003; Spira et al. 2004). Because thioredoxin reductases of higher eukaryotes, which are selenium-dependent dimeric flavoproteins, show a broad substrate spectrum, including nondisulfide substrates such as hydroperoxides and vitamin C (Arnér and Holmgren 2000), it appears economically more reasonable to upregulate this more pleiotropic antioxidant tool, rather than its substrate thioredoxin.

Based on its biological significance, there is clearly more experimental work needed to evaluate the role of the thioredoxin/thioredoxin reductase system in CS-related pathobiologic effects. Exploring this role, which appears to be a double-edged sword, as indicated by its potential to act as stress sensor and cellular defense system contrasted with its strong antiapoptotic activities, might also include studies on the chemopreventive potential of selenium, which is currently under debate (Ganther 1999). Finally, the capacity of thioredoxin to release biologically and pharmacologically relevant molecules from their corresponding GSH conjugates, as demonstrated for GSNO (Nikitovic and Holmgren 1996), appears worth investigating in the context of CS exposure.

4.2.3 Lipids and Membrane Constituents

In addition to protein and nonprotein thiols, components of the cellular membrane, such as phospholipids, are targeted by CS-dependent ROS, which may result in the generation of intermediates exhibiting strong proinflammatory signaling character (Niki et al. 1993). Examples of biologically active compounds formed from membrane lipids by CS-induced lipid peroxidation include various oxidized phospholipids that mimic the signaling activities exhibited by platelet-activating factor (PAF), F_2-isoprostanes, and 4-hydroxy-2-nonenal (4-HNE), as well as various other aldehydes, such as acrolein, which are all implicated in the development of CS-related diseases, particularly for COPD (for review, see Barnes 2004) and atherosclerosis (for review, see Stocker and Keaney 2004).

As summarized elsewhere in this book (see Chapter 3), lipid peroxidation reflects the uncontrolled breakdown of polyunsaturated fatty acids by a peroxidation mechanism believed to be induced by free radicals. Radical species involved in these processes either are released directly from CS (Pryor and Stone 1993) or are produced indirectly by their reaction with other biomolecules in the O_2-containing aqueous cellular milieu, leading to the intermediate formation of lipid hydroperoxides (Blair 2001). Hence, the list of CS-derived compounds potentially initiating lipid peroxidation includes hydroxyl radicals (·OH), which appear in aqueous solutions of CS via Fenton/Haber-Weiss chemistry, and/or peroxynitrite ($ONOO^-$), which is formed by the reaction of nitric oxide (NO·) with superoxide anion (O_2^{-}·) (Pryor and Stone 1993). Other radicals may po-

tentially be formed from the reaction of these compounds with other biomolecules, resulting in the formation of alkoxyl, peroxyl, and nitronium radicals. If not detoxified by PHGPx or GPx1/phospholipase activity (Thomas et al. 1990), such lipid hydroperoxides are further degraded, releasing products with strong proinflammatory signaling activity. For example, oxidation of polyunsaturated fatty acids by this mechanism may produce compounds with PAF-like activities, resulting in the uncontrolled activation of PAF receptors on platelets, monocytes, and leukocytes, which is a powerful trigger of proinflammatory signaling cascades. Eventually, this mechanism favors the formation of atherogenic low-density lipoprotein (LDL) particles as evidenced by the presence of CS-induced breakdown products of polyunsaturated fatty acids exhibiting PAF-like activity in these structures (Marathe et al. 2001).

Other CS-dependent ROS-induced lipid peroxidation products of major biological significance are the F_2-isoprostanes, which arise from uncontrolled arachidonic acid degradation. Because these compounds exhibit thromboxane A_2- and $PGF_{2\alpha}$-like activities and thus induce severe vaso- and bronchoconstriction as well as other lung inflammatory effects, they are supposed to be of key relevance in the development of COPD and CVD (for review, see Cracowski et al. 2002). In fact, increased levels of isoprostanes were identified in the exhaled air of smokers and in COPD patients (Montuschi et al. 2003), implicating a synergistic effect in the pathogenic process. Other end products of lipid peroxidation, i.e., aldehydes (particularly 4-HNE) are highly diffusible thiol-reactive compounds known to exert pleiotropic proinflammatory activities, which may be based, at least in part, on their ability to trigger several growth-related signaling pathways (for review, see Leonarduzzi et al. 2004). For example, it has been demonstrated that 4-HNE binds to, and subsequently activates, the epidermal growth factor (EGF) receptor (Suc et al. 1998) and the platelet-derived growth factor (PDGF) receptor (Escargueil-Blanc et al. 2001), which may catalyze the formation of atherogenic areas induced by oxidized LDL particles. Moreover, 4-HNE has been shown to affect protein kinase C (PKC) activity, especially PKC β_1 and β_2 isozymes (Leonarduzzi et al. 2004), and the stress-related signaling MAPKs JNK and p38MAPK, the latter of which was found to be involved in 4-HNE-induced expression of proinflammatory cyclooxygenase 2 (COX-2) (Kumagai et al. 2000; 2002), the rate-limiting enzyme during prostaglandin and thromboxane synthesis. Although the proinflammatory character of 4-HNE is not under debate, it is surprising that it does not, at least not directly, affect the proinflammatory transcription factor NF-κB, but rather may exert an inhibitory effect on NF-κB (Leonarduzzi et al. 2004). However, this might be explained by the -SH reactivity linked to the aldehyde nature of 4-HNE, because aldehydes have been shown to negatively affect NF-κB activity (Horton et al. 1999), most probably by inactivating the -SH residue of Cys 62 of its p50 subunit, which is required for efficient DNA-binding (Hayashi et al. 1993; Matthews et al. 1992; Okamoto et al. 1992).

Beyond the generation of compounds that cause strong proinflammatory signaling activities by lipid peroxidation, membrane damage itself impairs membrane functions, e.g., by the inactivation or uncontrolled activation of membrane-bound receptors and enzymes. Although no direct data are available for CS or CS-derived fractions, other redox-active chemical and physical treatments have been linked, in mammalian cells for example, with the clustering and internalization of membrane receptors, such as EGF, interleukin 1 (IL-1), and TNF-α coupled with JNK signaling (Rosette and Karin 1996). In addition, environmental stresses such as heat shock, UV irradiation, and oxidative stress caused by H_2O_2 treatment were shown to directly activate membrane-bound acid pH-dependent sphingomyelinase (ASMase), resulting in enhanced levels of the second

messenger lipid intermediate ceramide from sphingomyelin. Ceramide generation because of ASMase activation has been correlated with JNK activation and apoptosis in human myeloid leukemia U937 cells (Verheij et al. 1996). In fact, a gradual increase in ceramide levels followed by apoptosis was detected in this cell line on exposure to aqueous extracts of CS (T. Müller, unpublished results). Altogether, these results provide direct and indirect evidence that by interfering with lipid and protein components of biological membranes, CS-derived ROS and reactive nitrogen species (RNS) produce signaling intermediates that may promote the development of a proinflammatory environment.

4.2.4 Redox-Sensitive Molecules in Signal Transduction

Consistent with an apparent biological principle, pathways that transduce signals arising from an impaired redox balance are generally equipped with redox-sensitive components that potentially function as (oxidative) stress sensors. Hence, these pathways are kept silent in the nonstressed cell when facing a sufficiently low redox potential, but in the case of oxidative disturbance become activated through chemical modification(s) of these particular components (for review, see Haddad 2002). Paradigms of signaling pathways that are regulated by such a mechanism are those resulting in the activation of the transcription factors NF-κB, AP-1, and particularly Nrf2 (Fig. 4.1), which mainly orchestrate the cellular stress and antioxidant/phase II-related defensive programs, respectively (Nguyen et al. 2003).

In the unstressed cell, the cap 'n' collar/basic region leucine zipper (CNC/b-Zip) transcription factor Nrf2 is inhibited by a dual mechanism involving its retention in the cytoplasm and its proteolysis through proteasomal degradation. Central to the repression of Nrf2 activity is the actin-binding protein Kelch-like ECH-associating protein 1 (Keap1), which anchors Nrf2 to the cytoplasm via interaction with a specific four amino acid stretch within the Neh2 domain of Nrf2 (for review, see Motohashi and Yamamoto 2004), while the broad complex-tramtrack-bric-a-brac (BTB) and the intervening region (IVR) domains of Keap1 recruit Cullin3, a subunit of the E3 ligase complex, resulting in the ubiquitination and subsequent proteolytic degradation of Nrf2 (Cullinan et al. 2004; Kobayashi et al. 2004; Zhang et al. 2004) (Fig. 4.1b). The efficiency of this regulatory mechanism is reflected by a rapid turnover of Nrf2 in unstressed cells, as expressed by half-lives of less than 20 min. However, if, owing to changes in their microenvironment, cells and tissues are confronted with an imbalanced redox homeostasis arising from electrophiles and oxidants, then this inhibitory mechanism is abruptly abrogated. Because Keap1 is a cysteine-rich molecule, it was hypothesized early on that at least some of these -SH residues might function as sensors for oxidant and electrophile compounds (Dinkova-Kostova et al. 2002). In fact, further investigations revealed direct evidence that -SH-reactive compounds activate Nrf2 through modification (alkylation, oxidation) of essentially two cysteine thiol residues, both located in the IVR domain of Keap1 (Wakabayashi et al. 2004). Mechanistically, modifications of these crucial -SH residues are assumed to induce conformational changes that eventually result in the abrogation of ubiquitin-dependent degradation of Nrf2 and its release from Keap1. Beyond this redox-dependent mechanism, other phosphorylation-dependent activation mechanisms have been reported for Nrf2 (summarized in Motohashi and Yamamoto 2004; Nguyen et al. 2004; see below and Fig. 4.1).

Although the activation of Nrf2 by CS through the Keap1-dependent cysteine thiol-sensitive mechanism outlined above has not been demonstrated directly, there is nevertheless sufficient evidence that the Nrf2/Keap1 system represents a major target for CS-dependent oxidants, as indicated by the finding that *nrf2*$^{-/-}$ mice are highly susceptible to CS-related inflammatory diseases such as emphysema (Rangasamy et al. 2004), and, more generally, by gene expression studies showing the strong upregulation of the phase II-related gene spectrum in response to CS exposure in vitro (Bosio et al. 2002) and in vivo (Gebel et al. 2004; Spira et al. 2004b). In principle, every CS-dependent -SH-reactive compound (see above) may potentially directly interfere with the specific cysteine thiol sensors provided by Keap1, resulting in the activation of Nrf2, as, for example, has been specifically demonstrated for the CS-dependent metal carcinogen cadmium (Stewart et al. 2003) and the gas-phase component acrolein, which was seen to strongly induce phase II-related genes by a mechanism involving Nrf2 (Tirumalai et al. 2003). Moreover, the interference of electrophilic lipid oxidation products, such as isoprostanes (Levonen et al. 2004), and the end products of lipid peroxidation, such as 4-HNE, with the reactive thiols of Keap1 may represent an indirect mechanism of CS-provoked activation of Nrf2. Finally, in addition to the -SH-sensitive activation mechanism mediated by Keap1, the DNA-binding efficiency of Nrf2 seems, itself, to be dependent on a crucial cysteine thiol, as evidenced by experimentally induced mutagenesis of cysteine to serine in the DNA-binding domain of Nrf2 (Bloom et al. 2002). Considering the strong overall -SH reactivity of CS (see above) together with the paramount relevance of Nrf2 in the cellular defense against oxidative stress, the reactive cysteine thiols of Keap1 represent potential central targets of CS-derived oxidants and electrophiles.

Similar to Nrf2, the other paradigmatic (oxidant) stress responsive transcription factor, i.e., AP-1, is also subject to tight redox regulation (for review, see Dalton et al. 1999; Eferl and Wagner 2003; Karin 1995). However, unlike Nrf2, the redox sensitivity of AP-1 is confined to its DNA-binding activity, which depends on -SH functions provided by critical cysteine residues within the b-Zip proteins potentially forming AP-1, such as proteins of the Jun, Fos, and ATF families. For example, oxidant conditions interfere with the DNA-binding activity of Jun/Fos heterodimers, which led to the identification of Cys-154 in Fos and Cys-272 in Jun as crucial determinants of this parameter (Abate et al. 1990). In this regard, redox regulation of AP-1-related transcription factors is akin to NF-κB, where the -SH function of Cys-62 within the p50 subunit is required for efficient DNA binding (Hayashi et al. 1993; Matthews et al. 1992; Okamoto et al. 1992; see above and Fig. 4.1c). It is therefore not surprising that AP-1 and NF-κB show similar direct effects in response to acute CS exposure, as expressed by significantly reduced DNA-binding capacities, most probably based on the presence of α,β-unsaturated aldehydes such as crotonaldehyde and acrolein (Freed et al. 2003; Gebel and Müller 2001; Kehrer and Biswal 2000; Vayssier et al. 1998b). The apparent paradoxical finding of an increased activity of these transcription factors in CS-related diseases such as COPD may therefore be explained by indirect mechanisms such as chromatin remodeling effects and invasion of proinflammatory cells during chronic CS exposure (Marwick et al. 2004).

In contrast to oxidatively modified -SH functions of Cys-62 within p50 of NF-κB, which are directly dependent on thioredoxin for rereduction, transcription factors of the AP-1 family show an indirect dependence on thioredoxin. In fact, the redox factor Ref-1 was identified as controlling the redox status of cysteine residues relevant for DNA binding in AP-1-related transcription factors, whereas, in turn, oxidized Ref-1 is dependent on thioredoxin for rereduction (Hirota et al. 1997). Notably, in addition to the cysteine-reducing activity, which is not only relevant for AP-1 function but also for p53 and HIF-

1α DNA binding (Gaiddon et al. 1999; Lando et al. 2000), Ref-1 shows the characteristics of a multifunctional protein because it also acts as an apurinic/apyrimidinic endonuclease (Demple et al. 1991). Because this enzyme activity is integral to DNA repair, Ref-1 may represent a central factor in orchestrating/coordinating stress signaling and DNA repair processes. However, despite this obvious functional relevance, no data are available on the direct effects of CS or CS-related components on the activity of Ref-1.

In summary, data gathered over several decades indicate that CS exposure affects the cellular redox potential by mainly interfering with "defensive" nonprotein (i.e., GSH) and critical functional thiol groups of proteins involved in stress sensing and signal generation. The sum of these effects obviously prompts the cell to a comprehensive response at the transcriptional level, resulting in an extensively altered gene expression pattern as described below.

4.3 Activation of Signal Transduction Pathways in CS-Exposed Cells

According to the dimension of alterations seen in the gene expression pattern (see below), strong signaling activities can be expected to occur in CS-exposed cells and tissues. However, somewhat surprisingly, the number of reports in the literature describing the activation of specific signal transduction pathways because of exposure of CS or CS-related compounds is limited and concentrates mainly on the activation of the aryl hydrocarbon (Ah) receptor or on the signaling activities initiated by nicotine and its derivatives.

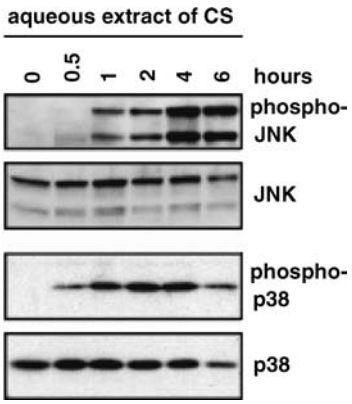


Fig. 4.2 Activation of c-Jun N-terminal kinase (*JNK*) and p38 mitogen-activated protein kinase (MAPK) signaling pathways in Swiss 3T3 cells exposed to aqueous extracts of cigarette smoke. Activation of JNK and p38 MAPK in Swiss 3T3 cells was demonstrated by Western blot analysis using antibodies against activated p38 MAPK (phospho-p38) and JNK (phospho-JNK) and the respective antibodies against the whole proteins

4.3.1 Stress Signaling Pathways

Regarding the induction of stress-related signal transduction pathways, activation of JNK signaling and AP-1 has been observed, for example, in the context of mucin production in the lungs of smokers, which was found to be independent of upstream epidermal growth factor receptor (EGFR) activation (Gensch et al. 2004). Evidence of JNK signaling was also obtained for the CS-induced expression of the AP-1 component *fra-1*, which in this case was dependent on EGFR function based on matrix metalloproteinase (MMP) activation (Zhang et al. 2005), thus confirming that CS-dependent alterations in the extracellular matrix are a trigger of CS-induced cell signaling. There is also evidence for the CS-dependent activation of p38MAPK, which was also demonstrated for the CS-dependent carcinogen cadmium (Alam et al. 2000; Chuang et al. 2000). In fact, our own laboratory observed a sustained activation of both JNK and p38MAPK in Swiss 3T3 cells exposed to aqueous extracts of cigarette smoke (unpublished results; Fig. 4.2). Thus, although data are limited, these results suggest that CS-derived ROS, directly or indirectly (e.g., through lipid peroxidation products such as 4-HNE or by interfering with the extracellular matrix), trigger classical stress-related signal transduction pathways, resulting in the activation of JNK and p38MAPK (Fig. 4.1a).

Beyond JNK and p38MAPK activation, CS and CS-derived compounds have also been reported to activate the other route of MAPK signaling, i.e., the extracellular signal-regulated kinase (ERK) pathway, which, in contrast to JNK and p38MAPK signaling, is mostly involved in transferring cell survival signals (Xia 1995) (Fig. 4.1a). An example of CS as a trigger of ERK signaling has recently been reported by Mercer et al. (2004), who showed that CS-induced expression of the gene encoding matrix metalloproteinase 1 (*MMP1*) was specifically dependent on ERK1/2-activation in human airway epithelial cells in vitro. In vivo experiments supported the assumption that ERK1/2 signaling may be involved in the development of CS-dependent emphysematous symptoms, as expressed by the identification of activated ERK in both airway lining cells and alveolar macrophages of mice exposed to CS as well as in the same cell types in smokers suffering from emphysema (Mercer et al. 2004). In this context it is intriguing to note that the expression of the proinflammatory cytokine IL-8 in alveolar macrophages of smokers was found to be mainly dependent on ERK (and p38MAPK) activation rather than related to the activation of NF-κB (Koch et al. 2004).

In contrast to JNK, p38MAPK, and ERK MAPK signaling, which may enhance inflammatory as well as apoptotic responses, e.g., by activating certain subpopulations of the AP-1 family of transcription factors, activation of the also stress-responsive Nrf2 pathway stimulates cell survival and anti-inflammatory functions. In addition to the mechanism involving the oxidative modification of specific -SH functions of Keap1 as outlined above, phosphorylation by upstream signaling kinases is also believed to be sufficient for activation of Nrf2 as a potent transcription factor of antioxidant and phase II-related genes (reviewed in Motohashi and Yamamoto 2004; Nguyen et al. 2004) (Fig. 4.1b). Accordingly, activation of Nrf2 through phosphorylation of N-terminal serine residues by PKC has been shown in response to oxidative stress and electrophiles (Bloom and Jaiswal 2003; Huang et al. 2000, 2002). Phosphorylation-dependent activation of Nrf2 has also recently been reported to be controlled by PERK, a kinase that becomes induced in response to certain types of stress, resulting in the abrogation of protein synthesis at the endoplasmic reticulum (ER) (Cullinan et al. 2003). Because nicotine (Jin et al. 2004; Mai et al. 2003) and CS-related dihydroxybenzenes such as hydroquinone

and catechol (Gopalakrishna et al. 1994) have been described to activate PKC, while at the same time CS acutely interferes with the translational efficiency at the ER (Müller and Gebel 1994), these pathways further represent potential routes for Nrf2 activation by CS. As a consequence of activation, whether via -SH oxidation of specific cysteine residues within Keap1 and/or phosphorylation by upstream signaling kinases such as PKC and PERK, Nrf2 is rapidly released from its negative regulator Keap1 and migrates to the nucleus to initiate, in conjunction with an appropriate dimerization partner (e.g., members of the group of b-Zip small Maf proteins), the antioxidant cellular response at the transcriptional level. In mechanistic terms, this is accomplished by binding of the activated heterodimeric transcription factor to the antioxidant response element (ARE) present in the control region of antioxidant and stress-responsive genes (Motohashi and Yamamoto 2004).

The duality of the cellular defense strategy against environmental stresses is well established. In addition to the expression of antioxidant and phase II genes, which is aimed at inactivating reactive cell damaging species, there is also the activation of phase I-related genes, which code for drug-metabolizing enzymes, such as monooxygenases and dehydrogenases, and help the cell prevent damage from harmful xenobiotics by rendering these compounds accessible to cellular excretion mechanisms (for review, see Nguyen et al. 2003). Typically, the expression of phase I-related genes is orchestrated by cytoplasmic receptors, which, on activation via ligand binding, translocate to the nucleus, where they function as transcription factors in a *cis* element-dependent context. The most prominent representative of this group of transcription factors is the aryl hydrocarbon receptor (AhR) (Denison and Nagy 2003; Nebert et al. 2000, 2004), which is activated by binding to a broad spectrum of xenobiotic ligands, such as polyaromatic hydrocarbons (PAH) and aryl amines. On forming a complex with a particular dimerization partner, i.e., AhR nuclear translocator (ARNT), AhR controls a broad spectrum of genes, including those known to be differentially regulated by CS (see below), by addressing a specific control element known as the xenobiotic responsive element (XRE) (Fig. 4.1b). Interestingly, the efficiency of AhR to function as a transcription factor was recently found to be partly controlled by activated ERK, because ERK activity facilitated ligand-initiated AhR transcriptional activation while targeting the receptor for degradation (Chen et al. 2005).

However, the expression of certain enzymes in response to AhR activation, e.g., cytochrome P450 1A1 (Cyp1A1) (see below), is considered a double-edged sword because there is a risk of reactive (oxygenated) intermediates appearing, which may be harmful to the cell, for example, by forming bulky DNA adducts that may ultimately lead to cancer-initiating mutations (discussed for CS exposure in Bartsch et al. 1992). Thus, inhibition of AhR activity in vivo by 3'-methoxy-4'-nitroflavone, a potent AhR antagonist, completely abolished the DNA genotoxicity induced by CS condensate and benzo[a]pyrene (BaP), a PAH known to be present in CS, in C57B1/6J mice (Dertinger et al. 2001). This effect was attributed to the abrogated expression of Cyp1A1 in response to AhR inactivation. However, these results should be interpreted with caution because paradoxical effects were reported for murine lines with disrupted *cyp* genes. For example, on exposure to BaP (125 mg/kg/day), *cyp1a1*$^{-/-}$ mice exhibited a strict lethal phenotype within 30 d, whereas wild-type mice did not show any lethality within 1 year of treatment. Even more remarkable is the finding that *cyp1a1*$^{-/-}$ mice generally harbored much higher amounts of BaP DNA adducts in comparison to their wild-type counterparts (Uno et al. 2004). Hence, these results indicate that the potential detoxification provided by AhR-stimulated Cyp enzymes "is more important than metabolic activation" (Uno et al. 2004).

Clearly, the obvious and important role played by the AhR in cell cycle regulation and apoptosis is only just beginning to emerge (Nebert et al. 2000).

The inducibility of phase I-related genes in the respiratory tract by CS, especially those encoding the cytochrome P450 isozymes, discussed below, was recently described in a detailed review by Ding and Kaminsky (2003).

4.3.2 Cell Growth-Related Signaling Pathways

According to several studies, nicotine is responsible for a strong antiapoptotic cell growth signal delivered by CS. Specifically, nicotine and nicotine-derived compounds, e.g., the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), have been reported to activate survival and cell growth signaling pathways, including PKC (Heusch and Maneckjee 1998; Jin et al. 2004; Mai et al. 2003; Maneckjee and Minna 1994) and protein kinase B/Akt (PKB/Akt), most presumably by binding to and activating α_7, α_4, or α_3 variants of nicotinic acetylcholine receptors (nAChR) (Maneckjee and Minna 1994; Schuller et al. 2000; West et al. 2003) (Fig. 4.1c). According to these investigations, nAChRs are not restricted to neuronal cells but, as a result of nicotine exposure, are also expressed in the membranes of cells in other tissues, such as airway epithelial cells and keratinocytes of the oral mucosa, where nicotine specifically interacts with the α3β2 subtype of nAChR, resulting in the activation of survival factors such as Bcl-2, NF-κB, and STAT-1 (Arredondo et al. 2005). Data describing the effects of nicotine-induced activation of PKC support the phosphorylation-dependent induction of antiapoptotic or inactivation of proapoptotic factors controlling the mitochondrion-related intrinsic apoptotic pathway. Accordingly, nicotine-induced PKC has been reported to trigger the activation of the Raf/Erk MAPK pathway, resulting in the phosphorylation-dependent activation of the apoptosis antagonist Bcl2 (Mai et al. 2003) and/or inactivation of the proapoptotic factor Bad (Jin et al. 2004).

Another route of nicotine- and NNK-dependent cell survival signaling affects the activation of PKB/Akt via nAChR binding and phosphatidylinositol 3-kinase (PI3K) activation as described in nonimmortalized human airway epithelial cells in vitro (Fig. 4.1c). This sequence of signaling steps favors the phosphorylation-dependent inactivation of proapoptotic factors, such as glycogen synthase kinase-3 (GSK-3), FKHR, a member of the fork head transcription factor family (West et al. 2003), and Bax (Xin and Deng 2005). As a consequence, nicotine and NNK attenuated the apoptosis induced by model treatments including exposure to etoposide or UV irradiation. Although these in vitro results, which are partially paralleled in vivo by activated PKB/Akt in human lung cancers in smokers (West et al. 2003), point to a growth-stimulating, tumor-promoting potential of nicotine, its role during carcinogenesis remains unclear because nicotine is considered to be neither genotoxic nor carcinogenic (for review, see Hecht 1999).

4.3.3 NF-κB

Regarding the proinflammatory signaling activities of CS, a controversy has emerged on the question of whether CS or CS-derived fractions are capable of *directly* activating the redox-sensitive and proinflammatory transcription factor NF-κB. Several reports de-

scribe a direct inhibitory effect of CS on NF-κB in vitro (Favatier and Polla 2001; Freed et al. 2003; Moodie et al. 2004; Vayssier et al. 1998b). As outlined above, this inhibitory effect was attributed mainly to the oxidative modification of Cys 62 of the p50 subunit of NF-κB by α,β-unsaturated aldehydes in CS, such as crotonaldehyde and acrolein, resulting in an impaired DNA-binding activity. In accordance with this concept (Fig. 4.1c), data describing the kinetics of the effect of CS on the DNA-binding activity of NF-κB in Swiss 3T3 fibroblasts in kinetic terms revealed a complex behavior with significantly decreased binding activities seen within the first 2 h of exposure (Gebel and Müller 2001). After 4–6 h of exposure, NF-κB DNA binding recovered, showing binding rates that were slightly elevated over controls (~2-fold). Rebinding of NF-κB was independent of inhibitory protein of κBα (IκBα) degradation but, as described above, correlated with the expression of thioredoxin reductase and the reavailability of reduced thioredoxin (Gebel and Müller 2001), the functional reductase of oxidized Cys 62 within the p50 subunit of NF-κB. Moreover, further studies revealed that aqueous extracts of CS significantly impaired the DNA-binding activities of NF-κB in 3T3 fibroblasts stimulated with tumor necrosis factor α (TNF-α) (Müller and Gebel, unpublished results), a phenomenon that was also described for A549 cells exposed to CS condensate (Moodie et al. 2004). These data clearly indicate that the *direct* response of NF-κB to CS is controlled by redox-dependent mechanisms rather than upstream signaling mechanisms, as has been described for proinflammatory cytokines such as TNF-α. Therefore, chronic inflammation related to CS exposure is, at least according to these data, of an *indirect* nature, e.g., caused by chromatin remodeling effects or cellular damage, resulting in necrosis and the consequent invasion of proinflammatory cells during chronic CS exposure. Finally, even nicotine was shown to interfere with NF-κB activation and cytokine expression in response to lipopolysaccharide (LPS) exposure in monocytic U937 cells, which, as stated by the authors, might explain the immunosuppressive activities observed in the context of CS exposure (Sugano et al. 1998).

The results of a CS-dependent inhibition of NF-κB DNA binding are, however, diametrically opposite to data showing that CS condensate *directly* activates NF-κB in cells of different cell lines (Anto et al. 2002). According to this report, the CS-dependent activation of NF-κB follows the "conventional" route, involving activation of IκB kinase (IKK) followed by degradation of IκBα (Fig. 4.1c). In fact, using various dominant negative mutants of signaling elements known to reside upstream from NF-κB activation, a CS-specific pathway very similar to the pathway of NF-κB stimulation by proinflammatory cytokines such as TNF-α was delineated. As evidence of a direct activation of NF-κB by CS condensate, the collinear expression of COX-2 was reported in this study (Anto et al. 2002). Although NF-κB is a key regulator of *cox-2* expression, it is worth noting in this context, that in both the human system and the murine system, the 5'-flanking promoter region of *cox-2* is equipped with several other transcription factor consensus elements in addition to NF-κB-binding sites, including recognition sites for CAAT enhancer-binding protein-β (C/EBPβ) (Caivano et al. 2001; Nie et al. 2003). In fact, the gene coding for C/EBPβ has been shown to become significantly expressed by aqueous extracts of CS in 3T3 cells (Bosio et al. 2002), suggesting that this transcription factor might also be a potential candidate for *cox-2* upregulation seen in CS-exposed cells.

If, as indicated by the results of Anto et al. (2002), CS exposure is a direct inducer of NF-κB, these data would mean that cells and tissues might start a proinflammatory response essentially independent of inflammation-related cytokines released by cells of the immune system. Thus, the question of whether CS is a direct or indirect inducer of an inflammatory response is of fundamental significance, and further efforts should be made to reconcile the conflicting data described above.

4.4 CS-Induced Differential Gene Expression In Vitro and In Vivo

Since entering the post–genome era, with the parallel development of sophisticated tools such as real-time PCR and gene chip/microarray technologies to routinely characterize gene expression on a comprehensive and high throughput basis, the number of publications reporting on the effect of CS on the gene expression profile has steadily increased. As discussed below, in vitro and in vivo investigations making use of these new technologies have not only broadened our understanding of the mechanism of CS-dependent disease formation, but have also raised new questions. Before the scientific and methodological breakthroughs mentioned above, the affect of CS on transcriptional activity in CS-exposed cells and tissues focused mainly on the expression of single (model) genes, which was—and still is—often used as a tool in mode-of-action analysis, i.e., as a molecular reporter aimed at identifying cellular targets as well as CS-dependent compounds involved in the mechanism of gene expression.

4.4.1 CS-Induced Differential Gene Expression of Single (Model) Genes

One of the first genes identified as being strongly induced by CS was *hmox1*. The pronounced sensitivity of this antioxidant gene to CS was deduced from [^{35}S]-methionine protein labeling experiments performed in 3T3 fibroblasts exposed to aqueous extracts of CS (Müller and Gebel 1994). Although CS was found to generally exert a transient inhibitory effect on translation efficiency, the CS-dependent principal expression of a cytoplasmic protein of 32 kDa was observed, which was subsequently identified as HO-1 by Western blotting. These studies also demonstrated that CS-induced HO-1 expression is regulated at the transcriptional level (Müller and Gebel 1994). In fact, almost all studies addressing CS-dependent alteration in the gene expression profile in vitro and in vivo revealed *hmox1* (with some remarkable exceptions, see below) as the most efficiently upregulated gene within the spectrum of antioxidant and phase II-related genes (Table 4.1). In direct agreement with this emerging biological concept, the potential physiological relevance of *hmox1* expression consequent to CS exposure recently became evident when it was found that smokers exhibiting a genetically compromised inducibility of *hmox1* show an increased susceptibility to develop emphysema (Yamada et al. 2000). Based on the paramount antioxidant nature of the enzyme products provided by the HO pathway, i.e., biliverdin and bilirubin (via ubiquitous biliverdin reductase activity), which supply the cell with a kind of SOS redox buffer against an imbalanced redox homeostasis, and apparently because of the release of CO, which has been implicated in the resolution of inflammatory responses (Otterbein et al. 2000), several signaling pathways converge on diverse *cis* regulatory elements identified upstream to the transcriptional start site of *hmox1*. In mechanistic terms, these regulatory elements are part of a complex system consisting of a promoter with a proximal enhancer (E_p) and two distal enhancers (E1 and E2), which, in the murine system, are distributed over more than 10 kb upstream of the transcriptional start site (reviewed in Alam and Cook 2003; Choi and Alam 1996). This ternary system harbors a whole array of *cis* regulatory motifs addressing numerous transcription factors known to be involved in oxidative stress and inflammatory responses, including NF-κB, AP-1, ATF/CREB, Nrf2, and heat

Table 4.1 Selection of genes differentially expressed in connection with cigarette smoke exposure in respiratory tissue samples from animal and human studies, and from one in vitro study

Antioxidant				Xenobiotics metabolism			Inflammation/ emphysema			Miscellaneous		
	I.v.	Animal	Human		Animal	Human		Animal	Human		Animal	Human
hmox1	1	2, 3, 4		*cyp1A1*	2, 3, 3k, 4	6, 7	*gro1/ cxcl1*	2		*fgfbp1*	3	
nqo1		2, 3	7	*cyp1B1*	2, 3, 3k, 4	6, 7	*c3*		7↓, 9, 10	*fgfr1*	4	
gclc		2, 3	6, 7	*cyp-2B1/2*	2↓		*cx3cl1*		7↓, 8	*gadd45 G*	2, 3	
gclm		3	6, 7	*cyp1A2*	5		*cxcl14*	3		*slc2a1*	3	
gsr		3	6	*aldh3A1*	2, 3, 3k	7	*S100A9*	3		*slc1a4*	2, 3	
txnrd1		3	6, 7	*adh7*	3	6, 7	*S100A8*	2, 3		*cdkn1A (p21)*	3, 3k, 5	
txn	1		7				*IL1R2*	2, 3		*IFi204*	3k	
prdx1		2, 3	7	*ugt1A7*	2		*IL4Rα*	3, 3k		*cdc25b*	4	
ftl1		3		*ugt2B12*	2		*IL6 st*	5		*cyclin D1*	2↓, 5	
fth1	1	2	7	*ugt1A6*	2, 3, 4		*tnfrsf7*	4				
				ugt1A2	4, 5		*cd3z*	4		*cct1*	4, 5	
gpx2		3	6, 7	*ugt1A10*		7	*ptgs2*	3, 3k		*cct3,4,6,8*	4	
gpx3			6, 10↓				*elf1*	4		*cct5*	5	
				akr1B8	3							
pgd		3	6, 7	*akr1C1*		7	*egr1*	3k	8, 9	*hspb1*	4, 5	
g6pd		2, 3	6	*akr1C2*		7	*fos*	2↓, 3k	8, 9	*hsf2*	5	
				akr1C3		6, 7				*dnajb4*	5	
sod 2		2, 4		*akr1B1*		6	*lox*	3, 3k		*dnajc1*	5	
sod3		3					*eln*	3, 3k		*dnajb9*	3	
cat1		4		*gstα4*	3		*col4a2*	3				
				gstα2	3		*spi2-2*	3		*casp7*	4	
sqstm1		3, 4		*gsto2*	3		*spi1-2*	4↓		*casp2*	5	
				gstpi2	3		*pai1*	4		*cad*	5	
map3k6		3, 3k		*gstpi1*	2, 4		*prtn3*	4				
map2k4		4		*gstA2*		6	*spi F1*		9	*ang2*	5	
epas1		3					*timp1*	2	9	*angpt1*	5	
mafF		3		*esd*	3		*muc-5AC*		7			

Antioxidant				Xenobiotics metabolism			Inflammation/ emphysema			Miscellaneous		
	I.v.	Animal	Human		Animal	Human		Animal	Human		Animal	Human
mafG		2	7	*aox1*	3		*cyr61*	3k	8	*pirin*		7
mafK	1			*pdk4*	3, 3k		*ctgf*		8	*ca12*		7
				lpl	3, 3k					*ceacam6*		7
mt1	1	3, 3k		*fmo3*	3k		*mmp10*		7↓			
mt2	1	3, 3k		*tst*	4		*mmp2*		9	*slit2*		7↓
mt1F			7↓	*mdr1*	4, 5		*mmp7*		10	*slit1*		7↓
mt1X			7↓	*taldo1*		6, 7	*ctsB1*	4	10	*gas6*		7↓
mt1G			7↓	*tkt*		6, 7	*ctsK*		9	*tu3a*		7↓

I.v. In vitro, ↓ repressed, *3k* data from nuclear factor erythroid 2-related factor 2 (Nrf2)$^{-/-}$ mice

Genes: ***aldh3A1*** aldehyde dehydrogenase family 3, subfamily A1; ***adh7*** alcohol dehydrogenase 7; ***akr*** aldo-keto reductase; ***ang2*** angiogenin-related protein; ***angpt1*** angiopoietin-1; ***aox1*** aldehyde oxidase 1; ***c3*** complement 3; ***ca12*** carbonic anhydrase XII; ***cad*** caspase-activated DNAse; ***casp*** caspase; ***cat1*** catalase 1; ***cct T***-complex protein 1; ***cd3z*** CD3 antigen; zeta polypeptide; ***cdc25b*** M-phase inducer phosphatase 2; ***cdkn1A*** cyclin-dependent kinase inhibitor 1A (P21); ***ceacam6*** carcinoembryonic antigen-related cell adhesion molecule 6; ***col4α2*** procollagen, type IV, α2; ***ctgf*** connective tissue growth factor; ***cts*** cathepsin; ***cyp*** cytochrome p450; ***cyr61*** cysteine-rich protein 61; ***cx3cl1*** chemokine (C-X3-C motif) ligand 1; ***cxcl14*** chemokine (C-X-C motif) ligand 14; ***dnaj*** DNAJ (Hsp40) homolog; ***egr1*** early growth response protein 1; ***elf1*** Ets-related transcription factor; ***eln*** tropoelastin; ***epas1*** endothelial PAS domain protein 1; ***es10*** esterase 10; ***fgfr1*** fibroblast growth factor receptor 1; ***fgfbp1*** fibroblast growth factor binding protein 1; ***fmo3*** flavin-containing monooxygenase 3; ***fos*** c-*fos* oncogene; ***fth1*** ferritin, heavy chain 1; ***ftl1*** ferritin, light chain 1; ***g6pd*** glucose-6-phosphate 1-dehydrogenase; ***gadd45G*** growth arrest and DNA-damage-inducible 45γ; ***gas6*** growth arrest-specific 6; ***gclc*** γ-glutamylcysteine synthetase (catalytic); ***gclm*** γ-glutamylcysteine synthetase (regulatory); ***gpx*** glutathione peroxidase, ***gro1/cxcl1*** chemokine (C-X-C motif) ligand 1; ***gsr*** glutathione reductase; ***gst*** glutathione *S*-transferase; ***hmox1*** heme oxygenase-1; ***hsf2*** heat shock transcription factor 2; ***hspb1*** heat shock 27-kDa protein; ***IFi204*** interferon-activated gene 204; ***IL1R2*** interleukin 1 receptor type II; ***IL4Rα*** interleukin 4 receptor α; ***IL6st*** interleukin 6 signal transducer; ***lox*** lysyl oxidase; ***lpl*** lipoprotein lipase; ***maf*** transcription factor MAF; ***map2k4*** mitogen-activated protein kinase kinase 4; ***map3k6*** mitogen-activated protein kinase kinase kinase 6; ***mdr1*** multidrug resistance protein 1; ***mmp*** matrix metalloproteinase; ***mt*** metallothionein; ***muc5AC*** mucin 5, subtypes A and C; ***nqo1*** NAD(P)H quinone oxidoreductase; ***pai1*** plasminogen activator inhibitor, serpine1; ***pdk4*** pyruvate dehydrogenase kinase, isoenzyme 4; ***pgd*** phosphogluconate dehydrogenase; ***prdx1*** peroxiredoxin 1; ***prtn3*** trypsin-chymotrypsin-related serine protease; ***ptgs2*** prostaglandin-endoperoxide synthase 2 (cyclooxygenase-2); ***S100A9*** S100 calcium-binding potein A9 (MRP-14); ***S100A8*** S100 calcium-binding protein A8 (MRP-8); ***slc*** solute carrier family; ***slit*** slit homolog (Drosophila); ***sod2*** superoxide dismutase 2 [mn]; ***sod3*** superoxide dismutase 3 (extracellular); ***spi1-2*** serine proteinase inhibitor 1-2, serpina1b; ***spi2-2*** serine proteinase inhibitor 2-2, serpina3n; ***spiF1*** serine proteinase inhibitor F1, serpinf1; ***sqstm1*** sequestosome 1; ***taldo1*** transaldolase 1; ***timp1*** tissue inhibitor of metalloproteinases 1; ***tkt*** transketolase; ***tnfrsf7*** tumor necrosis factor receptor superfamily, member 7, CD27; ***tst*** thiosulfate sulfurtransferase; ***tu3A*** tu3A protein; ***txn*** thioredoxin; ***txnrd1*** thioredoxin reductase 1; ***ugt*** UDP glycosyltransferase

References: *1* Bosio et al. (2002), *2* Gebel et al. (2004), *3* Rangasamy et al. (2004), *4* Izzotti et al. (2004a), *5* Izzotti et al. (2004b), *6* Hackett et al. (2003), *7* Spira et al. (2004b), *8* Ning et al. (2004), *9* Spira et al. (2004a), *10* Golpon et al. (2004)

shock factor-1. Current investigations in our laboratory have identified the activation of the transcription factor Nrf2 and its binding to three canonical ARE-like response elements, referred to as stress-responsive elements (StREs), present in both E1 and E2 as major (but not exclusive) contributing mechanism of the CS-induced *hmox1* expression (Knörr-Wittmann et al. 2005). These data correspond directly to recent experiments showing that CS-exposed *nrf2*$^{-/-}$ mice are prone to emphysema (Rangasamy et al. 2004), which, in turn, confirms the aforementioned observation that smokers harboring a genetic defect in the inducibility of *hmox1* are susceptible to emphysema (Yamada et al. 2000). In summary, these results clearly identify the Nrf2/*hmox1* pathway as a critical defense tool with respect to the development of CS-dependent emphysema and possibly other diseases.

Further characterization of the CS-induced *hmox1* expression in 3T3 fibroblasts (Müller and Gebel 1994) revealed that radicals produced by Fenton/Haber-Weiss chemistry, i.e., hydroxyl radicals, which are potential inducers of *hmox1* (Keyse and Tyrrell 1990) and which were formerly identified as causative principles in CS-mediated DNA strand break formation (Nakayama et al. 1985), could be excluded as a major source of activation. However, increasing the intracellular GSH content by the addition of NAC to the culture medium significantly attenuated the CS-dependent activation of *hmox1*, thus underlining once again the notion that thiol oxidation/modification plays a major role in CS-dependent stress signal initiation and gene expression.

Similar to *hmox1* expression, CS-induced activation of the c-*fos* protooncogene is not affected by inhibitors of hydroxyl radical formation produced by Fenton/Haber-Weiss chemistry but is sensitive to the exogenous addition of NAC (Müller 1995; Müller and Gebel 1998). The c-*fos* gene belongs to the family of stress-, growth-, inflammation-, and differentiation-related genes, whose protein product together with the protein encoded by the c-*jun* protooncogene forms the b-Zip transcription factor AP-1. Expression of c-*fos* represents one of the earliest measurable cellular responses to a variety of chemical and physical stimuli including growth factor treatment, exposure to tumor promoters, and oxidative stress (for review, see Karin 1995). Intriguingly, microarray-based experiments have demonstrated that upregulated expression of c-*fos* has been linked to the pathogenesis of CS-induced COPD (Ning et al. 2004; Spira et al. 2004a).

Studies on the mechanism of c-*fos* expression in 3T3 fibroblasts exposed to aqueous extracts of CS, in comparison to serum (growth factor)-stimulated cells, unraveled a complex regulation characterized by a moderate induction at the transcriptional level and paralleled by a significantly increased half life of the c-*fos* message ($t_{½}$ ≥2 h vs <20 min) (Müller 1995). These data suggest that water-soluble compounds delivered by CS interfere with the regulation of (protoonco)gene expression at at least two different regulatory levels, i.e., by promoter activation as a result of upstream stress signaling and, most probably, by interfering with enzymatic systems involved in controlling RNA turnover. For example, some crucial, growth-related mRNA species are subject to rapid turnover via distinct regulatory (AU-rich) elements present in the 3' untranslated region (3' UTR), which promote their deadenylation and eventually rapid degradation (for review, see Shim and Karin 2002).

Further investigations demonstrated that the complex mechanism of CS-dependent c-*fos* expression could be mimicked by exposing the cells to peroxynitrite, a highly reactive nitrogen species (Müller et al. 1997). Peroxynitrite, which is formed in aqueous solution from NO· and O_2^-·, was assumed to be a likely candidate in the c-*fos*-inducing mechanism because of large amounts of NO· delivered by the gas phase of CS and the formation of O_2^-· from quinone/hydroquinone-like redox systems, e.g., provided by dihy-

droxybenzenes in CS, such as catechol (Pryor and Stone 1993). Evidence for the involvement of peroxynitrite was corroborated by experiments showing that the CS-dependent activation of c-*fos* was sensitive to the presence of specific scavengers of NO· and O_2^-·, i.e., oxyhemoglobin and superoxide dismutase (SOD), respectively (Müller et al. 1997). The peroxynitrite concentration calculated as being present in aqueous solutions of CS prepared from the standard reference cigarette used in these investigations proved to be insufficient for inducing c-*fos* on its own. However, the addition of tobacco-related aldehydes, i.e., formaldehyde, acetaldehyde, and acrolein, also at CS-relevant concentrations, reestablished the effects originally seen with aqueous fractions of CS (Müller and Gebel 1998). Further mechanistic studies finally indicated that the presence of tobacco-related aldehydes significantly decreased the intracellular GSH content, which is obviously a prerequisite for peroxynitrite to interfere with crucial target molecules involved in the control of c-*fos* expression (Müller and Gebel 1998). Taken together, these data prove the complexity of the CS-dependent affects on diverse crucial cellular functions, i.e., regulation of transcriptional activity and RNA turnover, even by following the relatively simple experimental approach of exposing cells in culture to aqueous extracts of CS.

Beyond *hmox1* and c-*fos*, several other genes were identified as being induced by CS or CS-related fractions. For example, using cultured human monocytes, it was shown that aqueous extracts of CS upregulate a broad spectrum of genes coding for heat shock proteins (HSPs), including HSP70, HSP90, and HSP110, which, however, did not show any protective effect on CS-induced cytotoxicity (Pinot et al. 1997; Vayssier et al. 1998a). Surprisingly, the CS-dependent expression of *hsp* genes was not sensitive to inhibition by the addition of NAC, indicating that stress signaling pathways upstream to *hsp* expression are activated by CS-related compounds other than those involved in the CS-mediated -SH reactivity. A further interesting observation regarding CS-mediated cytotoxicity made during these investigations is the finding that the expression of the antiapoptotic protein Bcl2 was only seen in cells undergoing necrosis but not in apoptotic cells. In addition, although expression of HSP70 did not show a net effect on CS-mediated cell death, overexpression of HSP70 shifted the mechanism of cell death from necrosis to apoptosis (Vayssier et al. 1998a). However, regarding the ability of CS to induce HSP70, the data reported in these in vitro investigations (Pinot et al. 1997; Vayssier et al. 1998a) are in contrast to a report showing that CS does not induce HSP70 in vivo (Wong et al. 1995).

Finally, the CS-dependent expression of certain inflammation-related genes was also reported before gene expression could be studied on a comprehensive basis using DNA chip technology. For example, the expression and release of the chemoattractant cytokine IL-8 was reported in human bronchial epithelial cells exposed to aqueous extracts of CS in culture and confirmed by increased IL-8 concentrations detected in the bronchoalveolar fluid (BALF) of smokers (Mio et al. 1997). Interestingly, the IL-8 inducing activity of CS was partly attributable to the tobacco-related aldehydes acrolein and acetaldehyde. In the context of CS-induced expression of inflammation-related genes, it should be noted once again that CS is an inducer of the gene encoding the proinflammatory enzyme COX-2, see above.

4.4.2 CS-Induced Differential Gene Expression Studied by Gene Chip/Microarray Analysis

The deciphering of the whole human and several other genomes along with the development of highly sophisticated microarray technologies for routine application has revolutionized many areas of biological sciences, particularly the field of explorative gene expression. As a result, a steadily increasing number of manuscripts have been published reporting on the effect of CS on the human transcriptome and on the transcriptome of rodent model systems, including specific transgenic and knockout models displaying a genotype designed to provide information relevant to the elucidation of the mechanism of CS-dependent disease onset and development at the gene expression level. An attempt to provide an overall summary of the key findings reported in these manuscripts is presented in Table 4.1. However, it should be noted that the comparability of these data is limited by the different experimental approaches used, particularly with regard to the dose and type of CS (mainstream, sidestream, or a mixture of the two) and the study design (e.g., whether the exposure protocols included recovery periods or not) used for exposure, as well as with regard to the diversity of chip technologies applied (whether macroarray, cDNA microarray, or oligonucleotide-based microarray technology).

4.4.2.1 Studies in Rodent Systems

One of the first studies utilizing DNA chip technology to explore differential gene expression induced by CS was performed in murine 3T3 fibroblasts (Bosio et al. 2002). During this kinetic experiment, cells were exposed to subcytotoxic concentrations of aqueous extracts of CS for up to 24 h and profiled for differential gene expression by using a relatively small DNA chip covered with ~500 cDNA probes. As expected, the expression of antioxidant genes, such as *hmox1*, and genes encoding HSP105 and HSP90, was predominant (Bosio et al. 2002), thus confirming the data obtained from investigations on single target genes induced by CS (e.g., Müller and Gebel 1994; Pinot et al. 1997). In kinetic terms, the activation of antioxidant genes was preceded by the upregulation of genes encoding transcription factors implicated in a cellular stress response, such as members of the Jun family of transcription factors, whereas after 24 h, almost all genes returned to normal expression rates. However, the most important finding of this relatively simple approach in vitro was the discovery of an inflammation-related response, which was highlighted by the activation of *kc/gro1* (Bosio et al. 2002), the purported murine homologue of human *Il-8* (Bozic et al. 1994). The CS-dependent expression of this chemoattractant cytokine was subsequently confirmed at the protein level by a gradual increase of KC protein released by CS-exposed cells into the medium (Bosio et al. 2002). From an overall perspective, it appears important to note, that in principle, the CS-dependent signature of differential gene expression in vitro featuring a distinct antioxidant and inflammation-related response was generally observed in almost all microarray studies investigating the CS-induced differential gene expression in vivo.

Recently, in vivo rodent smoking models, including disease-prone transgenic and knockout murine lines, have been used to study the impact of CS (mainstream and sidestream CS) on the differential gene expression in cells of tissues of the respiratory tract. In one of these approaches, Sprague Dawley rats were exposed to relatively low doses

of mainstream CS (100 µg TPM/l) following an acute (3 h)- and subchronic (3 h/day, 5 days/week, 3 weeks)-exposure design, with killing of the animals either immediately or after a recovery period of 20 h after (the last) exposure (Gebel et al. 2004). The analysis of differential gene expression induced by CS in the respiratory nasal epithelium (RNE) and in the lungs using a cDNA microarray covered with >2,000 cDNA probes revealed a distinct pattern, which, in general agreement with the results obtained in vitro (Bosio et al. 2002; see above), showed a similar, pronounced antioxidant character, extended by the complexity inherent to an in vivo environment. The antioxidant response was hallmarked by the strong expression of *hmox1*, but also included phase II-related genes such as *nqo1*, *γgcs* (as represented by the catalytic subunits *gclc* and *gclm*), and several genes encoding various isozymes of UDP-glucoronosyltransferases (Table 4.1), which are all, at least in part, transcriptionally regulated by Nrf2 (Nguyen et al. 2003; see above). However, in quantitative terms, there was a striking tissue-dependent difference in the strength of CS-induced antioxidant and phase II-related gene expression, with, in principle, significantly lower expression rates observed in the lungs in comparison to the RNE (Gebel et al. 2004). An obvious explanation for this phenomenon is the deposition gradient of CS-dependent Nrf2/stress gene-inducing compounds from the upper to the lower respiratory tract, whereas tissue-specific differences in the potential inducibility of these genes represent an alternate mechanism to account for this effect.

A further intriguing observation related to the CS-dependent activation of antioxidant and phase II-related genes is the finding that genes in this category demonstrate an adaptive expression behavior during repeated exposures, as indicated by significantly (2- to 5-fold) lower induction rates seen after subchronic versus acute exposure (Gebel et al. 2004). In mechanistic terms, this effect may be explained by a steadily decreasing sensitivity of Nrf2 to become activated by CS-dependent stressors over prolonged exposure periods. Consequently, this effect may gradually compromise the ability of cells of the respiratory tract to adequately respond to CS-dependent (oxidative) stress during chronic exposure, and may therefore be critically involved in CS-dependent diseases such as emphysema (see below). Because, alternatively, the expression activity of this particular subset of genes could also be controlled by the intracellular concentration of the corresponding proteins, quantitative protein analysis and determination of the enzymatic activities are required to clarify this critical issue.

The suggested critical role of the Nrf2-controlled antioxidant and phase II-related gene response as a major defensive cellular tool in CS-dependent disease development was recently elegantly demonstrated by using *nrf2*$^{-/-}$ mice (Rangasamy et al. 2004). Exposure to an artificial CS sidestream surrogate (89% sidestream smoke and 11% mainstream smoke) for up to 6 months showed that mice with a disrupted *nrf2* gene, in comparison to their wild-type littermates, were significantly more prone to developing emphysema, both with regard to the time of disease onset as well as to the severity of the disease. This was based on a more pronounced bronchoalveolar inflammation, increased levels of oxidant DNA damage, i.e., 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OH-dG), and an increased number of apoptotic alveolar septal cells in CS-exposed *nrf2*$^{-/-}$ mice as compared with CS-exposed wild-type mice (Rangasamy et al. 2004). Using microarray analysis, ~50 genes were identified, which remained unaffected in CS-exposed *nrf2*$^{-/-}$ mice but were relatively strongly induced in CS-treated wild-type littermates, including several phase II-related genes, such as those coding for enzymes involved in GSH metabolism, protective proteins, transcription factors, and, particularly, various antioxidant genes such as *hmox1* and thioredoxin reductase (Table 4.1). Thus, these data directly correspond to the finding that smokers exhibiting a genetically compromised induc-

ibility of *hmox1* show an increased susceptibility to emphysema (Yamada et al. 2000). Scrutinizing the 5' flanking region of the transcriptional start site revealed that the vast majority of the genes presumptively controlled by Nrf2 are equipped with at least one ARE *cis* regulatory consensus sequence site (Rangasamy et al. 2004).

It should be noted, however, that a more thorough analysis of this report (Rangasamy et al. 2004) (including supplementary material) showed that some proinflammatory genes, such as *cox-2*, *cxcl14*, and *IL-1R*, were even more strongly induced in CS-exposed wild-type littermates rather than in smoke-treated *nrf2*$^{-/-}$ mice, indicating that the inflammation-related phenotype induced by CS in *nrf2*$^{-/-}$ mice is the result of a complex transcriptional network that is far from being completely understood. Nevertheless, data presented by Rangasamy et al. (2004) represent a major breakthrough in the efforts to link CS-dependent alterations in the gene expression pattern with CS-dependent disease formation, and future chronic studies using this knockout model will demonstrate whether extended exposure might also result in other CS-specific diseases.

In comparison to the distinct expression pattern—in kinetic and quantitative terms—observed for antioxidant and phase II-related genes in CS-exposed rats (Gebel et al. 2004), a completely different expression profile was obtained in the same study for CS-induced genes coding for phase I xenobiotic metabolizing enzymes. The differential expression of these genes was related to *cyp1b1*, *cyp2b1/2*, and, as described for the first time in the context of CS exposure, to the gene coding for aldehyde dehydrogenase type 3 subfamily A1 (ALDH3A1), as well as to *cyp1a1*, which in quantitative terms turned out to be the most strongly CS-dependently induced gene in CS-exposed Sprague Dawley rats, displaying an almost 200-fold induction in lung cells, (Gebel et al. 2004) (Table 4.1). Importantly, in contrast to the various antioxidant and phase II-related genes, the CS-dependent expression pattern of phase I-related genes, which are controlled by cytoplasmic receptors, such as AhR functioning through XRE (see above), does not show any indication of either a deposition gradient or an adaptive response. In this context, it is of note that, in principle, similar results for the expression of phase I-related genes were obtained in CS-exposed *nrf2*$^{-/-}$ mice, independent of the *nrf2* status (Rangasamy et al. 2004). In view of the paradoxical effects reported for AhR activation and *cyp1A1* expression (see Sect. 4.3.1), it is necessary to identify the role of these genes in the transcriptional network underlying CS-dependent disease onset and development. In this context, the identification of CS-dependent ligands resulting in the activation of, e.g., AhR, as well as the characterization of the CS-induced phenotype in *cyp1a1* knockout and *cyp1a1* overexpressing murine lines might be beneficial.

A further remarkable finding in the respiratory tract of CS-exposed rats (Gebel et al. 2004) was that both antioxidant and phase II- as well as phase I-related genes are controlled in a strict time-dependent manner. This conclusion was based on the fact that the CS-induced transcriptional changes observed immediately after exposure returned almost completely to normal in rats allowed to recover for 20 h, even after 3 weeks of repeated CS exposure. Finally, it should be stressed that subchronic exposure (3 weeks) to relatively low doses of CS is obviously not (yet) sufficient to induce inflammation-related genes. As expressed by a 4-fold upregulation of *gro1*, i.e., the gene encoding the functional rat homologue of *Il-8*, a weak proinflammatory response was only detectable in the lungs after 3 weeks of exposure, which, however, becomes strongly enhanced by the expression of a whole plethora of proinflammatory genes after extended exposure periods (Gebel et al., in preparation).

In two publications, Izzotti et al. (2004 a, b) report on the effect of CS in different specific murine model systems, using the same CS mixture as utilized by Rangasamy

et al. (2004) and cDNA macroarray technology for monitoring gene expression. In the first study, SKH-1 hairless mice were exposed for 4 weeks to light and/or CS followed by determination of differential gene expression in the skin and in the lungs. Interestingly, regarding CS exposure, apart from a common phase I response, including the upregulation of *cyp1a1*, clear differences in the pattern of upregulated genes were observed in the two different tissues. In the skin, the expression pattern was characterized by the induction of DNA repair activities aimed at addressing the repair of 8-OH-dG lesions and genes involved in cell cycle regulation, e.g., *GADD45* and *GADD153*, whereas in the lungs, activities aimed at counteracting (oxidative) stress, such as catalase 1 and *SOD2* precursor, and at activating the immune response were more prevalent (Izzotti et al. 2004a). In addition, a significant downregulation of the α-1-antitrypsin 1-2 precursor gene was observed in the lungs of CS-exposed mice, which, together with the upregulation of the neutrophil-specific genes cathepsin b (*ctsb*) and proteinase 3 (*prtn3*) (encoding a serine protease), clearly points to an emphysematous response. Although these findings demonstrate tissue-specific effects in the response to cell-stressing microenvironmental changes, it should be noted that statistically significant induction factors were usually between two and three, possibly because of the limited dynamic range provided by cDNA macroarray technology.

In the second study published by Izzotti et al. (2004b), effects of CS on the gene expression in lung tissues are described for F_1 mice produced from crossing lung tumor-prone A/J mice, a proposed surrogate animal model for CS-dependent lung tumor formation (e.g., Witschi et al. 1997), with mice carrying a dominant negative germline *p53* mutation. It was shown that lung tissue cells of the $p53^{+/-}$ offspring, when exposed for 4 weeks to CS, are compromised in upregulating key genes in executing apoptosis, such as caspase-2 precursor, whereas genes coding for growth factors (e.g., vascular endothelial growth factor [VEGF] and PDGF), growth-related transcription factors (various isozymes of PKC), and inflammatory proteins, such as MIP1α, were significantly (2- to 3-fold) induced (Izzotti et al. 2004b). However, the *p53* genotype obviously does not interfere with the CS-triggered transcriptional response of the differential expression of phase I-related genes, as exemplified by the similar high expression rates seen for *cyp1a2* (not seen in CS-exposed rats [Gebel et al. 2004] in mutant [$p53^{+/-}$] and wild-type [$p53^{+/+}$] mice [Izzotti et al. 2004b]). Thus, these results further support the outstanding role of the p53 tumor suppressor in cell cycle regulation and apoptosis induction in stressed cells and tissues in general and for CS-exposed systems in particular, even in a gene dosage-dependent context.

4.4.2.2 Studies in Humans

The first study investigating differential gene expression in smokers and nonsmokers, utilizing microarray technology, focused on differences in the antioxidant gene expression profile in tissues of the upper respiratory tract (Hackett et al. 2003). By analyzing fresh samples of airway epithelium obtained by airway brushing, 44 antioxidant genes were identified to be differentially expressed in phenotypically healthy chronic smokers (>20 pack years) and nonsmokers. Out of these 44 genes, significantly upregulated genes in smokers mainly encode proteins involved in GSH metabolism, such as γGCS, glucose metabolism, such as alcohol dehydrogenase 7 and aldo-keto reductase (also seen in CS-exposed mice [Rangasamy et al. 2004]), and redox homeostasis, including thioredoxin

reductase 1 (Hackett et al. 2003) (Table 4.1). Because the gene encoding thioredoxin was not differentially expressed, the latter result corresponds well to data from in vitro and in vivo smoking models (Gebel and Müller 2001; Gebel et al., in preparation; Rangasamy et al. 2004). Surprisingly, there was no mention of the expression of other major CS-related antioxidant target genes identified in rodent smoking models, e.g., *hmox1* and *nqo1*. Finally, a noteworthy finding made during these investigations is the high interindividual variability regarding the magnitude of the response.

The most extensive and thorough study on smoking-related differential gene expression in humans so far has been reported by Spira et al. (2004b). These authors, by also using sample material obtained from bronchoscopy, analyzed the airway transcriptome from current chronic smokers, former smokers, and never smokers, in the context of several variables, such as cumulative exposure, sex, age, and race. Employing DNA microarrays covering ~2,500 transcripts, 97 genes were identified to be significantly differentially regulated in current smokers and nonsmokers, which, according to hierarchical cluster analysis, revealed a specific signature of CS exposure.

A principal finding of this study (Spira et al. 2004b) showed that the spectrum of CS-dependently upregulated genes encoding phase I as well as phase II xenobiotics metabolizing and antioxidant enzymes is consistent with in vitro and in vivo rodent smoking models (Gebel et al. 2004 and references cited therein), thus underlining the suitability of these animal systems for investigating the toxicological effects of CS exposure at the transcriptional level. For example, the antioxidant and phase II-related gene expression in smokers covered, in addition to the several genes implicated in GSH metabolism, the upregulation of *nqo1* and thioredoxin reductase, whereas the phase I response was hallmarked by the strong induction of *cyp1b1* and, most intriguingly, of *aldh3a1* (Table 4.1). Interestingly, *cyp1a1* expression was obviously subject to strong interindividual variability, thus pointing to a high polymorphic variability. However, it is worth noting that, in contrast to the data obtained from rodent smoking models, *hmox1* is missing from the spectrum of differentially expressed genes in human smokers, which, based on the fact that the samples were derived from chronic smokers, might be explained by the adaptation phenomenon seen in rats subchronically exposed to CS (Gebel et al. 2004). Moreover, also in contrast to data from animal studies, thioredoxin was found to be slightly but significantly induced in the airway epithelium of human smokers. Most importantly, however, genes differentially expressed between smokers and never smokers also included several putative oncogenes, which were found to be upregulated in smokers, whereas, conversely, candidate tumor suppressor genes tended to be suppressed, as was also seen for genes supposed to be involved in the regulation of inflammation (Table 4.1). However, the expression of cystatin, which has been shown to correlate with tumor growth and inflammation, significantly correlated with the number of pack years, which renders this gene a potential biomarker of dose and effect.

Apart from these differences between smokers and never smokers, the work by Spira et al. (2004b) uncovered some remarkable characteristics in the gene expression profile of former smokers. For example, cluster analysis of the expression profiles of smokers with a smoking cessation period of more than 2 years were closely related to those of never smokers, whereas profiles of smokers with a smoking cessation period of less than 2 years were closely related to those of current smokers. These results show that most of the CS-specific effects induced at the transcriptional level are reversible, and, therefore, correlate with epidemiological data showing a decreasing relative risk of lung cancer formation in quitters, which is directly proportional to the period after terminating CS exposure (Peto et al. 2000). However, independent of the length of smoking cessation,

the relative risk to former smokers of developing lung cancer does not completely return to the level of never smokers (but asymptotically decreases to a relative risk of ~2 [Peto et al. 2000]), which is indicative of at least some irreversible changes induced during chronic CS exposure. Intriguingly, the expression profiles recorded from former smokers uncovered 13 genes that did not return to normal but retained the expression behavior specific for current smokers, even in those individuals who had stopped smoking for 20–30 years (Spira et al. 2004b). Most interestingly, beyond several putative oncogenes and tumor suppressor genes, this particular group of genes also includes three metallothionein genes (four metallothionein isoforms are known so far in humans), which remain decreased in former smokers. The activation of metallothionein genes, which was reported for CS-exposed model systems in vitro (Bosio et al. 2002) and in vivo (Gilks et al. 1998; Rangasamy et al. 2004; Gebel el al. in preparation), has been implicated as a transient response to any form of stress or injury providing cytoprotective action. Based on its unique zinc/cysteine thiolate coordination, which renders the complex oxidoreductive (Maret and Vallee 1998), metallothionein proteins provide antioxidant protection, particularly regarding the binding and detoxification of heavy metal ions such as cadmium. In addition, metallothionein proteins have been shown to be involved not only in cell proliferation and apoptosis, but also in pathophysiologic processes such as chemoresistance and radiotherapy resistance (for review, see Theocharis et al. 2003). Hence, because of the obvious inability to express efficiently these cytoprotective proteins, former smokers may be compromised in adequately responding to any stressing situation, whereas current smokers, lacking metallothionein expression, are at further increased risk of developing CS-related diseases.

In summary, data presented by Spira et al. (2004b) show that CS exposure activates antioxidant, metabolizing, and host defense functions in the airway epithelium of the upper respiratory tract. In general, these findings are consistent with data obtained from rodent smoking models.

Finally, in addition to the reports describing the effects of CS exposure at the transcriptional level in phenotypically healthy smokers, other publications compare the gene expression profile of diseased tissue with healthy or only mildly affected tissues in smokers with regard to emphysema (Golpon et al. 2004; Ning et al. 2004; Spira et al. 2004a) or diseased tissues in smokers and nonsmokers suffering from lung adenocarcinoma (Powell et al. 2003). Accordingly, expression data reported in these publications are mostly confined to the pathophysiologic process of disease formation and do not reflect the *direct* affect of CS or CS constituents on the gene expression profile. For example, gene expression patterns obtained from emphysematous tissues revealed the upregulation of c-*fos* and *egr1* (Ning et al. 2004; Spira et al. 2004a), which was not seen in tissue samples obtained from healthy smokers or CS-exposed wild-type animals. However, most importantly, both genes were significantly increased in emphysematous-prone *nrf2*$^{-/-}$ mice (Rangasamy et al. 2004), thus impressively corroborating the relevance of the Nrf2 pathway in the cellular defense against CS-induced stress.

4.5 Concluding Remarks

CS harbors a pronounced (oxidative) stress-inducing potential, which, based on its complex composition, affects exposed cells in a pleiotropic way. Hence, CS-caused effects are reported on small biomolecules, proteins, DNA, higher organized complex structures

(such as the cytoskeleton and membranes), and organelles (such as mitochondria and the ER), thereby affecting all tissue and cellular compartments from the extracellular matrix to the nucleus. Moreover, CS-specific effects are *direct*, e.g., as reflected by damage inflicted on proteins, membrane lipids, or DNA, as well as *indirect*, e.g., resulting from imbalance of redox homeostasis or signaling activities. It can be assumed that the sum of these effects determines the pathobiologic activity of CS and, according to improved medicinal and extended (molecular) epidemiological research, it is not surprising that the number of diseases linked to CS exposure has considerably increased (US Department of Health and Human Services 2004). However, it is also obvious that because of the chemical complexity inherent in CS, any mode-of-action analysis must be limited. This dilemma is further intensified by the lack of suitable animal model systems, especially with regard to CS-induced lung cancer formation.

Nevertheless, research over the past decades, profiting from immense progress in genome research and the development of sophisticated technical tools, has unraveled some principal mechanisms that provide a first insight into the biological activities of CS at the subcellular level. For example, previous and recent experiments have demonstrated over and over again that the -SH reactivity inherent to CS, which is characteristic to a large extent for components of both the gas phase and the particulate phase, plays a major role in interfering with crucial cellular and tissue functions. In fact, cells are particularly sensitive to this xenobiotic activity, because myriad cellular functions are controlled by functional -SH groups presented by critical protein cysteine residues. Hence, with the development of DNA microarray technologies and by using techniques to selectively knockout or silence specific genes, the Nrf2 pathway, which is controlled via the -SH-sensitive cytosolic inhibitor Keap1, was identified to be a major target of CS exposure (Knörr-Wittmann et al. 2005) and could be directly linked to a CS-related disease phenotype, i.e., emphysema (Rangasamy et al. 2004). Clearly, this experimental approach, which would be even more improved by integrating proteomics research tools, may be paradigmatic for the investigation of other CS-related pathophysiologic effects, for example, to elucidate the yet unsolved issue of how the CS-induced phase I response affects CS-dependent lung tumor formation. Finally, using this paradigmatic approach, we should be able to define specific biomarkers of disease by scrutinizing gene expression profiles using bioinformatics tools.

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Chapter 5

Redox Effects of Cigarette Smoke in Lung Inflammation

5

Irfan Rahman

Contents

Contents

5.1 Introduction

A multitude of lung diseases such as chronic bronchitis, emphysema, bronchiolitis and small airway diseases has been found to be a direct or indirect consequence of cigarette smoking. These are now classified collectively under chronic obstructive pulmonary

disease (COPD), characterized by airflow limitation that is not reversible and usually progressive. Cigarette smoking is also associated with impairment in the pulmonary immune function, pulmonary infections, and bronchooncogenesis. Cigarette smoking also initiates such lung inflammatory responses through a wide variety of mechanisms such as the activation of redox-sensitive transcription factors such as nuclear factor-κB (NF-κB) and activator protein 1 (AP-1), signal transduction (activation of mitogen-activated protein kinase [MAPK] pathways, phosphoinositide 3 [PI-3-kinase, PI-3K] and PI-3K-activated serine–threonine kinase, Akt, and chromatin modeling (histone acetylation/deacetylation), leading to gene expression of proinflammatory mediators (Rahman et al. 1998; (Fig. 5.1).

Cigarette smoke is a complex mixture of various noxious gases and condensed tar particles. These components elicit oxidative stress in lungs by continuous generation of reactive oxygen species (ROS) and various other inflammatory mediators (Chapter 1). Cigarette smoke exposure alters redox glutathione status, and causes oxidation of proteins, DNA, and lipids, which may cause direct lung injury or induce a variety of cellular responses through the generation of secondary metabolically reactive species. Cigarette smoke may induce alterations both within and outside the lung cells in the form of remodeling of the extracellular matrix, mucus hypersecretion, plasma exudation/epithelial permeability, cell death, mitochondrial respiration, cell proliferation, maintenance of surfactant and the antiprotease screen, effective alveolar repair response, and immune modulation in the lung (Gutteridge et al. 2000; Rahman et al. 2000; Richer et al. 1995).

This chapter encompasses the redox effects of cigarette smoke-mediated oxidative stress in lung inflammation, the redox molecular mechanisms of gene expression (cell signaling and chromatin remodeling), and pathophysiologic consequences of oxidants and altered redox balance in these conditions.

5.2 Cigarette Smoke and Oxidative Stress

5.2.1 Composition of Cigarette Smoke

Cigarette smoke contains many potent oxidants, carcinogens/mutagens, and chemicals that stand out as major risk factors for the development of COPD and lung cancer (Rahman and MacNee 1996a, 1999). Mainstream cigarette smoke is a complex mixture of over 5,000 chemical compounds, including high concentrations of oxidants (10^{17} per puff) (Church and Pryor 1985; Pryor and Stone 1993). The aqueous phase of the cigarette smoke condensate (CSC) may undergo redox recycling (because of the presence of free iron) for a considerable time in epithelial lining fluid (ELF) of smokers (Nakayama et al. 1989; Zang et al. 1995). The tar phase of cigarette contains a high concentration of radicals (10^{17} spins per gram), which are relatively stable, e.g., semiquinone radical. The tar phase is also an effective metal chelator and can bind iron (released from the activated macrophages) to produce tar semiquinone and tar Fe^{2+}, which can generate millimolar levels of H_2O_2 for at least 24 h (Nakayama et al. 1989; Zang et al. 1995). Sidestream cigarette smoke contains more than 10^{15} reactive organic compounds per puff, and comprises carbon monoxide, ammonia, formaldehyde, *N*-nitrosamines, benzo[*a*]pyrene, benzene, isoprene, ethane, pentane, nicotine, acrolein, acetaldehyde, and other genotoxic and carcinogenic organic compounds.

5.2.2 Oxidants in the Cigarette Smoke

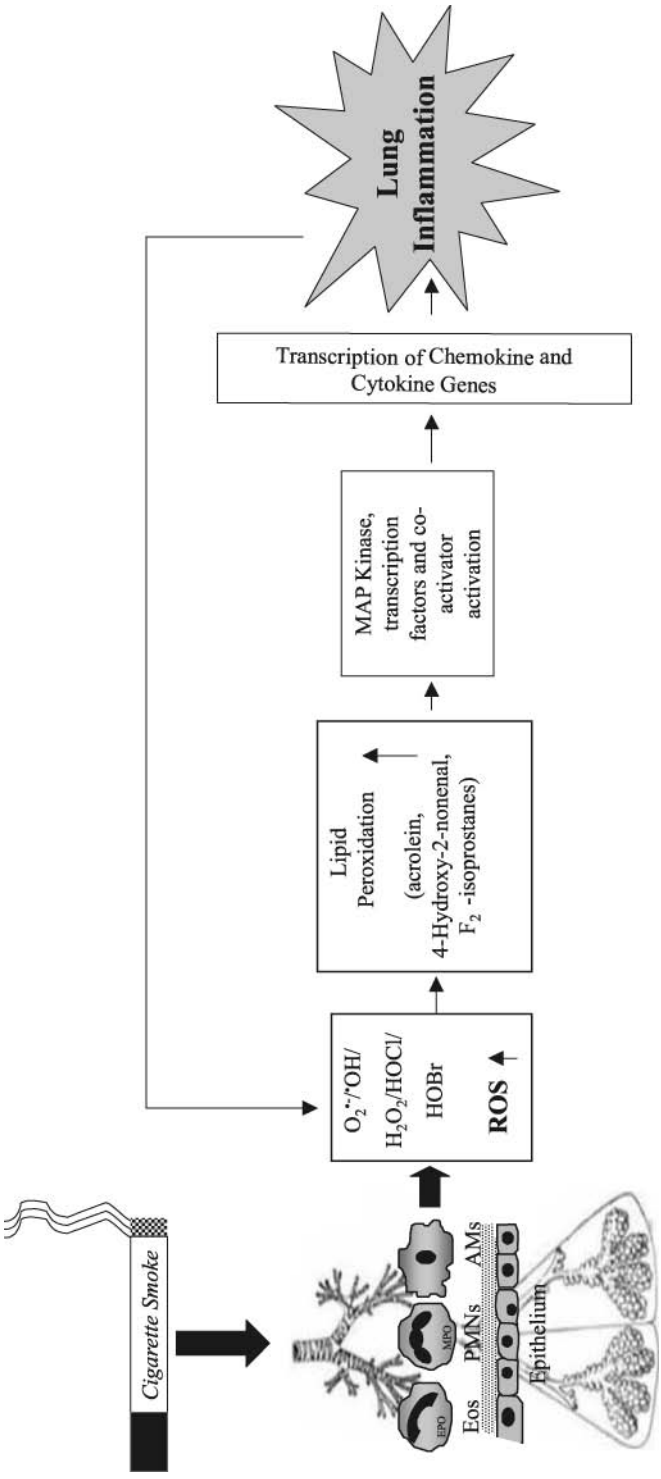
Short-lived oxidants, such as superoxide anion ($O_2^{-}\cdot$) and nitric oxide (NO·), are predominantly found in the gas phase of cigarette smoke. NO· and $O_2^{-}\cdot$ immediately react to form the highly reactive and toxic peroxynitrite ($ONOO^{-}$) molecule. The semiquinone radicals in the tar phase of cigarette can reduce oxygen to produce ROS, such as $O_2^{-}\cdot$, ·OH and H_2O_2 (Pryor and Stone 1993). Oxidants present in cigarette smoke can further augment alveolar macrophage production of ROS and a host of other mediators, some of which are chemotactic and recruit neutrophils and other inflammatory cells into the lungs. Both neutrophils and macrophages, which are known to migrate in increased numbers into the lungs of cigarette smokers, compared with nonsmokers, can generate ROS via the activation of NADPH oxidases (Bosken et. al. 1992; Chan-Yeung 1988; Chan-Yeung and Dybuncio 1984; Muns et. al. 1995; Rahman and MacNee 1996a; Rahman et. al. 1996b, 1997; Richards et. al. 1989; Van Antwerpen et al. 1995).

5.3 ROS-Mediated Lipid Peroxidation Products and Their Role in Biochemical Processes

5.3.1 Membrane Lipid Peroxidation

$O_2^{-}\cdot$ and ·OH generated and released by activated immune and inflammatory cells are highly cytotoxic and when generated in close proximity to a cell, oxidize membrane phospholipids (lipid peroxidation), which may initiate a chain reaction. A direct result of such a process is the generation of toxic downstream lipid peroxidation products, such as malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE), acrolein, and F_2-isoprostanes (Fig. 5.2). Some of these products, e.g., 4-HNE and F_2-isoprostanes, may be involved in certain signaling processes in a given cell (Uchida et al. 1999). The peroxidative alteration of polyunsaturated fatty acids severely impairs functions of the membrane, membrane-bound receptors, enzymes, and receptor/enzyme translocation. In addition, there is increased tissue permeability because of loss of membrane fluidity, which has been implicated in the etiogenesis of diverse lung injuries (Morrison et al. 1999; Rahman and MacNee 1996a). Endogenous generation of aldehydes because of lipid peroxidation has been found to underlie many of the pathophysiologic events associated with oxidative stress in cells and tissues (Gutteridge 1995). Lipid peroxidation may also have a role in the signaling events in the molecular mechanisms involved in the lung inflammation observed in COPD.

Fig. 5.1 Mechanisms of reactive oxygen species (*ROS*)-mediated lung inflammation in chronic obstructive pulmonary disease (COPD). Inflammatory response is mediated by oxidants either inhaled and/or released by the activated neutrophils, alveolar macrophages, eosinophils, and epithelial cells, leading to production of ROS and membrane lipid peroxidation. Activation of transcription of the proinflammatory cytokine and chemokine genes, upregulation of adhesion molecules, and increased release of proinflammatory mediators are also involved in the inflammatory responses in patients with COPD


Lung Inflammation
Transcription of Chemokine and Cytokine Genes
MAP Kinase, transcription factors and co-activator activation
Lipid Peroxidation (acrolein, 4-Hydroxy-2-nonenal, F_2 -isoprostanes)
$O_2^{\bullet-}$/$^{\bullet}OH$/ H_2O_2/HOCl/ HOBr
ROS
Cigarette Smoke
MPO
EPO
Eos
PMNs
AMs
Epithelium

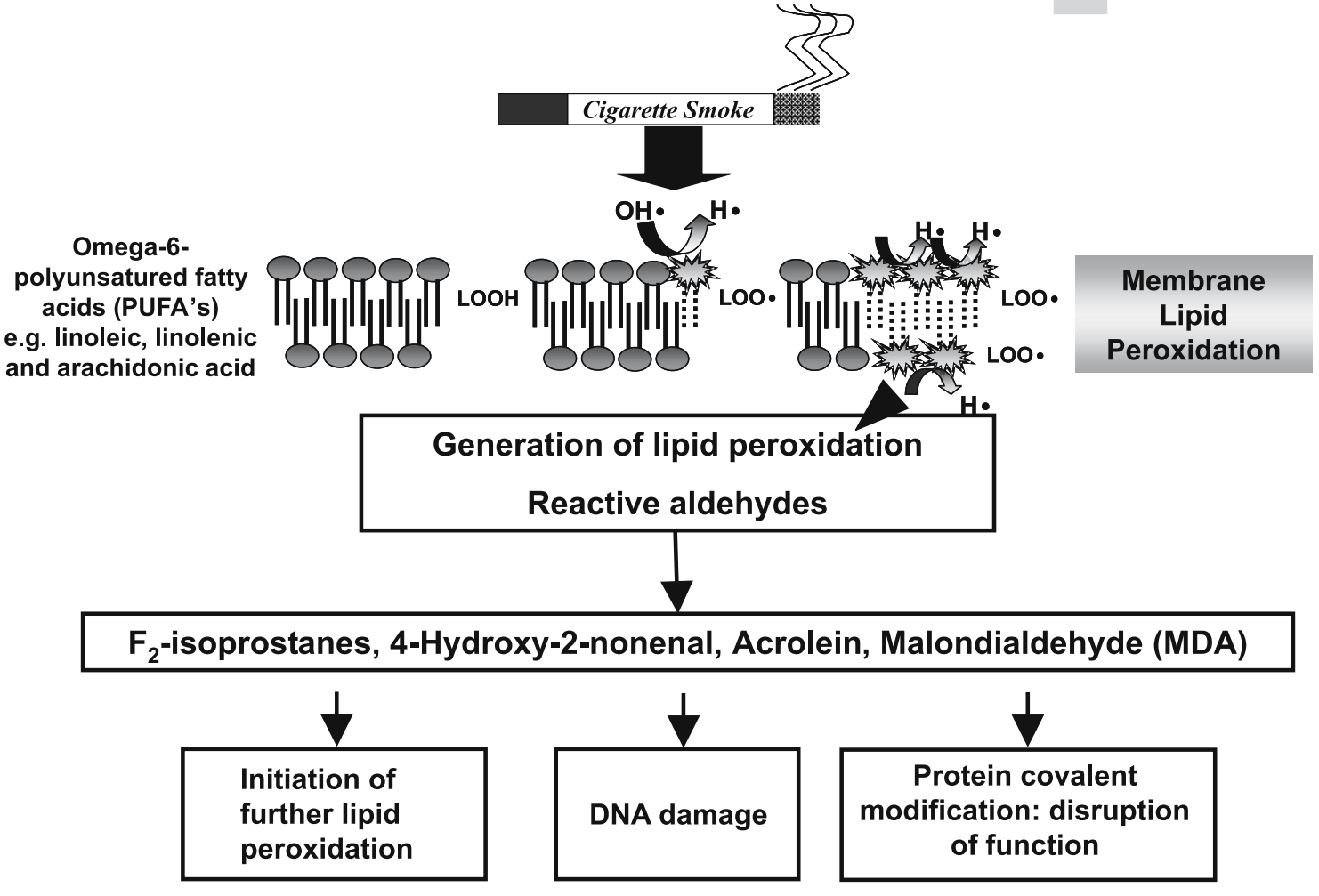

Cigarette Smoke
OH•
H•
Omega-6-polyunsatured fatty acids (PUFA's) e.g. linoleic, linolenic and arachidonic acid
LOOH
LOO•
H•
H•
LOO•
LOO•
H•
Membrane Lipid Peroxidation
Generation of lipid peroxidation
Reactive aldehydes
F2-isoprostanes, 4-Hydroxy-2-nonenal, Acrolein, Malondialdehyde (MDA)
Initiation of further lipid peroxidation
DNA damage
Protein covalent modification: disruption of function

Fig 5.2 Mechanism of membrane lipid peroxidation of polyunsaturated fatty acids by cigarette smoke, leading to generation of various reactive aldehydes. The reactive aldehydes triggers several redox signaling events

5.3.1.1 F_2-Isoprostanes

Despite circumstantial data available from in vitro studies suggesting that cigarette smoke may cause oxidative injury, whether this process occurs in vivo has been controversial (Harats et al. 1989). This controversy could largely be attributed to the fact that most methods previously available to assess oxidative stress in humans have been inaccurate and unreliable (Gutteridge and Halliwell 1990). Morrow et al. (1990) then reported a series of bioactive prostaglandin F_2-like compounds (termed F_2-isoprostanes) that are produced as a result of peroxidation of arachidonic acid catalyzed by free radicals and were found to be independent of the cyclooxygenase enzyme in humans. F_2-isoprostanes are initially formed in situ on phospholipids and are subsequently released preformed (Morrow et al. 1992). The level of F_2-isoprostanes is almost two-fold greater in magnitude as compared with cyclooxygenase-derived prostanoids in normal human biological fluids. In addition, a dramatic increase in the levels of F_2-isoprostanes has been observed in the circulation and in association with tissue phospholipids in animal models of oxidant injury.

F_2-isoprostanes are potent smooth muscle constrictors and mitogens. They modulate platelet as well as other cell functions in vitro via membrane receptors (thromboxane A_2) for prostaglandins (Lawson et al. 1999; Morrow and Roberts 1997). The levels of lipid peroxidation end products, such as F_2-isoprostanes and the hydrocarbons ethane and pentane, are increased in exhaled air condensate in smokers and in patients with COPD (Euler et al. 1996; Montuschi et al. 2000; Paredi et al. 2000).

5.3.1.2 4-Hydroxy-2-nonenal

4-HNE is a highly reactive diffusible end product of lipid peroxidation. Increased production of 4-HNE has been associated with several pathological conditions (Grune et al. 1997) and oxidant challenge (Bedossa et al. 1994; Kruman et al. 1997; Mark et al. 1997). In view of an increased 4-HNE production during oxidant challenge, it has been suggested that 4-HNE might act as a mediator in the induction of gene expression by oxidants. A recent screening of oxidized fatty acids in RAW264.7 cells has revealed that 4-HNE might be a major inflammatory mediator in development and progression of atherogenesis (Kumagai et al. 2004). 4-HNE was found to induce the cyclooxygenase 2 (*COX-2*) genes in these cells, thus reflecting the potential role of 4-HNE as perpetrator of inflammation. Cigarette smoke increases phosphorylation of ERK1/2 and nuclear translocation of p50 and p65 subunits of NF-κB, which are important elements involved in COX-2 expression and hence, prostaglandin E2 (PGE2) formation (Martey et al. 2004). Such an inflammatory condition may be an initial step toward epithelial cell transformation. Furthermore, exogenous micromolar 4-HNE increases the expression of several genes, e.g., *HO-1*, collagen a1(I) and aldose reductase, (Basu-Modak et al. 1996; Bedossa

et al. 1994; Parola et al. 1993; Spycher et al. 1997). The above results suggest that 4-HNE is involved in the regulation of gene expression even under physiological conditions.

4-HNE also serves as a chemoattractant for neutrophils both in vitro and in vivo (Schaur et al. 1994). In addition, 4-HNE has been reported to have chemotactic, cytotoxic, and immunogenic properties (Steinerova et al. 2001), and these effects were achieved in vitro with 4-HNE concentrations as low as 2.5 μ*M* (Muller et al. 1996). Recent findings from the authors' laboratory indicate increased 4-HNE-modified protein levels in airway and alveolar epithelial cells, endothelial cells, and neutrophils in subjects with airway obstruction, compared with subjects without airway obstruction (Rahman et al. 2002a). This is indicative of not only a definite generation of 4-HNE in COPD subjects, but also corroborates the observation of an increased levels of 4-HNE-modified proteins in lung cells in such subjects. On the physiological front, increased level of 4-HNE-adducts in alveolar epithelium, airway endothelium, and neutrophils was found to be inversely correlated with forced expiratory volume in one second, (FEV_1), further suggesting a role for 4-HNE in the pathogenesis of COPD. 4-HNE is known to induce/regulate various cellular events especially proliferation, apoptosis and activation of signaling pathways (Parola et al. 1999; Uchida et al. 1999). 4-HNE has a high affinity toward cysteine, histidine, and lysine residues; alters protein function; and forms direct protein adducts. An important outcome of 4-HNE generation is its interaction with the important thiol antioxidant glutathione (GSH) (Tjalkens et al. 1999). The conjugation of 4-HNE with GSH might be one of the important mechanisms whereby a cell may lose its antioxidant prowess, leading to oxidative stress. Interestingly, increased formation of 4-HNE has also been reported to induce expression of the glutamate cysteine ligase (*GCL*) gene, which increases synthesis of GSH (Rui-Ming et al. 1998). This might be an important cellular antioxidant adaptation during oxidative stress. Inhibition of lipid peroxidation, specifically the pathways leading to the production of 4-HNE and F_2-isoprostane, may therefore be a target for antioxidant therapy in inflammation and injury in patients with COPD.

ROS-mediated lipid peroxidation has been shown to be involved in epithelial remodeling during lung injury (Gutteridge and Halliwell 2000). The levels of 4-HNE adducts are increased in epithelial cells in patients with COPD, which may be of relevance for the understanding of epithelial changes in this disease. In addition to its ability to increase the expression of proinflammatory mediators, 4-HNE has also been shown to induce apoptosis in T cells (Liu et al. 2000) and cause activation of the epidermal growth factor receptor (EGFR) in human epidermoid carcinoma cells, which results in growth inhibition (Liu et al. 1999). It has recently been shown that 4-HNE also inactivates proteases such as cathepsin B (Crabb et al. 2002), various thiol antioxidant enzymes such as glutathione peroxidase, and key mitochondrial enzymes (Euler et al. 1996; Humphries and Szweda 1998). This may be because of the high affinity of 4-HNE for the sulfhydryl groups present within the active sites of these enzymes.

5.3.1.3 Acrolein

Acrolein (2-propenal) is another thiol reactive, α,$\tilde{\beta}$(beta)-unsaturated aldehyde, which is derived from various environmental sources and combustion of organic systems such as cigarette smoke (Lagrue et al. 1993). Acrolein has a very high affinity for nucleophilic targets, e.g., sulfhydryl groups. Inhalation of acrolein is known to induce changes in rat

lung structure and function. Previous studies have shown that acrolein exposure depletes glutathione and inhibits the activity of various glutathione redox system enzymes in the nasal mucosa of rats (Cassee et al. 1996; Lam et al. 1985) and in alveolar A549 epithelial cells in vitro (Kehrer and Biswal 1999). Acrolein inhibits the activity of redox-sensitive transcription factors such as NF-κB and AP-1 by virtue of inducing thiol imbalance as well as covalent modification of cysteine. In a study conducted using alveolar epithelial cells A549, Horton et al. (1999) have demonstrated that acrolein may decrease NF-κB activity by a mechanism independent of I-κB, which normally regulates NF-κB activity.

Exposure of human type II lung epithelial (A549) cells to a nonlethal dose of acrolein (150 fmol/cell for 1 h) depletes 80% of intracellular GSH and increases the transcription of GCL after 6–12 h posttreatment, as an adaptive feedback for replenishing the GSH to normal level. Acrolein exposure of 2 ppm to rats causes bronchioles to be filled with desquamized cells along with isolated peribronchial monocytes (Arumugam et al. 1999). Exposure to acrolein has been shown to reduce ciliary beat frequency in cultured bovine bronchial epithelial cells. In vitro exposure of bovine tracheal epithelial cells to acrolein caused increased release of a series of eicosonoids, such as PGE_2, $PGF_{2\alpha}$, etc. (Cassee et al. 1996; Kehrer and Biswal 1999). Alkylating agents, including acrolein, are versatile mutagens and/or carcinogens because they can react with a variety of nucleophilic sites in DNA, forming adducts with DNA bases (Arumugam et al. 1999). In general, acrolein treatment activates phase II genes transcription as evident by an increase in mRNA for NAD(P)H:quinone oxidoreductase (NQO1). Western blot analysis revealed increased level of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) in the nuclear extract from acrolein-treated cells, and a human NQO1-human antioxidant-response element (ARE) reporter assay has confirmed the involvement of Nrf2 in ARE-mediated transcriptional activation in response to acrolein. Furthermore, increased binding of nuclear proteins to the ARE consensus sequence has been reported in cells treated with acrolein. Enhanced phase II enzyme gene expression by acrolein may form the basis of resistance against cell death and can have implications in cigarette smoke-related lung carcinogenesis. In human lung type II epithelial (A549) cells, acrolein has been shown to induce transcriptional induction of phase II genes by activation of Nrf2. The importance of Nrf2 can be gauged from a recent report by Rangasamy et al. (2004), where they have shown that disruption of the *Nrf2* gene in mice leads to early and more intense emphysema in response to cigarette smoke. In the same study, they have shown that the expression of nearly 50 antioxidant and cytoprotective genes in the lungs may be transcriptionally controlled by Nrf2, and all genes may work in concert to overcome the effects of cigarette smoke. Very recently, Pagnin et al. (2005) have reported that acrolein could inhibit inflammatory responses in the human bronchial epithelial cell line (HBE1) by decreasing interleukin 8 (IL-8) generation via direct or indirect modulation of NF-κB activity.

5.4 GSH and Cellular Redox Regulation

5.4.1 GSH Metabolism

Evidence is rapidly accumulating as to the importance of intracellular redox environment in maintaining proper cellular homeostasis and function. The cells, in particular the lungs, have evolved elaborate mechanisms that ensure proper balance between the

prooxidant and antioxidant molecules as defense against constant oxidative challenge. GSH is the most important non–protein sulfhydryl in the cells and plays a key role in the maintenance of the cellular redox status. The redox potential is defined as the ratio of the concentration of oxidizing equivalents to that of reducing equivalents (Forman and Dickinson 2003). Two major redox forms of GSH has been identified in the cells, i.e., reduced GSH (GSH) and oxidized GSH (GSSG) or GSH disulfide. Recently, the two forms were implicated in a range of cellular processes such as cellular signaling, gene expression, and apoptosis (Rahman et al. 2005). GSSG represents a small fraction (1/10th) of the total GSH pool. The normal GSH content of a cell ranges from 1 m*M* to 10 m*M* and is a function of the balance between its depletion and synthesis. It is imperative for a cell to maintain this level of GSH for normal functioning. Cells can either excrete GSSG or reduce it back to GSH at the expense of NADPH, the reaction being catalyzed by GSH reductase (GR). However, de novo synthesis of GSH from its amino acid constituents is essential for the elevation of GSH that occurs as an adaptive response to oxidative stress. GSH synthesis involves two enzymatic steps catalyzed by GCL (formerly called as γ-glutamylcysteine synthetase) and glutathione synthetase (Huang et al. 1993). The enzyme GCL is the rate-limiting component of GSH synthesis (Huang et al. 1993). The de novo rate modulation of GSH synthesis is also determined by the cellular levels of the amino acid cysteine. The plasma membrane ectoenzyme γ-glutamyl transpeptidase (γ-GT) plays an important role in the supply of cysteine by hydrolytic release of this amino acid from cysteine-linked sources. γ-GT is the only enzyme that can break the γ-linkage found in GSH and GSH conjugates and release cysteine. It metabolizes the extracellular GSH and preferentially forms γ-glutamylcysteine, which is taken up by cells, bypassing its production by GCL. GCL is composed of a heterodimer containing a 73-kDa heavy catalytic subunit (GCLC) and a 30-kDa light modifying subunit (GCLM) (Huang et al. 1993) (Fig. 5.3). Although the heavy subunit contains all of the catalytic activity, the association of the heavy subunit with the regulatory light subunit can modulate GCL activity. The ratio of the two subunits for physiological function has long been assumed to be 1:1; however, in tissues the ratio varies significantly, and usually GCLC:GCLM is significantly greater than 1:1 (Krzywanski et al. 2004). GCL is regulated by GSH through feedback inhibition. 4-HNE has a high affinity toward cysteine, histidine, and lysine residues; alters protein function; and forms direct protein adducts. As stated earlier, 4-HNE can conjugate with GSH (Tjalkens et al. 1999). The conjugation of 4-HNE with GSH might an important mechanism whereby GSH depletion may occur during oxidative stress. Interestingly, increased formation of 4-HNE has also been reported to induce expression of the *GCL* gene, which increases synthesis of GSH (Rui-Ming et al. 1998) This might be an important cellular antioxidant adaptation during oxidative stress.

5.4.2 GSH and Oxidative Stress

The GSSG/2GSH ratio can serve as a good indicator of the cellular redox state (Park et al. 1998). This ratio in GSH parlance may be determined by the rates of H_2O_2 reduction by glutathione peroxidases (GPx) and GSSG reduction by GSH reductase. Thus, antioxidant enzymes play a critical role in the maintenance of the cellular reductive potential. Several enzymes/proteins involved in the redox system of the cell and their genes such as *MnSOD*, *GCLC*, *GPx*, thioredoxin reductase, and metallothionein are induced by modu-

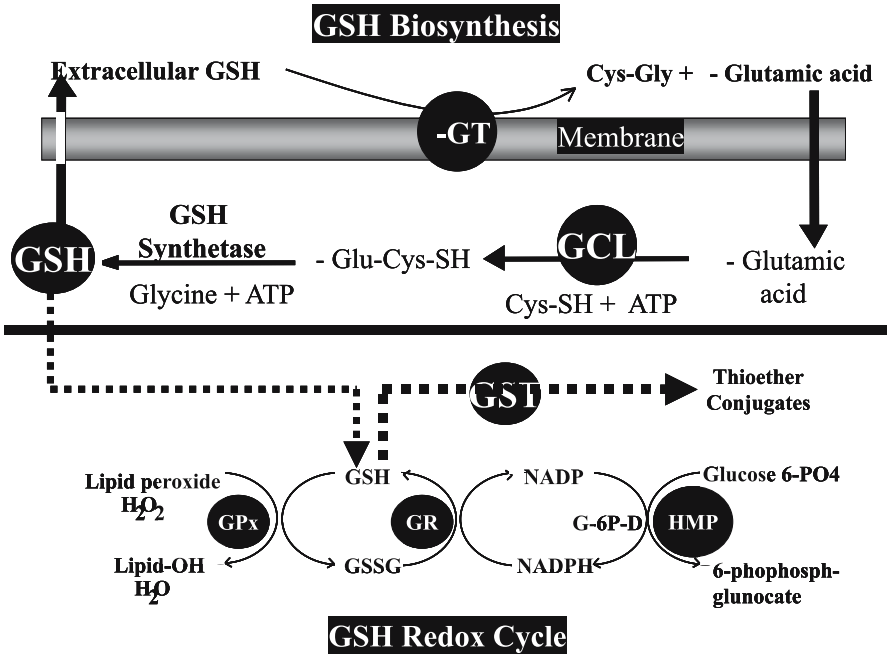


Fig. 5.3 Glutathione (*GSH*) biosynthesis and its redox cycle in lung cells. The steps involved in de novo GSH synthesis and breakdown of extracellular GSH are shown. GSH converts hydrogen and lipid peroxides to nontoxic hydroxy fatty acids and/or water. Glutathione disulfide (GSSG) is subsequently reduced to GSH in the presence of NADPH and glutathione reductase, which are linked with a hexose monophosphate shunt

lation of cellular GSH/GSSG levels in response to various oxidative stresses, including hyperoxia and inflammatory mediators such as tumor necrosis factor-α (TNF-α) and lipopolysaccharide in lung cells (Cotgreave and Gerdes 1998; Das 2001; Das et al. 1995). Intracellular redox status of lung epithelial cells has been shown to be a critical factor in determining cell susceptibility or tolerance to oxidative insults. It has been shown that GSH depletion because of GCL inhibition by buthionine sulfoximine (BSO) sensitizes both A549 and 16-HBE cells to the injurious effects of hyperoxia and H_2O_2, resulting in an increased membrane permeability and activation of NF-κB (Rahman et al. 2001). In contrast, pretreatment of these cell lines with hyperoxia before H_2O_2 exposure protects against the cytotoxic effects of H_2O_2 as well as preventing NF-κB activation. These protective effects were because of an adaptive increase in GSH in response to pretreatment with hyperoxia. Therefore, modulation of intracellular GSH can determine the course of tolerance to subsequent oxidant exposure.

The relationship to redox signaling is provided by the production of GSSG during the enzymatic reduction of hydroperoxides or $ONOO^-$ by GSH peroxidases. Normally, GSSG represents less than 1% of the total GSH pool. When H_2O_2 or $ONOO^-$ is transiently elevated, an elevation in GSSG, also transient, can occur, providing a possible

mechanism for signaling by means of thiol–disulfide exchange. In this scenario, signaling is indirectly dependent on ROS generation. Nonetheless, as this mechanism requires a change in GSSG that is usually only observed during oxidative stress, such signaling is more likely an oxidative stress response rather than physiologic redox signaling. Interesting to note is a recent report wherein it was shown that the sputum levels of GSSG and nitrosothiols are elevated in COPD subjects; the increase was associated with neutrophilic inflammation (Beeh et al. 2004). Therefore, increased GSSG in the sputum may serve as a marker of oxidative stress in lung diseases.

5

5.4.3 Protein-Thiol Alterations: a Novel Redox Signaling Mechanism and Adaptive Stress Response

5.4.3.1 Protein-Thiol Oxidation

The first evidence that cells may resist oxidative stress via protein thiolation was provided by Dominici and his coworkers (1999). In an elegant study, they showed *S*-glutathiolation of γ-GT on the surface of U937 lymphoma cells in response to oxidative stress. Initially, an *S*-glutathiolation-dependent loss of activity was observed for the enzyme wherein the free surface thiols of the enzyme were *S*-glutathiolated and may be correlated with a concurrent generation of H_2O_2, which is a by-product of γ-GT function. Reversible posttranslational modification of specific amino acid residues on proteins have now been identified as one of the important regulatory mechanism of protein function. Proteins bearing cysteine-thiol (SH) (Cys–SH) residues in the thiolate form (S^-) are considered prone to oxidative modification. Oxidation of protein-Cys-SH (PSH) may interfere with biological functions either as "damage" or in context to oxidant-dependent signal transduction. Although PSH behave like non–protein thiols, their biochemistry is much complicated by their accessibility, steric interference, and charge distribution (Di Simplicio et al. 2003). The response of PSH and their reaction mechanisms vary depending on the source of the PSH, and are influenced by the pK_a, disulfide susceptibility/accessibility to oxidants, and the conformation of the protein at a given time. Oxidative stress can be generated as a result of excessive ROS and or reactive nitrogen species (RNS) production. It is to be noted that NO· may be scavenged with a variety of ROS to form a range of RNS, which may lead to enhanced nitration of protein tyrosine in the lungs and hence, may play a pivotal role in airway inflammation (reviewed in Reynaert et al. 2005). Initially, oxidative thiolation of PSH was recognized as an aftermath of oxidative stress, but recent evidence suggests that such transformations may be of greater biochemical consequence, both as a protective and a signaling mechanism. Therefore, protein *S*-thiolation and protein *S*-nitrosation have emerged as a novel area of oxidative biochemistry. A great number of cells have now been recognized to respond to oxidative stress by *S*-thiolation and *S*-nitrosation. Although the major mediator of protein thiolation is the thiol antioxidant GSH (which promotes *S*-glutathiolation), its catabolite-dependent metal reduction has recently been identified to act as a prooxidant capable of modulating redox balance, signal transduction pathways, and transcription factors (Paolicchi et al. 2002). It has been hypothesized that GSH may also be involved as a physiological buffer for controlling cellular oxygen tension (Del Corso et al. 2002).

In a community-based study, cigarette smokers were found to have about 20% more glutathiolated proteins in their plasma, compared with nonsmokers (Muscat et al. 2004). Therefore, *S*-thiolation measurement has been hypothesized to be a prognostic marker for cigarette smoke-dependent oxidative stress. Similarly, cigarette smoking was associated with oxidation of GSH and cysteine in human plasma in smokers of ages 40–85 years, compared with nonsmokers (Moriarty et al. 2003). Cigarette smoke extract causes increased formation of *S*-adenosylmethionine and cystathionine by the transulfuration pathway in A549 lung epithelial cells (Panayiotidis et al. 2004). Analogous to ROS-dependent oxidative stress, Hausladen and Stamler (1999) have coined the term nitrosative stress for RNS-mediated oxidative stress. The excessive production of ROS or RNS or the failure of a cell's defense and repair mechanisms lead to a condition known as oxidative or nitrosative stress. Such a situation may lead to an irreversible loss or a reversible modulation of a protein function.

5.4.3.2 Cysteine Sulfoxidation as Protein Function Modulator

Cysteine thiolates ($Cys\text{-}S^-$) but not cysteine thiol (Cys-SH) can be readily oxidized to a sulfenic acid (-SOH), which is a relatively reactive form that can quickly form a disulfide with a nearby thiol. Strong oxidants will oxidize either $Cys\text{-}S^-$ or Cys-SH to sulfinic ($Cys\text{-}SO_2H$) and/or sulfonic ($Cys\text{-}SO_3H$) acid derivatives (Claiborne et al. 1999). This difference in the generation of a particular cysteine thiol species provides a basis for distinguishing redox signaling from oxidative stress. Whereas oxidative stress generally involves nonspecific oxidation of wide variety molecules, redox signaling is gradually being recognized to involve oxidation of those cysteines that are located in an environment promoting dissociation of thiols. The higher oxidation states in the form of sulfinic and sulfonic derivatives have essentially been considered as irreversible modifications under biologically relevant conditions and associated with oxidative injury. On the other hand, protein-cysteine-sulfenic acids are unstable and may be further oxidized to sulfinic or sulfinic species or scavenged by GSH or vicinal thiols to form intramolecular disulfides or mixed disulfides (Claiborne et al. 1993). Therefore, it is evident that cysteine may be recycled between a reduced (Cys-SH) and its oxidized forms (sulfinate, sulfinic, or sulfinic derivatives). This transition between the reduced and oxidized forms by itself may represent a regulatory mechanism of protein function. Most noteworthy examples of such a regulation are the ROS-dependent sulfinic acid formation-dependent inhibition of tyrosine phosphatase-IB (PTP-IB) and modulation of insulin receptor kinase activity (Denu et al. 1998; Lee et al. 1998). Recently GSSG reductase, cathepsin *S*-nitrosoglutathione (GSNO) and other NO· donors, and glyceraldehydes-3-P-dehydrogenase (GAPDH) have been identified as potential inducers of sulfinate species formation. Poole et al. (2004) have considered the idea that Cys-sulfenic acid might have an important role in the catalytic centers of the respective enzymes. These workers further suggest that Cys-sulfenates might be useful as sensors of both oxidative and nitrosative stress that affect enzymes and transcriptional regulators. Because the formation of sulfenic, sulfinic, or sulfonic species depends on the degree of oxidative stress, the presence and stoichiometry of these species may yield useful information regarding the exact status of the prevailing oxidative stress. However, much work is needed to establish emphatically protein function modulation via cysteine–sulfoxidation pathways.

5.4.3.3 Protein Function Modulation by Disulfide/Mixed Disulfide Formation: Role of Peroxiredoxin, Sulfiredoxin, and Thioredoxin

The concept that sulfinic and sulfonic acid derivatives of protein cysteines are irreversibly damaged has been contradicted by the work of Biteau et al. (2003), who have reported the presence of an enzyme capable of reducing sulfinic acid derivatives. These workers detected a protein in yeast that can reduce the sulfinic derivative of yeast peroxiredoxin Tsa1. This enzyme was termed sulfiredoxin, and is found to be highly conserved in eukaryotes. The evolutionary conservation of the enzyme might be an important indicator of its importance in the recovery of oxidatively modified proteins, which might be vital to cell signaling and/or functioning. Biteau et al. (2003) further proposed that sulfiredoxin might catalyze a multistep reduction process in view of its intrinsic phosphotransferase and thioltransferase activities. Sulfiredoxin may apparently overcome the energy barrier that normally prevents the reduction of protein-Cys-SO_2H by a transient introduction of a phosphate group in the peroxiredoxin–sulfinate moiety to make sulfinic phosphorylester in the presence of ATP and Mg^{2+}. Similarly, Woo et al. (2005) have shown that reduction of Cys-sulfinic acid by sulfiredoxin is specific to 2-Cys-peroxiredoxins, suggesting that the process is specifically reversible (Fig. 5.4). A thiolsulfinate disulfide is formed with another sulfiredoxin molecule, followed by replacement of the phosphate group to form reduced and stabler forms of the enzyme. The thiolsulfinate can also be reduced to peroxiredoxin-sulfenate and a sulfiredoxin-disulfide by reducing agents such as DTT or thioredoxin (Trx). Thus, peroxiredoxin inactivation may facilitate H_2O_2 signaling, whereas its reverse activation by sulfiredoxin may add a new dimension in the regulation of such a signaling. In contrast, disulfide bonds and protein sulfenic acid moieties can be easily reduced and are often considered as the mediators of redox signaling (Claiborne et al. 1999; Stamler and Hausladen 1998; Suzuki et al. 1997). Nonetheless, it is essential to the understanding of redox signaling to remember that not all cysteine residues are equal. GSH and most protein cysteines cannot react at a biologically significant rate with H_2O_2 unless they are in close association with a metal or exist in the form of a thiolate anion, $-S^-$ (Winterbourn and Metodiewa 1999). Indeed, the GSH peroxidase and peroxiredoxins that involve reaction of H_2O_2 with GSH never involve such a direct interaction. Instead, the GSH peroxidase reaction involves the interaction of H_2O_2 with the selenocysteine of that enzyme, whereas the peroxiredoxin reactions involve interaction of H_2O_2 with a Cys-thiolate residue of the peroxiredoxin. When ionized to the thiolate form, cysteine reacts quite rapidly with H_2O_2. The pK_a of cysteine being normally around 8.3, its ionization will only occur in unusual environments as a function of the surrounding residues. For example, when a cysteine is near a positively charged amino acid, its pK_a is lowered to below 5.0. Such a cysteine is deprotonated and becomes a prime target for H_2O_2 oxidation. Thus, only cysteine-containing proteins located in such an environment will be affected by H_2O_2 directly.

Cysteine in its thiolate form could also participate in thiol-disulfide exchange if there is no interference by steric hindrance:

$$R_1S^- + R_2SSR_3 \longleftrightarrow R_1SSR_3 + R_2S^- \quad (1)$$

In contrast, exchanges between thiols and disulfides are very slow and must be catalyzed by glutaredoxin or other protein disulfide isomerases that have a thioredoxin-like

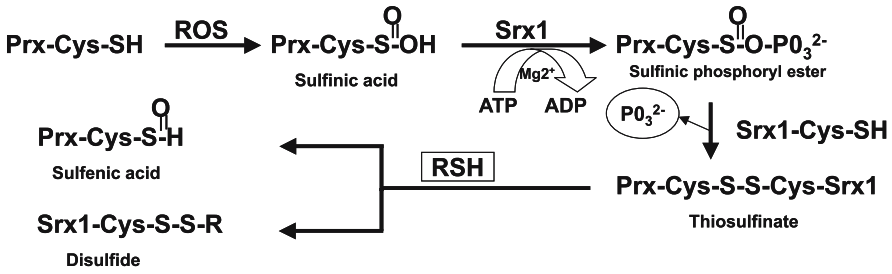


Fig. 5.4 The mechanism of peroxiredoxin sulfinic acid reversal by sulfiredoxin. The thermodynamic energy barrier for reduction of sulfinic acid species can be overcome by the help of a novel class of dual enzyme sulfiredoxin (*Srx*). This enzyme first catalyzes a phosphate group transfer from ATP to peroxiredoxin (*Prx*)-sulfinate (*Prx-Cys-SOH*) as a part of its phosphotransferase activity to form an intermediate disulfide (*Prx-Cys-S-S-Cys-Srx*). The latter is then reduced to a sulfenate and a corresponding disulfide, depending on the reducing group involved

structure in their active sites. Such disulfide exchange is a potential signaling mechanism because of its capacity for modifying cysteine residues in enzymes.

The redox status of proteins outside and within the cells is maintained by several different mechanisms. Whereas the proteins present on the extracellular face of a cell are stabilized by disulfide bonds (S-S) between two protein molecules, the proteins within the cells are redox stabilized by free sulfhydryl (-SH) moieties. Regulation of such redox states of proteins is carried out by a special class of proteins known as thioredoxins (Trx, mol. wt. 10–12) (Miranda-Vizuette et al. 1998). Trx are dithiol [$(SH)_2$]-disulfide oxidoreductases and catalyzes reduction of disulfides to their corresponding sulfhydryls. Trx systems comprises Trx and thioredoxin reductase (TrxR) components and need NADPH for their function. Mammalian TrxR are selenoenzymes that reduce oxidized thioredoxin and other protein disulfides. Trx of lower and higher animals are quite different, mammalian thioredoxins being closely related to the enzyme glutathione reductase. Along with GSH and glutaredoxin, Trx modulate the thiol:disulfide status of many signaling proteins (Holmgren 2003). Normally, Trx functions by reducing the disulfide bonds to dithiols [S-S to 2(-SH)]. However, Trx has been reported to introduce disulfides into proteins during oxidative stress (Watson et al. 2003). At the present juncture, this latter mechanism does not seem to be physiologically significant because such a reaction would possibly be inhibited by the presence of another dithiol motif at Cys-62 and Cys-69 of the Trx-1.

Trx are involved in a wide variety of cellular phenomena such as cell proliferation, reduction of ribonucleotide reductase, thioredoxin peroxidase, thiol–dithiol exchange between cysteine residues of key transcription factors, and protection against exogenous oxidants (Watson et al. 2004). In healthy lungs, Trx and TrxR are expressed in bronchial and alveolar macrophages. Their distribution may be altered in diseases such as usual interstitial pneumonia (UIP); desquamative interstitial pneumonia (DIP); UIP associated with collagen diseases, sarcoidosis, allergic alveolitis, granulomatous disease; and other lung disorders. Thioredoxin reduces the viscosity of sputum in cystic fibrosis patients. Exogenous thioredoxin has been shown to reduce ischemia reperfusion in the lungs. Trx also plays protective role against oxidative stress, bleomycin–induced lung damage, and doxorubicin-induced cardiotoxicity (Andoh et al. 2002). The major role of Trx ap-

pears to be through transcriptional modulation (Nishiyama et al. 2001). In one of the identified mechanisms, Trx in its reduced form reduces Ref-1, which in turn reduces Jun/Fos. Reduction of the dimer Jun/Fos facilitates NF-κB binding to the DNA. Because Trx is present in very low concentrations as compared with other cellular antioxidants, it probably exerts its influence via signaling pathways rather than scavenging mechanisms observed for other gross antioxidants (Watson et al. 2004). Interestingly, TRx enzyme activity can be ablated itself by introduction of a GSH-dependent disulfide bond at its Cys-72 position (Casagrande et al. 2002). Glutathiolated TRx then regains activity by an autodeglutathiolation mechanism. Thus, a reversible disulfidation mechanism is evident that might be an important adaptive and signaling cellular response during oxidative stress. A recent report has suggested that disulfide bond formation within particular families of cytoplasmic proteins may depend on the nature and extent of the oxidative stress and may serve as a focal point of control mechanisms involved in multiple physiological processes (Cummings et al. 2004).

5

5.4.3.4 Protein-*S*-Glutathiolation and Oxidative Stress

The term *S*-thiolation often refers to the phenomena wherein a mixed disulfide is formed between a protein and a cysteine or other thiols.

A cell may experience a redox imbalance either because of oxidative and or nitrosative stress, which can render a host of proteins nonfunctional. The adaptive response of a cell to such an environment is reflected by the modulation of structure and functions of its proteins. In addition to the mechanisms described in the preceding sections, to counter a general stress situation, a cell may employ a variety of other adaptive mechanisms such as acetylation, acylation, proteolytic processing, allosteric modulation, phosphorylation, alkylation, and a host of other mechanisms (Klatt and Lamas 2000). However, the mechanisms as to how proteins are protected and modulated during an oxidative or a nitrosative stress require deeper understanding. A large body of work in the past two decades has amply demonstrated the formation and accumulation of protein-mixed disulfides, both in intact tissues and cell cultures challenged with oxidative stress (Cotgreave and Gerdes 1998; Gilbert 1984; Thomas et al. 1995; Ziegler 1985). In addition, observation of accumulation of protein-mixed disulfides in rat lungs exposed to cigarette smoke further emphasizes the importance of these species in not only adaptive protection against oxidative stress, but also in cellular signaling (Park et al. 1998).

5.4.3.5 *S*-Glutathiolation-Dependent Redox—Adaptive and Signaling Mechanisms

5.4.3.5.1 Role of *S*-Glutathiolation in Cellular Resistance to Oxidative Stress

Physiological redox signaling disulfides are more likely formed by reaction of the thiolate with H_2O_2, forming a relatively unstable sulfenic acid intermediate followed by conjugation with GSH to form a mixed disulfide (Fig. 5.5). Apart from providing the proper cellular reducing environment, there is growing evidence that the GSH redox couple

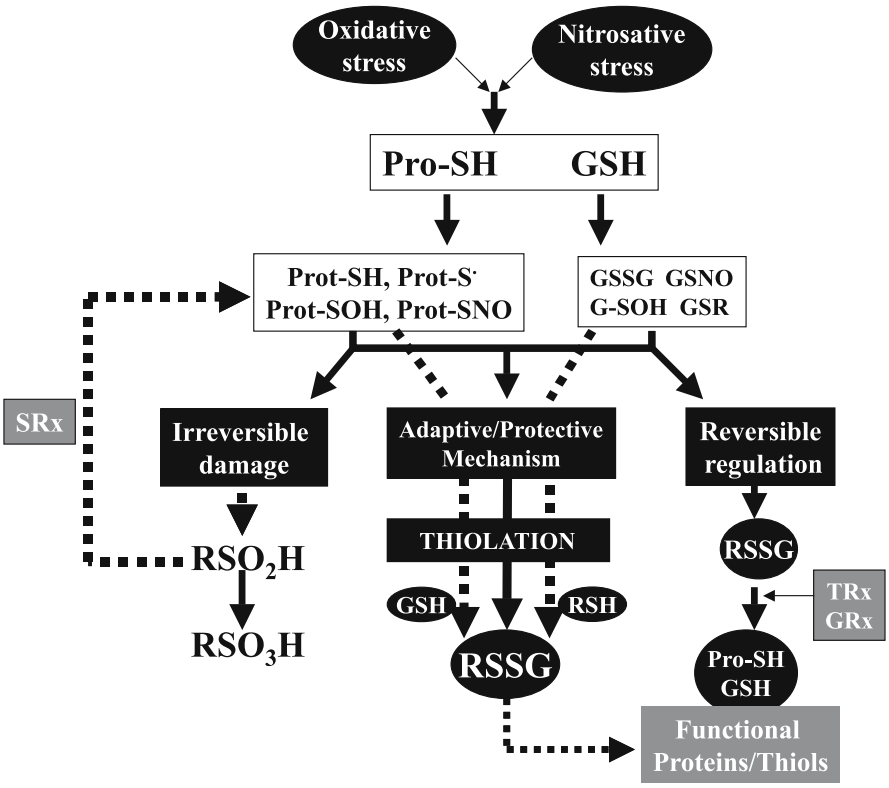


Fig. 5.5 Scheme summarizing protein thiolation. Oxidative or nitrosative stress oxidize thiols (*-SH*) of various proteins (*Pro-SH*) and glutathione (*GSH*). Two series of oxidized species arise, depending on the starting target. Oxidation/nitrosation of Pro-SH leads to the formation of Prot-S, Prot-SOH, Prot-SNO, and that of GSH yields GSNO, G-SOH, and GSR. All these oxidized species may (1) further be oxidized irreversibly to sulfinic (*RSO_2H*) and then sulfonic (*RSO_3H*) derivatives; (2) undergo *trans*-thiolation/reduction (*broken lines*) either by GSH or other thiol species (*RSH*) to form a mixed disulfide, RSSG; and (3) the RSSG formed may be reduced by various agents such as peroxiredoxin, thioredoxins, sulfiredoxins, or glutaredoxins to form active thiols (*Pro-SH/GSH*). The RSO_2H species, earlier thought to be irreversibly damaged, are now known to be reversibly modified by sulfiredoxins to form the unstable sulfenic species which may then be reduced back to their respective active forms. Formation of RSSG appears to be a protective mechanism during an oxidative/nitrosative stress and may be an important mechanism for preventing loss of important proteins because of oxidative damage

dynamically regulates protein function by a reversible formation of mixed disulfides between protein cysteines and GSH (Cotgreave and Gerdes 1998; Gilbert 1984; Thomas et al. 1995.). A specific mixed disulfide formation in conjunction with GSH is termed *S*-glutathiolation. Protein *S*-glutathiolation has been implicated in redox buffering of oxidative stress, extracellular protein stabilization, protection of proteins against irreversible oxidation of their critical cysteine residues, and regulation of enzyme activity (Cotgreave and Gerdes 1998; Thomas et al. 1995). ROS and RNS and alterations in intracellular redox potential have been reported to induce protein *S*-glutathiolation. These reversible chemical modifications of the thiols can result in a conformational change that

may affect DNA binding of transcription factors or enzymatic activities or the formation or release of protein complexes. In addition, these changes are transient, with the duration of the intermediate determined by the ratio of GSH/GSSG and reduced/oxidized thioredoxin.

Classically, phosphorylation of proteins has been considered the major mechanism of cellular homeostasis. *S*-glutathiolation has now been recognized as a potential modulator of redox-sensitive thiol proteins, especially those involved in signal transduction and protein translocation pathways (Shelton et al. 2005). The role of cellular redox alterations is increasingly being recognized in the control of transcriptional factor binding to DNA (Klatt et al. 1999). Actin, PTP-1B, Ras, and several other proteins are now known to be regulated by *S*-glutathiolation. Decomposition of GSNO formed as a consequence of nitrosative stress, has been found to induce *S*-glutathiolation of various proteins such as human Cu,Zn superoxide dismutase (SOD), brain calbindin D, rabbit muscle GAPDH and bovine serum albumin (reviewed in Tao and English 2004), creatine kinase, carbonic anhydrase, actin, and glycogen phosphorylase b (reviewed in Klatt and Lamas 2000). $ONOO^-$, a toxic product of NO·, has recently been implicated in inducing *S*-glutathiolation of proteins (Adachi et al. 2004). It was observed that sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA or Ca-pump) activity was increased by *S*-glutathiolation of the enzyme because of $ONOO^-$ derived from NO·. This is an interesting observation, especially in the light that NO· is a known smooth muscle relaxant. Involvement of $ONOO^-$-dependent *S*-glutathiolation in muscle relaxation is therefore an important indication of the role of *S*-glutathiolation in oxidative stress response and cell signaling.

5.4.3.5.2 *S*-Glutathiolation and Phosphorylation/ Dephosphorylation: a Possible Crosstalk

Protein tyrosine phosphatases play an important role in the dynamics of cell regulation because of phosphorylation–dephosphorylation mechanisms during extracellular signaling (Tonks and Neel 1996). Phosphorylation of tyrosine residues of various target proteins has been recorded in response to cytokines and growth factors and has been found to be at least partly mediated by the generation of ROS (Bae et al. 1997). A recent report has suggested that protein-tyrosine phosphatase 1B (PTB-1B) is oxidized at Cys-215 in a redox-dependent manner (Lee et al. 1998). This suggestion was later confirmed in a study wherein purified PTP-1B when treated with H_2O_2 was irreversibly inactivated, and reversibly inhibited when treated with O_2^{-}· (Barret et al. 1999a). It was observed during these experiments that PTP-1B was oxidized at its Cys-215 to sulfenic acid, which reacted with GSH to form a stable mixed disulfide (Barret et al. 1999b). Furthermore, the *S*-glutathiolated PTP-1B thus formed was found to undergo reduction to an active form when reacted with glutaredoxins (Barrett et al. 1999a). Importantly, *S*-glutathiolation of PTP-1B has also been demonstrated in intact cells (Barrett et al. 1999b). Interestingly, not only ROS, but also GSSG was found to induce PTP-*S*-glutathiolation (Degl'Innocenti et al. 1999). Overall, *S*-glutathiolation appears to be a protective/adaptive mechanism during oxidative stress and may also modulate signal transduction through the tyrosine phosphorylation pathway via modulation of the PTP-1B activity status. Several signal transduction pathways involving cell cycle progression, growth and differentiation, and cytoskeletal function have been found to be modulated by *S*-glutathiolation of proteins. A redox dependent β2-integrin (CD11b/CD18 or Mac-1)-medi-

ated H_2O_2 and TNF-α promoted activation of neutrophil adhesion and recruitment was found to involve *S*-glutathiolation of a component of this signaling pathway (Blouin et al. 1999). Therefore, inactivation of PTP-1B by *S*-glutathiolation might be an important point of cellular signaling mechanisms where protein phosphorylation and oxidative stress may crosstalk.

5.4.3.5.3 *S*-Glutathiolation and the Proteasome Pathway

Ubiquitination of proteins is an important mechanism of posttranslational modification of proteins. Proteins, once ubiquitinated, are directed to the proteasomes for proteolytic cleavage in a cascade involving the action of three enzymes: E1, E2, and E3. It is now understood that ubiquitination of proteins protects the cell from accumulation of oxidatively damaged molecules via their degradation through this pathway (Kornitzer et al. 2000). Various components of cell cycle progression, differentiation, and death are also recognized to be regulated by ubiquitination. The ubiquitin–proteasome pathway is now known to be redox regulated and has been shown to be modulated by GSH (Figueiredo-Pereira et al. 1998). It has been demonstrated that the E1 and E2 components of the ubiquitin pathway are reversibly inhibited by *S*-glutathiolation (Jahngen-Hodge 1997; Obin et al. 1998). This protects and repairs the signaling functions of the ubiquitin pathway. Inhibition by *S*-glutathiolation of the ubiquitin–proteasome enzymes during oxidative stress is thus considered to prevent loss of reversibly oxidized and reparable proteins.

5.4.3.5.4 *S*-Glutathiolation Modulation of Transcription Factors

The evidence of *S*-glutathiolation involvement in transcription was first obtained from a study wherein it was shown that binding of the nuclear factor 1 (NF-1) to DNA required a particular ratio of GSH/GSSG, and that oxidative inactivation of NF-1 because of mixed disulfide formation was reversed by glutaredoxin (Bandyopadhyay et al. 1998). More information on the subject was provided by the work of Klatt et al. (1995) who showed that binding of AP-1–c-Jun subunit to the DNA depended on the cellular GSH/GSSG ratio. The GSH/GSSG ratio provided a redox potential that determined the oxidation of c-Jun via the formation of a mixed disulfide as well as its *S*-glutathiolation at the conserved cysteines of the dimerization domain of the DNA-binding site. Mixed disulfide formation led to the inhibition of DNA binding by c-Jun. Molecular characterizations have further revealed that the susceptibility of c-Jun to form mixed disulfide under mild oxidizing conditions depended on the structural environment of the target cysteine residue (Klatt et al. 1999). Interestingly, because various transcription factors such as NF-κB, members of Jun/Fos, activating factor (ATF)/cyclic AMP response element-binding proteins (CREB), and c-Myb exhibit a common putative GSH-binding domain, it appears that *S*-glutathiolation may represent a general mechanism of redox signal transduction leading to suppression of gene expression (Rokutan et al. 1998). The NF-κB subunit p50 has further been shown to be modulated by the GSH/GSSG ratio by a mixed disulfide formation at the cysteine residue of the DNA-binding domain. This observation was found to be in agreement with an in vitro finding of an inhibition of

AP-1 activation because of decrease in the GSH/GSSG ratio (Rokutan et al. 1998). The finding of a nuclear glutaredoxin has further emphasized the importance of a reversible and enzymatic modulation of mixed disulfide-dependent alterations of nuclear protein thiols. Thus, protein function modulation by *S*-glutathiolation spans a wide variety of cellular functions ranging from resistance to oxidative stress, phosphorylation-dependent signal transduction, and posttranslational protein modification to transcriptional activation and inhibition.

5

5.5 Involvement of Cigarette Smoke in Redox Signaling and Gene Transcription

5.5.1 Signal Transduction

ROS derived from both inflammatory cells and the environment can activate and phosphorylate a wide variety of signal transducing molecules via oxidation-prone cysteine-rich domains and the sphingomyelinase–ceramide pathway, leading to increased gene transcription (Adler et al. 1999; Guyton et al. 1996; Rahman and MacNee 1998; Sen 1998). These include the MAPK family, extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 kinase, and PI-3K/Akt. Depending on the cell type and the cell's oxidative status, activation of members of the MAPK family leads to a complex array of transactivation of transcription factors, such as c-Jun, ATF-2, CREB-binding protein (CBP) and Elk-1 (Adler et al. 1999; Sen 1998; Thannickal and Fanburg 2000). This eventually results in chromatin remodeling, which in turn modulates the expression of a battery of distinct proinflammatory and antioxidant genes involved in several cellular events, including apoptosis, proliferation, transformation, and differentiation. Aldehydes, generated in part because of lipid peroxidation by cigarette smoke, have been shown to signal transcriptional activation and gene expression, leading to inflammatory responses (Parola et al. 1999; Uchida et al. 1999). Hoshino et al. (2005) showed that cigarette smoke extract specifically induces the JNK pathway, leading to endothelial cell injury. Stimulation of a metabolically important cellular signaling pathway mediated by protein kinase C (PKC) has been reported in response to cigarette smoke (Wyatt et al. 1999). Such activation may possibly be attributed to the formation of aldehyde/lipid peroxidation products in human bronchial epithelial cells.

5.5.2 NF-κB and AP-1 Activation

NF-κB regulates the expression of many inflammatory mediator genes, such as those for the cytokines, IL-8, TNFα, and NO· (Fig. 5.6). NF-κB, kept inactivated in the cytosol by its inhibitory subunit (IκB), may be stimulated by diverse stimuli including cytokines and oxidants. This results in ubiquitination, followed by dissociation of IκB from NF-κB and further destruction of IκB in the proteasome. These critical events during an inflammatory response are generally redox sensitive. Recently, Bai et al. (2005) have reported that ROS can directly activate receptor activator of NF-κB ligand (RANKL) and induce osteoclastic activity, leading to bone resorption in rats. It was found that ROS first promoted phosphorylation of CREB/ATF-2, which then binds to the CRE-binding

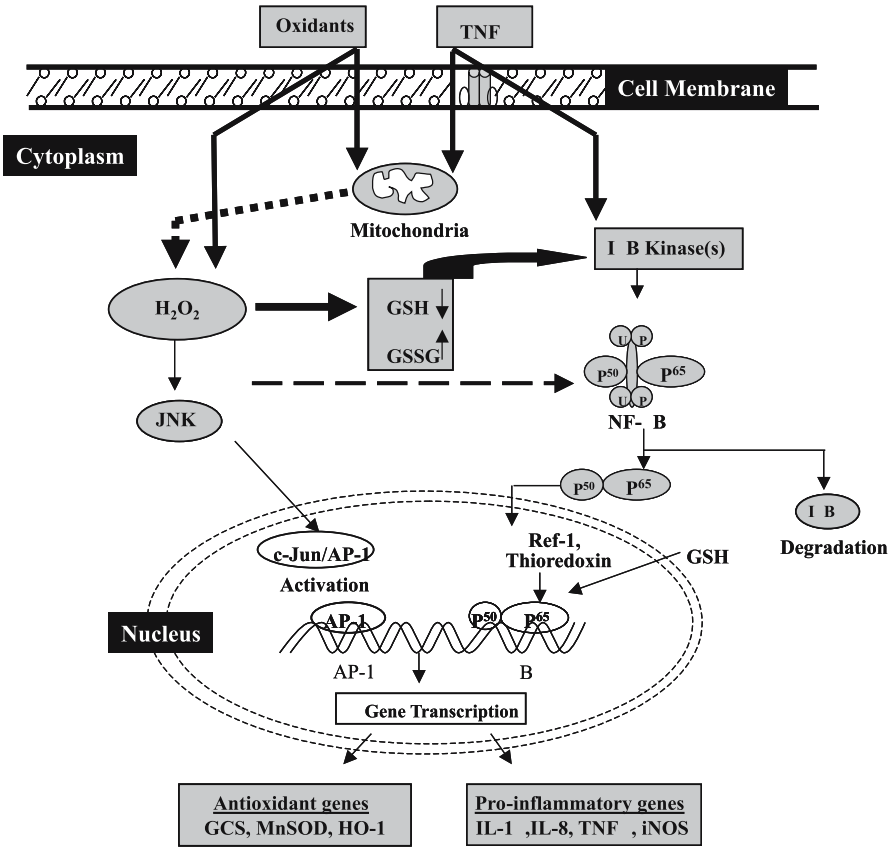


Fig. 5.6 Model for the mechanism of nuclear factor-κB (*NF-κB*) and activator protein-1 (*AP-1*) activation, leading to gene transcription. Tumor necrosis factor-α (TNF-α)/oxidants act on mitochondria to release hydrogen peroxide (and possibly peroxynitrite), which is involved in the activation of NF-κB and AP-1. Superoxide does not leave mitochondria unless generated by an outer mitochondrial membrane oxidoreductase. Hydrogen peroxide (H_2O_2) can leave mitochondria, as enough is generated to overcome the mitochondrial peroxidase activity. Activation of NF-κB involves the phosphorylation, ubiquitination, and subsequent proteolytic degradation of the inhibitory protein IκB. Free NF-κB then translocates into the nucleus and binds with its consensus sites. Intracellular redox ratio of glutathione (*GSH*)/oxidized GSH (*GSSG*) levels and intranuclear presence of Ref-1, and thioredoxin can modulate AP-1 and NF-κB activation. Similarly, AP-1 either c-Jun/c-Jun (homodimer) or c-Fos/c-Jun (heterodimer) is activated by the phosphorylation of c-Jun N-terminal kinase (*JNK*) pathway, leading to the activation AP-1, which binds with its TRE consensus region. Activation of NF-κB/AP-1 leads to the coordinate expression of protective antioxidant and proinflammatory genes

domain on the RANKL promoter. In the same study using human MG63 cells, it was revealed that ROS also triggered HSF2 binding to the heat shock element on the RANKL promoter domain. Thus, it appears that ROS can activate the NF-κB pathway via direct activation of RANKL via ERK/CREB-PKA and HSF2/ERK pathways in mouse and humans, respectively.

Activation of transcription factors and gene expression, leading to an inflammatory response, has been suggested to be because of the aldehydes, generated because of peroxidative alterations by cigarette smoke. A ten-fold higher activation of NF-κB was reported by Mochida-Nishimura et al. (2001) in bronchial alveolar lavage (BAL) cells from smokers in response to lipopolysaccharide (LPS), compared with that of nonsmokers. This was possibly because of enhanced release of inflammatory mediators that may activate NF-κB. However, a differential regulation of activation was recorded for MAPK-ERK, stress-activated protein kinase (SAPK), and p38. In contrast to ERK and SAPK, activation of p38 was found to be more rapid in BAL cells from smokers. The differences in activation of NF-κB and MAPKs in BAL cells from smokers and nonsmokers were attributed to the differences in their microenvironment, which is affected by chronic exposure to cigarette smoke. The activation of p38, therefore, may be responsible for the elevated levels of TNF-α and IL-8 seen in BAL fluid and sputum of patients with COPD (Keatings et al. 1996; Wesselius et al. 1997). In a study involving exposure of normal bronchial epithelial cells and A549 cells to Cigarette Smoke Condensate (CSC), Hellermann et al. (2002) showed an increased activation of NF-κB and phosphorylation of ERK1 and -2. The activation of NF-κB was confirmed by an increased reporter activity of NF-κB-luciferase promoter–reporter cassette transfected in A549 cells in response to CSC. The activation of NF-κB could further be associated with an increased expression of human soluble intercellular adhesion molecule-1 (sICAM-1), IL-1β, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF), suggesting that activation of NF-κB and MAPK pathways may play a role in the proinflammatory effects of CSC in epithelial cells. Involvement of IL-1β in cigarette smoke-mediated lung inflammation has recently been provided more impetus by the study of Castro et al. (2004). Similar effects of CSC via NF-κB activation were also observed in other cell lines, such as human histiocytic lymphoma U-937 Mono Mac 6 cells, Jurkat T cells, and H1299 lung cells (Anto et al. 2002; Yang et al. 2006). Furthermore, the activation of NF-κB by CSC corroborated well with time-dependent phosphorylation and degradation of IκBα and activation of IκBα kinase. In vitro experiments with CSC and cultured epithelial cells have revealed a distinct pattern of stress response in cultured epithelial cells, which may be related to the reported proinflammatory activities of CSC (via the formation of ROS/lipid peroxidation products) both in vitro and in vivo (Gebel and Muller 2001). Cigarette smoke exposure led to a decreased DNA binding of NF-κB during the first 2 h of exposure, followed by a further increase (>2-fold) over control after 4–6 h in Swiss 3T3 cells (Gebel and Muller 2001). This was independent of IκB-α, as evidenced by the lack of phosphorylation and degradation of IκB (Muller and Gebel 1998). In another elegant study, the DNA binding of NF-κB was effectively blocked by an anti-inflammatory celecoxib (Shishodia and Aggarwal 2004). In this study, it was observed that the celecoxib interfered with NF-κB binding to the COX-2 promoter region, inhibited p65 phosphorylation, nuclear translocation of NF-κB to the nucleus, and suppressed activation of IκBα and thus prevented activation of COX-2 by NF-κB and therefore, further inflammatory responses.

It has been shown that thioredoxin, a thiol-dependent protein, could regulate NF-κB DNA binding in a redox-dependent manner (Gebel and Muller 2001). Therefore, the initial loss of DNA-binding activity may also be because of the decreased level of reduced thioredoxin. However, within hours, the cells overcame the oxidative stress by induction of thioredoxin reductase mRNA, elevation of GSH levels, and restoration of NF-κB/thioredoxin complexes in nuclear extracts. An implication of this study is that the binding of NF-κB in response to cigarette smoke may be a redox-controlled mechanism depending

on the availability of reduced thioredoxin in the nucleus and furthermore, that the redox status of thioredoxin or glutathione may be involved in the transcriptional regulation of gene expression by cigarette smoke. It is interesting to note that in vitro treatment with various constituents of cigarette smoke, such as nicotine, acrolein, hydroquinone, catechol, and 4-HNE, inhibits either basal or LPS-induced NF-κB activation and the expression of NF-κB-dependent genes such as *IL-1*, *IL-2*, *IF-γ*, *TNF-α*, and *IL-8* in the U937 cell line or peripheral blood-derived monocytes (Ouyang et al. 2000; Sugano et al. 1998). It is postulated that the inhibitory effects of nicotine may contribute to cigarette smoke-induced immunosuppression. Similarly, Vayssier et al. (1998) have shown that cigarette smoke itself inhibits both spontaneous and LPS-induced NF-κB activation and cytokine release in human peripheral blood monocytes, which may be related to the altered cytokine profile observed in alveolar macrophages of smokers. In line with this observation, Laan et al. (2004) have recently uncovered a novel mechanism wherein CSC was found to inhibit bacterial LPS-dependent neutrophil–chemotactic cytokine production via downregulation of AP-1 activation in airway epithelial cells. Thus, it appears that cigarette smoke-mediated regulation of NF-κB may be cell type specific.

The c-*fos* gene belongs to a family of growth and differentiation-related immediate early genes, the expression of which generally represents the first measurable response to a variety of chemical and physical stimuli (Luo 1994). Studies in various cell lines have shown enhanced expression of the c-*fos* gene in response to cigarette smoke (Luo 1994; Muller 1995). These effects of cigarette smoke can be mimicked by peroxynitrite and smoke-related aldehydes (4-HNE, acrolein, acetaldehyde) in concentrations that are present in aqueous cigarette preparations (Muller et al. 1997). AP-1 (c-Fos/c-Jun) DNA binding is increased in epithelial and endothelial cells in response to CSC (Rahman et al. 1996c; Wodrich and Volhm 1993) and NF-κB, AP-1, and various AP-1 components are increased in lungs of smokers and patients with COPD (Crowther et al. 1999). In a study employing rat lungs, cigarette smoke was found to trigger costimulation of both c-Fos and iNOS along with enhancement in protein-tyrosine phosphorylation and induction of MAPK-ERK kinase 1 (MEK1)/ERK2 pathways. The latter may then trigger processes leading to lung pathogenesis (Chang et al. 2001).

5.5.3 Gene Transcription

There is now a spate of evidence that oxidative stress may modulate the expression of both proinflammatory and protective antioxidant genes. A particular balance or a ratio of oxidants to antioxidants may therefore regulate the expression of pro- and anti-inflammatory genes in response to ROS and during inflammation (Rahman and MacNee 2000a). This oxidant/antioxidant ratio may therefore be critical to cell injury/protection against the aftermath of inflammation. It is likely that antioxidant genes expression during chronic inflammation might precede the expression of proinflammatory genes. The expression of the latter may overtake the adaptive/protective antioxidant responses with persistence of inflammation with a resultant irreversible lung damage seen in various chronic lung diseases including COPD.

5.5.3.1 Antioxidant Protective and Stress Response Genes

Among the antioxidant enzymes, GSH synthesizing and associated redox enzymes appear to play a crucial protective role in the airspaces and epithelial cells (Fig. 5.3). To this extent, the cytoprotective role of GSH has been amply demonstrated both in vivo in the rat and in vitro, using monolayer cultures of alveolar epithelial cells exposed to cigarette smoke/oxidants (Lannan et al. 1994; Li et al. 1994, 1996). This was evidenced by a profound increment in the GSH levels in response to acute intratracheal instillation of CSC in the rat, and exposure of epithelial cell monolayers to cigarette smoke in vitro (Li et al. 1996). This increment in GSH levels was further emphasized by a rebound adaptive increase of GSH levels and GCL heavy subunit (GCL-HS) mRNA expression in both rat lungs and epithelial cell lines (Lannan et al. 1994; Rahman et al. 1995). This finding was mirrored in humans, where GSH was found to be elevated in ELF associated with an increased expression of GCL mRNA in lungs of chronic cigarette smokers (Cantin et al. 1987; Morrison et al. 1999; Rahman and MacNee 2000b). Such a situation was not the case in acute smokers as compared with nonsmokers. Thus, cells respond to an oxidative stress, including that generated by cigarette smoking, by upregulation of an important antioxidant gene involved in the synthesis of GSH as an adaptive mechanism against subsequent oxidative stress. However, such an adaptive response may not entirely counteract the potential burden of proinflammatory mediators and oxidants released therein during inflammation.

A recent study has shown that the expression of GCL mRNA is elevated in smokers' lungs and is even more pronounced in smokers with COPD (Rahman et al. 2000c). This implies that GCL expression might be upregulated (GSH levels were not studied) in response to an ongoing inflammatory response and oxidative stress in lungs of smokers with and without COPD. However, Harju et al. (2002) have found that the GCL immunoreactivity was decreased (again, GSH levels were not measured) in the airways of smokers, compared with nonsmokers. This suggested that cigarette smoke predisposes lung cells to ongoing oxidant stress. Neurohr et al. (2003) showed that decreased GSH levels in BAL cells of chronic smokers were associated with a decreased expression of γ-GCS light subunit (γ-GCS-LS) without a change in γ-GCS-HS expression. This highlighted the fact that increased GSH levels in the ELF of chronic smokers were not associated with increased GSH levels in alveolar macrophages. Furthermore, rats exposed to cigarette smoke have shown increased expression of manganese superoxide dismutase (*MnSOD*), *NAO1*, *CINC-1*, metallothionein (*MT*), and *GPx* genes in the bronchial epithelial cells, suggesting the importance of the antioxidant gene adaptive response against the injurious effects of cigarette smoke (Gilks et al. 1998; Stringer et al. 2004). Therefore, from the above discussion, cell response to a given oxidant stress appears to be quite intricate and delicately balanced and therefore merits further investigation in lungs of smokers with and without COPD.

Comhair et al. (2000) showed over a 2-fold increase in extracellullar glutathione peroxidase (eGPx) mRNA in human airway epithelial cells and alveolar macrophages in response to cigarette smoke, but without any iNOS expression. On the other hand, hyperoxic exposure increased iNOS mRNA in airway epithelial cells by 2.5-fold, without an increase in eGPx mRNA. They suggested that molecular responses by the lung cells to an oxidant may vary with the type of inhaled ROS, which are likely to influence the susceptibility of the airway to oxidative injury in vivo. In view of the possible dif-

ferential regulation of eGPx in acute and chronic smokers, it would be an interesting proposition to investigate the expression of eGPx in airway epithelial cells of smokers with and without COPD. In a recent study by the same group, eGPx was found to be increased in asthmatic airways (bronchial epithelial cells) in comparison to healthy controls, which might be an important defensive strategy of a cell against hydroperoxide-mediated injury to the airway surface in asthmatic individuals (Comhair et al. 2001). Comhair (2005) has further emphasized that induction of eGPx in the airways may be an important defense arsenal against oxidative injury to airway surface cells. Another antioxidant enzyme, GSH *S*-transferase P1 (GSTP1), was shown by Ishii et al. (2001) and coworkers to act in against cigarette smoke in the airway cells. Similarly, Maestrelli et al. (2001) have suggested that induction of heme oxygenase-1 (HO-1) in alveolar spaces of smokers may lead to increased levels of exhaled CO. Cigarette smoke also induces heat shock protein 70 (HS 70) in human monocytes and HO-1, which have been implicated in the regulation of cell injury and cell death and, in particular, modulation of apoptosis in human endothelial cells and monocytes (Favatier and Polla, 2001; Vayssier et al. 1998). The induction of HSP70 may stabilize IκBα, possibly through the inhibition of IκB kinase activation.

5.5.3.2 Pro- and Anti-Inflammatory Genes

TNF-α and LPS are important stimuli for inflammatory responses in COPD. Airway epithelial cells can respond to these stimuli by a concurrent increased generation of both intracellular ROS and RNS (MacNee and Rahman 1999; Rahman and MacNee 2000c; Rochelle et al. 1998). This intracellular production of oxidants and the subsequent changes in intracellular redox status are important in the molecular events (MAPK signaling and chromatin remodeling) controlling the expression of genes for inflammatory mediators (Richer et al. 1995). Animal studies have clearly demonstrated that cigarette smoking induces neutrophil influx to the airspace, increased IL-8 release, and NF-κB activation in the lungs. (Manwick et al. 2004) These observations were further supported by several other investigators who demonstrated that IL-8 release from human bronchial, alveolar epithelial, and endothelial cells increased in response to cigarette smoke, which led to airway inflammation (Masubuchi et al. 1998; Mio et al. 1997; Wang et al. 2000). The increased IL-8 paralleled the increased neutrophil counts in bronchial samples of BAL fluid. Abolition of these effects by recombinant SOD treatment suggested that cigarette smoke-mediated peroxidation/oxidative stress regulates the molecular events in lung inflammation (Nishikawa et al. 1999). Cigarette smoke-induced IL-8 release in turn may be mediated via oxidative stress because of a Fenton reaction–catalyzed peroxidation reaction, because aldehydes such as acrolein and acetaldehyde augmented IL-8 release (Masubuchi et al. 1998; Mio et al. 1997; Wang et al. 2000). A report by Saetta et al. (2002) lends credence to this observation by their demonstration of an increased expression of the chemokine receptor CXCR3 and its ligand CXCL10 in peripheral airways of smokers with COPD. Because the CXCR3/CXCL10 axis is associated with T-cell response, their increased expression might be involved in T-lymphocyte recruitment during airway inflammation because of increased levels of chemokines.

Another consequence of cigarette smoking is an increased adherence of leukocytes to vascular endothelial cells (Noguera et al. 1998). According to Shen et al. (1996), CSC in-

duces leukocyte adherence by increasing the expression of a wide variety of cell adhesion molecules. Adhesion molecules such as ICAM-1, endothelial leukocyte adhesion molecule 1 (ELAM-1), and vascular cell adhesion molecule-1 (VCAM-1) in human umbilical vascular endothelial cells have been associated with an increase in the binding activity of NF-κB. Such an expression of adhesion molecules has been implicated in an increased transendothelial migration of monocytes by cigarette smoking. Exposure of bronchial epithelial cells cultured from biopsy from patients with COPD to cigarette smoke, exhibited increased release of proinflammatory mediators, such as IL-1β and sICAM-1, as compared with smokers (Rusznak et al. 2000). This implies that patients with COPD have a greater susceptibility to the effects of cigarette smoke.

Transforming growth factor-β_1 (TGF-β_1) is a multifunctional growth factor that modulates cellular proliferation, differentiation, and tissue repair (Border and Ruoslahti 1992; Clark and Cocker 1998). In addition, TGF-β_1 acts as a chemoattractant and mitogen for fibroblasts and fibroblast-like cells and stimulates the synthesis and deposition of extracellular matrix. Recently, increased TGF-β_1 expression was demonstrated in bronchiolar and alveolar epithelium in subjects with COPD (de Boer et al. 1998; Takizawa et al. 2001). Increased TGF-β_1 expression associated with fibrosis in the basement membrane in the lungs could be suggestive of an abnormal repair process after exposure to cigarette smoke (Chan-Yeung 1988). TGF-β_1, which decreases GSH synthesis, has been associated with increased ROS production in human alveolar epithelial cells and pulmonary artery endothelial cells in vitro (Das and Fanburg 1991; Jardine et al. 2002; White et al. 1992) and thus may a play a role in membrane lipid peroxidation (Fig. 5.7). Conversely, lipid peroxidation products may in turn affect the expression of TGF-β_1, as has been recorded in experiments with 4-HNE (Leonarduzzi et al. 1997; Parolla et al. 1999). 4-HNE has been shown to induce cellular stress responses via the MAPK pathways, leading to the induction of AP-1-mediated genes (Leonarduzzi et al. 2000) such as GCL mRNA in alveolar epithelial cells (Liu et al. 2001). Smokers and subjects with COPD have been found to express increased GCL in lung alveolar epithelial cells (Rahman et al. 1996c). Hence, 4-HNE may play a role in the regulation of AP-1-dependent TGF-β1 and *GCL* gene expression in patients with COPD. It may therefore be surmised that increased 4-HNE because of membrane lipid peroxidation may be one factor signaling the expression of TGF-β_1 and GCL in lungs of COPD patients. Such a possibility is based on the observation of increased levels of 4-HNE adducts being associated with increased levels of TGF-β_1 in bronchiolar and alveolar epithelium and macrophages in subjects with COPD as compared with those without COPD (MacNee and Rahman 1999; Rahman et al. 2002a). The observed induction of GCL may be an important adaptive response of the alveolar epithelium to oxidative stress. A recent study by Sano et al. (2002) has shown that another antioxidant gene, thioredoxin, is induced by 4-HNE in response to LPS challenge in mice and may impart endotoxin tolerance. This suggests that 4-HNE triggers a second messenger response that upregulates expression of the protective GCL and thioredoxin genes as well as a variety of other genes like TGF-β_1, matrix metalloproteinase-1, cyclooxygenase 2 (*COX-2*), monocyte chemoattractant protein-1 (*MCP-1*) and *VEGF*, all of which have been implicated in the pathogenesis of COPD (Belloeq et al. 1999; Kasahara et al. 2000; Kumgai et al. 2000; Sano et al. 2002) (Fig. 5.8). Similarly, 4-HNE has been also shown to induce VEGF expression in retinal pigment epithelial cells (Ayalasomayajula and Kompella 2002), and such an induction has been linked to the pathogenesis of inflammatory lung diseases such as emphysema and COPD (Kasahara et al. 2000). Cigarette smoke exposure resulted in increased expression of VEGF and

its flk-1 receptor in main pulmonary arteries and in intraparenchymal arteries (Wright et al. 2002). Such an imbalance of an array of redox-regulated antioxidant versus pro-inflammatory genes therefore might be associated with the susceptibility or tolerance to disease (MacNee and Rahman 1999). Ward et al. (2000) have shown an upregulation of the protective antiapoptotic protein bcl-2 in response to transgenic IL-6 expression against hyperoxic acute lung injury and lipid peroxidation in animal models. Other investigators have reported that the activated Akt signaling pathway protects the lung from oxidant-induced injury in mice (Lu et al. 2001). However, the implications of these relatively novel antioxidant protective signaling mechanisms in various chronic lung inflammatory diseases are yet to be fully explored.

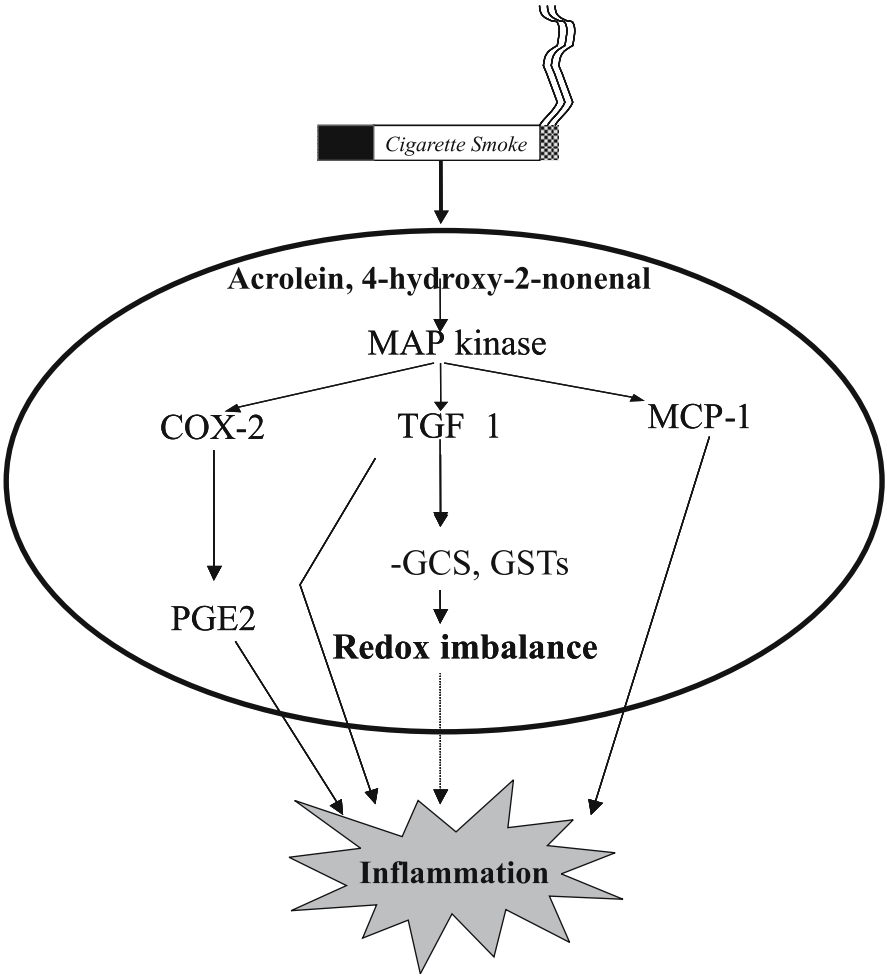


Fig. 5.7 Proposed model of the mechanism of cigarette smoke-mediated oxidative stress (formation of 4-hydroxy-2-nonenal) in redox-mediated lung inflammation

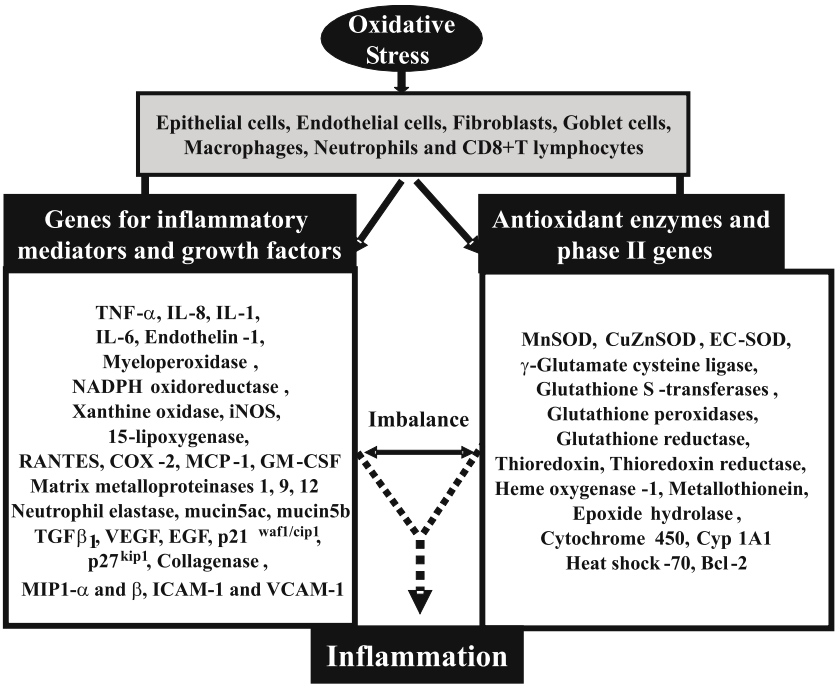


Fig. 5.8 Various inflammatory and structural cells are activated by oxidative stress, leading to transcription of various proinflammatory and antioxidant genes. It may be possible that induction of antioxidant enzymes might provide initial adaptive or protective responses against oxidative stress and inflammatory mediators. However, during sustained/chronic inflammation, the balance between genes for inflammatory mediators and antioxidant/phase II enzymes may be tipped in favor of proinflammatory mediators

5.5.3.3 Aldehyde/Lipid Peroxidation Product-Mediated Gene Expression

Acrolein, being an electrophilic agent, may induce a variety of genes regulated via the electrophilic response element or antioxidant response element (ARE). GCL and GST, which are under regulation by ARE, may be induced by acrolein (Biswal et al. 2002). Li et al. (1999) reported that acrolein-mediated induction of IκB occurred within 15 min of exposure to 5 μ*M* acrolein in rat alveolar macrophages that subsequently led to the inhibition of NF-κB activity. However, acrolein did not induce IκB in A549 epithelial cells, suggesting that the inhibition of NF-κB activation by acrolein may be IκB independent and cell-type specific (Horton et al. 1999). Similarly, 4-HNE induction of JNK-mediated AP-1 activation may be involved in transcriptional activation of ARE-dependent xenobiotic-metabolizing genes. 4-HNE also inhibits NF-κB activation and LPS-induced TNF-α expression by inhibiting I-κB phosphorylation and subsequent proteolysis in THP-1 monocytic cells (Page et al. 1999) This role of 4-HNE has been refuted by Okada et al. (1999), who have shown that 4-HNE impairs ubiquitin/proteasome-dependent intracellular proteolysis, a mechanism involved in NF-κB activation. Later studies have

shown that 4-HNE-mediated NF-κB inhibition was because of inhibition of IκB kinase in a human lung carcinoma cell line (H1299) (Ji et al. 2001) that may be accompanied by the inhibition of IκBα degradation. The differential activation of *NF-κB* and *AP-1* genes by aldehydes in cigarette smoke may reflect an imbalance of the genes induced by cigarette smoking.

5.5.3.4 Mucin Genes

Mucins are complex glycoproteins secreted by the goblet cells, which impart viscoelastic properties to the mucus and play a crucial role in airway defense. Although the regulation of mucins in lungs of COPD patients is not well understood, many chronic inflammatory diseases of the airway are associated with mucus hypersecretion. Smokers express more goblet cells than do nonsmokers, and activation of these cells results in mucus hypersecretion leading to airway plugging (Nadel 2001). Cigarette smoke can activate epidermal growth factor (EGF) receptors via tyrosine phosphorylation, resulting in the induction of *MUC5AC* gene expression leading to mucin hypersynthesis in epithelial cells and in lungs (Chang et al. 2005; Takeyama et al. 2001). Acrolein has been shown to induce the *MUC5AC* gene in the airway epithelial cell line NCI-H292 (Leikauf et al. 2002; Takeyama et al. 2001). This induction was accompanied by EGF ligand formation and the activation of EGF-dependent pathways by TNF-α-converting enzyme and amphiregulin (Lemjabbar et al. 2003; Shao et al. 2004). Neutrophil elastase also increases the expression of *MUC5AC* by enhancing mRNA stability (Fischer and Voynow 2002). Neutrophil release of elastase also impairs mucociliary clearance and stimulates goblet cell metaplasia and therefore, mucin production. Furthermore, inhibition of elastase-induced *MUC5AC* gene expression by antioxidants in human bronchial and alveolar epithelial cells suggests that neutrophil elastase-mediated *MUC5AC* gene expression is oxidant dependent (Kasahara 2000). Therefore, enhanced levels of lipid peroxidation products may be of etiological importance for mucus cell hyperplasia, as observed in smokers with airflow obstruction (Leikauf et al. 2002). This hypothesis is strengthened by the observation that cigarette smoke-mediated *MUC5AC* gene expression could be inhibited by selective EGFR tyrosine kinase inhibitors and antioxidants (Takeyama et al. 2001). In a recent study, it was reported that cigarette smoke-generated ROS upregulates *MUC5AC* gene expression via AP-1-JunD and Fra-2 or Fra-1 pathway (Gensch et al. 2004; Zhang et al. 2005). These transcription factors are known to require phosphorylation by upstream kinases such as JNK and ERK, respectively. Activation of JNK was found to be Src dependent and did not require EGFR. Understanding the EGF receptor signaling pathway in cigarette smoke-mediated upregulation of mucin gene expression could therefore lead to targeted inhibition of mucus hyper-production in epithelial cells.

5.5.3.5 DNA Microarray Profile: Cigarette Smoke-Mediated Gene Expression

A comprehensive gene expression profile of COPD has been carried out by Ning et al. (2004). Serial analysis of gene expression (SAGE) and microarray analysis were used to

compare the gene expression patterns of lung tissues from smokers with and without COPD. A total of 261 transcripts were differentially expressed in a total of 26,502 transcripts that were sequenced. The genes encoding for transcription factors (*EGR1* and *FOS*), growth factors, or related proteins (*CTGF*, *CYR61*, *CX3CL1*, *TGFB1*, and *PDGFRA*) and extracellular matrix proteins (*COL1A1*) were induced. Most recently, Shah et al. (2005) have compiled and analyzed over 22,500 transcripts of human thoracic airway cells in a study termed SIEGE (Smoking-Induced Epithelial Gene Expression database), using Affymetrix HG-U133A gene chips. (This microarray data along with relevant patient information is available on http://pulm.bumc.bu.edu/siegeDB.) Similarly, Golpon et al. (2004) have demonstrated a global decrease in gene expression associated with increased abundance of genes encoding proteins involved in inflammation, immune responses, and proteolysis in emphysematous lung tissues. Zhang et al. (2001) have also identified a number of genes that are modulated by nicotine in human coronary artery endothelial cells, using a cDNA microarray approach to evaluate over 4,000 genes. This observation is particularly interesting because nicotine can induce CBP coactivator and MAPK cascade genes that are also involved in cigarette smoke-related pathologies. Izzotti et al. (2004) have shown alterations of gene expression in skin and lungs of mice exposed to light and cigarette smoke. It has been shown that sunlight-mimicking light induces genotoxic damage not only in skin, but also in lung, bone marrow, and peripheral blood of mice. By using cDNA-arrays of 746 toxicologically relevant genes, they have identified glutathione-*S*-transferase-Pi, catalase, and COX-2 expression in both skin and lung. In another elegant study, Basio et al. (2002) described the kinetics of gene expression profiling up to 24 h, using glass chips containing 513 different cDNA probes in Swiss 3T3 cells exposed to aqueous extracts of cigarette smoke. The cigarette smoke-induced stress response genes related to antioxidant response elements studied were heme oxygenase-1, metallothionein 1 and 2, and heat shock proteins (*hsp90* and *hsp105*), genes coding for transcription factors, e.g., JunB, c-*maf*, sarp1 (antiapoptotic protein, secreted apoptosis-related protein) and CAAT/enhancer protein (*C/EBP*), cell cycle-related genes, e.g., *gadd34* and *gadd45*, and genes for inflammatory/immune-regulatory response, e.g, *st2* (IL-1-like receptor), *kc* (chemoattractant cytokine involved in neutrophil activation), and *id3* (induction of growth arrest/apoptosis in B-lymphocyte progenitor cells). Gebel and colleagues (2004), by using DNA microarray chips covering 2,031 cDNA probes, demonstrated a differential gene expression in tissues of rat respiratory tract after acute and subchronic cigarette smoke exposures. They show the induction of oxidative stress-responsive and phase II drug-metabolizing enzymes, such as heme oxygenase-1, NAD(P)H:quinine oxidoreductase, and Nrf2, without any change in phase I genes cytochrome P450 1A1 and aldehyde dehydrogenease-3 after acute cigarette smoke exposure. Taken together, these studies lay further emphasis on the potential imbalance of pro- and anti-inflammatory/antioxidant genes induced by cigarette smoking. It may now be possible in the near future to study the gene expression profile in a specific region of inflamed lungs by laser capture microdissection combined with real-time reverse transcriptase-polymerase chain reaction (Betsuyaku et al. 1996).

5.6 Role of ROS and Cigarette Smoke-Induced Oxidative Stress in Chromatin Modeling: Role for Histone Acetylation/Deacetylation and DNA Methylation

5.6.1 Role of ROS in Chromatin Remodeling and Gene Transcription: Epigenetics

Epigenetics is defined as the inheritance of information based on gene expression, in contrast to *genetics*, which is described as the inheritance of information based on DNA sequence. Histone acetylation/deacetylation and DNA methylation are two important epigenetic events that play an important role in inflammatory lung responses.

5.6.1.1 Chromatin Remodeling (Histone Acetylation and Deacetylation)

Many factors, including specific DNA sequences, histones, nonhistone chromosomal proteins, transcriptional activators/repressors, and the transcription machinery are all necessary for the assembly of an active transcriptional complex (Sternglanz 1996). Condensation of eukaryotic DNA as a tightly coiled structure in chromatin suppresses gene activity through the coiling of DNA on the surface of the nucleosome core and folding of nucleosome assemblies, thus denying accessibility to the transcriptional apparatus (Wu 1997). Tightly bound DNA around a nucleosome core (by histone residues H2A, H2B, H3, and H4) suppresses gene transcription by decreasing the accessibility of transcription factors, such as NF-κB and AP-1, to the transcriptional complex. Uncoiling of the DNA via acetylation of lysine (K) residues in the N-terminal tails of the core histone proteins allows increased accessibility for transcription factor binding (Imhof and Wolffe 1998). Acetylation of K residues on histone 4 (K5, K8, K12, K16) is thought to be directly related to the regulation of gene transcription (Bannister and Miska 2000; Imhof and Wolffe 1998). Histone acetylation is reversible and is regulated by a group of histone acetyltransferases (HATs), which promote acetylation, and histone deacetylases (HDACs), which promote deacetylation.

The nuclear receptor coactivators, steroid receptor coactivator 1 (SRC-1), CBP/adenoviral protein E1A (p300) protein, CBP/p300-associated factor (P/CAF), and ATF-2, all possess intrinsic HAT activity (Fig. 5.9) (Kawasaki et al. 2000; Ogryzko et al. 1996; Pham and Sauer 2000). Of these, CBP/p300 and ATF-2 are vital for the coactivation of several transcription factors, including NF-κB and AP-1, and are regulated by the p38-MAPK pathway (Kawasaki et al. 2000; Ogryzko et al. 1996; Pham and Sauer 2000; Thomson et al. 1999). These activation complexes act in consonance with RNA polymerase II to initiate transcription (Carrero et al. 2000; Kamei et al. 1996; Ng et al. 1997). Thus, it is likely that histone acetylation of H4 via CBP/p300 and/or ATF-2 has a significant role in the activation of NF-κB/AP-1-mediated gene expression of proinflammatory mediators (Carrero et al. 2000; Kamei et al. 1996; Kawasaki et al. 2000). However, the precise molecular mechanisms involved in such transcriptional regulation warrant more investigations.

The family of HDAC enzymes consists of at least ten distinct deacetylases (Tong et al. 2002). Several distinct HDACs are now recognized, and these are differentially expressed

and regulated in different cell types. HDACs catalyze the removal of the acetyl moieties from the ε-acetamido groups of lysine residues of histones, causing DNA rewinding and silencing of gene transcription. Recently, it has been shown that HDACs (1 and 2) play a crucial role in the regulation of cell proliferation and corticosteroid-mediated inhibition of proinflammatory mediators (Ito et al. 2000; Sambucetti et al. 1999). Disruption of the nucleosome or DNA unwinding caused by deacetylation inhibitors has been shown to facilitate AP-1 binding. HDACs not only cause the inhibition of gene transcription by deacetylation and, therefore, limiting coactivator access to target sites of DNA, but also directly affect the nuclear activity of transcription factors such as NF-κB. The duration of the NF-κB nuclear activation has been shown to be dependent on the activity of HDAC3, which provides an acetylation balance-dependent mechanism for the regulation of NF-κB mediated transcription (Chen et al. 2001).

It has been suggested that oxidant-generating systems and proinflammatory mediators influence histone acetylation/phosphorylation via a mechanism dependent on the

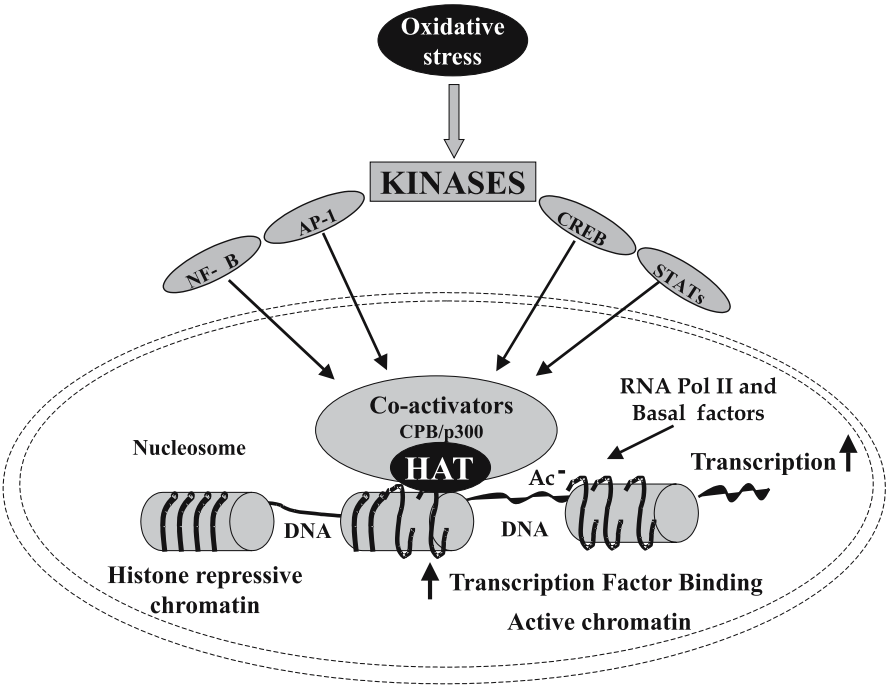


Fig. 5.9 Histone acetylation and deacetylation: oxidative stress and other stimuli, such as cytokines, activate various signal transduction pathways (Janus kinase [JAK], c-Jun N-terminal kinase [JNK], and IκB kinase [IKK]), leading to activation of transcription factors, such as nuclear factor-κB (*NF-κB*), activator protein-1 (*AP-1*), cyclic AMP response element-binding proteins (*CREB*), and signal transducers and activators of transcription (*STAT*) proteins. Binding of these transcription factors leads to recruitment of CREB-binding protein (*CBP*) and/or other coactivators to the transcriptional initiation complex on promoter region of various genes. Activation of CBP leads to acetylation (*Ac*) of specific core histone lysine residues by an intrinsic acetyltransferase activity (histone transacetylase [*HAT*]). Histone acetylation (active chromatin) leads to loosening of nucleosome, which enables access to basal factors and RNA polymerase II for gene transcription to occur

activation of the MAPK pathway (Bohm et al. 1997; Miyata et al. 2001; Tikoo et al. 2001). Recent evidence has shown that oxidative stress induced by H_2O_2 and TNF-α increases the activation of AP-1 and NF-κB and may regulate chromatin remodeling, leading to IL-8 expression (Rahman et al. 2002b). This may be pivotal in cell proliferation, apoptosis, and imbalance in gene transcription for proinflammatory mediators and antioxidant protective genes.

5.6.1.1.1 Gene Transcription

Inflammatory mediators play a crucial role in chronic inflammatory processes and appear to determine and direct the nature of the inflammatory responses via selective recruitment and activation of inflammatory cells and their perpetuation within the lungs. In several in vitro studies employing macrophages, alveolar, and bronchial epithelial cells, ROS have been shown to cause increased gene expression of inflammatory mediators, such as IL-1 and TNF-α. Direct or indirect oxidant challenge to the airway epithelium and alveolar macrophages is also known to generate cytokines, such as TNF-α (which in turn can affect airway epithelial cells to induce proinflammatory genes), TNF-α, IL-8, IL-1, inducible nitric oxide synthase (iNOS), COX-2, ICAM-1, IL-6, macrophage inflammatory protein 1 (MIP-1), GM-CSF, stress response genes (*HSP-27*, *-70*, *-90*, *HO-1*), and antioxidant enzymes (γ-glutamylcysteine synthetase γ-GCS, MnSOD, and thioredoxin) (Gutteridge and Halliwell 2000) (Fig. 5.8). The genes for these inflammatory mediators are regulated by redox-sensitive transcription factors, such as NF-κB and AP-1 (Anto et al. 2002; Hellerman et al. 2002). However, the expression/induction of these genes is now known to be regulated by their acetylation/deacetylation status. Acetylation of histones has been associated with the transcription of a range of inflammatory mediators including IL-8 (Hoshimoto et al. 2002), eotaxin, IL-1β, and GM-CSF (Adcock and Caramori 2001), MIP-2 (Ohno et al. 1997), and IL-6 (Berghe et al. 1999). Acetylation can occur specifically at the promoter sites of these genes as shown by chromatin immunoprecipitation (CHiP) assays for IL-8 (Ashburner et al. 2001), CYP1A1 (Ke et al. 2001), myeloperoxidase (Miyata et al. 2001), and 15-lox-1 (Shankaranarayanan et al. 2001) gene promoters, indicating acetylation specificity.

5.6.1.2 Mechanisms of Transcriptional Regulation

5.6.1.2.1 MAPK- and NF-κB-Mediated Histone Acetylation and Deacetylation

Oxidative stress and proinflammatory mediators have been suggested to influence histone acetylation and phosphorylation by ADP ribosylation, via a mechanism dependent on the activation of a MAPK pathway (Bohm et al. 1997; Miyata et al. 2001; Tikoo et al. 2001). Recently, work in our laboratory and by other investigators has shown that both H_2O_2 and TNF-α caused an increase in HAT activity in alveolar epithelial cells (Ito et al. 2001a; Moodie et al. 2004; Rahman et al. 2002b). The exact mechanism of increased histone acetylation in response to these agents is, however, yet to be fully decrypted. Reports are nonetheless available wherein oxidants and TNF-α have been shown to ac-

tivate MAPK pathways, specifically ERK and JNK. Activation of these redox-dependent pathways may then modulate ATF-2 and CBP coactivators that possess intrinsic HAT activity (Carrero et al. 2000; Kawasaki et al. 2000; Tong et al. 2002). ROS and TNF-α increase the activation of AP-1 and NF-κB and regulate chromatin remodeling, leading to IL-8 expression in lung cells (Berghe et al. 1999; Lakshminarayan et al. 1998; Rahman et al. 2002b). Recently, Ito and coworkers (2001b) have shown a role for histone acetylation and deacetylation in IL-1β-induced TNF-α release in alveolar macrophages derived from cigarette smokers. They have also suggested that oxidants may play an important role in the modulation of HDAC and inflammatory cytokine gene transcription. They further demonstrated increased expression of p65 protein of NF-κB in bronchial epithelium of smokers and patients with COPD (Di Stefano et al. 2002). The increased expression of p65 in epithelial cells was correlated with the degree of airflow limitation in patients with COPD. NF-κB is known to be associated with coactivators such as CBP and p300 that have HAT activity (Berghe et al. 1999). Their recruitment by transcription factors is thus an important link between promoter activation and transcriptional machinery. Shenkar et al. (2001) have shown that the p65 subunit of NF-κB interacts with CBP after hemorrhage and endotoxemia-induced acute lung injury. However, it is still unclear as to the status of the NF-κB signaling pathway and chromatin remodeling in small airways and parenchyma of COPD patients. We have also shown that CSC increased the acetylation of histone 4 associated with decreased levels of HDAC-2 levels in alveolar epithelial cells, monocytes and in vivo in rat lungs in response to cigarette smoke exposure (Marwick et al. 2004; Moodie et al. 2004; Yang et al. 2006). We also showed that inhibiting HDACs alone resulted in enhanced activation of AP-1 and NF-κB and increased histone acetylation, culminating in increased IL-8 release (Rahman et al. 2002, 2004) (Fig. 5.10). This observation is corroborated by previous studies showing that acetylation of histone proteins is associated with increased binding of the transcription factors AP-1 and NF-κB (Fusunyan et al. 1999; Ng et al. 1997). IL-8 release was also augmented on histone deacetylase inhibition by trichostatin A, when combined with TNF-α or H_2O_2. This increase in IL-8 release was associated with NF-κB binding, suggesting that inhibition of HDAC may promote NF-κB retention in the nucleus, triggering augmented TNF-α or H_2O_2-mediated gene transcription. In addition, NF-κB itself can be acetylated while in the nucleus by virtue of its interaction with CBP, which may lead to further transcriptional activation (Saccani et al. 2001). Once the acetylated active NF-κB dimers are localized in the nucleus, they scan the chromatin for any exposed transcriptional sites. This mechanism was supported by Saccani et al. (2002), who have shown bimodal (two temporally distinct phases) recruitment of NF-κB to target promoters by LPS stimulation in the Raw 264.7 murine macrophage cell line. They suggested that a subset of target genes, whose promoters are already heavily acetylated (H4 acetylation) before stimulation, are constitutively and immediately accessible to NF-κB. Such genes (*MnSOD*, *MIP2*, *IκB-ą* , *IL-2*) are subject to immediate transcription post-NF-κB recruitment, whereas other target genes (*MCP-1*, *RANTES*, *IL-6*) are not immediately accessible. Recruitment of NF-κB (p38 dependent) to late accessible gene promoters occurs after nuclear entry and is preceded by the formation of an initial transcription factor complex that directs the hyperacetylation of the promoter and makes it accessible to NF-κB. This shows the selectivity of stimulus-specific p38-dependent and NF-κB-mediated histone acetylation, leading to a subset of gene transcription.

It is known that p65, a component of the NF-κB transcription factor, has intrinsic HAT activity, and transactivation of p65 is independent of nuclear translocation (Ashburner et al. 2001; Chen et al. 2001; Pham and Sauer 2000). It has been shown that

HDAC1 can interact directly with the p65 subunit of NF-κB to exert its corepressor function in the nucleus (Ito et al. 2001; Yang et al. 2006). Therefore, NF-κB interaction with HDAC (Rahman and MacNee 2000b; Rahman et al. 2004) proteins may be an additional mechanism whereby NF-κB can regulate transcription (Zhong 2002). HDACs may be prevented from binding to nuclear p65 by cigarette smoke/oxidants, leading to enhanced p65 acetylation/phosphorylation, resulting in *IL-8* gene expression (Fig. 5.10). Zhong et al. (2002) have demonstrated yet another mechanism whereby the phosphorylation status of NF-κB determines its association with HAT (CBP/p300) or HDAC-1, a leading to gene transcription of various proinflammatory mediators in transformed human embryonic kidney HEK293 cells. These workers have proposed that transcriptionally inactive nuclear NF-κB in resting cells consists of homodimers of either p65 or p50 complexed with HDAC-1. Only the p50–HDAC-1 complex binds to the DNA and suppresses NF-κB-dependent gene expression in unstimulated cells. Activation of cells with NF-κB-inducing agents leads to nuclear localization of active phosphorylated p65 that associates with CBP and displaces the p50–HDAC-1 complexes, leading to gene transcription. It remains to be determined, however, whether the above-proposed mechanisms for NF-κB activation (phosphorylation of p65/depletion of HDAC-1 or displacement of p50–HDAC-1 complex) are also operative under oxidative stress. Furthermore, the transactivation of NF-κB may not necessarily require the classical NF-κB/IκB kinase

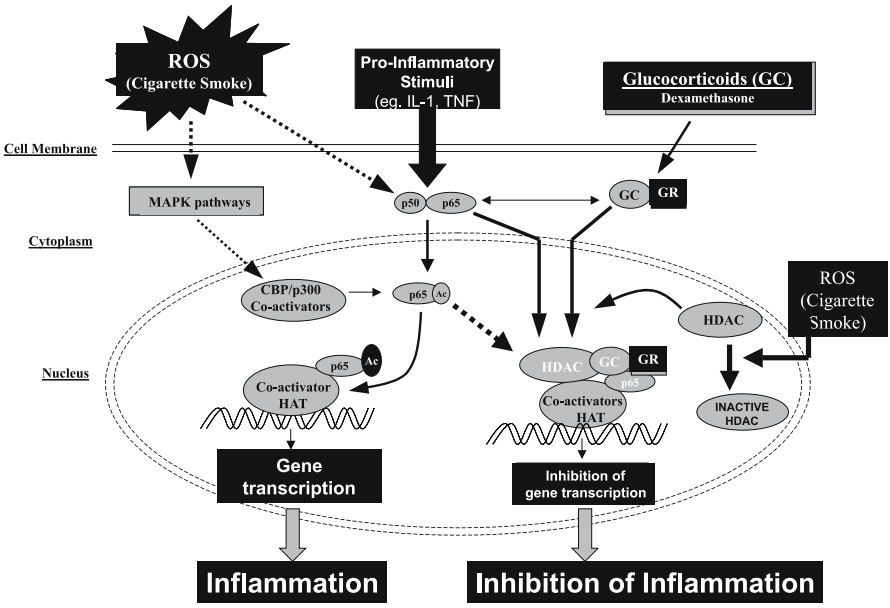


Fig. 5.10 Model showing the possible mechanism of histone acetylation by oxidative stress and its repression by corticosteroids (glucocorticoids [*GC*]), leading to inhibition of gene transcription. Mitogen-activated protein kinase (*MAPK*) signaling pathways may be activated by oxidative stress, leading to histone acetylation. Direct interaction between coactivators (histone transacetylase [*HAT*]), histone deacetylase, and the glucocorticoid receptor (*GR*) may result in repression of the expression of proinflammatory genes. Histone deacetylase (*HDAC*) forms a bridge with HAT to inhibit gene transcription. However, when the HDAC is inhibited by oxidants or the NF-κB subunit p65 is acetylated, steroids may not be able to recruit HDACs into the transcriptional complex to inhibit proinflammatory gene expression

pathway (Ashburner et al. 2001; Ito et al. 2001; Madrid et al. 2001; Pham and Sauer 2000; Saccani et al. 2002; Zhong et al. 2002). IL-8 and IL-6 release have been reported to be enhanced by HDAC inhibitors in intestinal epithelial cells and in murine fibrosarcoma L929sA cells (Gerritsen et al. 1997; Pender et al. 2000). The HDAC inhibitors were also found to prime the effect of IL-1 or TNF-α treatments and enhanced pulmonary cells responsiveness to H_2O_2 and TNF-α, leading to increased transcription factor DNA-binding and enhanced gene expression (Kuscher et al. 1996). On similar lines, Pender and coworkers (2002) have demonstrated that HDAC inhibitors enhance the levels of stromelysin-1 (matrix metalloproteinase 3) by augmenting histone acetylation by TNF-α or IL-1-stimulated mesenchymal cells. Similarly, IL-4 production from activated peripheral blood T cells was enhanced by the histone deacetylase inhibitor trichostatin A, and overexpression of HDACs 1, 2, and 3 inhibited transcription driven by the *IL-4* promoter in Jurkat T cells (Valapour et al. 2002). Potentiation of *IL-4* promoter activity because of cotransfection of HAT-CBP suggested that IL-4 expression is controlled at the transcriptional level by chromatin remodeling. It has been shown that *IL-4* gene expression was increased in lung cells obtained from smokers with COPD (Zhu et al. 2001). Thus, the gene expression of these proinflammatory mediators has important implications for inflammatory lung disease states where the HDAC enzyme is inactivated (Cosio et al. 2004; Ito et al. 2001). In these cases, ROS and TNF-α would lead to an augmented inflammatory response from the tissue. However, it is important to bear in mind that another HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA), inhibited release of key proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IFN-γ, in monocytes/macrophages both in vitro and in vivo (Leoni et al. 2002). Thus, HDAC inhibitors may also exhibit anti-inflammatory properties through suppression of cytokine expression. At present, however, the molecular mechanism for the inhibition of these cytokines by SAHA is not yet known.

5.6.1.2.2 Glucocorticoids and Chromatin Remodeling

Corticosteroids are potent anti-inflammatory agents. However, they do not significantly inhibit the inflammatory responses in patients with COPD. Recently, it has been suggested that oxidative stress may induce inflammation and influence glucocorticoid sensitivity via remodeling of the chromatin structure (Adcock et al. 2005; Rahman et al. 2004). It has been shown that glucocorticoid suppression of inflammatory genes requires recruitment of HDAC-2 to the transcription activation complex by the glucocorticoid receptor (Fig. 5.10) (Berghe et al. 1999). This results in deacetylation of histones and a decrease in inflammatory gene transcription. HDAC-2 is inhibited by ROS and CSC, which is reflected by an increase in the expression of various proinflammatory mediators, such ICAM-1, IL-8, IL-6, TNF-α, IL-1β, monocyte chemoattractant protein-1, matrix metalloproteinases, and heat shock proteins, in BAL fluid of smokers (McCrea et al. 1994; Ragione et al. 2001) and in various other cells (Berghe et al. 1999; Pender et al. 2000; Rahman et al. 2001). A reduced level of HDAC-2 was associated with increased proinflammatory response and reduced responsiveness to glucocorticoids in alveolar macrophages obtained from smokers and in rat lungs in response to cigarette smoke exposure (Ito et al. 2001a: Marwick et al. 2004). Similarly, reduced levels of HDAC activity and HDAC1 and HDAC2 proteins were found in bronchial biopsies obtained from asthmatics (Ito et al. 2002). This was partially restored in patients who received inhaled

steroids, suggesting that steroids either induce HDAC or decrease HAT activity in asthmatics. However, this mechanism may not be fully operational in case of COPD. In a recent study, Culpitt and coworkers (2002) have shown that release of IL-8 and GM-CSF was not inhibited in cigarette smoke solution-stimulated alveolar macrophages obtained from COPD subjects, compared with that of smokers. They suggested that the lack of efficacy of corticosteroids in COPD might be because of steroid insensitivity of macrophages in the respiratory tract. Thus, the cigarette smoke/oxidant-mediated reduction in HDAC-2 levels in alveolar epithelial cells and macrophages will not only increase inflammatory gene expression, but will also cause a decrease in glucocorticoid function in patients with COPD (Cosio et al. 2004; Rahman et al. 2004). This may be one of the potential reasons for the failure of glucocorticoids to function effectively in reducing inflammation in COPD (Fig 5.10). Other plausible mechanisms that would explain glucocorticoid inefficacy in COPD may be p65 acetylation by cigarette smoke-derived oxidants, elevated levels of hepatocyte nuclear factor-6, or enhancement of macrophage migration inhibitory factor (MIF), which can antagonize/override glucocorticoid-stimulated gene transcription (Chen et al. 2001; Ito et al. 2001b; Lolis 2001; Pierreux et al. 1999). It is also interesting to note that certain other translocatory HDACs, such as HDAC3 and HDAC5, which shuttle between cytoplasm and the nucleus, may also be important in regulating the transcriptional initiation complex by the glucocorticoid receptor. The exact signaling mechanisms that may be involved in the cigarette smoke-mediated chromatin remodeling and glucocorticoid insensitivity are currently unknown. It may be possible that cigarette smoke-mediated formation of aldehydes could be responsible for oxidation/nitrosylation/phosphorylation of HDACs 1, 2, and 5 during inflammation (Manwick et al. 2004; Yang et al. 2006). This line of thought is supported by a report of the ability of theophylline, a polyphenol antioxidant, to restore HDAC activity in alveolar macrophages from patients with COPD (Cosio et al. 2004). Furthermore, theophylline improved the response to dexamethasone in these subjects. Nevertheless, in general, oxidative stress that results in an imbalance between histone acetylation and deacetylation may account for the enhanced expression of inflammatory mediators, leading to amplified lung inflammation. This may serve as a potential mechanism for therapeutic intervention to ameliorate chronic inflammatory response that occurs in the development of smoking-induced chronic inflammatory lung diseases, such as COPD.

5.6.1.2.3 Coactivator Adenoviral E1A and Chromatin Remodeling

Adenoviral DNA can integrate itself into the human genome, and this can lead to the amplification of viral oncoproteins, such as adenoviral protein (E1A). The major role of E1A is to induce entry into the S phase of the cell cycle so that the conditions are optimal for viral replication. CBP and adenovirus E1A-associated protein p300 play essential coactivator roles for a number of transcription factors, including CREB, NF-κB, and signal transducers and activators of transcription (STAT). Therefore, the presence of E1A has been suggested to be a possible factor in susceptibility to inflammation caused by cigarette smoke. It is understood that E1A subverts cellular processes by displacing cellular transcription factors from CBP (Yang et al. 1996). The *E1A* gene has been found to be expressed at a higher frequency in the lungs of COPD patients than in smokers without COPD (Hogg 1999). Retamales and colleagues (2001) suggests that cigarette smoke-

induced inflammatory processes that underlie emphysematous destruction of the lung in COPD are amplified in smokers with advanced disease, compared with those with similar smoking histories and preserved lung structure and function. This enhanced response to a similar degree of stimulation was attributed to a latent adenoviral infection of the alveolar surface epithelium. The presence of E1A also enhances the inflammatory response of cells to endotoxins and oxidative stress (Keicho et al. 1999). NF-κB activation, expression of IL-8, TGF-β and ICAM-1 were all enhanced in E1A+ve lung epithelial cells in response to oxidative stress, compared with E1A–ve cells (Gilmour et al. 2001; Higashimoto et al. 2002). Stimulation of E1A-transfected cell lines (THP-1 and Jurkat) led to an increased synthesis of TNF-α, compared with cells transfected with a control plasmid, suggesting that the presence of E1A may induce histone acetylation by increasing the intrinsic HAT activity of CBP. Thus, the presence of transactivating E1A primes the transcriptional machinery for oxidative stress signaling and in turn facilitates persistent amplification of proinflammatory responses. This may also interfere in the action of glucocorticoids in reducing the inflammatory responses in COPD.

5.7 Conclusions

Cigarette smoke-derived ROS are important in the pathogenesis of COPD and may be critical not only to the inflammatory response to cigarette smoke/environmental oxidants, through the activation of redox-sensitive transcription factors, DNA nicking, alteration in histone acetylation/deacetylation and hence, proinflammatory gene expression, but may also be involved in the protective mechanisms against the effects of cigarette smoke by the induction of antioxidant genes. Further understanding of the effects and roles of cigarette smoke-mediated ROS in basic cellular redox functions, such as amplification of proinflammatory and immunological responses, signaling pathways, activation of transcription factors, chromatin modeling (histone acetylation and deacetylation) and gene expression, will provide important information regarding basic pathological processes contributing to smoking-induced lung diseases.

GSH is an important protective antioxidant in the lungs, which is altered in lining fluid in several inflammatory diseases. The glutathione redox status plays an important role in protein modifications and signaling pathways, including a dual effect on redox-sensitive transcription factors. Protein *S*-glutathiolation and mixed disulfide formation as candidate mechanisms for protein regulation during intracellular oxidative stress have gained a renewed impetus in view of the involvement of oxidants/antioxidants in various disease processes. The upstream regulation of the MAPKs, involvement of the oxidative species in cell proliferative mechanisms, and transient silencing of various protein function by sulfenic-species formation are all important indicators as to how thiol-dependent reactions involving GSH, cysteine, methionine, and other small-molecular-weight thiols may determine the overall outcome of an oxidative stress imposed by cigarette smoke-derived. ROS can influence the inflammatory response through its impact on signal transduction mechanisms, activation of NF-κB, and chromatin modifications resulting in pro-inflammatory gene expression. Understanding of the molecular mechanisms of ROS-mediated cell signaling pathways could provide information for development of novel antioxidant therapeutic targets to prevent smoking-induced diseases. As our understanding of gene expression/epigenetics/genomics increases, further clinical targets and therapeutic strategies are likely to emerge.

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Chapter 6

Oxidative Stress in the Pathogenesis of Chronic Obstructive Pulmonary Disease

6

Irfan Rahman

Contents

Contents

6.1 Introduction

The lung is the only organ that has the highest exposure to atmospheric oxygen and together with its large surface area and blood supply is susceptible to injury mediated by reactive forms of oxygen species.

In situ lung injury because of reactive oxygen species (ROS) is linked to oxidation of proteins, DNA, and lipids. These oxidized biomolecules may also induce a variety of cellular responses through the generation of secondary metabolic reactive species. Physiologically, ROS inflict their effects by altering remodeling of extracellular matrix and blood vessels and stimulate mucus secretion and alveolar repair responses. On the biochemical level, ROS inactivate antiproteases, cause apoptosis, and regulate cell proliferation (Rahman and MacNee 1996, 1999) (Fig. 6.1) and modulate the immune system in the lungs. At the molecular level, increased ROS levels have been implicated in initiating inflammatory responses in the lungs through the activation of transcription factors such as nuclear factor-kappaB (NF-κB) and activator protein 1 (AP-1), signal transduction, chromatin remodeling, and gene expression of proinflammatory mediators (Rahman and MacNee 1998).

This chapter brings forth the evidence for the role of ROS pertaining to chronic obstructive pulmonary disease (COPD) and attempts to unravel the cellular and molecular mechanisms and pathophysiological consequences of increased ROS release in COPD.

The relevance of noninvasive oxidative stress biomarkers in the pathophysiology of COPD is also discussed.

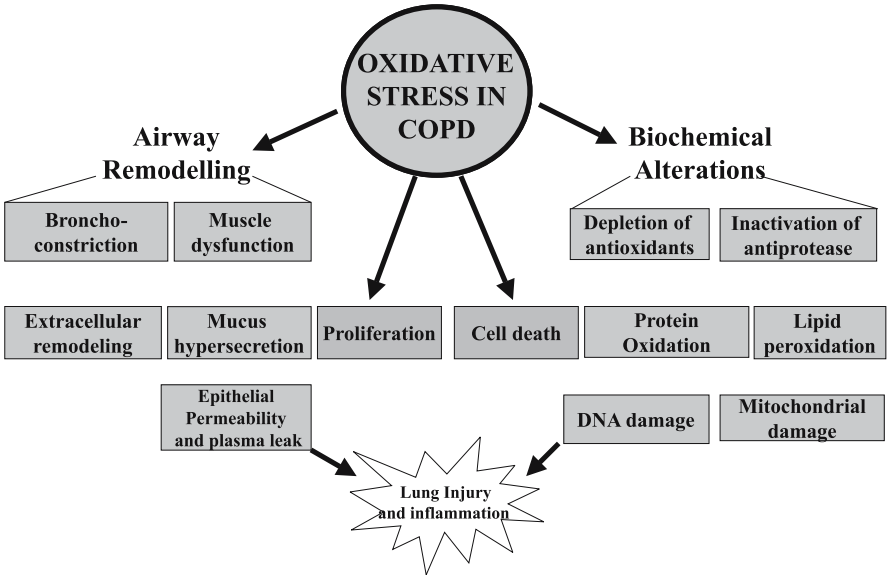


Fig. 6.1 Reactive oxygen species (ROS)-mediated cellular responses in chronic obstructive pulmonary disease (*COPD*)

6.2 Sources of Oxidants in the Lungs

6.2.1 Cell-Derived ROS/Oxidants

COPD is pathologically characterized by an inflammatory response, involving activation of epithelial cells, resident macrophages, and the recruitment and activation of pulmonary phagocytic cells and lymphocytes. Once recruited in the airspace, these cells become activated and generate ROS in response to inflammatory mediators. The primary ROS-generating enzyme is NADPH oxidase, a complex enzyme system that is present in phagocytes and epithelial cells. Following activation, macrophages, neutrophils, and eosinophils generate $O_2^{-}\cdot$, which is rapidly converted to hydrogen peroxide (H_2O_2) under the influence of superoxide dismutase (SOD). ·OH may be formed nonenzymatically in the presence of free catalytic iron (Fe^{2+}) as a secondary reaction. ROS and reactive nitrogen species (RNS) can also be generated intracellularly from several sources such as mitochondrial respiration, the NADPH oxidase system, and xanthine/xanthine oxidase. In addition to NADPH oxidase, phagocytes employ other enzymes to produce ROS and other potent oxidants such as hypochlorous acid (HOCl) and hypobromous acid (HOBr). The later two species are generated by the action of heme peroxidases (myeloperoxidase [MPO] or eosinophil peroxidase [EPO]).

6.2.2 Inhaled Oxidants and Cigarette Smoke

A direct consequence of cigarette smoking, inhalation of airborne pollutants, such as oxidant gases (ozone, nitrogen dioxide [NO_2], sulphur dioxide [SO_2]) or particulate in air, is lung damage and elevated inflammatory responses in the lungs. These environmental noxious agents are implicated in the pathogenesis and exacerbations of COPD. In the gas phase, the smoke contains high concentrations of oxidants/free radicals (>10^{15} molecules per puff) (Church and Pryor 1985), short-lived oxidants such as $O_2^{-}\cdot$, and nitric oxide (NO). NO and $O_2^{-}\cdot$ immediately react to form the highly reactive peroxynitrite ($ONOO^{-}$) molecule. The tar phase of cigarette smoke contains organic radicals, such as long-lived semiquinone radicals, which can react with molecular oxygen in a redox-dependent manner to form $O_2^{-}\cdot$ to form ·OH and H_2O_2 (Nakayama et al. 1989). The cycle can be sustained by biological reducing equivalents (ascorbate, NAD[P]H, glutathione [GSH], etc.), which reduce the oxidized quinoid substances back to their reduced states, enabling them to reproduce the superoxide radical. The aqueous phase of cigarette smoke condensate may undergo redox recycling for a considerable period of time in the epithelial lining fluid (ELF) of smokers (Nakayama et al. 1989). The tar phase is also an effective metal chelator wherein iron is chelated to produce tar semiquinone plus tar Fe^{2+}, which can generate H_2O_2 continuously (Nakayama et al. 1989). Sidestream cigarette smoke contains more than 10^{17} reactive organic compounds per puff, such as carbon monoxide, nicotine, ammonia, formaldehyde, acetaldehyde, crotonaldehyde, acrolein, *N*-nitrosamines, benzo[*a*]pyrene, benzene, isoprene, ethane, pentane, and other genotoxic and carcinogenic organic compounds. The concentrations of these reactive compounds present in the ELF following inhalation of cigarette smoke have been calculated by other workers (Eiserich et al. 1995).

6.3 COPD

6.3.1 Clinical Features

COPD is a gradually progressing condition characterized by airflow limitation, which is largely irreversible (Pauwels et al. 2001), and its diagnosis usually is based on the history of exposure to toxic stimuli (mainly tobacco smoke) and abnormal lung function tests. Because of variable pathology and only partial understanding of the molecular mechanisms involved in the disease process, a straightforward definition of COPD is yet to be understood. However, the diagnosis of COPD still based on the persistent presence of airflow obstruction in a cigarette smoker (Celli et al. 2004).

Cigarette smoking has been implicated as the major etiological factor underlying the disease. Although more than 90% of patients with COPD are smokers, not all smokers develop COPD (Pauwels et al 2001). However, 15–20% of smokers appear to be susceptible to the ill effects of smoking and show a rapid decline in lung function measured as forced expiratory volume in one second (FEV_1). This category of smokers is at an increased risk of developing the disease. Other factors that may exacerbate COPD are air pollutants, infections, and occupational dusts, which also have the potential to produce oxidative stress (Fig. 6.2). Based primarily on FEV_1, the Global Initiative for Chronic

Obstructive Lung Disease (GOLD) guidelines have classified the severity of COPD into four stages (Pauwels et al 2001). However, mere emphasis on FEV_1 alone as an index of severity for COPD has been criticized. A composite measure essentially based on clinical parameters (Body-Mass Index, Airflow Obstruction, Dyspnea, and Exercise Capacity [BODE] Index) has been shown to be better at predicting mortality than does FEV_1 (Celli et al. 2004).

Although the development of COPD in terms of deterioration of FEV_1 is yet to be confirmed, there is, however, a tendency to classify the stages as evolving from stage 0 to stage 4. In the context that not all smokers develop COPD, it is similarly possible that the disease may not progress from one stage to the next in an established sequence. In relatively young patients with severe COPD, it is yet to be established if early stages of their disease are similar to those found in patients with mild COPD. With COPD being a heterogeneous disease, several outcomes may be seen at each of the stages. For example, recently, Hogg and coworkers (2004) have shown that the progression of COPD was strongly associated with an increase in the volume of tissue in the wall and the accumulation of inflammatory mucous exudates in the lumen of the small airways. The percentage of the airways that contained polymorphonuclear neutrophils, macrophages,

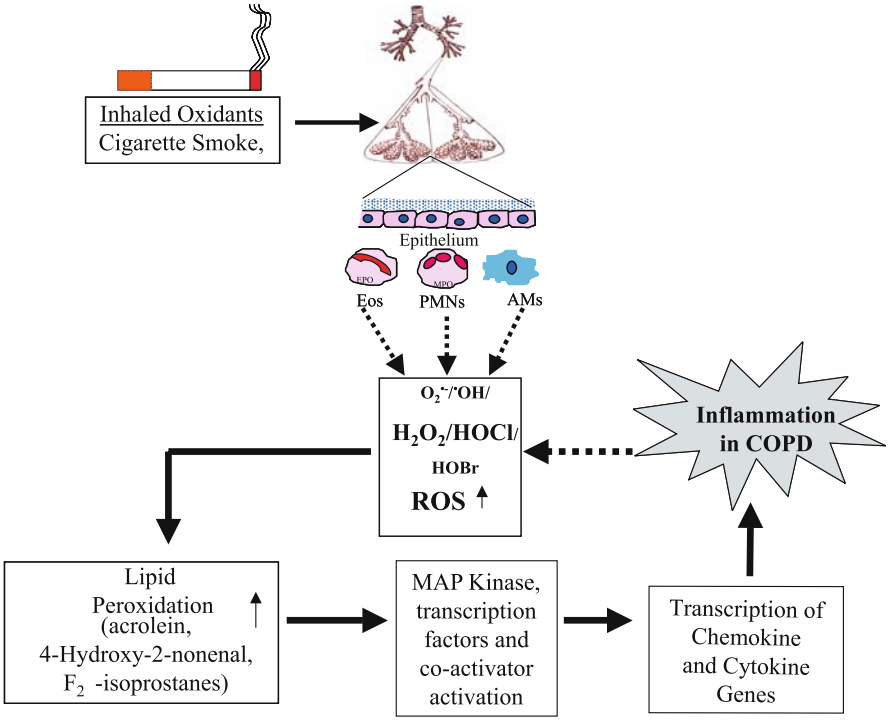


Fig. 6.2 Mechanisms of reactive oxygen species (ROS)-mediated lung inflammation in chronic obstructive pulmonary disease (*COPD*). Inflammatory response is mediated by oxidants either inhaled and/or released by the activated neutrophils, alveolar macrophages, eosinophils, and epithelial cells, leading to production of ROS and membrane lipid peroxidation. Activation of transcription of the proinflammatory cytokine and chemokine genes, upregulation of adhesion molecules, and increased release of proinflammatory mediators are involved in the inflammatory responses in patients with COPD

CD4, CD8, and B cells increases as COPD progresses. Systematic experimental modeling of each stage of severity might provide a better insight into this issue (Groneberg and Chung 2004).

6.4 Morphological/Cellular Alterations in COPD

6.4.1 Airspace Epithelial Injury/Permeability

An important early consequence of inflammation because of cigarette smoke is airspace epithelial injury that leads to an increase in airspace epithelial permeability. ^{99m}Technetium-diethylenetriaminepentacetate (^{99m}Tc-DTPA) lung clearance studies in humans have revealed increased epithelial permeability in chronic smokers, compared with nonsmokers. ^{99m}Tc-DTPA clearance was further increased following acute smoking (Morrison et al. 1999). Extra- and intracellular levels of GSH appear to be critical for the maintenance of epithelial integrity following exposure to cigarette smoke. Studies involving epithelial cell monolayers in vitro and in rat lungs in vivo following exposure to cigarette smoke condensate have demonstrated increased epithelial permeability. This increase in epithelial permeability was related to profound changes in the antioxidant GSH (Rahman and MacNee 1999, 2000). Treatment of cells with GSH markedly diminished cigarette smoke-induced epithelial injury/permeability, suggesting that the injurious effects were in part oxidant-mediated (Rahman and MacNee 1999).

6.4.2 Neutrophil Sequestration and Migration in the Lungs

The pathophysiology of COPD includes an important role of neutrophils. These cells can release a multitude of mediators and tissue-degrading enzymes such as elastases, which can orchestrate tissue destruction and chronic inflammation (Chung 2001; Stockley 2002). A large number of mediators including cytokines released from resident lung cells, alveolar macrophages, epithelial, and endothelial cells can activate neutrophils while in transit in the pulmonary microcirculation. Inhaled oxidants such as those contained in cigarette smoke and other air pollutants could influence the transit of cells in the pulmonary capillary bed by decreasing neutrophil deformability (shape alterations in neutrophils to pass the smaller capillary segments).

Increased neutrophils and macrophages have been reported in bronchoalveolar lavage fluid (BALF) from cigarette smokers (Hunninghake and Crystal 1983). Induced sputum neutrophilia has been found to be higher in patients with a high degree of airflow limitation than in subjects without airflow limitation. Increased sputum neutrophilia is also related to an accelerated decrease in FEV_1. Subjects with chronic cough and sputum production have a greater incidence of sputum neutrophilia (Stanescu et al. 1996). Studies in humans, using radiolabeled neutrophils and red cells, show a transient increase in neutrophil sequestration and decreased neutrophil deformability in the lungs during smoking/oxidative stress (Rahman and MacNee 1996) and returns to normal on cessation of smoking. A similar decrease in deformability is demonstrated in vivo for neutrophils from the blood of subjects who are actively smoking (Rahman and MacNee 1996). Activation of neutrophils sequestered in the pulmonary microvasculature could

induce the release of ROS and proteases within the microenvironment, leading to destruction of the alveolar wall that occurs in emphysema.

6.5 Biochemical Features of COPD

6.5.1 Antioxidant Status in COPD

6.5.1.1 Systemic and Local Depletion of Antioxidants

The antioxidants armamentarium of ELF is composed of low-molecular-weight antioxidants, metal-binding proteins, antioxidant enzymes, sacrificial reactive proteins, and unsaturated lipids. The major antioxidants in ELF include mucin, reduced GSH, uric acid, protein (largely albumin), ceruloplasmin, and ascorbic acid (Cross et al. 1994). Depending on the redox environment, the concentrations of nonenzymatic antioxidants may vary in ELF. Some of these antioxidants, such as GSH and ascorbate, are concentrated in ELF, compared with plasma. This indicates their relative importance in the antioxidant defenses of ELF. Smoking and exacerbations of COPD are reflected in the plasma by decreased antioxidant capacity, associated with depleted protein sulfhydryls in the plasma (Rahman et al. 1996, 1997). The decrease in antioxidant capacity in smokers occurred transiently during smoking and resolved rapidly after smoking cessation (Rahman et al. 1996). In exacerbations of COPD, however, diminished antioxidant capacity continued for several days after the onset of the exacerbation. The levels tended towards controls only at the time of recovery from the exacerbation (Rahman et al. 1997). The depletion of antioxidant capacity could be partially explained by increased release of ROS from peripheral blood neutrophils. ROS thus released are neutralized by the antioxidants, which leads to the reduction of antioxidant levels. This observation is further emphasized by a significant negative correlation between neutrophil superoxide anion release and plasma antioxidant capacity (Rahman et al. 1996).

Depletion of total antioxidant capacity in smokers is associated with depletion of major plasma antioxidants, i.e., ascorbic acid, vitamin E, β-carotene, and selenium in the serum of chronic smokers (Mezzetti et al. 1995). No such consensus has been established for changes in other antioxidants and antioxidant enzymes in response to cigarette smoke. Reduced levels of vitamin E in the BALF of smokers and a marginal increase in vitamin C in BALF of smokers, compared with nonsmokers have been shown (Rahman and MacNee 1996). Thus, there is clear evidence that oxidants in cigarette smoke markedly decrease plasma and BALF antioxidants.

6.5.1.2 Depletion of Lung GSH

GSH homeostasis may play a pivotal role in maintaining the integrity of lung airspace epithelial barrier. This is supported by the observation that a decrease in the levels of GSH in epithelial cells alters barrier function and increases epithelial permeability (Morrison et al. 1999; Rahman and MacNee 2000; Rahman et al. 1995). There is, however, limited information as to the status of respiratory epithelial antioxidant defenses in smokers, and even less for patients with COPD. Several studies have shown that GSH is

elevated in BALF in chronic smokers (Cantin et al. 1987; Morrison et al. 1999). However, this increase is not observed immediately after acute cigarette smoking (Morrison et al. 1999). Interestingly, Harju and colleagues (2002) have found that γ-glutamylcysteine synthetase, ([γ-GCS] now called γ-glutamale cysteine ligase [GCL]) immunoreactivity was decreased (GSH levels were not measured) in the airways of smokers, compared with nonsmokers, suggesting that cigarette smoke predisposes lung cells to ongoing oxidant stress. GCL has light regulatory and heavy catalytic subunits. This indicates that a twofold increase of GSH in BALF in chronic smokers may not suffice for the excessive oxidant burden recorded during smoking. In such a condition, an acute depletion of GSH may be observed (Morrison et al. 1999; Rahman and MacNee 2000). Neurohr and colleagues (2003) recently attributed decreased GSH levels in BALF cells of chronic smokers to a decreased expression of the GCL light subunit. However, no change was observed in GCL heavy subunit expression. This highlight the fact that increased GSH level in ELF of chronic smokers may not be associated with increased GSH levels in alveolar macrophages. Thus, further in-depth studies are needed to understand the regulation of GSH levels in the lungs of smokers and COPD subjects in order to devise appropriate antioxidant GSH therapy.

6.6 Oxidants and Oxidative Stress in COPD

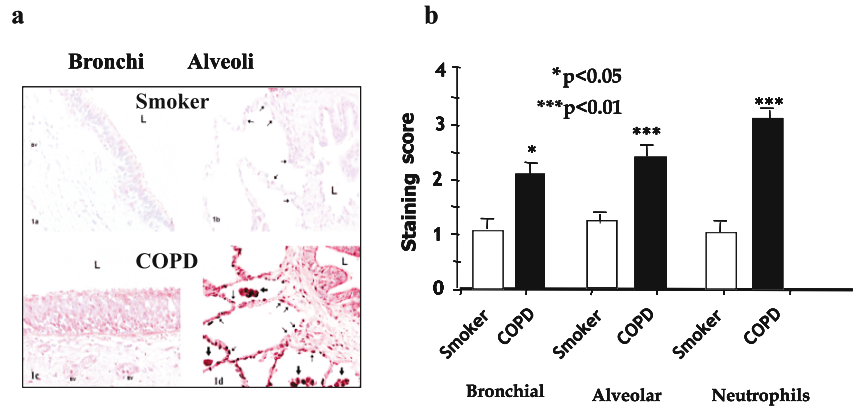
6.6.1 Oxidative Stress in the Alveolar Space

Increased oxidant burden in the lungs is characteristic of COPD. In smokers, the oxidant load is enhanced by the release of ROS from macrophages and neutrophils, which are known to migrate in increased numbers into the lungs of cigarette smokers. Once activated in the lungs, these cells can generate ROS via the NADPH oxidase system (also known as respiratory burst oxidase). The respiratory burst is an important step implicated in a sudden surge in the generation of $O_2^{-}\cdot$. Circulating neutrophils from cigarette smokers and patients with COPD release more $O_2^{-}\cdot$ (Rahman et al 1996). Increased content of MPO in neutrophils from smoker has been found to correlate with the degree of pulmonary dysfunction (Gompertz et al. 2001), suggesting that neutrophil MPO-mediated oxidative stress may be important in the pathogenesis of COPD. Macrophages obtained from BALF of smokers are activated, compared with those from nonsmokers. One manifestation of this is the release of increased amounts of ROS such as $O_2^{-}\cdot$ and H_2O_2 (Morrison et al. 1999; Rahman and MacNee 1996).

COPD subjects manifest higher intracellular iron content in alveolar macrophages, which is increased in cigarette smokers. The iron level is further increased in chronic bronchitis, compared with nonsmokers (Lapenna et al. 1995). High iron levels augment the generation of ROS in ELF in the airspaces of smokers and COPD subjects. This is further supported by the observation that lungs of smokers contain darkly pigmented areas. These pigmented areas yield high electron spin resonance (ESR) signals because of heme/nonheme iron and carbon-centered radicals (Church and Pryor 1985). This may relate to excessive iron accumulation in the alveolar macrophages of smokers and COPD subjects.

6.6.2 Alterations in Lung Tissue

An important link between oxidative stress and the pathogenesis of COPD has emerged from the demonstration of ROS-dependent oxidative modification of target molecules and their presence in increased amounts in the lungs of smokers, particularly those who develop COPD. ROS such as $O_2^{-}\cdot$ and ·OH, generated and released by activated immune and inflammatory cells, are highly reactive and when generated in close vicinity to cell membranes, initiate membrane phospholipids peroxidation (lipid peroxidation). Lipid peroxidation then may continue as a chain reaction, leading to membrane and cell damage. Many of the effects of ROS in airways may be mediated by the secondary release of inflammatory lipid mediators such as 4-hydroxy-2-nonenal (4-HNE), a footprint of oxidative stress/lipid peroxidation. 4-HNE, a highly reactive and diffusible end product of lipid peroxidation, is known to induce/regulate various cellular events, such as proliferation, apoptosis, and activation of mitogen-activated protein kinase (MAPK) signaling pathways (Uchida et al. 1999). 4-HNE has a high affinity towards cysteine, histidine, and lysine residues and also conjugates with GSH. Conjugation of 4-HNE with amino acid residues in proteins may render a protein nonfunctional. It is also chemotactic to neutrophils in vitro and in vivo. The levels of 4-HNE-modified protein were increased in airway and alveolar epithelial cells, endothelial cells, and neutrophils in subjects with airway obstruction, compared with otherwise normal subjects (Rahman et al. 2002) (Fig. 6.3). Furthermore, Aoshiba and colleagues (2003a) have demonstrated that cigarette smoking enhanced the levels of 4-HNE adducts in bronchiolar epithelial and alveolar type cells in mice. Thus, evidence is accumulating that cigarette smoke triggers oxidative



Photographs from immunostaining for 4-HNE in lung tissue from subjects with and without COPD. 1b: Non-COPD; 2b: COPD, L=Lumen. Original magnification: 200X

Fig. 6.3 a Photographs from immunostaining for 4-hydroxy-2-nonenal (*4-HNE*) in lung tissue from subjects with and without chronic obstructive pulmonary disease (*COPD*). *1a* Non-COPD, bronchial; *1b* Non-COPD, alveolar; *2a* COPD, bronchial; *2b* COPD, alveolar. *L* lumen. Original magnification, ×200. b Individual immunostaining scores for 4-HNE-adducts in bronchial and alveolar lung tissue in epithelial cells, endothelial cells, and neutrophils. The mean is indicated, as well significance levels (*p*) for differences between the indicated groups

stress, leading to lipid peroxidation in lungs of smokers and patients with COPD. Lipid peroxidation may further herald extensive damage to the lung architecture.

6.6.3 Systemic Oxidative Stress

In addition to in the lungs, COPD is now considered to have systemic manifestations. One manifestation of a systemic effect is the presence of markers of oxidative stress in the blood in patients with COPD. Various studies have demonstrated increased production of $O_2^{-}\cdot$ from peripheral blood neutrophils obtained from patients during acute exacerbations of COPD, which returned to normal when the patients were clinically stable (Noguera et al. 1998; Rahman et al. 1996, 1997). Another biological source of $O_2^{-}\cdot$ and H_2O_2 is the cellular xanthine/xanthine oxidase (XO) system, which has been shown to be increased in cell free BALF and plasma from COPD patients. (Rahman et al.1996) This observation has been further associated with increased $O_2^{-}\cdot$ and lipid peroxide levels in COPD subjects (Pinamonti et al. 1998).

6.7 Noninvasive Biomarkers of Oxidative Stress

6.7.1 ROS and RNS as Surrogate Markers in Plasma and Exhaled Breath Condensate

Assessment of oxidative end products as biomarkers by both noninvasive and semi-invasive techniques has recently emerged to be of interest for understanding the status of COPD. Measurements of surrogate biomarkers oxidative stress have been made in blood, urine, breath, or exhaled breath condensate or in induced or spontaneously produced sputum of smokers and patients with COPD. Oxidative stress biomarker assessment in exhaled breath condensate (EBC) is emerging as a promising area of future research in COPD (Kharitonov and Barnes 2001; Rahman and Kelly 2003). The relative concentrations of various oxidant/antioxidant biomarkers detected in EBC are listed in Table 6.1 (Kharitonov and Barnes 2001; Rahman and Kelley 2003) There is, however, a limitation of oxidative stress biomarkers at present because of the lack of correlation with disease severity or outcome and the variations in the control subjects. Furthermore, the validity of EBC as a tool for the assessment of airway oxidative stress is still questionable, owing to limitations in the reproducibility of analyzed oxidative biomarkers, with respect to intra- and interindividual variability, sampling time, and variability in dilution of respiratory droplets by water vapor, sensitivity, and specificity of the assays used (Rahman 2004; Rahman and Biswas 2004). Other confounding factors contributing to variation include smoking, consumption of caffeine, alcohol, and intake of diet rich in antioxidants. Identification of specific and reproducible biomarkers of oxidative stress and inflammation in EBC would be of great value for noninvasive investigations of the natural history and epidemiology of COPD and for phenotyping in genetic perspectives. Exhaled NO has also been used as a marker of airway inflammation and as an alternate measure of oxidative stress. Sporadic reports of increased levels of NO in exhaled breath in patients with COPD are available, but not to the extent reported in asthmatics (Rahman et al. 1996). Although smoking increases NO levels in breath, the spontaneously

rapid reaction of NO with $O_2^{-}\cdot$ (which forms $ONOO^{-}$), however, limits the usefulness of this marker in COPD. Generation of RNS because of cigarette smoke results in nitration and oxidation of various cellular and plasma proteins at their tyrosine residues. The levels of nitrated proteins (3-nitrotyrosine) were conspicuously higher in smokers, compared with nonsmokers (Petruzzelli et al. 1997). NO and $ONOO^{-}$-mediated formation of 3-nitrotyrosine in plasma and Fe^{2+} levels in ELF) are elevated in chronic smokers (Ichinose et al. 2000; Kanazawa et al. 2003). The levels of nitrotyrosine were negatively correlated with the FEV_1%. Also, NO generated in the cells has been found to be involved in the depletion of GSH levels and may be important because their binding to the thiol to form *S*-nitrosoglutathione (GSNO). Of interest is the recent finding that sputum concentrations of nitrosothiols and oxidized GSH (GSSG) are increased in patients with COPD and associated to neutrophilic inflammation (Beeh et al. 2004).

These data further underline the role of oxidative stress in the pathogenesis of COPD and suggest that GSH is important to scavenge both reactive oxygen and nitrogen species. Because GSSG and nitrosothiols can be detected in sputum, this could be a simple, noninvasive source of a COPD marker. $ONOO^{-}$, on the other hand, can bring about thiol oxidation, lysosomal degranulation, and various oxidative changes that are potentially injurious to the cells. This indicates that the increased level of RNS plays a role in

Table 6.1 Oxidant/antioxidant biomarkers in exhaled breath condensate and their measurements

Biomarker	Analysis	Values in EBC		
		Nonsmoker	Smoker	COPD
Reactive oxygen species				
Hydrogen peroxide (μ*M*)	Spectroscopy, Fluorometry, chemiluminesce	0.01–0.09	0.10–0.75	0.2–2.6
Reactive nitrogen species				
Nitrite (μ*M*)	Electrometry, spectroscopy,	0.64	0.44–2.4	2.6
Nitrite and nitrate (μ*M*)	ELISA, GC/MS, HPLC	20.2	20.2–29.2	–
Nitrotyrosine (ng/ml)		0.66–6.3	7.2	–
Nitrosothiols (μ*M*)		–	0.1–0.46	0.24
Lipid peroxidation products				
TBARS (μ*M*)	Colorimetry, ELISA	–	–	0.48
Malondialdehydes (nmol/l)	EIA, GC/MS, HPLC	17.2–19.4 nmol/l by LC/MS	–	57.2 by LC/MS
F_2-Isoprostanes (pg/ml)	LC/MS	3.9–15.8 by EIA, and 7±4 by GC/MS	–	42.5
Antioxidant				
Glutathione, n*M*	Spectrophotometry, HPLC, LC/MS	14.1	–	–

Values are adapted in part from Rahman and Biswas 2004

EBC exhaled breath condensate, *COPD* chronic obstructive pulmonary disease, *ELISA* enzyme-linked immunosorbent assay, *GC/MS* gas chromatography/mass spectrometry, *HPLC* high-performance liquid chromatography, *TBARS* thiobarbituric acid reactive substances, *EIA* enzyme immunoassay, *LC/MS* liquid chromatography-tandem mass spectrometry

the abnormal inflammatory response that occurs in COPD and may be involved in tissue damage.

6.7.2 H_2O_2

H_2O_2, measured in EBC, is a direct measurement of oxidant burden in the airspaces. The source of H_2O_2 in EBC is unknown, but may in part derive from the release of $O_2^{-}\cdot$ from alveolar macrophages. Smokers and patients with COPD have higher levels of exhaled H_2O_2 than do nonsmokers, and levels are even higher during exacerbations of COPD (Kharitonov and Barnes 2001; Rahman and Kelly 2003). However, H_2O_2 levels vary considerably in healthy, young nonsmokers and smokers from 0.01 to 0.09 μ*M* and 0.10 to 0.75 μ*M*, respectively (Rahman 2004). This variation (60–80%) in H_2O_2 concentrations may be attributed to different storage conditions and/or analytical techniques used for EBC H_2O_2 assay. Thus, the variability of the measurement of exhaled H_2O_2 because of its highly volatile nature, along with the presence of other confounding factors, e.g., increased generation of ROS by cigarette smoke-mediated redox cycling, has led to concerns about its reproducibility as a marker of oxidative stress in smokers and in patients with COPD (Rahman 2004; Rahman and Biswas 2004). Recently, Gerristen et al. (2005) have demonstrated increased levels of exhaled H_2O_2, interleukin 8 (IL-8) and the soluble cell adhesion molecule (sICAM), and sE-selectin in serum of freshly admitted COPD patients. On medication with prednisolone, the levels of H_2O_2 were found to be significantly reduced in the exhaled breath and a concomitant decrease in the serum levels of IL-8 and sICAM was found. However, no appreciable alterations were observed for sE-selectins and spirometry. This further emphasizes the importance of certain cell adhesion molecules as biomarkers of COPD for diagnosis and prognosis of the disease (Gerritsen et al. 2005). A study characterizing the relationship between oxidative stress and inflammatory components in induced sputum and blood of COPD patients has been conducted by Sadowska et al. (2005). In their study, the authors have established a correlation between inflammatory markers such as IL-8, eosinophil cationic protein (ECP), sICAM-1, and oxidative stress markers such as SOD, GSH peroxidase (GPx), Trolox-equivalent antioxidant capacity (TEAC), albumin, vitamins E, and A. Treatment with *N*-acetyl-L-cysteine and/or inhaled corticosteroids was found to significantly modulate the correlation of these markers of COPD.

6.7.3 Lipid Peroxidation Products

Evidence of aldehydes being generated endogenously during the process of lipid peroxidation has been amply demonstrated. These aldehydes are being implicated in many pathophysiological processes and are associated with oxidative stress in cells and tissues. In addition to their cytotoxic properties, lipid peroxides are now recognized as mediators of inflammatory response via the signal transduction pathways.

Isoprostanes are products of nonenzymatic lipid peroxidation. They have also been used as markers of oxidative stress. The levels of 8-iso-prostaglandin F_2 (8-isoprostane) in EBC are elevated in healthy smokers and more markedly in patients with COPD reflecting the degree of oxidative stress (Kharitonov and Barnes 2001; Montuschi et al.

2000). 8-Isoprostane levels were elevated in the plasma and BALF in chronic smokers and patients with stable COPD, as compared with healthy, age-matched nonsmoking subjects (Rahman and Biswas, 2004). Furthermore, F_2-isoprostanes were even higher in plasma of patients with acute exacerbations of COPD than in patients with stable COPD, indicating that increased oxidative stress occurs in exacerbations. Increased levels of these markers of lipid peroxidation products have been further correlated with airway obstruction. Because of its consistent association with the disease process, 8-isoprostane is currently considered to be a reliable oxidative stress biomarker that can be easily detected in plasma, EBC, and BALF of patients with COPD. In a more recent study, Santus et al. (2004) have reported an increased urinary excretion of the isoprostane 8-iso-prostaglandin $F_2\alpha$ as an index of in vivo oxidant stress. Overnight urinary excretion of the isoprostane was found to be significantly higher in COPD patients than in controls. Treatment with polyphenol extract resulted in a significant decrease in isoprostane excretion, which was found to be correlated with significant increase of partial pressure of arterial oxygen. Furthermore, changes in FEV_1 significantly correlated with the changes in isoprostane urinary excretion observed from enrollment to the end of treatment.

Indirect and nonspecific measurements of lipid peroxidation products, such as thiobarbituric acid reactive substances (TBARS) have also been shown to be elevated in breath condensate and in lungs of patients with stable COPD (Fahn et al. 1998; Nowak et al. 1999). The level of plasma lipid peroxides (TBARS) is elevated in COPD and has been negatively correlated with the FEV_1. Other specific products of lipid peroxidation such as malondialdehyde (MDA) and 4-HNE, derivatized immediately after sampling, can be measured successfully by a more reliable method employing high-performance liquid chromatography (HPLC). Using liquid chromatography-tandem mass spectrometry (LC/TMS), Corradi and coworkers (2003) have recently shown higher concentrations of MDA in the EBC of COPD. Further longitudinal and cross-sectional studies of well-characterized smokers with and without COPD are needed to evaluate the correlation of a broad array of putative oxidative markers of COPD to susceptibility, severity, exacerbations, progression, and status of the disease.

6.8 Consequences of Oxidative Stress in COPD

6.8.1 Protease/Antiprotease Imbalance

An imbalance between protease and antiprotease enzymes has been hypothesized with respect to the pathogenesis of emphysema (Shapiro 2003). This hypothesis was based on the observation that α1-antitrypsin (α_1-AT)-deficient subjects develop severe emphysema and that protease–antiprotease imbalance was later demonstrated in animal models of COPD (Hautamaki et al. 2004; Shapiro et al. 2003). Although α_1-AT deficiency is a very rare cause of emphysema (Parfrey et al. 2003), it implies the importance of proteases and proteolysis in COPD (Barnes et al. 2003). Studies have shown that mice deficient in neutrophil elastase were significantly protected from emphysema from chronic cigarette smoke exposure (Shapiro et al. 2003). Similarly, mice deficient in the macrophage elastase gene also were protected from emphysema induced by cigarette smoke (Hautamaki et al. 1997). Each of these elastases were found to cross-inactivate the endogenous inhibitor of the other, with macrophage elastase degrading α_1-AT

and neutrophil elastase degrading tissue inhibitor of metalloproteinase-1 (Shapiro et al. 2003). Animals with elastase gene-deficiency when exposed to cigarette smoke showed impaired recruitment of neutrophils and monocytes. In addition, decreased macrophage elastase activity was observed, which was attributed to a decreased macrophage influx in these animals. Thus, it appears that neutrophil and macrophage elastase may play a major role in the mediation of alveolar destruction in response to cigarette smoke (Hautamaki et al. 1997; Shapiro et al. 2003).

In the case of smokers having normal levels of α_1-AT, the elastase burden may be increased as a result of increased recruitment of leukocytes into the lungs. A further functional deficiency of α_1-AT may be developed because of oxidative inactivation of α_1-AT in the lungs because of oxidative modification of methionine at the active site of the enzyme. Such oxidative inactivation may be carried out by the oxidants present in the cigarette smoke. Furthermore, oxidation of the methionine residue in α_1-AT was confirmed in the lungs of healthy smokers (Carp et al. 1982). These studies supported the concept of inactivation of α_1-AT by oxidation of the active site of the protein. Early studies showed that the function of α_1-AT in BALF was decreased by approximately 40% in smokers, compared with nonsmokers (Gadek et al. 1979). Secretory leukoprotease inhibitor (SLPI), another major inhibitor of neutrophil elastase (NE), can also be inactivated by oxidants. In vitro studies have also shown loss of α_1-AT inhibitory capacity from oxidants or cigarette smoke. All the above studies and many more reports have thus supported the hypothesis that α_1-AT pays an important role in the early pathogenesis of emphysema in COPD subjects.

6.8.2 Mucus Hypersecretion

Mucins are complex glycoproteins that provide viscoelastic properties to the mucus and form an essential protective mechanism in the upper airways. Mucins are secreted by goblet cells in the airway tracts and are responsible for removal of many types of substances from the lungs. The regulation of mucin production has been found to be altered in the lungs of COPD patients. Goblet cell hyperplasia has been documented in smokers. Goblet cell activation results in mucus hypersecretion, leading to airway plugging. Cigarette smoke has been found to activate epidermal growth factor (EGF) receptors by tyrosine phosphorylation. This results in the induction of mucin gene (*MUC5AC*) expression and synthesis in epithelial cells and in vivo in lungs (Takeyama et al. 2001). Tobacco smoke-induced mucin transcription was directly controlled by an AP-1-containing response element located at –3700/–3337 in the *MUC5AC* 5' flanking region. Site-specific mutagenesis revealed that the major functional components of this region were two AP-1 sites. The ability of smoke to stimulate *MUC5AC* through AP-1 strongly depends on both c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) for transcriptional activity. Although ERK activation was a consequence of epidermal growth factor receptor (EGFR) activation, JNK activation by smoke was mediated by Src in an EGFR-independent manner triggered by ROS (Gensch et al. 2004). Inhibition of tyrosine phosphorylation by selective EGFR tyrosine kinase inhibitors and antioxidants inhibits cigarette smoke-mediated *MUC5AC* gene expression. This suggests that ROS have a role in the mucin gene expression (Takeyama et al. 2001). 4-HNE, a lipid peroxidation product, has been shown to induce release of mucus from airway epithelial cells and fibroblasts via activation of extracellular signal-related kinases (ERK1/2) (Tsu-

kagoshi et al. 2002). Furthermore, elastase released from neutrophils has been shown to impair mucociliary clearance and stimulates goblet cell metaplasia and mucin production. Neutrophil elastase increases the expression of *MUC5AC* by enhancing mRNA stability in the cytosol (Fischer and Voynow 2002). Detailed understanding of EGFR signaling pathway and regulation of *MUC5AC* expression by cigarette smoke could lead to targeted inhibition of mucus hyperproduction in epithelial cells.

6.8.3 Remodeling of Extracellular Matrix

Oxidative stress has been implicated in the remodeling of extracellular matrix in lung injury. Treatment of alveolar macrophages, obtained from COPD subjects with cigarette smoke, induced increased release of matrix metalloproteinase 9 (MMP-9), compared with that of nonsmokers. MMP-9 has extracellular matrix-degrading activity, thus suggesting the role of oxidative component of cigarette smoke in increased elastolytic enzyme activity (Russell et al. 2002). This increase in the elastolytic activity may have its genesis in the generation of hypochlorous acid or peroxynitrite by the alveolar macrophages, leading to extracellular matrix breakdown (Kirkham et al. 2003). MMP-9 synthesis and secretion has very recently been found to be triggered by IL-17 released by T lymphocytes (Prause et al. 2004). However, increased proteolytic load because of MMP-9 has been attributed to increased neutrophil recruitment in the lungs that triggers degradation of extracellular matrix and basement membrane in the airways and lungs. Factors other than oxidative stress, such as ozone and lipid peroxides, also induce matrix protein type I collagen and *MMP-1* gene expression (Choi et al. 1994). Other forms of oxidative stress derived from *tert*-butyl hydroperoxide and iron can also modify collagen synthesis by a mechanism presumably involving redox sensor/receptor. Wang and colleagues (2003) have recently documented that cigarette smoke produces airway wall remodeling in rat tracheal explants by induction of procollagen and NF-κB activation via a ROS-dependent mechanism. They suggested that transactivation of EGFR, rather than MAPK activation, was involved in airway remodeling. Furthermore, cigarette smoke-mediated activation of EGFR was found to be oxidant dependent.

Another important extracellular component is hyaluronan (HA) (Dentener et al. 2005). Extracellular matrix HA has proinflammatory role and has been found increased in sputum of COPD patients. Two categories of COPD subjects have been identified, one group having high HA levels and the other having moderate levels. COPD subjects exhibiting higher HA levels had low FEV_1 as compared with moderated and control categories. Furthermore, increased breakdown and therefore increased HA levels were further correlated with an increased expression of the *HYAL2* gene, and increased HA breakdown has been associated with local inflammation and severity of COPD.

6.8.4 Apoptosis

Alveolar epithelial lining cells have been found to undergo apoptosis in response to cigarette smoking, with consequent progressive cell loss and development of emphysema. Recent in vitro and in vivo studies in animals and humans have provided evidence of induction of apoptosis in cigarette smoke-treated macrophages, airway epithelial, den-

dritic cells, and lung fibroblasts (Carnevali et al. 2003; Hoshino et al. 2001). Therefore, it appears that oxidative stress may mediate apoptotic processes, particularly in airway epithelial cells. Cigarette smoke extract damages vascular endothelial cells through the JNK pathway, which is activated by oxidative stress (Hoshino et al. 2005). This may be because of oxidant dependent activation of caspases and/or apoptosis signal-regulating kinase-1 (ASK-1). The latter is held in an inactive form by thioredoxin, a redox-sensitive enzyme. Oxidant burden because of ROS triggers oxidation of thioredoxin, leading to an apoptotic cascade (Saitoh et al. 1998). Recently, both in vivo and in vitro investigations have revealed that cigarette smoke triggers endothelial cell apoptosis (Kasahara et al. 2000, 2001; Mullick et al. 2002; Wang et al. 2001), and that pulmonary vascular endothelial cell apoptosis is present in emphysematous lungs (Kasahara et al. 2003). Tuder and colleagues (2003) have shown that general inhibition of vascular endothelial growth factor receptor 2 (VEGF-KDR) leads to an increased oxidative stress that is mediated by reactive carbonyls and aldehydes (4-hydroxy-2-nonenal), leading to emphysema. They have further shown that both caspase inhibitor (Z-Asp-CH2) and a superoxide dismutase mimetic (M40419) blocked the development of emphysema and significantly reduced lung markers of oxidative stress and apoptosis (Mullick et al. 2002). Howeven, Mansick et al. (2006) have shown that cigarette smoke decreased VEGFR2 but it was not assonated with emphysema in that lungs or in patients with COPD. In a more recent study, Karnenburg et al. (2005) have demonstrated that VEGF and its receptors Flt-1 and KDR/Flk-1 may induce peripheral vascular and airway remodeling processes via an autocrine or paracrine mechanism. The expression of VEGF and its receptors were greatly enhanced in the vascular and airway smooth muscles (VSM and ASM) bronchial, bronchiolar and alveolar epithelium, and alveolar macrophages COPD subjects as compared with control subjects. Whereas KDR/Flk-1 was markedly enhanced in endothelial cells and intimal and medial VSMs, Flt-1 was more intensively expressed in the endothelial cells. VEGF expression was found to be high in pulmonary microvasculature and intimal and medial VSMs. The enhanced expression of VEGF and its receptors correlated inversely with FEV_1.

Tsuji and his colleagues (2004) have reported that sublethal concentrations of CS induce senescence phenotypes in alveolar epithelial cells both in vitro and in vivo. Cellular senescence may be involved in the cigarette smoke-related pulmonary diseases associated with chronic epithelial damage. Cigarette smoking may induce the senescence of alveolar epithelial cells. In addition, the continuous proliferation of alveolar epithelial cells required to regenerate their loss as a result of apoptosis or necrosis accelerates telomere shortening, which in turn leads to the senescence of alveolar epithelial cells. When the alveolar epithelial cells reach the senescence stage, epithelial proliferation ceases, and the alveolar damage in cigarette smokers is no longer repaired. This model provides a plausible explanation for the chronic nature of cigarette smoke-related pulmonary diseases, such as pulmonary emphysema and fibrosis, which evolve slowly over many years.

Investigations in various cell models have led to the proposal that cigarette smoke may induce necrosis by inhibition of caspase activation and hence, may prevent apoptosis (Wickenden et al. 2003). Aoshiba and colleagues (2003a) have shown that single intratracheal injection of active caspase-3 resulted in epithelial cell apoptosis and enhanced elastolytic activity in mice. Further confirmation of the role of apoptosis in emphysematous changes in a murine model was obtained by an intratracheal injection of nodularin, a proapoptotic serine/threonine kinase inhibitor (Aoshiba et al. 2004b). Surprisingly, this approach did not cause inflammation or other forms of lung pathology, a hallmark of emphysema. Nevertheless, it is more or less now clear that cells involved in

the lung architecture undergo apoptosis by cigarette smoke. However, the scenario may not be the same for inflammatory cells. It is likely that cigarette smoke and its constituents through their interaction with extracellular matrix may inhibit inflammatory cell (neutrophils) apoptosis, which renders them not to be recognized by macrophages. This phenomenon would then enhance or promote the inflammatory response in the lungs (Kirkham et al. 2004). Further studies are required to understand the mechanism of cigarette smoke-induced cell death, using both in vitro and in vivo models so as to define strategy for safe resolution of inflammation or inhibit alveolar wall destruction.

6.8.5 Muscle Dysfunction

Dysfunction of the respiratory and peripheral skeletal muscles along with weight loss and exercise limitations has been increasingly recognized as an important feature of systemic effects of COPD. Oxidative stress occurs in skeletal muscle during skeletal muscle fatigue, weakness, and sepsis, accompanied by an increased load imposed on the diaphragm in patients with severe COPD (Rabinovich et al. 2001). Hypoxia, impaired mitochondrial metabolism, and increased cytochrome C oxidase activity in skeletal muscle in patients with COPD have been implicated in the etiology of the aforementioned anomalies (Engelen et al 2000; Heunks and Dekhuijzen 2000). Ribera and colleagues (2003) have recently shown that mitochondrial electron transport chain (ETC) function is enhanced in inspiratory muscles of patients with COPD. Increased ETC activity was associated with an increase in functional demand on the muscles to endure training-like effects, leading to increased oxidative stress. Engelen and coworkers (2000) have correlated reduced muscle glutamate levels to increased muscle glycolytic metabolism in patients with severe COPD. Reduced glutamate levels (a precursor for GSH) were therefore associated with decreased GSH levels, suggesting that oxidant/antioxidant balance may be compromised in skeletal muscle dysfunction in patients with COPD (Agusti et al. 2002; Rabinovich et al. 2001). Apoptotic pathways have been implicated in skeletal muscle atrophy in patients with COPD (Agusti et al. 2002), linking oxidative stress-mediated muscle atrophy to apoptosis of skeletal myocytes/myofibers in patients with COPD. However, it still remains to be determined whether oxidative stress and/or poor nutrition (alterations in calorie intake or lowered basal metabolic rate) play a central role in mediating muscle mass wasting/apoptosis, particularly in susceptible subsets of patients with COPD. Whether susceptibility of subgroups of COPD patients to oxidative stress and injury in muscle wasting is because of their inability to boost endogenous protective and defect repair mechanisms warrants further examination.

6.9 Oxidative Stress and the Development of Airways Obstruction

Various epidemiological studies have shown a relationship between circulating neutrophil numbers and FEV_1 (Rahman and MacNee 1996; Rahman et al. 1996). Oxidative stress, measured as lipid peroxidation products in plasma, has also been shown to correlate inversely with the predicted FEV_1 in a population study (Celli et al. 2004). It is possible that differences in interindividual antioxidant capacity may contribute to the

differences in the susceptibility against cigarette smoke-induced COPD. An association between dietary intake of antioxidant vitamins and lung function has been established in general population. However, some epidemiological studies have demonstrated negative associations of dietary antioxidant intake with pulmonary function and obstructive airway disease (Grievink et al. 1998). Another study (Britton et al. 1995) involving 2,633 subjects has shown an association between dietary intake of the antioxidant vitamin E and lung function, supporting the hypothesis that vitamin E may have a protective role in the development of COPD. Another study has suggested that antioxidant levels in the diet could be a possible explanation for differences in COPD mortality in different populations (Sargeant et al. 2000). Furthermore, dietary polyunsaturated fatty acids have also been found protective against the development of COPD in cigarette smokers (Shahar et al. 1999). These studies support the concept that dietary antioxidant supplementation including polyphenols may be a possible therapy to prevent of oxidative stress and inflammatory response in COPD.

6.10 Molecular Mechanisms of Oxidative Stress effects in COPD

6.10.1 NF-κB Activation

NF-κB is an important cytosolic transcription factor that can translocate to the nucleus on activation. In normally growing cells, NF-κB is maintained in an inactive state in the cytoplasm by the inhibitor IκBα, which prevents NF-κB from binding DNA. However, because both NF-κB and IκBα are shuttling proteins that rapidly move back and forth between the cytoplasm and the nucleus, the apparent cytoplasmic retention of NF-κB may simply represent an equilibrium state of a highly dynamic process. There are several pathways that can trigger NF-κB activation, the classical one that occurs in the cytoplasm and finally ends the nucleus. NF-κB activators such as tumor necrosis factor-α (TNF-α) and IL-1β engage cell surface receptors and transmit signals through the cytoplasm to the IκB kinase (IKK) complex. Activation of the IKK complex results in post-translational modification and proteasome-dependent degradation of IκBα; as a result, NF-κB is then free to activate transcription of responsive genes.

It is now generally accepted that NF-κB regulates the expression of various inflammatory genes, such as those for the cytokines, IL-8, TNF-α, and inducible nitric oxide (iNOS). Di Stefano and colleagues (2002) have demonstrated an increased expression of p65 protein of NF-κB in bronchial epithelium of smokers and patients with COPD, which was negatively correlated with the degree of airflow limitation in patients with COPD. Similarly, Caramori and coworkers (2003) have reported increased levels of p65 subunit of NF-κB in sputum macrophages but not in sputum neutrophils during exacerbations of COPD. This suggests that inflammatory responses may vary with the cell type. The activation of NF-κB in monocytes/macrophages may then, via release of proinflammatory mediators in lung epithelial fluid, trigger an amplified inflammatory cascade by activation of epithelial cells and recruitment of neutrophils in the airways.

Mochida-Nishimura and coworkers (2001) have shown that cells obtained from BALF of smokers exhibited tenfold higher activation of NF-κB in response to lipopolysaccharide (LPS), compared with that of nonsmokers. This may be because of the amplification

of inflammatory mediators that may activate NF-κB. However, activation of the MAPK pathways, ERK, stress-activated protein kinase (SAPK), and p38 was differentially regulated. Activation of p38 was more rapid in BAL cells from smokers, compared with the activity of ERK and SAPK. They further suggested that the differences in activation of NF-κB and MAPKs in BAL cells from smokers and nonsmokers may be related to the differences in their microenvironment, which is affected by chronic exposure to cigarette smoke.

6.10.2 Proinflammatory Genes

A large number of studies have indicated that COPD is associated with airway/airspace inflammation and with the presence of markers of inflammation, including IL-8 and TNF-α, which are elevated in the sputum of patients with COPD (Keatings et al. 1996). In vitro treatment of macrophages, alveolar, and bronchial epithelial cells with oxidants has resulted in the release of inflammatory mediators such as IL-8, IL-1, and NO. The enhanced levels of these inflammatory markers paralleled increased transcription of their respective genes, and increased nuclear binding and activation of NF-κB (Antonicelli et al. 2002; Parmentier et al. 2000). Similarly, cigarette smoke-induced IL-8 release from human bronchial and endothelial cells, which may contribute to airway inflammation in smokers (Mio et al. 1997; Mochida-Nishimura et al. 2001). Increased IL-8 expression and release in COPD was found to parallel the increase in neutrophil counts in bronchial samples of BALF (Mochida-Nishimura et al. 2001).

Cigarette smoke has been implicated in vivo as a cause of increased leukocyte adhesion to vascular endothelium. Shen and coworkers (1996) have shown that cigarette smoke condensate induces the expression of a subset of cell adhesion molecules, such as ICAM-1, endothelial leukocyte adhesion molecule 1 (ELAM-1), and vascular cell adhesion molecule (VCAM-1) in human umbilical vascular endothelial cells associated with an increase in the binding activity of NF-κB. This suggests that increased transendothelial migration of monocytes occurs from cigarette smoking. The release of proinflammatory mediators, such as IL-1β and sICAM-1, was increased by cigarette smoke exposure to bronchial epithelial cells cultured from biopsy materials obtained from patients with COPD, compared with smokers (Rusznak et al. 2000; Witherden et al. 2004). Similarly, primary cultured human alveolar and bronchial epithelial cells obtained from patients COPD showed higher levels of TNF-α-induced release of IL-6 and IL-8, compared with nonstimulated COPD cells (Patel et al. 2003). These studies suggest that patients with COPD have a greater susceptibility to the effects of cigarette smoke.

6.10.3 Antioxidant and Stress Response Genes

An important effect of oxidative stress in the lungs is the upregulation of protective antioxidant and stress response genes. One of the important factors in this series is the upregulation of GSH levels in ELF in chronic smokers. Increased GSH has been correlated to the transcriptional activation of the gene for GSH synthesis (*GCL*) induced in the epithelial cells by cigarette smoke components (Cantin et al. 1987; Rahman and MacNee

2000; Rahman et al. 1995). Thus, upregulation of the gene(s) involved in GSH synthesis in response to oxidative stress, and cigarette smoke, may be protected by the cells. However, cigarette smoke may inflict injurious effects during intermittent smoking when the lung is already depleted of antioxidants because of earlier smoking. Importantly, GSH may also be one of the depleted antioxidants during such conditions.

TNF-α, which is an outcome of inflammation in COPD, has been shown to increase GCL mRNA expression in alveolar epithelial cells (Rahman et al. 1999). On the other hand, transforming growth factor-β_1 (TGF-β_1) has been shown to decrease antioxidant glutathione synthesis and is associated with increased ROS production in human alveolar epithelial cells and pulmonary artery endothelial cells in vitro (Jardine et al. 2002). Increased TGF-β_1 expression was associated with fibrosis in the basement membrane in the lungs and depletion of GSH, suggesting that cigarette smoking interferes in normal repair (Fig. 6.4).

Extracellular GPx (eGPx), another important antioxidant in the lungs, may be secreted by epithelial cells and macrophages, particularly in response to cigarette smoke or oxidative stress (Avissar et al. 1996). Ishii and coworkers (2001) have demonstrated the protective role of GSH *S*-transferase P1 (GSTP1) against cigarette smoke in the airway cells. Similarly, Maestrelli and coworkers have recently shown the induction of heme oxygenase-1 (HO-1) in alveolar spaces in response to smoking. This indicates that oxidative stress because of cigarette smoke may increase the gene expression of HO-1 (Maestrelli et al. 2001). Cigarette smoke also induces heat shock protein 70 (HS70) and HO-1 in human monocytes and has been implicated in the regulation of cell injury and cell death. In particular, HS70 and HO-1 has been implicated in the modulation of apoptosis in human endothelial cells and monocytes (Vayssier et al. 1998).

Thus, oxidative stress, including that produced by cigarette smoke, causes transcriptional activation of both proinflammatory genes and also of antioxidant and stress response genes. Therefore, a balance between proinflammatory and antioxidant gene expression may determine the susceptibility of a cell to injury in response to cigarette smoking. Such an imbalance of an array of redox-regulated antioxidant versus proinflammatory genes might therefore be associated with the susceptibility or tolerance to disease. It is possible that induction of antioxidant enzymes may provide initial adaptive or protective responses against oxidative stress and inflammatory mediators. However, during sustained/chronic inflammation, the balance between genes for inflammatory mediators and antioxidant/phase II enzymes may be tipped in favor of proinflammatory mediators (Fig. 6.4).

6.11 Chromatin Remodeling (Histone Acetylation and Deacetylation) and Glucocorticoid Inefficacy in Response to Smoking and in Patients with COPD

Tightly bound DNA around a nucleosome core (histone proteins) suppresses gene transcription by decreasing the accessibility of transcription factors, such as NF-κB and AP-1, to the transcriptional complex. A key regulator of gene transcription and expression is the balance of histone acetylases (or histone acetyltransferase [HAT]) and histone deacetylases (HDAC) (Rahman et al. 2004). The activity/expression of the two enzymes controls the access of the transcriptional machinery to bind to regulatory sites on DNA. Acetylation of core histones by HAT leads to a modification of chromatin structure

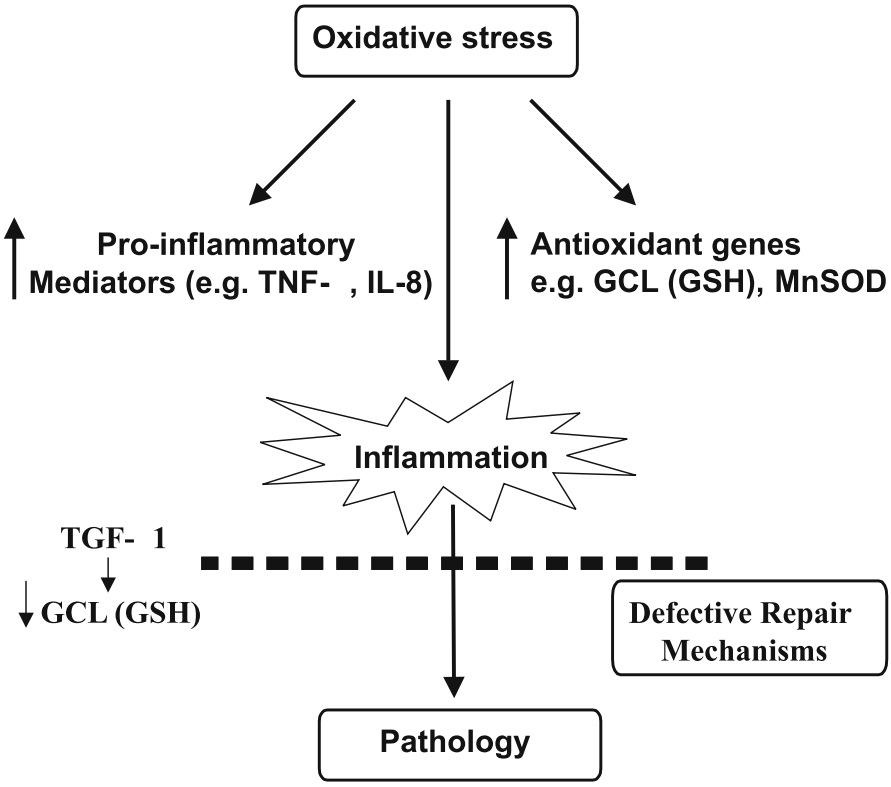


Fig. 6.4 Oxidative stress/cigarette smoke can cause increased gene expression of proinflammatory genes and also activation of protective genes, such as γ-glutamylcysteine synthetase/glutamate cysteine ligase. During sustained/chronic inflammation, the balance between genes for inflammatory mediators and antioxidant/phase II enzymes may be tipped in favor of proinflammatory mediators. It is possible that oxidative stress is enhanced during repair process by decreasing the glutathione (*GSH*) levels, leading to pathology

that affects transcription. Acetylation of lysine residues in the N-terminal tails of the core histone proteins results in uncoiling of the DNA, allowing increased accessibility for transcription factor binding. The acetylation status in turn depends on a balance of both HAT and HDAC. Exposure to cigarette smoke has been reported to alter chromatin remodeling by decreasing HDAC activity, resulting in increased transcription of proinflammatory genes in lungs of rats (Marwick et al. 2004). The above phenomena was linked to an increase in phosphorylated p38 MAPK in the lung concomitant with increased histone 3 phosphoacetylation, histone 4 acetylation, and elevated binding of NF-κB to the DNA, and AP-1. In addition, oxidative stress has also been shown to enhance acetylation of histone proteins and decrease histone deacetylase activity, leading to modulation of NF-κB activation, similar to the effect recorded with cigarette smoke (Moodie et al. 2004).

Ito and coworkers (2001) have shown a role for histone acetylation and deacetylation in IL-1β-induced TNF-α release in alveolar macrophages derived from cigarette smok-

ers. They have also suggested that oxidants may play an important role in the modulation of HDAC and inflammatory cytokine gene transcription. Furthermore, we have shown that both cigarette smoke/H_2O_2 and TNF-α caused an increase in histone acetylation (HAT activity), leading to IL-8 expression in monocytes and alveolar epithelial cells (Rahman et al. 2002, 2004).

It has been suggested that oxidative stress may have a role in the poor efficacy of corticosteroids in COPD. Glucocorticoid suppression of inflammatory genes requires recruitment of HDAC-2 to the transcription activation complex by the glucocorticoid receptor (Ito et al. 2001). This results in deacetylation of histones and a decrease in inflammatory gene transcription. A reduced level of HDAC-2 was associated with increased proinflammatory response and reduced responsiveness to glucocorticoids in alveolar macrophages obtained from smokers (Ito et al. 2001). HDAC-2 has been shown to be modified posttranslationally by 4-HNE and NO/$OONO^-$ (Yang et al. 2006; Rahman et al. 2004). Culpitt and coworkers (2002) have shown that cigarette smoke solution stimulated release of IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF), which was not inhibited by dexamethasone, in alveolar macrophages obtained from patients with COPD, compared with that of smokers. They suggested that the lack of efficacy of corticosteroids in COPD might be because of steroid insensitivity of macrophages in the respiratory tract. Thus, cigarette smoke/oxidant-mediated reduction in HDAC-2 levels in alveolar epithelial cells and macrophages will not only lead to an increase in inflammatory gene expression, but will also cause a decrease in glucocorticoid function in pa-

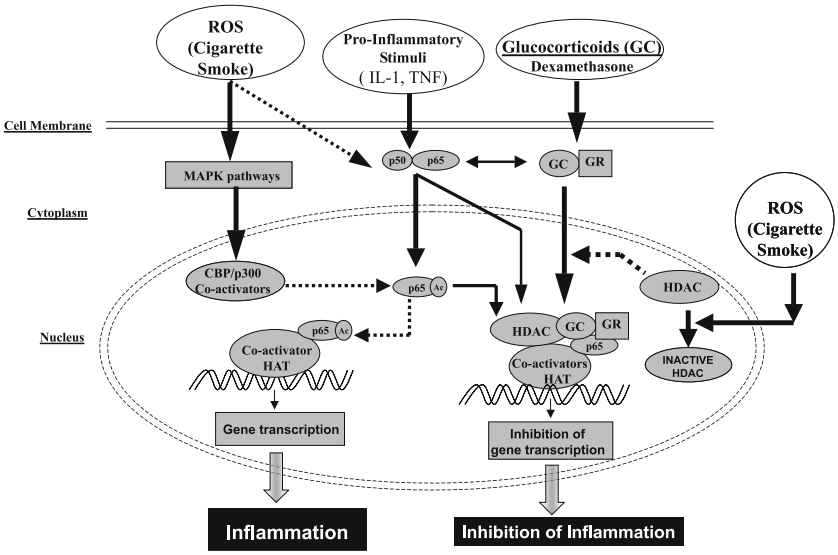


Fig. 6.5 Model illustrating the mechanism of corticosteroid (*GC*) action in suppressing proinflammatory gene expression and its impairment by oxidants. Direct interaction between coactivators histone acetyltransferase (HAT), histone deacetylase (*HDAC*), and the glucocorticoid receptor (*GR*), results in repression of proinflammatory gene expression. HDAC forms a bridge with HAT to inhibit gene transcription. The mitogen-activated protein kinase (*MAPK*) signaling pathways activated by oxidative stress can lead to p65 nuclear factor-kappaB (NF-κB) subunit and histone acetylation. Moreover, when HDAC is inhibited by oxidants/cigarette smoke or the p65 NF-κB subunit is acetylated, steroids may not be able to recruit HDACs into the transcriptional complex to inhibit proinflammatory gene expression

tients with COPD. (Yang et al. 2006) This may be one of the potential reasons for lack of effect of glucocorticoids in COPD (Fig. 6.5).

6.12 Genetic Polymorphisms as Markers of COPD

A genetic predisposition hypothesis has been suggested for COPD in view of the observations that approximately only 15–20% of smokers develop symptoms, and COPD is known to cluster in families. Although several candidate genes have been assessed, the data, however, often do not correlate, and more systematic studies are required to identify disease-associated genes. Understanding the genetic polymorphisms/mechanisms of susceptibility would provide an important insight into the pathogenesis of COPD (Sandford et al. 1997; Silverman and Speizer 1996). α1-AT deficiency has been widely studied as a genetic marker for COPD. Polymorphisms of various genes have been shown to be more prevalent in smokers who develop COPD (Barnes 1999). A number of these polymorphisms may have functional significance, such as the association between the TNF-α (*TNF2*) gene polymorphism, which is associated with increased TNF levels in response to inflammation, and the development of chronic bronchitis (Huang et al. 1997). Relevant to the effects of cigarette smoke is a polymorphism in the gene for microsomal epoxide hydrolase, an enzyme involved in the metabolism of highly reactive epoxide intermediates that are present in cigarette smoke (Smith and Harrison 1997). The proportion of individuals with slow microsomal epoxide hydrolase activity (homozygotes) was significantly higher in patients with COPD and a subgroup of patients shown pathologically to have emphysema (COPD 22%, emphysema 19%), compared with control subjects (6%) (Smith and Harrison 1997). These data have, however, not been reproduced in other patient populations (Yim et al. 2000). Genetic polymorphisms in matrix metalloproteinase genes *MMP1*, *MMP9*, and *MMP12* may also be important in the development of COPD. However, polymorphisms in the *MMP1* and *MMP12* genes, but not *MMP9*, appear to be related to smoking-related lung injury (Joos et al. 2002). They may also be in linkage disequilibrium with other causative polymorphisms (Sandford and Silverman 2002). An association between an *MMP9* polymorphism and the development of smoking-induced pulmonary emphysema was also reported in a population of Japanese smokers (Minematsu et al. 2001). Genetic polymorphism of antioxidant phase II detoxifying gene GSH *S*-transferase is associated with a decline in lung function in smokers (He et al. 2002). Variations in the levels of GSH and the genetic polymorphism of its synthesizing gene, *GCL*, and *HO-1* may represent new oxidative stress susceptibility factor in the pathogenesis in COPD (Cheng et al. 2004). It may be that a panel of "susceptibility" polymorphisms of functional significance in enzymes involved in xenobiotic metabolism or antioxidant enzyme genes may allow individuals to be identified as being susceptible to the effects of cigarette smoke. Nuclear factor erythroid 2-related factor 2 (Nrf2) is essential for the antioxidant-responsive element (ARE)-mediated induction of phase II detoxifying, *GCL* and *HO-1* genes in response to electrophiles present in cigarette smoke. Polymorphisms of GSH *S*-transferases (GSTs), *GCL*, and *HO-1* have been shown in smokers/patients with COPD. Recently, a genetic study has shown that *Nrf2*$^{-/-}$ mice were extremely susceptible to cigarette smoke and developed emphysema (Rangasamy et al. 2004). Hence, polymorphism of *Nrf2* may be present in susceptible smokers. Polymorphisms in the genes encoding for IL-11 (Klein et al. 2004), TGF-β1 (Wu et al. 2004), and the group-specific component of serum globulin (Ito et al. 2004)

have been associated with genetic predisposition for COPD. However, some of the gene polymorphism studies could not be replicated among different populations, thus warranting future studies. In the present context and with the availability of the human genome sequence, whole genome screening in patients and unaffected siblings by single-nucleotide polymorphisms (SNPs) might prove to be a promising genetic approach to identify genes associated with COPD.

6.13 Models for Studying COPD

COPD is a complex disease involving several biomolecular, histological, and molecular abnormalities. A systematic approach to the understanding of these aspects is therefore essential to have in-depth knowledge of the disease. To date, three major experimental approaches have been adopted for the study of COPD. COPD was either induced by inhalation of cigarette smoke, noxious stimuli, tracheal instillation of tissue-degrading enzymes to induce emphysema-like lesions, or gene-modifications leading to a COPD-like phenotype (Gorenberg and Chung 2004). These approaches in isolation or in combination may also be applied. Ideally, a number of potential markers for COPD as identified by the GOLD guidelines should be present in animal models of COPD. Because the definition of COPD still relies on lung function measures, ideally, it would be prudent to have reproducible lung function measurements in experimental models (Kips et al. 2003). Measurement of lung function in very small mammals such as mice is difficult, and the use of enhanced pause (Penh) in conscious mice as an indicator of airflow obstruction is not ideal. Therefore, invasive methods remain the method of choice and should be correlated with inflammatory markers and cellular remodeling.

Tobacco smoke has been routinely used as a noxious stimulant to induce COPD in a wide variety of animals. In addition to rabbits, mice, dogs, and rats, guinea pigs—being very susceptible species—were therefore an animal of choice. Within a few months of exposure to active tobacco smoke, guinea pigs develop COPD-like lesions and emphysema-like airspace enlargement (Wright and Churg 1990). In contrast, rats seem to be more resistant to the induction of emphysema-like lesions.

The most favored laboratory animal species with regard to the study of inflammatory and immune mechanisms are mice. This preference is because of the ease of manipulation of gene expression. However, it is more difficult to assess lung function in these animals, and moreover, rodents (including rats) do not have well-developed small airways. Mice are known to tolerate at least two cigarettes daily for a year with minimal alterations in body weight and carboxyhemoglobin levels. Mice differ considerably in respiratory tract functions and anatomy from to humans. They are obligate nose breathers and have lower numbers of cilia, fewer Clara cells, and the submucosal glands are restricted to the trachea. However, emphysema (increased mean linear intercept) is seen in mice after 4 to 6 months of chronic cigarette smoke exposure (≥ 300 mg/m^3 total particulate matter). Mice exhibit lesser pulmonary filteration of tobacco smoke and cough reflex. Mice also respond differently to histamine or tachykinins. The development of emphysema-like lesions in mice is strain dependent (Guerassimove et al. 2004). Another widely employed model of COPD is rats. However, they appear to be relatively resistant to the induction of emphysema-like lesions. Morphometry and histopathologic studies show significant differences in development of emphysema in mice and rats (March et al. 1999). Studies on rats exposed to cigarette smoke have revealed resistance to cortico-

steroids (Marvick et al. 2004), akin to that in patients with COPD (Culpitt and Rogers 2000; Pauwels et al. 1999).

Tobacco smoke exposure has been generally used to induce COPD and features such as emphysema, airway remodeling, and chronic inflammation. The alterations still differ from the human situation. Many mediators involved in the pathology may have different functional effects, especially in the murine respiratory tract. However, these models still provide useful approaches to investigate cellular and molecular mechanisms underlying the pathogenesis of COPD. In view of the considerable interstrain and interspecies variations found in the models used so far, selection of a strain needs great caution. Animal models of COPD still need to be precisely evaluated as to their ability to mimic features of human COPD, and their limitations must be appreciated. Observations recorded from one or a combination of models may provide in-depth understanding of novel mechanisms involved in COPD.

6.14 Conclusions

Oxidative stress has important implications in several events of lung physiology and for the pathogenesis and progression of COPD. There is now a spate of evidence supporting the notion that increased generation of ROS in COPD is an important factor determining the onset of pathogenesis and severity of this condition. ROS may be critical in amplifying the normal inflammatory response to cigarette smoke/environmental oxidants (noxious agents), through upregulation of redox-sensitive transcription factors and hence, proinflammatory gene expression. They also exert protection against the effects of cigarette smoke via induction of antioxidant genes. Several markers of COPD like 4-HNE, MDA, TBARS, H_2O_2 and isoprostanes, have been identified for diagnosis and prognosis of the disease. The variation in their levels because of several factors has warranted more caution in their interpretation and therefore requires an in depth study. ROS can influence the response at many levels through its impact on signal transduction mechanisms, activation of redox-sensitive transcriptions factors, and chromatin regulation resulting in pro-inflammatory gene expression. It is this impact of ROS on chromatin regulation by reducing the activity of the transcriptional co-repressor, histone deacetylase-2 (HDAC-2), that leads to the poor efficacy of corticosteroids in COPD. Polymorphisms in genes such as *TNF*-α, *MMP-1*, *MMP-9*, *MMP-12*, *IL-11*, *GST*s, GCL, *HO-1*, and microsomal epoxide hydrolase have been identified in COPD and other related conditions. However, vast variations in results obtained from population studies have hindered favorable attention for their use as genetic markers of COPD. Animal models of COPD still need to be precisely evaluated as to their ability to mimic features of human COPD, and their limitations must be appreciated. Various models such as rats, mice, guinea pigs, cell lines that are in vogue, and various agents such as gases, cigarette smoke, tracheal instillation, and proteases have been used to induce emphysema and COPD. The variations in susceptibility to different agents by different animals and cells have produced conflicting results. Therefore, observations recorded from one or a combination of models may provide in-depth understanding of novel mechanisms involved in COPD. Further understanding of the role of ROS in basic cellular functions and molecular mechanisms such as amplification of proinflammatory and immunological responses, defective repair mechanism, signaling pathways and apoptotic mechanisms will provide important information regarding basic pathological processes contributing to COPD.

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Chapter 7

Modulation of Cigarette Smoke Effects by Diet and Antioxidants

7

Marion Dietrich and Gladys Block

Contents

7.1 Introduction

Cigarette smokers as well as passive smokers are exposed to reactive free radicals present in cigarette smoke (Bermudez et al. 1994; Pryor and Stone 1993). Free radicals can cause oxidative damage to DNA, proteins, and lipids and may be involved in the development of chronic diseases like atherosclerosis and cancer (Diplock et al. 1998; Frei et al. 1991; Pryor 1986, 1987). In vitro studies have shown that antioxidants (AO) such as vitamin C, vitamin E, carotenoids, and thiols ameliorate free radical-induced oxidative damage (Burton et al. 1983; Frei et al. 1989, 1991; Pryor 1984; Khanna et al. 1999; Lykkesfeldt et al. 2000). There is evidence from epidemiological studies that persons who consume a diet rich in fruits and vegetables have a lower risk of cancer, cardiovascular diseases, and other diseases (Block et al. 1991, 1992). Because fruits and vegetables are major sources of antioxidants (as well as other factors), it has been hypothesized that antioxidants in fruits and vegetables are the protective compounds or major components of the protective effect (Diplock et al. 1998).

In vitro studies have shown that free radicals in cigarette smoke (CS) deplete certain plasma antioxidants (Frei et al. 1991; Eiserich et al. 1995). A number of studies have found lower plasma antioxidant levels in smokers in vivo (Jarvinen et al. 1994; Lykkesfeldt et al. 2000; Marangon et al. 1998; Norkus et al. 1987; Ross et al. 1995; Schectman et al. 1989; Stryker et al. 1988). Some information is available on the effect of CS exposure on plasma antioxidant concentrations in passive smokers (Alberg et al. 2000; Ayaori et al. 2000; Farchi et al. 2001; Tribble et al. 1993; Valkonen and Kuusi 1998; Dietrich et al. 2003b).

Antioxidant intervention trials in smokers and passive smokers have been conducted to investigate whether antioxidant supplementation can decrease biomarkers of oxidative stress (Davi et al. 1997, 1997; Gokce et al. 1999; Patrignani et al. 2000; Reilly et al. 1996; Dietrich et al. 2002; Dietrich et al. 2003a). Numerous biomarkers for oxidative damage have been used, including biomarkers for DNA damage and for alterations in proteins and lipids (Dean et al. 1997; Roberts and Morrow 2000; Shigenaga et al. 1989). One such biomarker is the F_2-isoprostanes (F_2-IsoPs), which are products of free radical-catalyzed lipid peroxidation of arachidonic acid (Morrow et al. 1990). They are formed in situ, esterified to phospholipids, and subsequently released by phospholipases into the plasma where they can be measured (Morrow et al. 1992). They can also be measured in urine. Increased F_2-IsoP levels in urine or plasma have been found in patients with atherosclerosis (Belton et al. 2000), severe heart failure (Cracowski et al. 2000), diabetes (Mezzetti et al. 2000), Alzheimer's disease (Pratico et al. 2000), as well as in smokers (Morrow et al. 1995; Obata et al. 2000).

This chapter reviews literature on the association between dietary antioxidant intake and chronic disease risks, as well as literature on antioxidant supplementation studies using lipid peroxidation biomarkers as indices for oxidative stress, in smokers, and if available, also in passive smokers.

7.2 Observational Studies: Modulation of Cigarette Smoking-Related Chronic Diseases by Dietary Antioxidants

7.2.1 Lung Cancer

Cigarette smoking is the primary cause of lung cancer. Observational studies have consistently shown an inverse association between fruit and vegetable intake and lung cancer risk (Ziegler et al. 1996). A large review of approximately 200 epidemiological case-control and prospective studies on fruit and vegetable intake and cancer prevention that included studies on lung cancer found significant protection of fruit and vegetable consumption in 24 of 25 studies after controlling for smoking (Block et al. 1992). In 1998, the International Agency of Research on Cancer (IARC) reviewed more than 20 case-control studies and reported that the majority of these studies found strong inverse trends in lung cancer risk with high fruit and vegetable intake (Vainio and Rautalahti 1998). Findings from the European Prospective Investigation into Cancer and Nutrition Study (EPIC Study) on the association of fruit and vegetable intake and lung cancer risk have recently been published (Miller et al. 2004). More than 500,000 individuals from ten European countries participated in the EPIC Study, and 478,021 were included in the analysis on fruit and vegetable intake and lung cancer risk. After adjustment for age, smoking, height, weight, and gender, there was a significant inverse association between fruit consumption and lung cancer risk (hazard ratio for the highest quintile of consumption relative to the lowest 0.60 [95% confidence interval {CI} = 0.46–0.78], *p*-value for trend = 0.0099). No association between vegetable consumption and lung cancer risk was observed.

Analyses from two reports on pooled analyses of seven or eight large prospective cohort studies including never, past, and current smokers, carried out in North America and Europe have recently been published. One of the analyses focused on fruit and vegetable intake and lung cancer risk (Smith-Warner et al. 2003); the other analysis focused on dietary carotenoid intake and lung cancer risk (Mannistoe et al. 2004). The results from the first study conducted by Smith-Warner et al. suggest that elevated fruit and vegetable consumption is associated with a modest reduction in lung cancer risk, mostly attributable to fruit, not vegetable, intake. When adjusted for smoking status and other lung cancer risk factors, a 21% lower risk of lung cancer was observed among men and women with higher intakes of total fruits and vegetables. Pooling these large cohort studies allowed the authors to analyze the data by smoking status. Among current smokers, a significant 16% lower risk was observed for comparison of the highest versus the lowest quartile of total fruit and vegetable consumption (0.84 [0.71–0.98, relative risk {RR} {95% CI}]; *p*-value = 0.03 [test for trend]).

The second study analyzing the same prospective cohort studies (Mannistoe et al. 2004) focused on dietary carotenoid intake and lung cancer risk. Carotenoids are fat-soluble compounds abundant in many fruits and vegetables and are hypothesized to be protective compounds against oxidative stress in vivo. Among current smokers, a significant trend for an inverse association between β-cryptoxanthin intake and lung cancer risk was observed (*p*-value, test for trend <0.001). β-Cryptoxanthin is one of the main carotenoids in citrus fruits, which also contain high amounts of vitamin C and other compounds that may decrease cancer risk. The authors therefore also adjusted the analysis for vitamin C, folate, other carotenoids, and multivitamin use. These adjustments did not alter the inverse association between β-cryptoxanthin and lung cancer risk much, but the authors note that it is possible that other substances than β-cryptoxanthin in

fruits and vegetables, particularly in citrus fruit, are primarily responsible for the inverse association observed. High lycopene intake was marginally associated with a lower risk of lung cancer (p-value, test for trend = 0.06). Significantly β-carotene intake was not associated with lung cancer risk (p-value, test for trend = 0.62).

The association of passive smoking with fruit and vegetable consumption, or with specific carotenoids, and lung cancer could not be investigated in these pooled analyses of prospective studies because most studies did not collect information on environmental tobacco smoke exposure.

A cohort study that was included in the two pooled analyses described above also analyzed the role of folate and vitamin C in the association of fruit and vegetable consumption and lung cancer risk (Voorrips et al. 2000). Results from that study showed highly significant inverse associations for vitamin C and folate with lung cancer risk (p-value, tests for trend <0.0001 for both micronutrients). The authors concluded that high folate and vitamin C intakes might be better protective compounds than the carotenoids. Several epidemiological studies have investigated whether dietary vitamin C intake is protective against lung cancer. A case-control study including smokers, past smokers, and nonsmokers conducted by Fontham et al. (1988) found a strong protective effect of vitamin C for lung cancer among men and women in Louisiana (odds ratio = 0.65, 0.50–0.87, high intake). This association was significantly stronger than the inverse association the authors observed for carotene (odds ratio = 0.84, 95% CI = 0.64–1.09). A more recent prospective study by Yong et al. (Yong et al. 1997) also found a significant inverse association between dietary vitamin C intake and risk of lung cancer in data from the First National Health and Nutrition Examination Survey Epidemiologic follow-up study. In a multivariate analysis adjusted for smoking status, the RR of lung cancer for subjects in the highest quartile of dietary vitamin C intake, compared with those in the lowest quartile was 0.66 (95% CI = 0.45–0.96). The authors also investigated the effect of supplement use but did not find additional protective effects of vitamin C supplements beyond that provided through dietary intake. Several other cohort studies observed inverse relationships between vitamin C intake and risk of lung cancer. For example, in the New York State Cohort Study, a prospective study including 48,000 male and female smokers and nonsmokers, an inverse dose–response relationship was observed in men. In males, the adjusted RRs for the medium and highest level of consumption, compared with the lowest one were 0.78 (CI = 0.62–0.98) and 0.63 (CI = 0.53–0.88), respectively. A similar protective relationship was observed for folate and carotenoids. In women, weaker, nonsignificant inverse relationships were observed for all of the investigated micronutrients. The authors also investigated the role of vitamin supplementation by adjustment for the corresponding supplement. Results were essentially the same (Bandera et al. 1997). Two large case-control studies conducted by Hinds et al. (1984) and Le Merchand et al. (1989) in Hawaii found an inverse association with lung cancer for total vitamin C (diet plus supplements) in men, but not in women.

7.2.2 Cardiovascular Diseases

Smoking and exposure to environmental tobacco smoke are associated with oxidative stress and with increased risk of atherosclerosis and cardiovascular diseases (Glantz and Parmley 1995; Wells 1994). Oxidative stress can lead to the oxidative modification of low-density lipoprotein (LDL), a process that has been strongly hypothesized to be involved

in the initiation of atherosclerosis. Antioxidants may ameliorate these effects. Epidemiologic studies have been conducted in order to investigate if dietary intake of antioxidants is associated with the reduction of cardiovascular and cerebrovascular events, such as stroke. In the Rotterdam Study, for example, Voko et al. (2003) investigated whether high intake of antioxidants from food is associated with the risk of ischemic stroke. A higher intake of antioxidants was associated with a lower risk of ischemic stroke among approximately 6,000 participants of this prospective study. The relationship was significant for vitamin C and most pronounced in smokers. These results indicate that a high dietary intake of vitamin C in smokers may reduce the risk of coronary artery diseases. Hirvonen et al. (2000) investigated the association between dietary antioxidants and risk of stroke in participants of the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (ATBC Study) during a 6.1-year follow-up using dietary baseline data. More than 26,000 male smokers, aged 50–69 years, were included in that analysis. Dietary intake of β-carotene was significantly inversely associated with the risk for cerebral infarction (relative risk [RR] of highest vs lowest quartile = 0.74, 95% CI = 0.60–0.91). No association was detected between other dietary antioxidants and risk for stroke. Results from these studies suggest that high dietary antioxidant intakes, especially of vitamin C and β-carotene, may have protective effects against stroke in smokers.

7.3 Primary Prevention Studies: Modulation of Cigarette Smoking-Related Chronic Diseases by Antioxidant Supplements

7.3.1 Lung Cancer and Cardiovascular Diseases

Two large primary prevention trials have been conducted with antioxidants specifically in smokers, the ATBC Study (The ATBC Cancer Prevention Study Group 1994) including more than 29,000 male smokers and the Beta-Carotene and Retinol Efficacy Trial (CARET study), which included more than 14,000 male and female heavy current and former smokers (Omenn et al. 1996). The antioxidants administered were α-tocopherol and/or β-carotene in the ATBC Study and β-carotene combined with retinol (vitamin A) in the CARET study. These intervention studies were initiated after epidemiologic studies linked vegetables high in carotenoids with lower lung cancer risk (National Research Council 1982; Peto et al. 1981). In the ATBC Study, after 5–8 years of treatment, no reduction in incidence of lung cancer was observed among men who were supplemented with α-tocopherol. A higher incidence of lung cancer was observed among subjects who received β-carotene. In the CARET study, after 4 years of supplementation, subjects in the β-carotene/vitamin A treatment group had a significantly higher relative risk of lung cancer of 28% when compared to the placebo group.

Toernwall et al. (2004) very recently published results from an evaluation of the 6-year posttrial effects of α-tocopherol and β-carotene supplementation on coronary heart disease in the ATBC Study. The authors report that β-carotene seemed to increase the posttrial risk of first-ever nonfatal myocardial infarction in male smokers, and state that their findings do not support the use of β-carotene or α-tocopherol supplements in the prevention of coronary heart disease among male smokers.

Another large randomized β-carotene intervention study on cancer and cardiovascular disease is the Physicians' Health Study (Hennekens et al. 1996). That cohort of male

subjects included 11% of current smokers and 39% former smokers. After a follow-up of 12.5 years, no effect of β-carotene supplementation on cancer or heart disease was observed, indicating that β-carotene supplementation had neither beneficial nor harmful effects with regard to cancer or cardiovascular disease. However, only a small proportion of the subjects were smokers and low numbers of lung cancer cases could have decreased the statistical power necessary to detect treatment effects.

7.4 Biomarker Studies: Modulation of Cigarette Smoking-Related Oxidative Stress by Antioxidant Supplements

As mentioned in the Introduction, a range of oxidative damage biomarkers can be applied to measure smoking-associated oxidative stress and the effects of antioxidants on those biomarkers. However, in this chapter, we focus mainly on biomarkers for lipid peroxidation because this is the research field where most of the work in our group has been conducted.

7.4.1 Vitamin C

Several studies investigated the effect of vitamin C in smokers, using a variety of lipid peroxidation biomarkers. We reviewed eight studies that investigated the effects of vitamin C on lipid peroxidation biomarkers in smokers (see Table 7.1 for details). Four of these eight studies found statistically significant protective effects, two observed no effects. One small study on eight subjects found a nonsignificant 26% reduction in malondialdehyde (MDA) in the vitamin C group, compared with a 9% reduction in the placebo group. One study that measured two different biomarkers found no effect on one biomarker but an increasing effect on the other biomarker (Nyyssoenen et al. 1997).

A relatively small study by Harats et al. (1990) investigated the acute effect of smoking on plasma lipoproteins in 17 smokers after vitamin C supplementation with 1,000 or 1,500 mg daily for 2 or 4 weeks. The authors found that vitamin C significantly decreased oxidation of plasma LDL measured by thiobarbituric acid-reactive substances (TBARS). A similar-sized study by Fuller et al. (1996) in which 19 healthy smokers were supplemented with 1,000 mg of vitamin C for 4 weeks found that the vitamin C supplementation led to a significant reduction in plasma LDL oxidative susceptibility as measured by TBARS. A study in smokers that used F_2-IsoPs as the marker for lipid peroxidation is the study by Reilly et al. (1996). These authors reported that a 5-day supplementation of smokers with 2,000 mg vitamin C significantly reduced their urinary excretion of F_2-IsoPs. We conducted a large double-blind, placebo-controlled vitamin C intervention study in 81 smokers, measuring plasma F_2-IsoPs (Dietrich et al. 2002). We also found a significant decrease in this biomarker by vitamin C, but only in smokers with a body mass index (BMI) above the median. Overweight/obesity is associated with elevated oxidative stress. Our finding of only a protective effect of vitamin C in overweight smokers might indicate that a certain threshold level of oxidative stress is necessary to see a treatment effect with antioxidants. Three studies did not observe statistically significant protective effects (Mulholland et al. 1996; Nyyssoenen et al. 1997; Samman et al. 1997). Two of these studies were very small, and their intervention period was shorter than that

Table 7.1 Antioxidant supplementation studies in smokers and passive smokers using biomarkers for lipid peroxidation

Reference	Subjects	Type and dose of antioxidant	Duration	Body fluid	Biomarker used and results
Smokers					
Harats et al. 1990	17 smokers	-Vitamin C (1,000 + 1,500 mg/day)	2 + 4 weeks	Plasma, in vitro	LDL TBARS: significantly decreased
Fuller et al. 1996	19 smokers	-Vitamin C (1,000 mg/day)	4 weeks	Plasma, in vitro	LDL oxidizability: significantly decreased (measured by TBARS)
Reilly et al. 1996	16 heavy smokers	-Vitamin C (2,000 mg/d, *n*=5) -Vitamin E (800 IU/d, *n*=7) -Vitamin C (2,000 mg/day) and vitamin E (800 IU/day) in combination (*n*=4)	5 days	Urine	F_2-IsoPs: -Vitamin C alone: significantly decreased F_2-IsoPs -Vitamin E alone: no effect -Vitamin C plus vitamin E combination: significantly decreased F_2-IsoPs
Mulholland et al. 1996	8 smokers (8 placebo)	-Vitamin C (1,000 mg/day)	2 weeks	Serum	TBARS: vitamin C group decreased TBARS 26%, placebo 9%, not statistically significant
Nyyssoenen et al. 1997	59 smokers (19 placebo, 19 plain vitamin C, 20 slow-release vitamin C)	-Vitamin C (500 mg/day)	8 weeks	Plasma, in vitro and in vivo	LDL oxidizability (measured by CD): no change when compared with placebo group Plasma MDA: increased significantly when compared with placebo group
Samman et al. 1997	8 smokers crossover, 8 day washout between periods	-Vitamin C (1,000 mg/day)	2 weeks	Plasma, in vitro	LDL oxidizability (measured by CD): declined, not statistically significant
Steinberg and Chait 1998	20 smokers (19 placebo)	-Vitamin C (600 mg/day), vitamin E (400 IU/day), and β-carotene (30 mg/day) in combination, in tomato juice	4 weeks	Plasma, in vitro	LDL oxidizability (measured by CD): combination of AO significantly decreased when compared with placebo group
Patrignani et al. 2000	34 smokers (12 placebo)	-Vitamin E (300 mg/day, *n*=11) -Vitamin E (600 mg/day, *n*=12) -Vitamin E (1,200 mg/day, *n*=11)	3 weeks	Urine	F_2-IsoPs: no significant effect on F_2IsoPs of either dose

Reference	Subjects	Type and dose of antioxidant	Duration	Body fluid	Biomarker used and results
Fuller et al. 2000	22 smokers (8 placebo)	-Vitamin C (1,000 mg/day, *n*=6) -Vitamin E (400 IU/day, *n*=8) -Vitamin C (1,000 mg/day) and vitamin E (400 IU/day) in combination (*n*=8)	8 weeks	Plasma, in vitro	LDL oxidizability (measured by CD): -Vitamin C alone: no changes -Vitamin E alone: significantly decreased LDL oxidizability -Vitamin C plus vitamin E combination: significantly decreased LDL oxidizability
Dietrich et al. 2002	81 smokers (45 placebo)	-Vitamin C (500 mg/day) (*n*=42) -AO cocktail containing vitamin C, vitamin E, and α-lipoic acid[a] (*n*=39)	8 weeks	Plasma	F_2-IsoPs: -Vitamin C alone: significantly decreased F_2-IsoPs[b] when compared with change in placebo -AO cocktail: no effect when compared with change in placebo
Passive smokers					
Howard et al. 1998	36 passive smokers	-AO mixture containing vitamin C, vitamin E, β-carotene, and minerals[c]	8 weeks	Plasma	TBARS: 7% decrease, statistical significance not provided
Valkonen and Kuusi 2000	10 passive smokers	-Vitamin C (3,000 mg/day)	Single dose	Plasma, in vitro	TRAP, LDL oxidizability, TBARS: significant protective effect of vitamin C on all of these measures
Dietrich et al. 2003	43 passive smokers (24 placebo)	-Vitamin C (500 mg/day, *n*=22) -AO cocktail containing vitamin C, vitamin E, and α-lipoic acid[a] (*n*=21)	8 weeks	Plasma	F_2-IsoPs: -Vitamin C alone: significantly decreased F_2-IsoPs when compared with change in placebo -AO cocktail: significantly decreased F_2-IsoPs when compared with change in placebo

LDL low-density lipoprotein, *TBARS* thiobarbituric acid reactive substances, *F_2-IsoPs* F_2-isoprostanes, *CD* conjugated dienes, *MDA* malondialdehyde, *AO* antioxidants, *TRAP* total peroxyl radical trapping potential of serum

[a]AO cocktail: 500 mg vitamin C, 371 mg α-tocopherol, 171 mg γ-tocopherol, 50 mg α-tocotrienol, 184 mg γ-tocotrienol, 18 mg δ-tocotrienol, 95 mg α-lipoic acid

[b]Only in smokers with body mass index above median

[c]3,000 μg β-carotene, 60 mg vitamin C, 30 IU α-tocopherol, 40 mg zinc, 40 μg selenium, 2 mg copper

of most of the other studies, which could have led to the negative result. However, the study by Nyyssoenen et al. (1997), which also did not observe a protective effect, was rather large with 59 subjects, and the intervention period was as long as other studies that did observe protective effects. In fact, Nyyssoenen et al. observed increased plasma MDA levels in the group of subjects taking ascorbic acid, compared with subjects in the placebo group. The authors note that this was an unexpected result and could not be explained by artificial formation of lipid peroxides during sample preparation or by interday laboratory variability. The authors conclude that long-term ascorbic acid supplementation alone without any other antioxidant might promote the formation of MDA. However, in our large intervention study of the same duration, we found a significant reduction in MDA in the vitamin C treatment group (unpublished data).

With regard to vitamin C supplementation and lipid peroxidation in passive smokers, only two studies have been published to date, and both showed protective effects of vitamin C. Valkonen and Kuusi (2000) conducted a small study with ten nonsmokers who were exposed to environmental tobacco smoke for 30 min on 2 consecutive days. Vitamin C significantly prevented the formation of serum TBARS. The other study was a randomized double-blind placebo controlled study including 67 healthy passive smokers and was conducted by our group (Dietrich et al. 2003). We found that vitamin C significantly decreased plasma F_2-IsoPs in passive smokers.

7.4.2. Vitamin E

Three studies reviewed here investigated the effect of vitamin E in smokers. One found a lipid peroxidation-lowering effect; two found no statistically significant effects. The study by Reilly et al. (1996), which did not find a protective effect of vitamin E alone, was a very short-term study of only 5 days. This might have not a long enough time to see any effects of vitamin E. However, they did find a positive effect when vitamin E was administered in combination with vitamin C, which suggests that vitamin C and vitamin E act in combination. On the other hand, Fuller et al. (2000) observed lipid peroxidation decreasing effects of vitamin E given alone or in combination with vitamin C, but no decreasing effects of vitamin C alone. Patrignani et al. (2000) conducted a study administering three different doses of vitamin E to a total of 34 subjects and measured lipid peroxidation in form of urinary F_2-IsoPs. The authors observed no effect of any of the given vitamin E doses.

7.4.3 Combinations of Antioxidants

Five studies investigated the effect of combinations of antioxidants on lipid peroxidation markers in smokers and passive smokers (Reilly et al. 1996, Steinberg and Chait 1998, Fuller et al. 1998, Dietrich et al. 2002, Dietrich et al. 2003). The combinations were either vitamin C and vitamin E combined, or these two vitamins combined in addition with β-carotene, minerals (Howard et al. 1998), or α-lipoic acid. One study administered an antioxidant combination in tomato juice (Steinberg and Chait 1998). Most of the studies observed lipid peroxidation-lowering effects in smokers and passive smokers (see Table 7.1 for details). However, in our intervention study, which was done in smokers

and in passive smokers, the antioxidant combination lowered F_2-IsoP in passive smokers (Dietrich et al. 2003), but not in smokers (Dietrich et al. 2002). This result was unexpected and is difficult to explain. It might be possible that components of the combination counteracted the effect of vitamin C on F_2-IsoPs, because, as mentioned above, significant treatment effects by the same dose of vitamin C were observed in the vitamin C treatment group.

7.5 Summary

In summary, epidemiologic studies consistently showed significant, though modest, inverse associations of high fruit and vegetable intake with lung cancer risk. Carotenoids found in fruits and vegetables have been significantly associated with lower lung cancer risks, such as, for example, β-cryptoxanthin. However, it cannot be ruled out that the protective effects seen for this specific carotenoid are indicative of other protective compounds abundant in fruits and vegetables, such as vitamin C or folate, or by a combination of those. Most of the studies that investigated dietary vitamin C and lung cancer risk found significant inverse associations.

Interestingly, two large cohort studies, the EPIC Study and the pooled analysis study by Smith-Warner et al. (Smith-Warner et al. 2003), observed stronger protective effects against lung cancer for high fruit intake than for high vegetable intake. This might indicate that micronutrients that are abundant in higher concentrations in fruits, compared with in vegetables might be responsible for that effect. However, the authors of the EPIC Study hypothesize that smoking as a high risk factor for lung cancer has overhelmed any protective associations of vegetable consumption (Miller et al. 2004).

It is notable that with respect to antioxidant nutrients, current smoking is an effect modifier of the role of nutrients on disease, rather than a confounder. That is, the effect of a given level of intake is likely to be different in current smokers versus nonsmokers, based on the fact that the blood antioxidant levels obtained on the same antioxidant intake are lower in smokers than in nonsmokers. Unfortunately most of the observational studies did not examine the relationships in this way, and few stratified on current smoking status.

Most of these cohort studies did not estimate the possible protective effect of total antioxidant intake, wich consists of intake from both food and supplements. This could underestimate the nutrient effect. Similarly, most studies did not stratify by antioxidant supplement use when examining the fruit and vegetable effect, which could lead to underestimations of the risk reduction by fruit and vegetable intake.

The discrepancy between the observational studies, which suggested a protective role for fruits and vegetables, and the ATBC and other randomized trials, which found null or even harmful effects, is troubling. The issues have been discussed elsewhere (Blumberg and Block 1994). However, the discrepancy does not necessarily invalidate the findings of the observational studies, for several reasons. The primary reason is that the randomized trial tested whether beginning high-dose treatment after smokers had been smoking for an average of 36 years could prevent progression to lung cancer. It does not provide any evidence regarding whether a diet rich in antioxidants throughout those 36 years might have prevented, or reduced the risk of, lung cancer. Furthermore, the dose of β-carotene used in the trials is at least five times higher than what is commonly achieved through diet.

With regard to the effects of antioxidants on oxidative stress biomarkers, the majority of intervention studies administering vitamin C found lipid peroxidation-lowering effects in both smokers and passive smokers. These results indicate that vitamin C ameliorates smoking-related oxidative stress. Results from studies that administered vitamin E were less consistent. However, most studies that administered combinations of antioxidants found lipid peroxidation-preventing effects.

7.6 Conclusion

In conclusion, despite the observed small to modest cancer risk reduction by high fruit and vegetable intake in smokers, and the lipid peroxidation lowering effect of supplemental vitamin C in smokers and passive smokers, the most efficient way of preventing smoking-related oxidative stress and diseases is smoking cessation. Therefore, smoking prevention and cessation should be the primary focus in public health for the prevention of lung cancer incidences.

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Chapter 8

Modulation of Cigarette Smoke Effects by Antioxidants: Oxidative Stress and Degenerative Diseases

8

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Contents

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8.1 Introduction

Smoking is an important risk factor in many diseases and disease states, for example, lung diseases. These include asthma, chronic obstructive pulmonary diseases (COPD), chronic bronchitis and emphysema, and lung cancer (Eiserich et al. 1995; Virtamo 1999). Smoking is also associated with some other cancers, such as cancers of the head and neck, bladder, esophagus, pancreas, stomach, and kidney (Kuper et al. 2002), and smoking elevates the risk of cardiovascular diseases (Ambrose and Barua 2004). There is an accumulating body of evidence demonstrating that smokers have a lower intake of fruits and vegetables, lower plasma antioxidant levels, and they are more susceptible to lipid and DNA oxidation, as compared with former smokers or nonsmokers (Alberg 2002; Morrow et al. 1995; Prieme et al. 1998).

In the above-mentioned degenerative diseases, oxidative stress/lipid peroxidation seems to play a vital role (Mayne 2003). The harmful effects of cigarette smoking are mediated via the carcinogens present in the smoke, such as polycyclic aromatic hydrocarbons (PAH compounds) (Pfeifer et al. 2002), but also via its prooxidant, lipid peroxidation-increasing effects.

Even though smokers are encouraged to break their habit, which is undoubtedly the best way to minimize the harmful effects of smoking, many smokers fail to refrain from smoking. It has been proposed that some of the harmful effects of the cigarette smoking could be reduced by antioxidant supplementation. To answer this question, we have reviewed the current literature concerning antioxidants, oxidative stress, and their interaction with smoking in different case-control, cross-sectional, intervention, and follow-up studies in smokers, somewhat emphasizing the research related to carotenoids and polyphenols.

8.2 Oxidative Stress and Lipid Peroxidation

8.2.1 Effects of Smoking on Lipid Peroxidation

It has been estimated that cigarette smoke contains 10^{14} free radicals per inhalation (Church and Pryor 1985). Hydroxyl radicals, aldehydes, and nitrogen oxides in cigarette smoke are thought to be significant contributors to biomolecular damage (Eiserich et al. 1995; Spencer et al. 1995). Cigarette smoking also increases the numbers of alveolar neutrophils and macrophages, which once activated, can produce strong oxidizing agents,

such as superoxide radicals (Halliwell and Gutteridge 1989). Thus, inflammation, as reflected in the elevated C-reactive protein (CRP) levels (Block et al. 2004), may be an important further mechanism increasing free radical stress in smokers. Dietary habits are less healthy in smokers, which means they have a lower intake of fruits and vegetables, and consequently, of antioxidants (Dyer et al. 2003; Walmsley et al. 1999). In addition, there are several studies that have reported that smoking itself can directly decrease antioxidant levels (Eiserich et al. 1995; Handelman et al. 1996). The in vivo mechanism is unclear, but it may involve increased consumption/cycling, lowered absorption, or increased elimination rate of the antioxidants. In in vitro models, the exposure of plasma to gas-phase cigarette smoke has evoked depletion of urate, ascorbate, ubiquinol 10, α-tocopherol, and a variety of carotenoids (Eiserich et al. 1995; Handelman et al. 1996). Lipid peroxidation end products can further mediate the harmful effects of free radicals. For example, there is some evidence that $F_{2\alpha}$-isoprostanes possess bioactive pulmonary and vasoconstrictive effects (Basu 2004).

8.2.2 Effects of General Factors on Lipid Peroxidation

There are several factors that can modulate the effects of cigarette smoking. The most recent evidence has dealt with genetic factors. Genotype variations between subjects in their antioxidant enzymes can confound the findings of population-based studies, i.e., individuals with certain genotypes may be more prone to the harmful effects of cigarette smoke. Polymorphism in the paraoxonase (*PON2*) gene in smokers is one example of such a phenomenon (Martinelli et al. 2004). Other general factors increasing lipid peroxidation include male gender, inflammation, high cholesterol levels, low plasma levels of different dietary antioxidants, such as vitamin C and β-carotene (Block et al. 2002), and recently performed exhaustive exercise (Kaikkonen et al. 1998), which is nevertheless quite an unusual event in smokers. It is still not clear whether age is a major primary factor associated with the status of lipid peroxidation, and the so-called free radical theory, which claims that as an individual ages, the radical burden increases still need to be confirmed (Wickens 2001). Also, the content of the ingested fatty acids may modify the findings. There are many studies showing that providing individuals with vitamin E supplements can lower plasma lipid peroxidation. However, Weinberg and coworkers (2001) found that smoking subjects supplemented with vitamin E and consuming a high-polyunsaturated fat diet were more prone to oxidation as measured by increased total F_2-isoprostane and prostaglandin (PG)$F_{2\alpha}$ levels, as compared with subjects receiving no vitamin E. Thus, studies investigating the effects of antioxidants in smokers should take these confounding factors into consideration, especially in epidemiological studies. Naturally, the randomization and the group size need to be sufficiently large to correct for these interfering factors in intervention studies.

8.2.3 Assessment of Lipid Peroxidation and DNA Oxidation

In general, it is quite difficult to assess oxidative stress in humans, partly because no standardized methods are available in this area. For example, it has been observed that several simultaneously performed measurements can give conflicting, even opposite, findings.

There are several approaches that can be used to assess lipid peroxidation and oxidative stress. First, the oxidation susceptibility of lipid-containing body liquids and liquid fractions can be assessed in test tubes ex vivo by using oxidizing agents, such as transition metals or azo-compounds, which act as initiators of lipid peroxidation. Copper ions, iron ions, 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH) and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) have been the most commonly used initiators. Accumulation of different propagation- and termination-phase products, such as conjugated dienes and lipid hydroperoxides, have been quantified in these induction experiments (Corongiu et al. 1989; Esterbauer et al. 1992; Neuzil et al. 1997; Rice-Evans and Miller 1994). Second, oxidation products formed in vivo in the human body can be determined. Thiobarbituric acid reactive substances (TBARS) have been measured either in vivo or after in vitro oxidation of lipoproteins (Esterbauer et al. 1984; Richard et al. 1992). One clear shortcoming of this method is that a part of the TBARS might be formed during the assay itself. Breath alkane excretion has been used as a marker of in vivo fatty acid peroxidation in lungs, but unfortunately, this marker is considered to be quite unspecific. Products of polyunsaturated fatty acid (PUFA) and monounsaturated fatty acid (MUFA) oxidation, i.e., hydroxy fatty acids, oxysterols, and total F_2-isoprostanes, or more specific $F_{2\alpha}$-isoprostanes (8-iso-$PGF_{2\alpha}$, $PGF_{2\alpha}$), have been mainly determined by gas or liquid chromatography/mass spectrometry (GC/MS or LC/MS) or by immunoassay. $F_{2\alpha}$-isoprostanes are considered as one of the most reliable and specific markers of in vivo lipid peroxidation (Basu 2004, Salonen 2000; Kaikkonen et al. 2004). Third, the activities of different antioxidant enzymes, such as blood/plasma glutathione peroxidase (GPx), superoxide dismutase (SOD), or catalase (CAT) can be measured, and antioxidants and their redox pairs assessed (Kaikkonen et al. 2004; Salonen 2000).

Because lipid peroxidation is thought to be high in smokers, it is plausible that the effects of antioxidant supplementation would be easier to identify in such subjects. For this reason, numerous supplementation trials have been carried out in smokers. Because $F_{2\alpha}$-isoprostanes are known to be one of the most reliable indicators of lipid peroxidation, we have mainly focused on studies that have concentrated on this compound, though other in vivo measurements of lipid and DNA oxidation have also been considered.

8.3 Antioxidants and Their Mechanisms of Action

8.3.1 Adaptive Mechanisms of Antioxidants in Smokers

There is some evidence that the human body can adapt to the cigarette smoke by increasing the recycling of the consumed antioxidants back to their active forms, e.g., ascorbic acid. Ascorbic acid recycling describes the process in which ascorbic acid is oxidized to dehydroascorbic acid by various pathways, and subsequently reduced back to ascorbic acid intracellularly, thereby preserving the ascorbic acid pool (Lykkesfeld et al. 2003). The kinetics of deuterium-labeled tocopheryl acetate has also been compared between smokers and nonsmokers. Traber and coworkers (2001) found that smoking appeared to increase the plasma vitamin E disappearance rate. Glutathione (GSH) is an endogenous water-soluble antioxidant, and it is unclear how it reacts to cigarette smoke. Hininger and colleagues (1997) noted that the whole-blood concentrations of reduced GSH were higher in smokers than in nonsmokers, and decreased to the levels measured from nonsmokers during a 2-week supplementation with fruits and vegetables. Recently, Moriarty and coworkers (2003) have reported that GSH levels were significantly lower in smokers

(*n*=43), compared with nonsmokers (*n*=78). Correspondingly, it is also unclear whether the expression/activities of antioxidant enzymes are lowered or increased as a result of smoking. Boemi and coworkers (2004) found that paraoxonase (PON1) mass and activity were lower in smokers, and smoking was an independent determinant of PON1 status. In another study, the expression of manganese SOD, copper zinc SOD, and extracellular SOD were investigated by immunohistochemistry in the airways of nonsmokers (*n*=13), smokers (*n*=20), and COPD patients (*n*=22). The expression of manganese SOD was higher in the central bronchial and alveolar epithelium among smokers, and in addition, the activity of the enzyme was higher in smokers than in nonsmokers (Harju et al. 2004).

8.3.2 Prooxidant Effects of Antioxidants

Both vitamin C and E possess prooxidant properties, at least in vitro, depending on their concentration, and the existence of regenerating coantioxidants, plus, for example, on the amount of polyunsaturated fatty acids. Carotenoids may act as prooxidants if the partial pressure of oxygen is above the oxygen pressure in the air (Burton and Ingold 1984; Chen and Djuric 2001; El-Agamey et al. 2004). Thus, exposure of lungs to elevated oxygen concentrations (Halliwell and Gutteridge 1989) might explain, at least partly, the harmful findings of long-term β-carotene supplementations in smokers. Most tissues, however, are exposed to normal oxygen pressure under physiological conditions (Burton and Ingold 1984; Krinsky 2001). The prooxidative effects of β-carotene may also be mediated via changes in the enzyme activity of cytochrome P450 (CYP) isoforms. In an animal model, β-carotene caused a booster effect on phase I enzymes, which are enzymes that can activate the formation of carcinogens in lungs (Paolini et al. 2003). In rat fibroblasts, tobacco smoke condensate increased significantly the production of 8-OHdG at physiological concentrations (0.75–4.0 μ*M*) of β-carotene. This effect of β-carotene on 8-OHdG production was dose and time dependent (Palozza et al. 2004). The role of carotenoid cleavage products might also be important as an inducer of these oxidative reactions (Siems et al. 2003).

8.4 Effects of Smoking and Quitting Smoking on the Plasma Levels of Antioxidants

There is a large body of evidence showing that smoking decreases the plasma levels of different antioxidants (Alberg 2002; Kaikkonen et al. 2001). A recent review evaluated antioxidant concentrations in smokers and former smokers in comparison with nonsmokers and never smokers. In general, smokers and former smokers have lower plasma concentrations of vitamin C and carotenoids, in particular β-carotene, but vitamin E concentrations do not usually differ significantly (Alberg 2002). Plasma γ-tocopherol levels have even been reported to be higher in active or passive smokers, as compared with nonsmokers (Dietrich et al. 2003a). Likewise, some studies have also noted that there are higher plasma concentrations of some individual carotenoids, such as lutein, zeaxanthine, lycopene, or β-carotene, in former smokers than in nonsmokers (Brady et al. 1996; Marangon et al. 1998; Theron et al. 1994).

It seems on average that the circulating levels of β-carotene are 34% lower in smokers

than in nonsmokers, whereas in former smokers, the concentration of β-carotene is 22% lower in contrast to never smokers. The corresponding values for vitamin C concentrations are –27% and –6%. Clear dose–response trends have been observed between decreased plasma concentrations of β-carotene and vitamin C, and the increased number of cigarettes smoked per day. Also, passive smoking seems to have a similar effect, indicating that already low-dose exposure to cigarette smoke has clear unfavorable effects (Alberg 2002; Alberg et al. 2000; Dietrich et al. 2003a; Farchi et al. 2001; Marangon et al. 1998; Polidori et al. 2003; Tröbs et al. 2002). In an experimental study (n=15, ≥7 cigarettes per day) quitting smoking seemed to elevate the vitamin levels by 4 weeks at near or equal to those found in nonsmokers, resulting in plasma that was less susceptible to oxidation (Polidori et al. 2003). In addition, in vivo markers of oxidative stress, i.e., 8-epi-$PGF_{2\alpha}$ (Pilz et al. 2000) and urinary 8-OHdG excretion (Prieme et al. 1998) have been noted to decrease as a result of smoking cessation.

Although the relation between smoking and plasma concentration of antioxidants seem to be quite clear, different findings have also been observed. The most important predictive factor for plasma/serum carotenoid concentrations in 3,043 European subjects from 16 different regions, including nine different European countries, was simply the region in which they lived, not the smoking status (Al-Delaimy et al. 2004). In Japanese men, the alcohol consumption exhibited an inverse association with serum carotenoid levels, and the levels were independent on the smoking status or on the number of smoked cigarettes per day (Fukao et al. 1996). Despite of these opposite findings, major part of the studies have reported dose–response-related strong associations between low circulating levels of antioxidants and smoking status or passive smoking.

8.5 Effects of Antioxidant Supplementation on Lipid Peroxidation and DNA Damage in Smokers

8.5.1 Vitamin C

The effects of vitamin C have been tested in smokers by using several different ex vivo analyses of lipid peroxidation, such as low-density lipoprotein (LDL) susceptibility to oxidation and unspecific TBARS/malondialdehyde (MDA). Findings from these studies suggest that vitamin C supplementation seems to be unable to increase oxidation resistance of isolated LDL or to result in lower TBARS/MDA levels in smokers (Kelly 2003). In addition to these studies, numerous antioxidant cocktail and disease association studies have been published related to vitamin C (see below).

8.5.2 Vitamin E

Patrignani and coworkers (2000) performed a randomized double-blind, placebo-controlled study of the effects of vitamin E (300, 600, and 1,200 mg of DL-α-tocopheryl acetate daily, each dose for 3 consecutive weeks) on 8-iso-$PGF_{2\alpha}$ in 48 moderate cigarette smokers (30 male and 18 female volunteers, aged 20–47 years, smoking 15–30 cigarettes per day). Vitamin E supplementation caused a dose-dependent increase in its plasma levels that reached a plateau at 600 mg (42±11 μmol/l, p<0.001). This increase was not

associated with any statistically significant change in urinary immunoreactive 8-iso-$PGF_{2\alpha}$.

Weinberg and coworkers (2001) reported in their crossover study that supplemented vitamin E (400 IU DL-α-tocopheryl acetate daily) could function as a prooxidant in ten smokers (seven female, three male, >20 cigarettes per day, aged 37 ± 1 years, [mean ± SE]), who consumed a high-polyunsaturated fat diet containing linoleic safflower oil. A diet of olive oil for 3 weeks was used as the control diet, following 3-week safflower oil and safflower plus vitamin E diets. In their study, the safflower oil diet increased total F_2-isoprostanes from 53.0±7.2 to 116.2±11.2 nmol/l and $PGF_{2\alpha}$ from 3.5±0.2 to 5.5±0.5 nmol/l, without changing LDL oxidation parameters. Addition of vitamin E further increased the total F_2-isoprostanes to 188.2±10.9 nmol/l and $PGF_{2\alpha}$ to 7.8±0.4 nmol/l.

In addition, the effect of vitamin E supplementation (600 IU/day for 4 weeks) on plasma total oxysterol levels have been investigated in 12 smokers (≥15 cigarettes per day, four males, eight females) and 14 controls (eight males, six females), aged 18–70 years. The levels of oxysterols were higher in smokers versus controls (354 ± 104 vs 265 ± 66 nmol/l, $p<0.05$), but vitamin E supplementation did not affect the plasma oxysterol levels (Mol et al. 1997).

8.5.3 β-Carotene

As far as we are aware, only one pure β-carotene supplementation study has been carried out using F_2-isoprostanes as the marker of lipid peroxidation. Mayne and coworkers (2004) studied the possible prooxidant effects of a high dose of β-carotene (50 mg/day) in a subpopulation (n=55) of a multiyear (median, 4.0 years) randomized placebo-controlled chemoprevention trial with supplemental β-carotene. The effects of β-carotene in smokers and former/never smokers were monitored with creatinine adjusted spot urine total and 8-iso-$PGF_{2\alpha}$ isoprostanes, which decreased during the supplementation period both in smokers (n=12) and former (n=36)/never (n=2) smokers, though not significantly. The number of the subjects in different groups was quite small, and therefore, it is difficult to draw any clear conclusions from these results.

Block and coworkers (2002) studied the associations between plasma antioxidants and biomarkers of lipid peroxidation in 298 healthy adults aged 19–78 years. The study subjects were smokers (>15 cigarettes per day), passive smokers, or nonsmokers. An inverse association was observed between plasma diet-derived β-carotene and plasma $F_{2\alpha}$-isoprostane levels. The contribution of β-carotene concentrations to the prediction of $F_{2\alpha}$-isoprostane levels was stronger than were current smoking status and age, but lower than were sex, race, body mass index (BMI) or plasma ascorbic acid.

8.5.4 Antioxidant Cocktails and Polyphenols

8.5.4.1 Antioxidant Cocktails

Reilly and coworkers (1996) studied in a crossover study the effects of 5-day dosing with vitamin E (800 IU per day), vitamin C (2 g per day), and their combination on urinary $F_{2\alpha}$-isoprostane levels in four to seven heavy-smoking subjects, aged 20–47 years, smok-

ing a mean of 38 cigarettes per day. There was a 2-week washout period between the treatments. Vitamin C ($p<0.05$) and the combination of vitamin C and E ($p<0.05$) suppressed significantly urinary excretion of 8-epi-$PGF_{2\alpha}$, whereas vitamin E alone had no effect (Reilly et al. 1996).

In a 2-month, randomized double-blind, placebo-controlled trial, Dietrich and coworkers (2003b) investigated whether supplementation with vitamin C (500 mg) or an antioxidant mixture containing vitamin C (500 mg), α-lipoic acid (95 mg), and vitamin E (371 mg of *RRR*-α-tocopherol, 171 mg of *RRR*-γ-tocopherol, 50 mg of α-tocotrienol, 184 mg of γ-tocotrienol, 18 mg of δ-tocotrienol) daily for 2 months could decrease plasma $F_{2\alpha}$-isoprostane levels in 126 smokers, aged 20–78 years, mean age of 46 years, smoking at least 15 cigarettes per day. In smokers with a BMI above the median, the vitamin C supplement did decrease plasma $F_{2\alpha}$-isoprostane levels by 28.8 pmol/l when compared with the placebo group ($p=0.001$). In subjects with BMI below the median, no effect was observed. This group restudied the effect of the antioxidant supplementation in nonsmokers exposed to environmental tobacco smoke and found changes in the same direction.

Kaikkonen and colleagues (2001) studied the effect of vitamin C (500 mg of slow-release ascorbate daily), vitamin E (200 mg of d-α-tocopheryl acetate daily), their combination and placebo on the plasma $F_{2\alpha}$-isoprostane levels in 100 men, aged 45–70 years. Half of the men were smokers (≥5 cigarettes per day). Only vitamin E lowered this index of lipid peroxidation. There was a tendency that the treatment effect was stronger in smokers as compared with nonsmokers. The increase in lipid-standardized vitamin E was associated with the decrease in $F_{2\alpha}$-isoprostanes, both in smokers and nonsmokers.

Jacob and coworkers (2003) tested whether moderate antioxidant supplementation with vitamin C (272 mg), *all-rac*-α-tocopherol (31 mg), and folic acid (400 mg) daily for 3 months could decrease lipid peroxidation in vivo in 38 male smokers (≥10 cigarettes per day) and in 39 nonsmokers, aged 20–51 years. The supplementation used did not lower the oxidative stress in either smokers or nonsmokers. There were several indicators of oxidative stress examined, e.g., F_2-isoprostanes, both total and 8-isoprostanes, TBARS, and protein carbonyls.

The effects of different vitamins on the oxidative DNA damage in smokers have been studied in small clinical trials. Fifteen smokers were divided into the following supplementation groups: vitamin E (200 IU/day), β-carotene (9 mg/day), vitamin C (500 mg/day), red ginseng (1.8 g/day), and placebo. Supplementation was given to smokers for 4 weeks. The carbonyl content of the proteins and 8-OHdG excretion were used to assess the effects of supplemented antioxidants on the oxidative damage of DNA in smokers. No effects in β-carotene or vitamin C group were observed, whereas vitamin E and ginseng extracts were effective in lowering both markers (Lee et al. 1998). Welch and colleagues (1999) studied the effects of ascorbic acid (350 mg/day), *RRR*-α-tocopherol (250 mg/day), β-carotene (60 mg/day), selenium (80 μg/day of sodium selenite), and ascorbic acid plus *RRR*-α-tocopherol (350+250 mg/day) supplementation on DNA damage in smokers ($n=9$) and nonsmokers ($n=12$). Supplementation with β-carotene for 4 weeks decreased the level of 8-OHdG in leukocytes of nonsmokers, but conversely increased its concentrations in smokers, whereas the other antioxidants had no effects.

The effects have also been investigated of combination of vitamin E (1,200 IU/day), C (1,500 mg/day) and β-carotene (22.5 mg/day) supplementation on the intracellular activities of different antioxidant enzymes, such as superoxide dismutase, GPx and catalase. Six-week supplementation increased the plasma levels of all of the ingested vitamins in 14 smokers and in 8 nonsmokers. The concentrations of all vitamins were much

higher in lung lavage cells of the smokers in contrast to nonsmokers, but no significant downregulation of the antioxidant enzymes was observed (Hilbert and Mohsenin 1996). Tomato oleoresin capsules, which contained lycopene (4.9 mg), α-tocopherol (1.2 mg), phytoene (0.5 mg), and phytofluene (0.4 mg), were given three per day for 2 weeks to 12 smokers and 15 nonsmokers, with the control groups receiving placebo capsules. The tomato oleoresin capsules were ineffective in protecting the smokers against the oxidative damage of DNA, which was determined by the number of DNA strand breaks, but in nonsmokers the amount of undamaged DNA was higher after the supplementation period (Briviba et al. 2004).

8.5.4.2 Polyphenols

Similar to the situation with epidemiological studies, there are only a few clinical trials that have examined the effects of polyphenol supplementation on the oxidative damage in smokers, and only one of these studies has utilized in vivo markers of lipid peroxidation (Caccetta et al. 2001), the other studies having resorted to the markers of antioxidant capacity (Young et al. 2002), the oxidation susceptibility of LDL (Princen et al. 1998, Vigna et al. 2003), and the markers of oxidative damage of DNA (Hakim et al. 2003).

Caccetta and coworkers (2001) studied the effects of consumption of red wine (375 ml, 450 mg of polyphenols), white wine (375 ml, 130 mg of polyphenols), and dealcoholized red wine (500 ml, 450 mg of polyphenols) on the production of F_2-isoprostanes in smoking men (n=18, aged 25–71 years). In their randomized trial, plasma free plus esterified F_2-isoprostanes decreased significantly (from 882.5 to 703.4 pmol/l) after 2-week consumption of dealcoholized red wine, but not after the consumption of red or white wine as such.

Vigna and coworkers (2003) studied the effects of grape polyphenol supplementation (300 mg of procyanidin extract) on TBARS and LDL susceptibility to oxidation in a randomized double-blind, crossover study in heavily smoking men (n=24, >50 years of age). Four-week polyphenol supplementation decreased the concentration of TBARS and prolonged the lag phase.

The effects of green tea extract on the markers of oxidative status were studied in a blind, crossover intervention study with nonsmokers (n=8) and smokers (n=8, 10–15 cigarettes per day), aged 20–31 years. Three-week consumption of green tea extract (18.6 mg of catechins/day, equivalent to one to two cups of green tea) did not have any effect on the markers of oxidative stress measured from fasting blood samples. However, green tea extract did increase the plasma antioxidant capacity in postprandial plasma, with this increase being most prominent in smokers (Young et al. 2002).

Princen and coworkers (1998) studied the effects of consumption of green tea (equivalent to 6 cups per day), black tea (equivalent to 6 cups per day), or high amount of isolated green tea polyphenols (3.6 g of tea polypheols, equivalent to 18 cups per day) on oxidative stress in male (n=32) and female (n=32) smokers (aged 34±12 years, at least 10 cigarettes per day) in a randomized placebo-controlled study. No differences in the concentration of plasma antioxidants or parameters of LDL oxidation were detected after 4 weeks of the intervention.

Hakim and coworkers (2003) tested the effects of 4 months' consumption of either black (four cups, 450 mg of polyphenols) or green tea (four cups, 580 mg of polyphenols) on the oxidative damage of DNA in female (n=100) and male (n=33) smokers

(>10 cigarettes per day), who were 18–79 years of age, in a randomized controlled study. Urinary excretion of 8-OHdG decreased (–31%) after 4 months of drinking decaffeinated green tea, whereas no effect was seen after the consumption of black tea.

The number of polyphenol supplementation studies in smokers is limited, and so far, only one study has reported results using F_2-isoprostanes as a marker of oxidative stress. Studies that have utilized ex vivo methodology have led to inconsistent findings. Therefore, no conclusions can be drawn about the effects of polyphenol supplementation on oxidative stress in smokers.

8.5.4.3 Fruit-and-Vegetable-Rich Diet and Oxidative Stress in Smokers

A diet supplemented with fruits and vegetables contains a great many potentially beneficial components, which is not the case when vitamin supplements are ingested. Thus, changes in the diet can affect the intake of several vitamins and polyphenols, which may cause problems in interpretation of the results.

The effects of the increased intake of vegetables and fruits on activities of various antioxidant enzymes in smokers have been determined (Hininger et al. 1997; van den Berg et al. 2001). Diet rich in fruits and vegetables provided 30 mg/day of carotenoids, which increased the plasma concentrations of carotenoids by 23% in smokers and by 11% in nonsmokers. The resistance of LDL to oxidation increased by 14% within 2 weeks in smokers, and by 28% in nonsmokers. However, no effect was observed on the activity of antioxidant enzymes (SOD and GPx) (Hininger et al. 1997).

The effects of vegetable burgers and fruit drinks on antioxidant enzymes and markers of lipid peroxidation were studied in 24 smoking men using a 3-week crossover design with a 2-week washout between the supplementation and control periods. The vegetable burger-and-fruit drink diet provided 18 mg/day of carotenoids, 118 mg/day of vitamin C, 13 mg/day of vitamin E and 28.5 mg/day of flavonoids. No effects were observed on any of the markers of lipid oxidation (TBARS/MDA and plasma 8-epi-$PGF_{2\alpha}$) protein and DNA oxidation (carbonyls and Comet assay), or antioxidant enzymes (GSH-*S*-transferase-α and -π), but the plasma concentrations of antioxidants did increase (van den Berg et al. 2001).

According to these results, dietary changes did affect the plasma concentration of vitamins and flavonoids in smokers, but altered poorly to the markers of lipid peroxidation or DNA damage.

8.6 Antioxidants and the Risk of Degenerative Diseases in Smokers

8.6.1 Clinical Outcome Studies with Antioxidant Supplements

Randomized placebo-controlled studies, which have evaluated the incidence of cardiovascular disease (CVD) and cancer and mortality from these diseases, have provided the strongest evidence for beneficial health effects of antioxidants. To date, several large-scale controlled clinical trials evaluating the effects of vitamin supplementation on the

prevention of cardiovascular diseases or cancer have been conducted. In this chapter, we concentrate on studies that have been conducted in smokers, or studies in which the findings concerning the subgroup of smokers have been presented separately. Four large antioxidant primary prevention supplementation trials have now been completed, and two trials have evaluated the effect on secondary prevention of cancer in smokers.

8.6.1.1 Alpha-Tocopherol, Beta-Carotene Study

The first study, the Alpha-Tocopherol, Beta-Carotene Study (ATBC Study), was a randomized double-blind, placebo-controlled supplementation trial designed to study whether supplementation of α-tocopherol (50 mg/day), β-carotene (20 mg/day), or their combination would reduce the incidence of different cancers in smokers (ATBC Study Group 1994). A total of 29,133 Finnish male smokers, aged from 50 to 69 (a mean age of 57 years) were recruited for the study. The average amount of cigarettes smoked per day was 20.4, and the average smoking history was 35.9 years. The follow-up time varied from 5 to 8 years (a median of 6.1 years), and during this time 876 new cases of lung cancer were diagnosed. The main finding of the study was that β-carotene supplementation increased the incidence of lung cancer by 16% (95% confidence interval [CI] = 2–33%), and also the total mortality was 8% (95% CI = 1–16%) higher in the β-carotene group (Albanes et al. 1996), but no separate effect on the primary coronary heart disease outcomes was observed (Virtamo et al. 1998). Vitamin E (α-tocopherol) supplementation had no significant effect on either the incidence of lung cancer or on the primary coronary heart disease outcomes (Virtamo et al. 1998). However, vitamin E (α-tocopherol) supplementation decreased the incidence of prostate cancer and mortality from that disease (ATBC Study Group 1994).

8.6.1.2 Carotene and Retinol Efficacy Trial

The Carotene and Retinol Efficacy Trial (CARET) was a randomized multicenter, placebo-controlled trial designed to study the effects of β-carotene (30 mg/day) and retinol (25,000 IU/day) on the incidence of lung cancer in smokers and/or workers with an occupational history of asbestos exposure in the United States. A total of 18,314 smokers (60%) and former smokers (39%), aged from 45 to 74 years, were recruited. The CARET study was stopped prematurely after a mean follow up time of 4.0 years because of the significant 28% (95% CI = 4–57%) increase in the lung cancer incidence. Also, the deaths from lung cancer (relative risk [RR]=1.46, 95% CI = 1.07–2.00), CVD (RR = 1.26, 95% CI = 0.99–1.61), and any reason mortality (RR = 1.17, 95% CI = 1.03 = 1.33) increased (Omenn et al. 1996).

8.6.1.3 Physicians' Health Study

The Physicians' Health Study (PHS) was a long-term randomized double-blind, placebo-controlled trial designed to study the effects of aspirin (325 mg/every other day),

β-carotene (50 mg/every other day), or their combination on the incidence of cancer, CVD, and mortality from those diseases. The study population consisted of a total of 22,071 male physicians, aged 40–84 years, of whom 11% were classified as being current smokers and 39% as former smokers. During the 12-year follow-up time, 125 lung cancer deaths occurred. β-Carotene had no significant treatment effect on the endpoints investigated. The results were similar in the subgroups of current and former smokers (Hennekens et al. 1996).

8.6.1.4 Women's Health Study

Lee and colleagues (1999) studied the effects of β-carotene (50 mg/every other day), aspirin (100 mg/day), and vitamin E (600 IU/day) supplementation in women in a randomized double-blind, placebo-controlled trial. Out of a total amount of 39,876 participants, 13% were classified as current smokers. Neither vitamin E nor β-carotene supplementation affected the incidence of cancer or cardiovascular disease in smokers or in nonsmokers. The median follow-up time was 4.1 years (Lee et al. 1999).

8.6.1.5 Antioxidant Polyp Prevention Studies

In addition to primary prevention supplementation studies, several secondary prevention studies have been conducted to evaluate whether the recurrence of different cancers can be prevented by vitamin supplementation, but as far as we are aware, only two of these studies have been conducted with smokers.

Baron and colleagues (2003) studied the effect of β-carotene (25 mg/day), vitamin C, and α-tocopherol (1,000 and 400 mg) supplementation on colorectal adenoma recurrence. A total of 864 subjects who had an adenoma removed participated in this double-blind, placebo-controlled clinical trial. Among the subjects who neither smoked nor drank alcohol, the β-carotene supplementation was associated with a decreased risk of recurrent adenomas (RR = 0.56, 95% CI = 0.35–0.89). However, in smokers, the supplementation was associated with a modest, but a nonsignificant increase in the risk of recurrence (RR = 1.36, 95% CI = 0.70–2.62). Similar findings were reported in the subjects who drank more than one serving of alcohol per day (RR = 1.13, 95% CI = 0.89–1.43). Among those participants, who both smoked and drank alcohol, the risk was almost doubled (RR = 2.07, 95% CI = 1.39–3.08). According to these results, alcohol intake and cigarette smoking appeared to modify the effects of β-carotene on colorectal adenoma recurrence. Vitamin C or E supplementation did not have any effects, positive or negative (Greenberg et al. 1994).

Mayne and colleagues (2001) studied the effects of β-carotene (50 mg/day) on the recurrence of head and neck cancers. The participants consisted of 264 patients who had been curatively treated for cancer of the oral cavity, pharynx, or larynx. At baseline, 48% of the participants receiving β-carotene were classified as current smokers and 45% as former smokers. After a median follow-up time of 51 months, β-carotene supplementation did not have any effect on the recurrence of cancers of the head and neck. The cumulative probability of survival was nonsignificantly better for both smokers and nonsmokers in the β-carotene supplemented group.

8.6.1.6 Summary and Discussion of Clinical Outcome Studies

The results of the randomized primary prevention trials have not supported the view that prolonged high dose of antioxidant supplementation would decrease the risk of lung cancer or cardiovascular diseases. In fact, the results in two of these studies (ATBC, CARET) pointed to potentially adverse effects of β-carotene supplementation in smokers, and this led to the premature termination of these studies. However, in both of these studies the supplementation of β-carotene did not seem to increase the risk of lung cancer in general. In the ATBC Study, the RR for lung cancer was 0.93 (95% CI = 0.65–1.33) for nondrinkers in the β-carotene supplemented group, but was 1.35 (95% CI = 1.01–1.81) among those who reported consuming one or more alcohol containing drinks per day (Albanes et al. 1996). The incidence of lung cancer was higher also among those subjects who smoked at least 20 cigarettes daily when compared with those who smoked 5–19 cigarettes per day. A similar trend was seen also in the CARET study in which the RR for nondrinkers was 1.07 (95% CI = 0.76–1.51), but 1.99 (95% CI = 1.28–3.09) among subjects in the highest quartile of alcohol intake (Omenn et al. 1996). These observations suggest that β-carotene may be detrimental only to those who are heavy smokers and/or regular consumers of alcohol (Mayne et al. 1996). The results of the PHS did not confirm these findings (Cook et al. 2000). The relative risk for lung cancer was 0.8 (95% CI = 0.5–1.2) among the daily consumers of alcohol. Several explanations have been proposed for the discrepancy between the results, e.g., simply because of chance, differences in the study populations, the dose (PHS: 50 mg in every other day, ATBC: 50 mg/day, and CARET: 30 mg/day), differences in the serum concentrations, or the duration of the supplementation (PHS, 13 years; ATBC, 6 years; and CARET, 4 years) (Cook et al. 2000).

There are several possible reasons why β-carotene has not been able to protect against chronic diseases, such as cancer, in large-scale supplementation studies (Omaye et al. 1997). It has been suggested that (1) β-carotene may not be the crucial protecting factor present in fruits and vegetables, (2) β-carotene has to interact with other nutrients in food, (3) β-carotene only has efficacy against precancerous lesions, (4) the dose used in the studies was too high or low, and/or (5) the populations may have had sufficient β-carotene status and additional supplementation would thus provide no further benefit. In addition, even though there are over 600 identified carotenoids, the studies have concentrated mainly on β-carotene. Other carotenoids present in fruits and vegetables may have an effect on the risk of chronic diseases.

The mechanism by which β-carotene supplementation could increase the lung cancer risk has not yet been firmly elucidated. The local concentrated oxygen concentration (Burton and Ingold 1984; Chen and Djuric 2001; El-Agamey et al. 2004) or a variation in the activity of CYP enzyme isoforms may play some role (Paolini et al. 2003). It has also been suggested that components of cigarette smoke may induce oxidation of β-carotene, resulting in the formation of oxidized metabolites or cleavage products with prooxidant capabilities (Mayne et al. 1996, Siems et al. 2003). This has been studied in vitro with human bronchial epithelial cells, where tobacco smoke did not have any prooxidant effects on β-carotene (Arora et al. 2001). On the other hand, in recent in vitro studies, some evidence for prooxidation has been demonstrated (Palozza et al. 2004; Paolini et al. 2003). It is obvious that further studies are needed to clarify the role of β-carotene in the etiology of lung cancer.

8.6.2 Dietary Intake of Polyphenols and the Disease Risk

There are several studies that have evaluated the association between flavonoid intake and the risk of chronic diseases, but only one prospective study (ATBC Study) has concentrated solely on smokers, whereas other studies have included both smokers and nonsmokers. In the ATBC Study, a moderate inverse association was found between the intake of flavanols and flavones and the risk of nonfatal myocardial infarctions (MI) in male smokers. The relative risk for nonfatal MI was 0.77 (95% CI = 0.64–0.93) for the men in the highest (18 mg/day) versus the lowest quintile (4 mg/day) of intake (Hirvonen et al. 2001a). No significant associations with the other cardiovascular disease endpoints were observed. A high intake of flavonoids was also associated with a decreased risk of lung cancer (RR = 0.56, 95% CI = 0.45–0.69), whereas no evident association with the risk of other cancers was found (Hirvonen et al. 2001b). In the Finnish Mobile Clinic Health Examination Survey, Knekt and colleagues (1997, 2002) found that a high intake of one flavonoid, quercetin, was associated with a reduced risk of lung cancer in smokers (RR = 0.49, 95% CI = 0.28–0.86), even though the association was stronger in nonsmokers (RR = 0.13, 95% CI = 0.03–0.57).

A number of case-control studies have also evaluated the effects of flavonoids and the risk of lung cancer in smokers. Many of these studies have reported some association between the high flavonoid or tea intake and decreased risk of lung cancer in smokers (Le Marchand et al. 2000; Mendilaharsu et al. 1998). These results have not been confirmed in other trials (Garcia-Closas et al. 1998; Zhong et al. 2001).

The results of the two prospective cohort studies suggest that a high-flavonoid intake may decrease the risk of lung cancer in smokers. However, the role of flavonoids in lung cancer or in the other chronic diseases cannot be adequately evaluated, because of the limited number of existing studies and partly because the intake of flavonoids depends clearly on the quality of the diet, e.g., a diet rich in flavonoids is usually rich in other antioxidants.

8.7 Conclusion

In this chapter, we have examined the effect of smoking on plasma vitamin levels, the capability of vitamin supplementation to attenuate oxidative stress, and the risk of degenerative diseases in smokers. Furthermore, analytical methods used for assessing lipid peroxidation/oxidative stress have been briefly reviewed.

In smokers, the plasma levels of all of the most important nutritional antioxidants are lower than in nonsmokers, though vitamin E represents an exception, revealing no difference between smokers and nonsmokers. It is evident that smoking itself is at least partly responsible for this decrease in antioxidant levels, and it cannot simply be attributed to the poorer dietary habits of smokers. Also, in passive smokers, one can observe a similar trend. One possible explanation for this finding could be that passive smokers belong to the same social class, family, or working environment as the smokers, and have therefore similar dietary habits. For these reason, it has been recommended that smokers should use antioxidant supplements to achieve the same plasma levels of vitamins as nonsmokers. In fact, daily recommendations have even been calculated for smokers, e.g.,

the daily dose of vitamin C should be as high as 124–200 mg to achieve the levels of non-smokers (Kelly 2003). The most frequently studied antioxidants are vitamin C and vitamin E, even though it seems that smoking does not decrease the plasma vitamin E levels. The results found are somewhat conflicting, with some groups claiming that vitamin C is capable of lowering oxidative stress in smokers, but some others attributing this effect to vitamin E. Similar conflicting results have also been found with carotenoids, such as β-carotene, but also with polyphenols. In fact, for the polyphenols, there is even more complexity as there is extensive structural variation because these compounds can exist as both free and conjugated forms and give rise to a variety of different metabolites.

When results of antioxidant cocktail studies are evaluated, one can quite clearly conclude that the tested antioxidant mixtures do not possess cumulative or interacting effects, as compared with single substances. Also, many of the tested antioxidants seem to possess prooxidant properties under certain circumstances. Markers of lipid peroxidation have been mainly used in small supplementation studies, whereas in larger clinical studies, only the disease endpoints have been assessed. β-Carotene has been a focus of interest since the Finnish finding (ATBC Study) that β-carotene supplementation in smokers tended to increase rather than decrease the risk of lung cancer (ATBC Study Group 1994). However, the pooled analysis of seven cohort studies assessing the dietary intake of β-carotene does not support this finding (Männistö et al. 2004).

It is difficult to compare different supplementation studies, as (1) the definition for a smoker differs between the studies, i.e., in some studies an individual smoking 5 or more cigarettes a day is a smoker, but in others, more than 20 cigarettes need to be inhaled; (2) there are differences in the structure of the supplements (for example slow-release and "normal" vitamin C) and in their doses; (3) there have been a variety of different supplement cocktails examined; and (4) there are a variety of analytical methods used to assess lipid peroxidation. For example, even though $F_{2\alpha}$-isoprostanes are considered as one of the most reliable indicators of lipid peroxidation, there are several modifications also from this single measurement, including enzyme-linked immunosorbent assays and gas and liquid chromatographic mass spectrometric methods. In addition, the forms of F_2-isoprostanes measured vary between studies, i.e., from free to esterified forms and from total concentration to specific 8-epi-$PGF_{2\alpha}$. In other analyses, such as in in vitro oxidation experiments, the variation is even higher. The possible publication bias can also lead to an underestimation of the real number of negative findings. In smokers, the role of the studied antioxidants is still unclear both in the inhibition of oxidative stress and in the etiology of degenerative diseases. Prooxidant or antioxidant, this is the dilemma, at least with respect to β-carotene.

As the findings concerning the effects of antioxidant supplementation in smokers are conflicting, it does seem that the best advice one can give to smokers if they wish to minimize the harmful prooxidant effects of smoking is that they should give up smoking.

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Chapter 9

Smoking Depletes Vitamin C: Should Smokers Be Recommended to Take Supplements?

9

Jens Lykkesfeldt

Contents

9.1 Introduction

Smoking has been identified as one of the major risk factors in human diseases such as atherosclerosis and several cancers (Doll et al. 1994; Mosca et al. 1997; Palmer 1985; Stein et al. 1993), yet approximately one third of the Western World's adult population continues to smoke (WHO Health for All Database 2000). Among other factors, oxidative stress has been suggested to play an important role as initiator of the pathological conditions resulting from tobacco smoking (Colditz et al. 1987; Frei et al. 1991; Genkinger et al. 2004; Gey 1986; Hirvonen et al. 2000; Macfarlane et al. 1995; Poulsen et al. 1998; Pryor and Stone 1993). Because cigarette smoke has been shown to result in increased oxidative stress as measured by a variety of biochemical markers, it has been speculated that increased consumption of fruits and vegetables rich in antioxidants or even specific antioxidant supplements could perhaps be particularly beneficial to smokers (Ames 1998; Ames and Gold 1998; Ames and Wakimoto 2002; Ames et al. 1995;). Indeed, numerous reports have shown that cigarette smokers have lower plasma concentrations of almost all low-molecular-weight antioxidants (Eiserich et al. 1995; Kallner et al. 1981; Lykkesfeldt et al. 1997; Munro et al. 1997). This condition results from at least two factors, one of diet and one of smoking (Dietrich et al. 2003; Lykkesfeldt et al. 1996, 2000; Schectman 1993; Schectman et al. 1991). Thus, because of the consumption of a diet containing more fat and less fruits and vegetables, smokers have a lower intake of a variety of phytonutrients, compared with nonsmokers (Dallongeville et al. 1998; Faruque et al. 1995; Jarvinen et al. 1994; Larkin et al. 1990; Ma et al. 2000; Marangon et al. 1998a; Morabia and Wynder, 1990; Preston, 1991; Serdula et al. 1996; Zondervan et al. 1996). However, in addition to dietary differences, it has been shown in studies correcting for dietary intakes of antioxidants, that in particular, vitamin C is further depleted by the smoke itself (Dietrich et al. 2003; Lykkesfeldt et al. 1996, 2000; Schectman et al. 1991). So the question remains: Should supplementation with vitamin C among smokers be a higher priority for health professionals?

A high intake of fruits and vegetables has long been associated with a reduced risk of developing cardiovascular disease and cancer (Knekt et al. 1991b). A critical evaluation of the cancer-related literature by the International Agency for Research on Cancer under the World Health Organization recently confirmed this and concluded that there was evidence, albeit limited, for a beneficial effect of fruits and vegetables on some types of cancers (mouth, pharynx, esophagus, stomach, colorectal, larynx, lung, ovary, bladder and kidney), but insufficient evidence for a beneficial effect on all other cancer types (International Agency for Research on Cancer WHO 2005). It has been suggested that particularly the antioxidant content of the fruits and vegetables could be responsible for the beneficial effect. In agreement with this notion, longitudinal studies have linked low plasma concentrations of antioxidants to increased risk of developing, e.g., lung and prostate cancer and cardiovascular disease (Eichholzer et al. 1996; Knekt et al. 1991a, 1994).

In contrast, large prospective studies using antioxidant supplements have been less promising. Thus, none of the major clinical studies using mortality or morbidity as end points has found significant positive effects of supplementation with such as vitamin C, vitamin E, or β-carotene (Blot et al. 1993; Heart Protection Study Collaborative Group 2002; Miller III et al. 2005). In contrast to the negative results of these larger studies, many smaller or more specific studies suggest a beneficial role of vitamin C supplemen-

tation. For example, the Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) Study found that in male smokers, a combination of vitamins C and E could retard atherosclerotic progression in hypercholesterolemic subjects as measured by carotid intima thickness (Salonen et al. 2000, 2003). Also, high doses of vitamin C given by infusion have been shown to improve the endothelial dysfunction typically observed in smokers (Traber et al. 2000). Moreover, numerous studies using surrogate markers of disease such as, e.g., lipid peroxidation have shown a positive effect of antioxidant supplementation (Carr and Frei 1999b). Finally, most in vitro studies have suggested a positive effect with increasing vitamin C concentrations (Carr and Frei 1999a).

Thus, whereas unequivocal evidence appears to exist supporting the observation that smoking results in lower plasma concentrations of vitamin C, the clinical significance of this depletion—beyond that of scurvy—remains to be clarified. Studies of the possible beneficial effect of antioxidant supplementation to smokers have produced ambiguous data. However, it should not be overlooked that a substantial part of the population in the developed countries apparently suffers from subclinical vitamin C deficiency that may well affect both short- and long-term health, and could easily be cured. This chapter examines the clinical significance of long-term low vitamin C status in smokers and the current pros and cons of supplementing smokers with vitamin C.

9.2 The Antioxidant Activity of Vitamin C

Vitamin C, or ascorbic acid, is a simple, low-molecular-weight carbohydrate, yet its enediol structure provides it with a highly complex chemistry. Of its many chemical properties, the monoanion ascorbate—the predominant form of vitamin C at physiological pH—fulfills the criteria of an effective antioxidant (Halliwell 1996). In fact, the electron donor properties of ascorbate account for all its known functions. It has a complicated redox chemistry that involves comparatively stable radical intermediates and is heavily influenced by the acidic properties of the molecule. It has been known for many years that ascorbate is easily oxidized by molecular oxygen. The two-electron oxidation product in this reaction is dehydroascorbic acid (DHA), which also has antiscorbutic properties and is readily converted back to amino acids in vivo by both chemical and enzymatic means (Poulsen et al. 2004). Further oxidation/hydrolysis renders the vitamin inactive by leading to the irreversible formation of 2,3-diketoglulonate as well as oxalate, threonate, and other products (Poulsen et al. 2004). DHA has a half-life of only a few minutes at physiological pH (Bode et al. 1990). Consequently, highly efficient ways of regenerating ascorbate in vivo have evolved. These processes are of major importance in maintaining ascorbate in its active reduced state—in particular in species that lack the ability to synthesize ascorbate such as humans.

Vitamin C plays a pivotal role in the antioxidant defense. The molecule has been called the most important water-soluble antioxidant in biological fluids (Frei et al. 1989, 1990) and has earned this honor for several reasons. Chemically speaking, both ascorbate and its one-electron oxidation product ascorbate free radical have remarkably low one-electron reduction potentials of +282 and –174 mV, respectively (Buettner 1993), placing ascorbate at the bottom of the antioxidant hierarchy. This means that on top of the ability to reduce virtually all physiologically relevant oxidants, ascorbate is capable of regenerating other antioxidants such as vitamin E from the α-tocopheroxyl radical and

glutathione from the glutathiyl radical back into their active states (Buettner and Schafer 2004). Equally importantly, the relative stability of the ascorbate free radical renders it a harmless intermediate incapable of inducing free radical damage itself. Instead, at physiological pH, the ascorbate free radical primarily disproportionates via dimer formation into one molecule of ascorbate and one of DHA (Buettner and Schafer 2004). DHA is subsequently recycled by means of either glutathione- or NADPH-dependent dehydroascorbic acid reductases (Del Bello et al. 1994; May et al. 1997, 2001; Park and Levine 1996; Wells and Xu, 1994; Wells et al. 1990, 1995).

Whereas the more electrochemical evidence outlined above suggests that ascorbate is indeed a unique antioxidant, in vitro experiments have confirmed that ascorbate plays a key role in the antioxidant defense as a whole. For example, Frei and coworkers have shown under a variety of oxidizing conditions—including that of cigarette smoke—that ascorbate is the only antioxidant capable of completely preventing lipid oxidation in plasma and once ascorbate is depleted; lipid hydroperoxides are formed despite the presence of other plasma antioxidants such as α-tocopherol and β-carotene (Frei et al. 1988, 1991; Gokce and Frei 1996; Lynch et al. 1994; McCall and Frei 1999). These and other findings suggest that the presence of adequate amounts of vitamin C in particular could be of major importance for the maintenance of redox homeostasis in vivo.

9.3 Vitamin C Homeostasis in Smokers

It has been known since the 1950s that smoking results in lower vitamin C in plasma (Pelletier 1968, 1970); however, the precise mechanism of this effect has still not been fully characterized.

Several epidemiological studies have demonstrated that smokers have a lower intake of phytonutrients—including antioxidants—compared with nonsmokers (Dallongeville et al. 1998; Faruque et al. 1995; Jarvinen et al. 1994; Larkin et al. 1990; Marangon et al. 1998b; Morabia and Wynder 1990; Preston 1991; Serdula et al. 1996; Zondervan et al. 1996). This has hampered the ability to distinguish between effects of smoking and diet on the depletion of antioxidants consistently observed among smokers. In a large epidemiological study, the National Health and Nutrition Examination Survey (NHANES), Schectman et al. compared the vitamin C intake and plasma vitamin C concentrations of smokers consuming >1, 1, or <1 pack of cigarettes per day, and nonsmokers, who had stopped smoking <1 year ago, >1 year ago, or had never smoked (Schectman et al. 1990). The results showed that smokers and nonsmokers who had stopped smoking within the last year had a markedly lower intake of vitamin C, compared with that of never smokers. However, as the effect of smoking on the plasma concentration of vitamin C persisted even when corrected for dietary intake, they concluded that depletion of vitamin C as a result of smoking presumably occurs predominantly via mechanisms independent of dietary intake (Schectman et al. 1990). In agreement with this conclusion, clinical studies adjusting for differences in vitamin C intake, and those assessing populations with similar fruit and vegetable intake have found that smokers have lower plasma vitamin C concentrations than have nonsmokers (Dietrich et al. 2001; Lykkesfeldt et al. 2000; Marangon et al. 1998a). Moreover, smoking cessation quickly results in a substantial elevation of plasma vitamin C concentrations, apparently independent of dietary changes (Lykkesfeldt et al. 1996). These results all suggest that smoking per se predisposes to lower vitamin C status.

In their latest report, the Food and Nutrition Board (2000) recommended that the RDA for vitamin C for smokers be increased to 125 and 110 mg/day for men and women, respectively, compared with those for nonsmokers of 90 and 75 mg/day. The basis for this recommendation was primarily the study by Kallner and coworkers (1981) showing increased metabolic turnover of vitamin C in smokers, compared with nonsmokers. They found a vitamin C turnover among smokers of 70 mg/day, compared with 36 mg/day in nonsmokers, suggesting that smokers need an additional 34 mg vitamin C per day.

As mentioned above, the actual mechanisms by which the vitamin C status is compromised remains less well characterized. However, many data point toward increased oxidative stress as the mediator of this effect. One puff of cigarette smoke has been estimated to contain as many as 10^{15} of gas-phase radicals and 10^{14} of tar-phase radicals (Pryor and Stone 1993). Evidently, it has been suggested that oxidative stress arising directly from the toxicity of the smoke itself can increase vitamin C turnover (Pryor 1997). Also, smoke-induced inflammatory responses may indirectly increase oxidative stress, thereby contributing to the turnover of vitamin C (Anderson 1991; Elneihoum et al. 1997; Langlois et al. 2001a; Lehr et al. 1997). Whether directly or indirectly, cigarette smoke results in an increase vitamin C oxidation ratio (%DHA of total vitamin C) among smokers (Lykkesfeldt et al. 1997). This suggests that vitamin C acts as a free radical scavenger in smokers but could also be because of an impairment of the enzymatic recycling of vitamin C. However, in a recent report, it was shown that recycling of vitamin C is, in fact, increased in smokers, and that this activity is not related to differences in vitamin C transport, glutathione concentration, or cellular energy status (Lykkesfeldt et al. 2003b). From these results, it may be concluded that the rate of vitamin C oxidation exceeds that of the DHA reductases in smokers (Lykkesfeldt et al. 2003b). Other studies have suggested that differences in vitamin C status between smokers and nonsmokers be unrelated to altered pharmacokinetics, although possible differences in sodium-dependent vitamin C transport has not been accounted for (Lykkesfeldt et al. 2003a; Viscovich et al. 2004). Taken together with the observed increased oxidative damage among smokers as measured by biochemical markers, the evidence is building up for an oxidative stress mediated depletion of vitamin C.

9.3.1 The RDA for Vitamin C in Smokers

In the debate against or in favor of antioxidant supplementation to smokers, one of the major points of discussion has been how to determine the proper RDA for smokers. Although smokers have been shown to have lower plasma concentrations of several antioxidants, only the RDA for vitamin C has so far been increased. Based on the data outlined above, this seems appropriate because the depletion of antioxidants other than vitamin C appears to result primarily from altered dietary habits. However, the question remains: How is the correct RDA for vitamin C in smokers determined?

As mentioned previously, the Food and Nutrition Board (2000) currently recommends an RDA for vitamin C for smokers of 125 and 110 mg/day for men and women, respectively. However, Schectman and coworkers proposed a higher RDA for smokers, based on their comprehensive material from the second NHANES (II) (Schectman 1993; Schectman et al. 1989, 1990, 1991). By simply plotting the serum level of vitamin C on the daily intake of vitamin C for smokers and nonsmokers separately, they were able to show that smokers would require a daily intake of 200 mg vitamin C to achieve

the serum concentration of nonsmokers consuming 60 mg vitamin C per day (the previous RDA for vitamin C), i.e., resulting in serum levels around 70 μ*M* (Schectman et al. 1990).

In another approach to this problem, Levine and coworkers (1995, 1996b, 1997) studied vitamin C pharmacokinetics in detail. They found that in nonsmokers, consumption of 60 mg of vitamin C per day resulted in a plasma concentration around 25 μ*M*, i.e., considerably less than the concentration indicated by Schectman's data, whereas ingestion of 200 mg vitamin C per day resulted in a plasma concentration of about 66 μ*M* (Levine et al. 1996b). Higher doses did not increase plasma concentrations considerably, and doses above 500 mg were largely excreted unabsorbed. Based on their studies, Levine et al. concluded that the RDA for vitamin C should be increased to 200 mg per day.

Using DHA as biomarker of smoking-induced oxidative stress added a new way of estimating an increased vitamin C requirement in smokers (Lykkesfeldt et al. 1997). Lykkesfeldt et al. (1997) found no DHA in the plasma of nonsmokers regardless of their vitamin C status, whereas a significant inverse correlation between plasma DHA and plasma vitamin C was observed in smokers. Complete reduction of DHA was maintained and not different from that of nonsmokers for plasma vitamin C concentrations higher than 70 μ*M*. Consequently, the data could be interpreted as supporting an RDA for smokers that result in a plasma concentration of about 70 μ*M*. Based on the data from Schectman et al. (1990) and in agreement with their conclusions, this would require an intake of 200 mg vitamin C per day.

An equally important aspect of an increased RDA for vitamin C for smokers is the possibility of getting the recommended amount of vitamin C through the regular diet. Dietary guidelines currently recommend five to nine servings per day of fruits and vegetables (National Research Council 1990). However, several studies suggest that only a relative small proportion of the US population routinely has an intake in the recommended range (Krebs-Smith et al. 1995; Munoz et al. 1997; Rogers et al. 1995). Moreover, as mentioned previously, considerable evidence has been presented to demonstrate that smokers constitute a relative large part of those having a low intake of fruits and vegetables (Dallongeville et al. 1998; Faruque et al. 1995; Jarvinen et al. 1994; Larkin et al. 1990; Marangon et al. 1998b; Morabia and Wynder 1990; Preston, 1991; Serdula et al. 1996; Zondervan et al. 1996). In contrast to this reality, a daily intake of 200 mg vitamin C has been estimated by the Food and Nutrition Board to require the consumption of a minimum of five servings per day of fruits and vegetables. Thus, conforming to the guidelines for fruit and vegetable consumption will result in nonsmokers getting more than 2-fold their current RDA for vitamin C, whereas smokers would get about 1.5 times their RDA and indeed, the 200 mg per day suggested above. However, a significant dietary modification would be required for most smokers to overcome their increased risk of hypovitaminosis C (Schectman 1993). As this may be practically impossible for many, supplementation with vitamin C may be necessary to reduce the prevalence of low plasma ascorbate concentrations of smokers to rates acceptable for nonsmokers. These considerations have warranted studies to investigate the effects of vitamin C supplementation in smokers in particular.

In the 2000 revision of the RDA for vitamin C from the Food and Nutrition Board (2000), the overwhelming evidence supporting an important role of vitamin C in the defense against oxidative stress was taken into account for the first time. Indeed, the current RDA for vitamin C is primarily based on the intake necessary to achieve about 80% saturation of neutrophils (Levine et al. 2004). In contrast, previous recommenda-

tions were based primarily on clinical evidence, i.e., the prevention of scurvy and hypovitaminosis C. This acceptance of biochemical evidence in support of a higher RDA for vitamin C may well result in the RDA for smokers being further increased in the future.

9.3.2 Repletion of Vitamin C in Smokers

Ever since it became clear half a century ago that smoking apparently predisposes to lower vitamin C status, there have been speculations regarding the possible beneficial effect of antioxidant supplementation. Although it would seem very unlikely that antioxidant supplementation alone should be able to prevent the multiple deleterious effects of smoking, there has been an obvious interest in trying to counter the depletion of antioxidants in smokers: (1) it fits the basic theory of smoking-induced oxidative stress and damage, (2) oxidative stress has been suggested to play an important role as initiator of the pathological conditions resulting from smoking, and (3) plasma concentrations of antioxidants are easily elevated by daily administration of a multivitamin pill. However, one adverse effect may be that these studies can unintentionally detract the focus from the only known effective remedy against smoking-related diseases: to stop smoking. In other words, the smokers may be waiting for a miracle.

As pointed out above, some controversy still exists on the proper RDA for vitamin C for smokers. However, the fact that an increased RDA for smokers exists may be interpreted as acceptance of the basic idea that vitamin C supplementation can be a treatment of oxidative stress caused by smoking. Numerous studies of vitamin C intervention in smokers have been published over the past four decades (Carr and Frei 1999b; Traber et al. 2000). Most studies have used doses between 500 mg and 2 g of vitamin C per day, and found plasma levels saturating around 70 μmol/l. Sodium-dependent vitamin-C transporter 1 (SVCT1) activity, responsible for both primary absorption in the intestine and secondary reabsorption in the kidney, is dependent on the concentration of vitamin C, and the kidney threshold for active tubular reabsorption is around 70μmol/l (MacDonald et al. 2002; Oreopoulos et al. 1993). Based on the detailed pharmacokinetics provided by Levine et al. (1996b) as well as what could realistically be obtained from a diet rich in fruits and vegetables, it has been shown that only 250 mg of vitamin C per day can restore plasma vitamin C levels in poorly nourished smokers from an average of only 23 μmol/l to that of near saturation around 70 μmol/l (Lykkesfeldt et al. 2000). These data also provide indirect evidence that intestinal and renal SVCT1 activity is not impaired by smoking, and that decreased SVCT1 capacity cannot explain the depletion of vitamin C among smokers.

9.4 Prevalence and Clinical Significance of Low Vitamin C Status

Although the definition of optimal vitamin C status remains a matter of some controversy, the opinions appear to converge around the level of apparent saturation, i.e., a plasma concentration of approximately 70 μmol/l (Carr and Frei 1999b; Levine et al. 1995, 1996b, 1997, 2004). Defining vitamin C deficiency is equally complex, because considerable individual variation exists regarding the relationship between the plasma

concentration of vitamin C and the classical hallmark of severe vitamin C deficiency: scurvy (Newton et al. 1985; Schorah et al. 1979). Moreover, the clinical significance of vitamin C deficiency—beyond that of scurvy—has not been clearly defined. Guidelines developed by the National Survey of Canada suggested categories of "severe vitamin C deficiency" (serum level <11 μmol/l) and "marginal vitamin C deficiency" (serum levels between 11 and 23 μmol/l) (Smith and Hodges 1987). As the RDA for vitamin C has been increased since these categories were put forward in 1987, and considering the believed optimal vitamin C level in plasma of 70 μmol/l, a new category (e.g., for serum levels between 23 and, e.g., 50 μmol/l) should be added and could be termed "suboptimal vitamin C status."

9.4.1 Severe Vitamin C Deficiency

Scurvy is normally the clinical manifestation of prolonged and severe vitamin C deficiency. In nonsmokers, scurvy is prevented by a daily intake of as little as 10 mg of vitamin C (Weber et al. 1996b). Clinical symptoms include follicular hyperkeratosis, petechiae, ecchymoses, coiled hairs, inflamed and bleeding gums, perifollicular hemorrhages, joint effusions, arthralgia, and impaired wound heeling (Chazan and Mistilis 1963). Other symptoms include dyspnea, weakness, fatigue, and depression. Scurvy usually occurs in individuals with plasma concentrations lower than 11 μmol/l, i.e., those diagnosed as having severe vitamin C deficiency. However, far from all individuals with plasma levels <11 μmol/l actually develop clinical scurvy (Newton et al. 1985; Schorah et al. 1979). Thus, the relationship between plasma vitamin C status and scurvy is not entirely clear.

Although the basic symptoms and cure of the disease have been known for centuries, a significant part of the population in developed countries—and the smokers in particular—suffer from severe vitamin C deficiency and thus have increased risk of experiencing scurvy-like symptoms. Data from the NHANES II (11,592 subjects, collected from 1976–1980) show that of all smokers (including those taking vitamin C supplements), 7.4% suffered from severe vitamin C deficiency, compared with 1.9% of the nonsmokers, giving rise to a risk odds ratio relative to smoking of 3.0 after adjusting for vitamin C intake (Schectman et al. 1989). A Swiss study of more than 4,000 employees came to the same conclusion (Ritzel and Bruppacher 1977). More recent data suggest that the incidence of severe vitamin C deficiency in developed countries is not declining, but may actually be increasing, although improved analytical methodology may also account for a more accurate categorization of the population as older methods may have overestimated vitamin C (Lykkesfeldt, 2002; Washko et al. 1992). Regardless, data from NHANES III (15,769 subjects, collected from 1988 to 1994) showed that in the United States, 14% of males and 10% of females suffered from severe vitamin C deficiency (Hampl et al. 2004). Of smokers, the numbers were 31 and 25%, respectively. The third Glasgow Multinational Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA) population survey (1,267 subjects) found 26% of males and 14% of females suffered severe vitamin C deficiency (Wrieden et al. 2000). Among the smokers alone, the numbers were 36 and 23% of men and women, respectively (Wrieden et al. 2000). A French population study (1,039 subjects) found severe vitamin C deficiency in 7 to 12% of men and 3 to 5% of women, depending on age group (Hercberg et al. 1994).

The clinical significance of severe vitamin C deficiency extends beyond that of scurvy. In clinical studies in which subjects were made vitamin C deficient, common complaints as gingival inflammation and fatigue were among the most sensitive markers of deficiency (Leggott et al. 1986, Levine et al. 1996b). In a prospective population study, Nyyssönen et al. (1997) found a higher risk of myocardial infarction (relative risk, 3.5) among men with severe vitamin C deficiency constituting about 6% of their Finnish cohort (1,605 subjects). Moreover, Langlois et al. (2001b) recently showed that 14% of patients with peripheral arterial disease suffered from severe vitamin C deficiency as compared with none of the healthy controls and suggested a relationship between vitamin C status and severity of atherosclerosis. In a study with advanced cancer patients, 30% had severe vitamin C deficiency and these patients had shorter survival (Mayland et al. 2005).

9.4.2 Marginal Vitamin C Deficiency

As defined above, plasma concentrations between 11 and 23 μmol/l constitutes a situation of marginal vitamin C deficiency. Hypovitaminosis C has been characterized as having a plasma concentration of vitamin C <23 μmol/l (Schectman 1993), i.e., encompassing both severe and marginal vitamin C deficiency. As with severe vitamin C deficiency, smokers also have increased risk of marginal vitamin C deficiency. Thus in the NHANES II, 19.7% of the smokers showed marginal vitamin C deficiency, compared with 8.2% of the nonsmokers (Schectman et al. 1989). The Scottish MONICA Study found marginal vitamin C deficiency among 30% of smoking men, compared with 22% of nonsmoking men and 30% of smoking women, compared with 16% of nonsmoking women (Wrieden et al. 2000). In the cohort as a whole, the numbers were 26 and 22% for men and women, respectively (Wrieden et al. 2000). In the NHANES III, marginal vitamin C deficiency was found in 20% of males and 17% of females, the upper limit of the group being set at 28 μmol/l (Hampl et al. 2004). In a Parisian cohort, 10–46% of males and 3–15% of females had plasma vitamin C concentrations between 11 and 19 μmol/l, depending on the age group (Hercberg et al. 1994). These data clearly demonstrate that a substantial part of the populations in the developed countries can be diagnosed with vitamin C deficiency.

The clinical significance of marginal vitamin C deficiency—as isolated from severe vitamin C deficiency—has not been thoroughly investigated. In most studies, upper and lower tertiles, quartiles, or quintiles are compared, making it difficult to compare groups between studies. Consequently, the category of marginal vitamin C deficiency can rarely be singled out from all vitamin C deficiency/hypovitaminosis C. With respect to scurvy, clinical cases among people with marginal vitamin C deficiency are rare, but do occur (Hodges et al. 1971; Reuler et al. 1985). Probably more importantly, considerable epidemiological evidence suggests that there may other clinical consequences of marginal vitamin C deficiency. Thus, in a recent reexamination of the NHANES II data combined with a follow-up on vital status 12–16 years later, Loria et al. (2000) found that men in the lowest (<28.4 μmol/l), compared with the highest (>73.8 μmol/l) serum ascorbate quartile had a 57% higher risk of death from any cause and a 62% higher risk of dying from cancer. A similar conclusion was reached by Simon et al. (2001), who also found that severe or marginal vitamin C deficiency was significantly associated with all-cause mortality while being weakly associated with death from cardiovascular disease.

In a 20-year follow study in Britain (730 subjects), significantly higher risk of mortality from stroke was observed in elderly men and women with severe and marginal vitamin C deficiency separately, compared with those with plasma concentrations of vitamin C >28 μmol/l (Gale et al. 1995). The authors concluded that vitamin C status was as strong a predictor of death from stroke as diastolic blood pressure (Gale et al. 1995). An inverse correlation between vitamin C status and stroke was also reported from a study (2,121 subjects) in a rural Japanese population, aged 40 years or more (Yokoyama et al. 2000). In the 12-year follow-up on the Basel Prospective Study, significantly increased risk of ischemic heart disease and stroke was found in individuals with plasma ascorbate <22.7 μmol/l, corresponding to severe or marginal vitamin C deficiency (Gey et al. 1987, 1993a, b).

9.4.3 Suboptimal Vitamin C Status

Based on the increased RDA for vitamin C as well as the indication that a plasma concentration of vitamin C of about 70 μmol/L is currently considered optimal for health, a new category of suboptimal vitamin C status is reasonable for those individuals with plasma concentrations between 23 and about 50 μmol/l. The obvious rationale for this additional category could be that if 70 μmol/l is optimal, e.g., 35 μmol/l is probably not, and therefore, investigations into the clinical significance of a suboptimal vitamin C status are warranted. However, limited data are available and need to be extracted from the few studies discriminating between the concentrations of suboptimal and optimal vitamin C status.

In the Coronary Artery Risk Development in Young Adults (CARDIA) Study, Simon et al. (2004) divided 2,637 subjects (originally enrolled as young adults, aged 18–30 years) into four groups with respect to plasma vitamin C levels, and their "low normal" group (between 23 and 45 μmol/l) corresponds approximately that of suboptimal vitamin C status. At the 10-year follow-up, 26% of the smokers suffered from severe or marginal vitamin C deficiency, whereas about 40% had suboptimal vitamin C status. Among the never smokers, the numbers were 8 and 33%, respectively (Simon et al. 2004). At a 15-year follow-up, they found that low vitamin C status (as measured at 10 years) was associated with a higher prevalence of coronary artery calcium among men but not women. Statistics was not performed individually on the male low-normal group having an odds ratio of 2.09 to 1, compared with the "saturation" group (plasma vitamin C level >62.5 μmol/l). In a larger population sample of 8,453 subjects, aged 30 years or older from the NHANES II, a similar prevalence for vitamin C deficiency or suboptimal vitamin C status among smokers was observed. Thirty percent of the smokers suffered from severe or marginal vitamin C deficiency, whereas 35% had suboptimal vitamin C status (plasma vitamin C between 23 and 55 μmol/l). Among never smokers, the numbers were 9 and 31%, respectively (Simon et al. 2001).

Several large prospective studies have shown an inverse relationship between plasma vitamin C status and risk of cardiovascular disease and/or all-cause mortality (Eichholzer et al. 1996; Gale et al. 1995; Khaw et al. 2001; Loria et al. 2000; Nyyssonen et al. 1997; Riemersma et al. 1991; Sahyoun et al. 1996; Singh et al. 1995). However, no studies have investigated the specific clinical significance of suboptimal vitamin C status as compared with the optimal. Thus, it remains to be established if the biochemical evidence pointing toward an optimal plasma level around 70 μmol/l can be backed up in larger epidemio-

logical studies or clinical trials. Clearly, the effects of suboptimal, compared with optimal vitamin C status are likely to be at most moderate and presumably relevant only in the long perspective, if at all. Thus, it is debatable if studies aimed at clarifying such a limited risk are feasible bearing the high cost in mind. On the other hand, the problems potentially associated with low vitamin C status affects a large percentage of the population and can be readily and inexpensively cured.

9.5 The Pros of Vitamin C Supplementation to Smokers

As outlined above, it is well established that smoking predisposes to low vitamin C status. However, whereas it is questionable if smokers in general are capable of conforming to such a significant modification of their diet as would be required to comply with the dietary guidelines (Schectman 1993), moderate amounts (250 mg) of vitamin C given as supplement saturates even poorly nourished smokers (Lykkesfeldt et al. 2000). Thus, the inability of most smokers to comply with dietary guidelines indirectly supports the recommendation of supplements. In addition, supplementing smokers with moderate amounts of vitamin C is safe. Moreover, the bioavailability of vitamin C from supplements is not different from that of vitamin C from natural sources (Johnston and Luo 1994; Mangels et al. 1993). The amount needed to supply smokers is far from the tolerable upper intake level (UL) of 2 g/day of the Food and Nutrition Board's most recent reference values (2000). A large body of evidence agrees that ingestion of <500 mg/day poses no significant risk to human health, and even several-fold higher daily doses of vitamin C have indicated low toxicity (Johnston 1999). Unfortunately, those currently eating supplements are the least likely to need them (Kirk et al. 1999; McNaughton et al. 2005; Sinha et al. 1994). One might add that they are also the least likely to benefit from them, as saturation kinetics will result in excretion of surplus amounts. Supplementing smokers with vitamin C will shift this balance toward those most likely to need and benefit from them. In the NHANES II, 63% of those suffering from severe or marginal vitamin C were smokers and in this group; only 1% of both smokers and nonsmokers used vitamin C or multivitamin supplements (Loria et al. 2000). In the NHANES III, only 7% of male and 5% of female users of any supplement suffered from severe vitamin C deficiency (Hampl et al. 2004). Thus, supplementing smokers with vitamin C will substantially lower the risk of smokers suffering from scurvy-like symptoms, including those of weakness, fatigue, and depression.

Another part of the rationale for supplementing smokers with vitamin C is based on the so-called oxidation theory of atherosclerosis in which oxidized low-density lipoprotein (LDL) lipid is thought to play a key role in the initiation and progression of the disease (reviewed recently by Witting and Stocker 2004). Other hallmarks of atherosclerosis include endothelial dysfunction and leukocyte adhesion to the endothelium, both of which are increased in smokers (Kalra et al. 1994; Lehr et al. 1993; Morrow et al. 1995; Vita et al. 1990; Weber et al. 1996a; Zeiher et al. 1991).

As mentioned previously, increased oxidative stress and damage have consistently been observed in smokers. This oxidative stress has been suggested to play an important role as initiator of the pathological conditions resulting from smoking (Colditz et al. 1987; Frei et al. 1991; Genkinger et al. 2004; Gey 1986; Hirvonen et al. 2000; Macfarlane et al. 1995; Poulsen et al. 1998; Pryor and Stone 1993). With respect to atherosclerosis, lipid oxidation has been suggested as an early marker (Steinberg 1997). Consistent with

this theory, increasing amounts of F_2-isoprostanes, a marker of lipid oxidation, have been found in smokers (Morrow et al. 1995). Increased amounts of malondialdehyde, another frequently used—albeit more unspecific—marker of lipid peroxidation, has also been found in smokers (Lykkesfeldt et al. 2004). In a recent survey, low vitamin C status was among the strongest predictors of lipid peroxidation (Block et al. 2002). In agreement, vitamin C supplementation has been shown to decrease lipid oxidation in smokers (Dietrich et al. 2002; Helen and Vijayammal, 1997; Motoyama et al. 1997; Nyyssonen et al. 1994; Panda et al. 2000; Valkonen and Kuusi 2000), although not all studies have found an effect (Jacob et al. 2003; Kaikkonen et al. 2001). However, the clinical significance of increased lipid oxidation remains to be further established.

Moving from biomarkers to clinically more relevant studies, vitamin C has been found to improve endothelial dysfunction in smokers, apparently by increasing the bioavailability of nitric oxide (NO) (Antoniades et al. 2003; Carr and Frei 2000; Frei 1999; Heitzer et al. 1996; Kaufmann et al. 2000; Lehr et al. 1997; Levine et al. 1996a; Salonen et al. 1991; Schindler et al. 2000) although conflicting reports also exists (Pellegrini et al. 2004; Raitakari et al. 2000; Scott et al. 2005; Van Hoydonck et al. 2004). The mechanism was initially thought to involve direct scavenging of superoxide radicals (May 2000). However, although intracellular concentrations of ascorbate could potentially be high enough for ascorbate to be able to compete with NO for the reaction with superoxide, the reaction kinetics are unfavorable for ascorbate (Jackson et al. 1998). More recently, it has been shown that vitamin C in physiological amounts can increase the production of NO substantially in human endothelial cell in culture (Heller et al. 1999). The mechanism is believed to involve a vitamin C mediated increase in tetrahydrobiopterin, which increases NO activity via endothelial NO synthase (eNOS) in a dose-dependent manner (Heitzer et al. 2000; Heller et al. 1999; Huang et al. 2000a). High plasma concentrations achieved by infusion have been reported to improve endothelial-dependent vasodilation in, e.g., smokers and patients with type 1 and 2 diabetes and coronary artery disease (Chambers et al. 1999; Gokce et al. 1999; Heitzer et al. 1996; Hornig et al. 1998; Ito et al. 1998; Kugiyama et al. 1998; Levine et al. 1996a; Motoyama et al. 1997; Solzbach et al. 1997; Taddei et al. 1998; Timimi et al. 1998; Ting et al. 1996, 1997). Although unphysiologic levels are achieved, this line of research shows interesting potential—in particular for smokers.

Clinical evidence that vitamin C can actually prevent atherosclerosis is limited, and studies have not been performed in particular with subjects selected for low vitamin C status. In the ASAP Study, the effect of vitamin C, vitamin E, or a combination on carotid intima thickness was studied as a marker of a atherosclerotic progression (Salonen et al. 2000). The authors found that 3 years of supplementation with vitamins C and E in combination—but not vitamin C alone—significantly decreased the intima progression rate in men but not women. Similar results were found at the 6-year follow-up, and the authors concluded that supplementation with vitamins C and E in combination slows down atherosclerotic progression in hypercholesterolemic men (Salonen et al. 2003). Also, another study in women found no effect of vitamins C and E in combination for almost 3 years on the progression of coronary atherosclerosis (Waters et al. 2002).

9.6 The Cons of Vitamin C Supplementation to Smokers

The main problem in recommending vitamin C supplements to smokers can be summarized by looking at two separate observations: (1) smoking predisposes to low vita-

min C status and (2) smoking increases the risk of developing chronic diseases such as atherosclerosis and cancer, and every second smoker will die from the habit (Doll et al. 1994; Mosca et al. 1997; Palmer 1985; Stein et al. 1993). The problem is that limited evidence links the two observations, a situation that mainly boils down to the classic issue in epidemiology: Is smoking-induced vitamin C depletion a cause or a consequence of smoking-related diseases?

The few large prospective intervention studies have consistently found no positive effect of vitamin C supplementation on morbidity and mortality (Blot et al. 1993; Greenberg et al. 1994; Heart Protection Study Collaborative Group 2002). However, none of them used vitamin C as a single substance but included β-carotene or vitamin E as well as other antioxidant vitamins and substances. Regardless, the use of vitamin C and other antioxidants for protection against, e.g., cardiovascular disease in high-risk individuals as those in the British Heart Protection Study is difficult to justify (Heart Protection Study Collaborative Group 2002). Notable for the present discussion is that the subjects included in the Heart Protection Study (20,536 subjects) did generally not suffer from severe or marginal vitamin C deficiency, but rather represented the general population in terms of vitamin C status and thus were less likely to benefit from the intervention. In contrast, the subjects included in the vitamin C part of the Linxian (China) Study (about 18,000 subjects) presumably suffered from vitamin C deficiency in general, although this assumption is based on screening of a sample of only 49 individuals showing an average plasma vitamin C concentration of 8.6 μmol/l (Blot et al. 1993). Following 5 years of intervention with 120 mg vitamin C and 30 mg molybdenum per day, the plasma concentration of vitamin C increased to an average of 46.6 μmol/l, i.e., far from saturation. No effect of the intervention was observed on cancer development or mortality while endpoints such as scurvy symptoms were not included. Only 1% of the deaths among the study participants were attributed to ischemic heath disease, thus limiting the evaluation of intervention effects on this endpoint. As pointed out by the authors, the 5-year duration of the study may have been too short to monitor any effects on cancer incidence, but it might be added that the 120 mg of vitamin C was apparently insufficient to saturate the intervention group with vitamin C, and this may also have impacted the results. Moreover, the main causes of death in Linxian differ from those of the developed countries in that the incidence of cardiovascular disease is considerably lower and that of, e.g., epithelial cancers is extraordinarily high presumably because of dietary and lifestyle differences.

With respect to cancer prevention, clinical intervention trials with vitamin C using surrogate endpoints such as oxidative DNA damage have also been unpromising (reviewed recently by Poulsen et al. 2004). Thus, most studies found no effect of vitamin C supplementation on markers of DNA damage, typically in urine or lymphocytes (Anderson et al. 1997; Brennan et al. 2000; Green et al. 1994; Huang et al. 2000b; Jacobson et al. 2000; Lee et al. 1998; Panayiotidis and Collins, 1997; Porkkala-Sarataho et al. 2000; Prieme et al. 1997; Proteggente et al. 2000; Vojdani et al. 2000; Welch et al. 1999; White et al. 2002; Witt et al. 1992), although positive reports also exist (de la Asuncion et al. 1996; Howard et al. 1998; Moller et al. 2004; Rehman et al. 1998; Schneider et al. 2001).

A different aspect of vitamin C supplementation is the actual timing. For example, should the dose be increase in case of disease or should supplementation be discontinued? High plasma concentrations achieved by infusion have been found to improve, e.g., endothelial-dependent vasodilation. However, vitamin C homeostasis is normally under a tight control, maintaining the concentration within a narrow range at steady state (Levine et al. 2004). This could suggest that higher "unphysiologic" levels are potentially either directly toxic perhaps because of the often-discussed prooxidant effect

of vitamin C, or indirectly because putative regulatory function could be lost when vitamin C concentrations in general approach regulatory levels. Excess levels of oxidants are important for, e.g., activation of internal cellular cascades of apoptosis that again are involved in the protective mechanisms that kill cancer cells and also critical for effective cancer treatment (Blumenthal et al. 2000; Kuipers and Lafleur 1998; Weijl et al. 1997). As pointed out by Zeisel (2004), indiscriminate use of high-dose antioxidant supplements should probably be avoided, at least until the potential risks and benefits have been more clearly characterized.

A completely different reservation against recommending vitamin C supplementation to smokers may be that such action could be interpreted as a potential "cure" for smoking-related diseases and thereby take the wind out of the long-preferred and simple message to the smokers: The only known effective remedy against smoking-related diseases is to quit in time. If vitamin C supplements are eventually recommended particularly to smokers, it is crucial that the potential perspectives—or lack of—are kept in mind and communicated unambiguously. Clearly, vitamin C supplements only have the potential of relieving a relative small part of the problems associated with smoking.

9.7 Conclusion

Whereas considerable literature has identified an inverse correlation between vitamin C status and mortality, the current evidence is primarily of epidemiological nature and thus lacks the ability to establish causality. None of the large prospective intervention studies with vitamin C have been able to establish this causality, which should be kept in mind regardless of the fact that the studies were not designed to look at poorly nourished people with a Western lifestyle, i.e., the group most likely to immediately benefit from such intervention.

However, the fact that a substantial part of the population of the developed countries are suffering from subclinical vitamin C deficiency taken together with the potential health problems and expenses for society associated with this condition as well as its easy and inexpensive cure, demonstrates that action is urgently needed to establish if vitamin C supplements to this part of the population is associated with improved health.

Those presumably in need of vitamin C supplements are the least likely to ingest them. Smokers that currently do not take supplements constitute the largest subpopulation that would potentially benefit from vitamin C supplements because a major proportion remains at increased risk of vitamin C deficiency because of poor dietary habits and the enormous voluntary oxidant exposure. A likely short-term benefit from such a supplementation may be a substantially decreased risk of suffering from scurvy-like symptoms, including weakness, fatigue, and depression. Whether a long-tern benefit exists in terms of lower incidence of cardiovascular disease and cancers can only be established by large controlled clinical trials. Thus, it is important that future studies focus on individuals with a low daily intake of vitamin C. Controlled studies are needed to clarify further the long-term consequences of low vitamin C status in both smokers and the general population as well as the clinical effects of moderate supplementation with vitamin C to these specific individuals. There appears to be a good chance of a value-for-money health benefit for a considerable part of the population.

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Chapter 10

Experimental In Vitro Exposure Methods for Studying the Effects of Inhalable Compounds

10

Michaela Aufderheide

Contents

10.1 Introduction

Analysis of the cellular reactions to complex mixtures such as ambient air, diesel exhaust, or cigarette smoke in vitro is a comprehensive approach because of the complexity of the test atmosphere. Smoke, in particular, represents a mixture of thousands of substances, including short- and long-living radicals in both the gaseous and particulate phases of the aerosol (Hoffmann and Wynder 1986). These compounds undergo chemical reactions that will change the mixture qualitatively and quantitatively in a short time after smoke generation (Pryor 1992, 1997). Thus, all toxicological investigations should be carried out under conditions that are as relevant as possible to the in vivo smoking situation.

The exposure of lung cells to such complex atmospheres will be responsible for either cell injury or cell activation associated with the overexpression of mRNA or the release of various mediators. Therefore, attention should be given to cellular reactions induced by cigarette smoke as a possible cause for the development of chronic lung disorders. Characterization of the early cellular and molecular events plays an important role in understanding the mechanisms involved in chronic lung diseases. Bronchial epithelial cells, alveolar macrophages, and alveolar epithelial cells are in close and permanent contact with inhalable compounds. They participate, at least in part, for example, in the development of inflammatory reactions, commonly described after exposure to air pollutants. The understanding of the functional and pathological disorders resulting from the inhalation of toxic gases and of complex mixtures like cigarette smoke in the respiratory tract requires investigations of the direct effect of such compounds concerning the state and activity of the cells. Here, in vitro systems offer (1) a variety of human cells including bronchial and alveolar cells as well as macrophages for use in monolayer or coculture systems; (2) the analysis of individual cell type responses to complex mixtures or fractions, allowing a better understanding of the independent contribution of this cell type to a particular response; and (3) in vitro exposure conditions that in general can be controlled and reproduced more easily (Leikauf and Driscoll 1993; Wallaert et al. 1996, 2000).

10.2 In Vitro Exposure Systems

10.2.1 Requirements for Exposure Systems

For the exposure of cultivated cells to native atmospheres, "several requirements must be met for in vitro exposure systems," as reported by Rasmussen (1984) and Ritter et al. (2004): (1) The precise control of smoke generation, (2) the age and the physicochemical properties of the smoke when brought into contact with the biological test system, (3) the test atmosphere should have as close as possible contact with the cells, and (4) the system should be designed to allow significant exposure times. Normally, the exposure of cells to ambient atmosphere results in their rapid inactivation because of drying. Therefore, methods have to be developed to maintain a humidified atmosphere or to moisten the cells to establish relevant exposure times.

10.2.2 Analysis of Endpoints

After exposure, representative endpoints should be analyzed as indicators of the toxicological effect, like reduced glutathione, which plays a key role in the cellular reactions following exposure to a wide range of reactive oxygen and nitrogen species (Comhair and Erzurum 2002) as well as to cigarette smoke (Rahman and MacNee 1999). Acute exposure to a variety of different oxidants, including cigarette smoke extracts, has been shown to induce a drastic depletion of the intracellular glutathione in vivo and in vitro (Li et al. 1994; Park et al. 1998; Rahman et al. 1995; Uejima et al. 1990). Following acute depletion, induction of glutathione biosynthesis may occur (Dickinson et al. 2003; Rahman et al. 1996) to remedy the situation.

Because of the need to test the toxic potency of complex mixtures using endpoints considered predictive, as demonstrated by in vivo studies or human evidence, a number of experimental approaches have been made to test the biological effects of such compounds in vitro. Nevertheless, most of them are limited to the testing of pure gases without particulate phase.

10.2.3 Exposure

The easiest way, without great technical effort, is to flush the cells with the test gas. Pace et al. (1961, 1969) measured the cytotoxic effects of nitrogen dioxide (NO_2) and ozone (O_3) on liver cells that were cultured in specially designed flasks (T-60 Pyrex) fitted with ground joints and tubes to permit continuous gas flow and exchange of culture medium. When the cells were covered with medium, little effect was produced by the NO_2 at concentrations below 2,400 ppm. When the medium was removed from the cells and the flasks inverted so that the gas could contact the cells more directly, then as little as 5 ppm NO_2 induced significant inhibition of cell proliferation. This effect was attributed to the physical protection of the liquid layer, as well as to the reaction of NO_2 with serum or other components of the medium. A major conclusion from these studies was that thin layers of liquid are sufficient to protect cells from NO_2, and therefore, it was recommended to use exposure systems that allow more-direct contact between the gases and the cells. These results were confirmed by experiments with O_3 (Pace et al. 1969).

Another possibility is the bubbling of a test atmosphere through a cell suspension. Voisin et al. (1974) used such a method for exposing alveolar macrophages to NO_2, whereby the gas was generated continuously by catalytic reactions with NO. Such an approach was also used in studies with O_3 (Cardile et al. 1995, Van der Zee et al. 1987). However, under these conditions, the cells are covered with culture medium, and consequently, there is no direct contact between cells and the test atmosphere.

To reduce the diffusion barrier for gas-phase toxicants by decreasing the thickness of the media overlay, various techniques have been developed to realize a more realistic and susceptible exposure condition. In this regard, in vitro systems have been described, incorporating roller bottles and rotating or rocking platforms (Baker and Tumasonis 1971; Bolton et al. 1982; Fischer and Placke, 1987; Friedman et al. 1992; Guerrero et al. 1979a,b; Madden et al. 1991; Valentine, 1985; Wenzel et al. 1979) where the culture medium is periodically placed over the apical surfaces of the cells, thus acting as a variable

diffuse barrier. Additional systems that perfuse media past cells attached to membrane filters have also been mentioned (Samuelson et al. 1978; Rasmussen and Crocker 1982).

In 1969, these concepts were used to expose cells to O_3 (Pace et al. 1969) or volatile compounds (Muckter et al. 1998) in culture flasks on rocker platforms. Bombick et al. (1997) used this approach to analyze the biological activity of whole smoke, including the vapour/gas phase under periodically submerged conditions. Typical exposure times needed in such setups are in the range of several hours to a number of days (Boland et al. 2000; Don Porto Carero et al. 2001).

Culture dishes were also integrated for such strategies. Placed inside an incubation chamber on a rocker platform or a rotating holder at an angle, they could be tilted back and forth to realize an intermittent exposure to the test atmosphere without medium overlay (Rusznak et al. 1996; Wenzel et al. 1979, 1982). Guerrero et al. (1979a) used such a system for exposing human fibroblasts to O_3. Under such conditions, cells were sensitive to killing by concentrations of around 1 ppm and showed effects on alkaline phosphatase as well as some indications of genotoxic effects (Guerrero et al. 1979b).

Roller bottles with adherent growing cells on the inside offer another possibility for a periodic exposure procedure. The bottles, filled only with a minimal amount of medium to supply the cells with nutrients, rotate, thus creating a situation where the cells are without a medium layer and could be exposed to gases for a certain length of time (Bolton et al. 1982). In addition, other investigators (Madden et al. 1991) used such a technique for the treatment of rat alveolar macrophages with O_3. The system (Friedman et al. 1985) allowed direct exposure of the cells (>90%) to O_3 at any time with minimal interaction of the toxicant with the culture medium.

10.2.4 Modified Exposure Systems

10.2.4.1 Rolling Inserts

Modified exposure systems were also used to study the toxicological effects of complex mixtures composed of particulate and gaseous compounds. Morin et al. (1999) developed an in vitro system for the continuous exposure of lung tissue slices to diesel exhaust. The design of such an exposure chamber is shown in Fig. 10.1. It consists of two concentric cylinders placed over a continuous Wheaton rolling system, placed in an incubator at 37 °C. The external cylinder contained a solution of 1% copper sulfate ($CuSO_4$) solution in water to achieve adequate hygrometry (85–90%) of the atmosphere. The internal cylinder had slits at the periphery, and it is possible that the humidity from the external cylinder reach the inside of the internal cylinder where a constant and controlled flow (gas debimeters) is applied. The whole-exposure system was placed in a slight vacuum to ensure that the flow through the chamber is without physical obstruction to the progression of particulate compounds of the test atmosphere.

Freshly prepared lung slices of female Wistar rats, transferred onto a titanium grid of a Teflon rolling insert, were placed in scintillation vials with opened caps to have free access to the gaseous and particulate phase of the exhaust, or with caps bearing a Paleflex filter for free exchange of the gaseous compounds but without the penetration of particles. Short-term exposure to diesel exhaust (1 h) resulted in a significant decrease of the intracellular ATP and glutathione (GSH) levels, whereas filtered exhaust showed less marked effects. An exposure of 3 or 6 h induced an inflammatory response (tumor

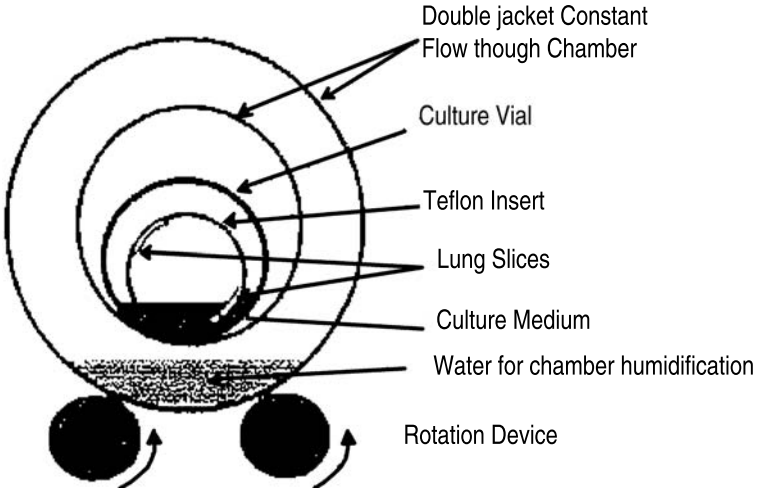

Fig. 10.1 Cross-section of the flow through exposure chambers. The two external concentric circles delimit the humidification compartment. The culture vial rotates freely on the internal wall of the chamber, and the lung slices are alternately fed by culture medium and exposed to the flow of complex atmosphere (Morin et al. 1999)

necrosis factor-α [TNF-α]) and apoptotic processes, as shown by the terminal transferase dUTP nick end-labeling (TUNEL) method and the determination of nucleosomes (Le Prieur et al. 2000). Here, under controlled conditions, a complex mixture containing particulate as well as gaseous compounds was tested, showing different activities of the whole and filtered smoke. All these systems were characterized by an undefined contact between cells, medium, and the test atmosphere, but they demonstrated the possibility to determine biological effects of inhalable test compounds such as gases and complex mixtures with regard to endpoints like cytotoxicity, inflammation, oxidative stress, and DNA damage.

10.2.4.2 Microporous Membranes

A further and more realistic strategy is to expose cells cultured on collagen gels (Jabbour et al. 1998; Zamora et al. 1986) or microporous membranes (Rasmussen 1984; Voisin et al. 1974, 1977).

In the first case, lung alveolar cells were grown on hydrated and nutrified collagen gel prepared from rat tail collagen (Zamora et al. 1986) and exposed to different NO_2 concentrations for 1 h in a modified modular exposure chamber (Fig. 10.2).

The exposure atmosphere was generated by metering 500 ppm of NO_2 in the air through a rotameter into a stream of 95% air-5% CO_2. This system supplied the desired NO_2 concentrations to the cells while maintaining environmental conditions necessary for cell survival (pH ~7.4, high relative humidity). The horizontal gas flow through the chamber was between 2.6 and 2.8 l/min.

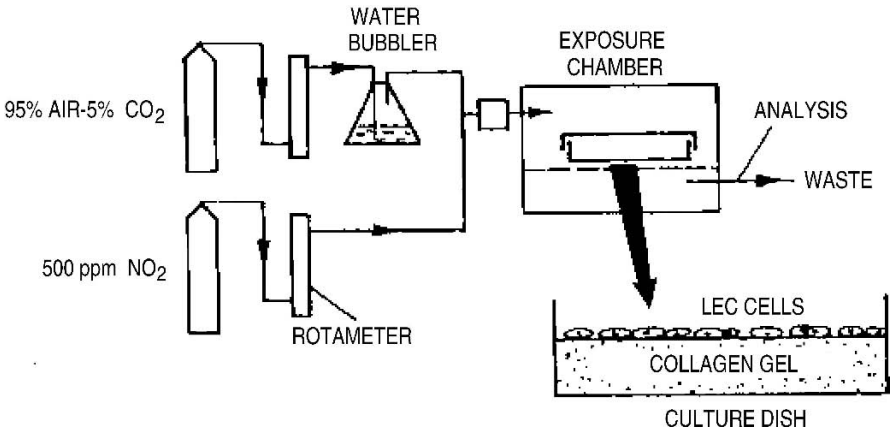

Fig. 10.2 Schematic drawing of the cell exposure system (Zamora et al. 1986)

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The introduction of microporous membranes for biphasic cultures offered a new way to make progress in the field of inhalation toxicology by using in vitro methods. In this strategy, called air–liquid exposure, the cells are fed and humidified from the basal side and exposed from the apical side. This method provides the best and closest contact between the test atmosphere and the cells, compared with the in vivo situation.

Voisin and coworkers (1975, 1977a, b) described a method where macrophages were layered on a polysulfone membrane of 0.2-µm porosity, which was placed on a plastic ring in a culture dish, filled with medium (Fig. 10.3).

In such a way, the cells can be exposed and nutrified. Gas exposure was conducted in an exposure chamber for various time durations to various gas concentrations in a continuous flow of 2 l/min. Control cells were exposed in the same manner to purified air with 5% CO_2. Immediately after gas exposure, cell injury was determined by measuring the ATP content and lactate dehydrogenase (LDH) release. The biological effects of gas exposure could also be estimated by the release of various bioactive mediators or cytokines into the medium.

10.2.4.3 Models for Epithelial Exposure

Another in vitro system (Adler et al. 1987; Whitcutt et al. 1988) was developed for maintaining guinea pig respiratory epithelial cells between the air and liquid phases. The cells, which were plated onto a collagen gel substratum, formed on the top of a nitrocellulose membrane and were fed from below through the membrane and collagen gel, whereas the upper surface was not exposed to medium, but to an air interface, as it occurs in vivo. The system is ideally suited for studies on airway epithelial function, such as secretory responses to irritants and mediators of inflammation, studies involving cell differentiation and gene expression, as well as exposure studies to study the effects of pollutants.

This cultivation method at the air–liquid interface was favored for the development of exposure strategies to analyze the biological effects of inhalable substances, especially

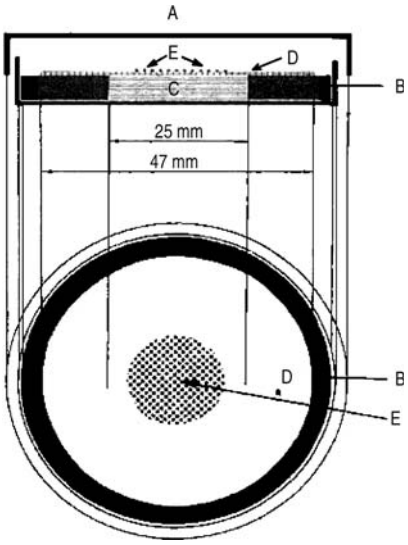

Fig. 10.3 **a** Petri dish. **b** Plastic ring. **c** Medium reservoir. **d** Microporous membrane. **e** Zone for the cultivation of the macrophages (Voisin et al. 1977)

on cells from the respiratory tract in order to simulate the in vivo situation. In general, mainly gases were analyzed for their adverse effects on alveolar macrophages, bronchial, and alveolar epithelial cells by using static or dynamic exposure conditions. However, each of these experimental approaches is complex, because of the biological and technical efforts that have to be made to create a reproducible exposure method for the determination of dose-dependent effects.

By using a biphasic culture system in aerobiosis, Aerts et al. (1992) demonstrated the roles of antioxidant enzymes and of GHS in providing protection against hyperoxia in alveolar type II cells of the guinea pig. In all studies, cell cultures were maintained at 37 °C in humidified airtight incubation chambers, which were flushed with gas mixture (containing 50 or 95% O_2, 5% CO_2, and balanced purified N_2) and then closed. Control cells were exposed to purified air under the same conditions. The chambers were then sealed and incubated for 2 days at 37 °C. Afterwards, cells were harvested for ATP cell content and biochemical analyses. Exposure of type II cells did not induce significant changes in the cell protein content, superoxide dismutase (SOD), catalase, GHS peroxidase (GPx), or GSH content when compared with control cells under normoxia. With ATP content expressed as a cell injury index (CII), type II cell injury was found to increase with increasing O_2 concentrations. Cell injury effects of hyperoxia did not correlate with the endogenous antioxidant enzyme activities (SOD, manganese [MN]-SOD, catalase), whereas a significant correlation between the CII and total GSH content of the cells was estimated. This correlation was largely because of the close relationship between CII and reduced GSH. Hyperoxic-induced cell injury was clearly associated with significantly lower intracellular GSH levels when compared with control cells under normoxia.

10.2.4.4 Delivery of Constant Gas Concentrations

With in vitro systems, a major problem is the proper design of the exposure system to deliver constant and reproducible concentrations of a test gas, especially when using a highly reactive chemical like O_3. Another challenge is to mimic in vivo conditions where the luminal surfaces of respiratory epithelial cells are exposed almost directly to the inspired air, except for a mucus or surfactant layer of variable thickness. Exposure of cell cultures through a stationary liquid layer is undesirable for the following reasons: (1) relatively insoluble gases such as O_3 have little effect except at very high concentrations (Pace et al. 1969, Hager et al. 1981), and (2) the mechanism of action may be different if an oxidant first reacts with the components of a liquid layer rather than reacting directly with the cell or its surface layer (Wenzel and Morgan 1982). To solve this problem, Whitcutt and coworkers (1988) developed a biphasic cell culture system where the cells are maintained between air and the liquid medium to realize a direct exposure of the apical surface of the epithelial cells, e.g., to O_3.

Based on this culture device, Tarkington et al. (1994) designed a new in vitro exposure system for the direct exposure of cultured airway epithelial cells and of tracheal explants in several replicate vessels to O_3, and simultaneously allowing cells to be exposed to an experimental atmosphere without O_3 as a control. This system was designed to generate and monitor consistent, reproducible levels of O_3 over a broad range of concentrations in a humidified atmosphere. Application of earlier versions of the exposure device to tracheal explants has been described by Nikula et al. (1988, 1990). A schematic diagram of the in vitro system is shown in Fig. 10.4.

One vessel for O_3 exposure and one control vessel were used, placed in an incubator. Lines pass through a port in the incubator and convey the gas stream to the exposure and control vessels (Fig. 10.5).

The gas streams are humidified by bubbling through bottles containing sterile distilled water. The humidified gases are then conveyed to jars, serving as exposure and control vessels that have a surface area of 75 cm^2, an appropriate volume to contain five culture vials (Fig. 10.6).

Inside each vessel, the gases enter at the top through a jet orientated in such a way that the atmosphere is injected tangentially to the wall and swirls across the tops of the culture vials. This promotes mixing and even exposure among each of the five vials. Exhaust from each vessel is taken from the bottom in the center. Here, for the first time, a system was described using a directed gas stream to the cells, whereas in other systems, the gas was guided horizontally above the cells. After exposing human tracheobronchial epithelial cells and rat tracheal epithelium to 0.1–1.0 ppm of O_3, cell viability was measured also dependent on the medium layer above the cells. As shown in Fig. 10.7, without any fluid on the top surface of the epithelial cells, O_3 caused substantial cell damage (cell viability 20%) in comparison with cultures exposed to filtered air (85%). When culture medium was added, viability increased, thus demonstrating the importance of direct exposure in order to assess the toxicity of O_3 in vitro. Furthermore, a good correlation between the duration of exposure and the loss of cell viability could be found. In summary, such an exposure method offers the possibility to study functional and morphological effects of gaseous compounds under controlled conditions also in human cell systems.

For the exposure of cells of the respiratory tract to reactive gases, exposure at the air–liquid interface was favored, based on the experience gained so far. Different experimental setups were described in the following years, based on a biphasic culture system.

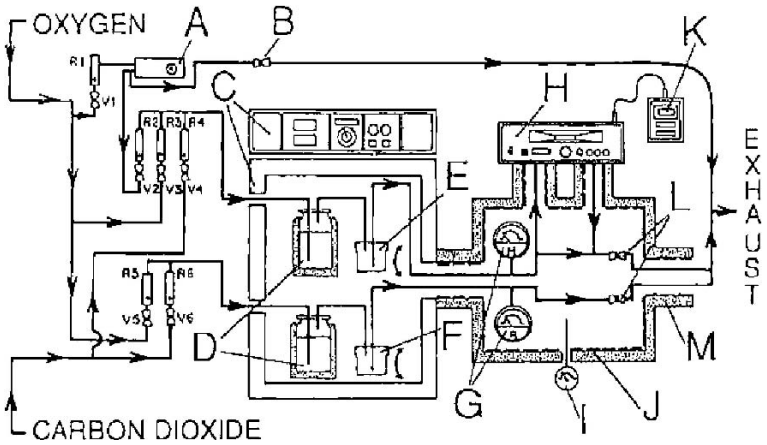

Fig. 10.4 Schematic diagram of in vitro exposure system. **a** Ozonizer. **b** Ozone bypass valve. **c** Incubator. **d** Humidification bottles. **e** Exposure vessel. **f** Control vessel. **g** Pressure gauges. **h** Ozone analyzer. **i** Thermometer. **j** Heating tape. **k** Thermometer. **l** Thermal insulation (Tarkington et al. 1994)

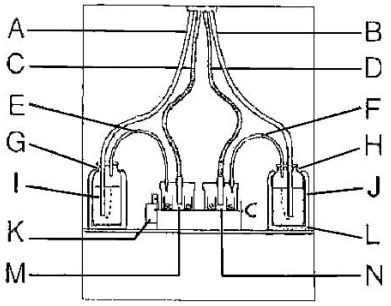

Fig. 10.5 Ozone exposure system: view within incubator. **a** Inlet exposure atmosphere. **b** Inlet control atmosphere. **c** Exhaust exposure atmosphere. **d** Exhaust control atmosphere. **e** Humidified exposure atmosphere. **f** Humidified control atmosphere. **g** Humidifier bottle, exposure. **h** Humidifier bottle, control. **i** Distilled water. **j** Heating mat. **k** Motorized rocker. **l** Thermal insulation. **m** Exposure vessel. **n** Control vessel (Tarkington et al. 1994)

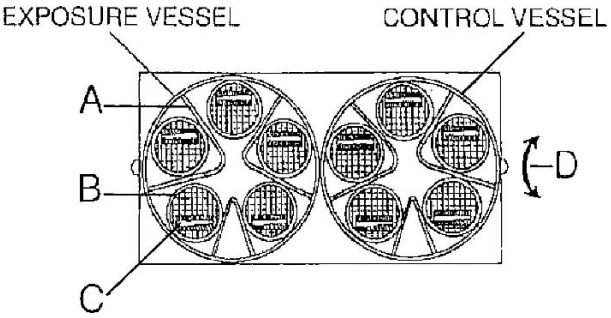

Fig. 10.6 Rocking platform supporting exposure and control vessels. (Tarkington et al. 1994)

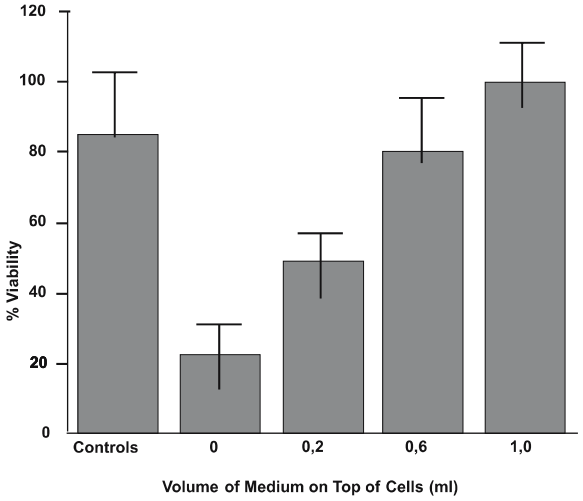

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Fig. 10.7 Effects of culture surface fluid volume on ozone toxicity. The human cell line S6 was maintained biphasically. Cultures were exposed to 1 ppm ozone or to filtered air for 2 h. Before ozone exposure, various volumes of cell culture medium, as shown in the figure, were added. (Tarkington et al. 1994)

Janic et al. (2003) studied the effects of O_3 and other gaseous agents in THP-1 cells (monocyte/macrophage lineage) also under dynamic exposure conditions, i.e., a continuous flow of the test atmosphere. The system used for in vitro exposure (Umstead et al. 2002) also delivered precisely controlled flow rates of gases to the exposure vessel, with precise O_3 concentrations and appropriate humidification. To avoid any interaction of O_3 with the components in the media, all the exposures were done using Hanks Balanced Salt Solution (HBSS). To facilitate direct contact of the test gas with cells and accurate O_3 doses, no HBSS remained on the top during exposures; cells were moistened from below with 1.6 ml HBSS. THP-1 cells exposed to different O_3 concentrations ranging from 0.1 to 0.5 ppm for 1 h were analyzed for cell viability and apoptosis either immediately after exposure or at later time points. The studies demonstrated the absence of apoptosis and a small decrease in cell viability after O_3 exposure. In addition, THP-1 cell expression of cell surface proteins (CD14 and CD11b) appeared to be unaltered. However, the response to a stimulus (i.e., lipopolysaccharide [LPS]) following gas exposure, resulted in a decrease in TNF-α levels, compared with filtered air/LPS-exposed cells.

10.2.4.5 Sidestream Tobacco Smoke Exposure

Encouraged by the positive results gained under the exposure conditions at the air–liquid interface, this experimental approach was also used to study the biological effects of complex mixtures. Sun et al. (1995) studied the effects of sidestream tobacco smoke (STS) as a surrogate for environmental tobacco smoke (ETS) under biphasic culture conditions. BEAS-2B cells (a human bronchial epithelial line immortalized by viral transformation), cultured in the biphasic Millicell-CM system, were exposed in an exposure

system described by Teague et al. (1994). The cells were placed in a 37 °C incubator (lid off) with apical exposure to a defined atmosphere (filtered air and 5% CO_2 saturated with water vapor) and the addition of STS generated from an ADL/II smoking system. The machine delivers a standard puff of 35 ml in 2 s at 30-s intervals on 1KR4F cigarettes. The proliferating cells were exposed to 1 mg/m³ RSP (total respirable suspended particles) STS for 0, 2, 4, or 6 h, and then their viability and functional status were evaluated by measuring DNA synthesis, the mitochondrial metabolic activity, and cell counting. The results clearly demonstrated that STS is cytotoxic to BEAS-2B cells; it inhibited the metabolism as well as the proliferative ability of the cells. The extent of cell injury was directly related to the concentration of STS to which the cells were exposed. Furthermore, exposed cells were analyzed for their protein synthesis pattern. In general, the exposure inhibited total cellular protein synthesis; however, there were several proteins whose synthesis was upregulated after smoke exposure. Among them, a 45-kDa protein was most significantly induced. Expression of classical heat shock proteins with molecular masses around 70 kDa was not increased in these cells. In addition, the exposure of BEAS-2B cells to mainstream smoke resulted in an increased synthesis of the 45-kDa protein, whereas the exposure to cigarette smoke condensate (CSC) showed no induction in protein synthesis. These results suggest that the particular chemical components responsible for this reaction probably exist in the gas phase of tobacco products or are chemically quite labile.

Especially when dealing with complex atmospheres, the biological activity of the particulate and gas phase has to be taken into consideration to characterize the toxicologic activity of such test compounds.

10.2.4.6 Filtered Mainstream Tobacco Smoke Exposure

For studying in particular the effects of the gas phase and the biological mechanisms of its action, Piperi and coworkers (2003) exposed mouse lung epithelial cells (LA-4 and NCI-H157 cells) to filtered cigarette smoke in a plastic chamber, containing the 96-well or 6-well plate cultures. The medium was replaced with serum-free medium before exposure and, after exposure, the cells were washed with phosphate-buffered saline (PBS), and complete medium was added. The dose ranged between one and nine puffs, generated according to International Organization for Standardization (ISO) rules. The cytotoxicity of the gas phase was found to be dose dependent. Exposure to low doses (one to three puffs) had minimal effects on cell proliferation (WST-1) and viability (LDH leakage), whereas higher doses were associated with a significant increase in cell mortality. These observations were evident even 2 and 8 h postexposure, thus pointing to significant effects of the gas phase that do not immediately cause loss of viability, but lead to progressive loss of metabolic activity.

The mechanisms of cell death were found to be both apoptotic and necrotic, depending on the concentrations used. Because of the high amount of oxidants and free radicals, the gas phase mediated its effects primarily by depleting cellular GSH or by modifying protein cysteine residues. Cells that have been exposed to the gas phase showed a rapid but dose-dependent depletion of GSH as measured immediately, 2 and 4 h after exposure. Dose dependency was identical to that observed in cell proliferation and viability assays, suggesting that gas-phase cigarette smoke exerts its effects via oxidative damage. In parallel, increased nitrotyrosine immunoreactivity and phosphorylation of p44/42

mitogen-activated protein kinase (MAPK) proteins were observed. Interestingly, activation of MAPK appeared to coincide with the depletion of cellular GSH, which suggests that the effects of the gas phase may be the results of redox changes of critical protein cysteine residues secondary to GSH depletion or to direct oxidation of protein thiol residues that may occur with, or subsequently to, the loss of GSH.
By using the same experimental setup, the gas phase of cigarette smoke deprived of its volatile constituents (VOCs) was used to study the role of the nonorganic compounds on the cytotoxicity of mouse lung epithelial cells (Pouli et al. 2003). The exposure of cells to this gas fraction showed no toxic effects, and cell viability was found to be equivalent to control cultures, indicating that the removal of the VOCs almost eliminated the cytotoxic potency of the gas phase.

10.2.4.7 Mainstream Cigarette Smoke Exposure

Taking into consideration the importance of the particulate and gas phase of cigarette smoke with regard to their qualitative and quantitative contribution to the biological effects, it would be desirable to analyze these compounds in biphasic systems where the cells are in direct contact with the test atmosphere.

In a specially designed exposure device, CULTEX® (Fig. 10.8), human cells of the respiratory tract (bronchial epithelial cell line, HFBE21; A549 cells) were exposed at the air–liquid interface (Ritter et al. 2003, 2004) to mainstream cigarette smoke as well as to its gas–vapor phase. To test the native atmosphere, exposures were done without heating, humidification, or addition of CO_2. This strategy has already been successfully used for the in vitro testing of native gaseous compounds (Ritter et al. 2001), complex mixtures like sidestream cigarette smoke (Aufderheide et al. 2001), and automobile exhaust (Knebel et al. 2002).

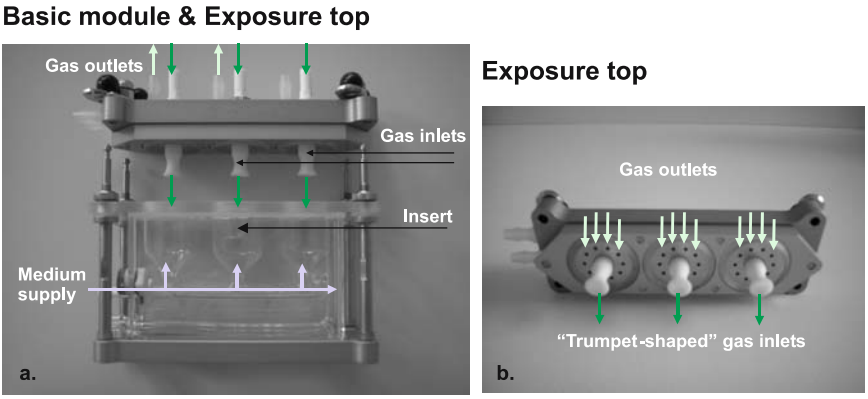

Fig. 10.8: CULTEX® exposure module. **a** The basic part of the exposure module housing the Transwell inserts in three vessels connected via a glass tube system for medium supply. The inner space of the module is insulated against the Transwell vessels to be filled with water at a defined temperature. **b** Exposure top with specially designed trumpet-shaped gas inlets for homogenous distribution of the test atmosphere above the cells and outlets placed in a circle around the inlets

Cells were seeded on Transwell membranes and placed in the basic part of the exposure unit (Fig. 10.8a) housing three Transwell inserts and exposed to the atmosphere without humidification or other modifications. The vessels for the inserts are insulated against the inner space of the module, which can be floated in warm water (37 °C). The vessels are filled with medium, thus providing the humidification and supply of the cells with nutrients. The exposure top of the module (Fig. 10.8b) has three specially designed inlet tubes that guarantee homogenous distribution of the test atmosphere above the cells. In contrast to systems described so far, the smoke is guided vertically, directly to the cells in a continuous flow. The smoke is sucked by negative pressure through the system, exiting the exposure chamber via several outlets. This exposure technique results in an effective and direct contact. By using the CULTEX® system, A549 cells, which are described to share several fundamental characteristics with human lung alveolar type II cells (Lieber et al. 1976; Mendelson and Boggaram, 1991; Nardone and Andrews 1979; Smith 1977; Smith et al. 1982; Young and Mendelson, 1997) and competence of xenobiotic metabolism (Hukkanen et al. 2000; Iwanari et al. 2002; Urani et al. 1998), were exposed to mainstream smoke and the gas phase of three cigarettes, two of comparable tar content (Ritter et al. 2004). Fresh cigarette smoke, generated according to ISO norms by the smoking robot VC10 (Vitrocell, Germany) was sucked at different dilutions (1.67 to 17.67) above the cells for 32 min. To characterize the biological effect of smoke, protein content and intracellular reduced GSH were analyzed directly after exposure. Cellular protein revealed no statistically relevant changes in comparison with control populations exposed to synthetic air throughout all tested cigarettes, dilutions, and exposures to whole and filtered smoke. Under these nontoxic conditions, all cigarettes induced a significant dose-dependent depletion of reduced GHS, which has been shown to be of high relevance with respect to human lung cells and cigarette smoke.

In addition, exposure to whole smoke throughout depleted the GSH more effectively than did exposure to filtered smoke (Fig. 10.9). Based on a detailed analysis of one of the concentrations, the quantitative effects of whole smoke and the gas phase were statisti-

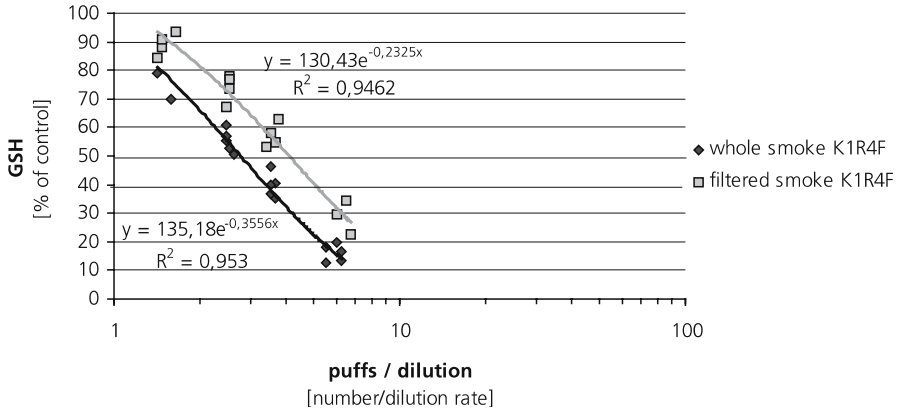

Fig. 10.9 Dose–response curves from exposures of A549 cells to fresh filtered or whole smoke from K1R4F. Intracellular glutathione contents were plotted as a percentage of control values against exposure dose calculated as the ratio of number of smoked cigarette puffs and dilution rate used. Each *dot* represents the result of a single exposure experiment. For determination of effective glutathione (GSH) dose for 50% of the cells ($ED_{50\text{-}GSH}$) values, an exponential regression (K1R4F) was applied (Ritter et al. 2004)

cally significantly distinguishable for the different cigarettes. In conclusion, these studies have shown that the experimental approach allows the investigation of complex mixtures composed of particulate matter and gaseous compounds. By using such a system with a directed flow of the test atmosphere to the cells on the basis of a biphasic cell culture, basic mechanisms of cigarette smoke can be evaluated, e.g., with human lung cells.

10.3 Conclusions

In summary, new developments in the cultivation of isolated cells and optimized exposure methods using direct exposure principles at the air–liquid interface open up new research fields for the analysis of complex mixtures like cigarette smoke. The complexity of such an aerosol, including short- and long-lived radicals in both the gaseous and particulate phase can now be described by analyzing biological effects after exposure to native, unmodified test atmospheres.

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Chapter 11

Oxidative Stress in Laboratory Animals Exposed to Cigarette Smoke, with Special Reference to Chronic Obstructive Pulmonary Disease

11

Chris Coggins

Contents

11.1 Introduction

The historical concept of oxygen as a major air pollutant has already been well documented (Halliwell and Gutteridge 1999), putting into balance the fact that virtually all life on Earth needs oxygen, with the fact that oxygen is biologically very reactive. The two are in balance because of the evolutionary development of antioxidant-defense mechanisms. Organisms that have antioxidant defense mechanisms can use oxygen for the production of energy, mainly in the cellular mitochondria.

Oxidative stress comes about when organisms are exposed to excessive amounts of oxygen, as higher atmospheric concentrations, higher partial pressures in such activities as diving, disease and/or malnutrition, and in the case of higher species such as vertebrates, by alterations in the oxygen transport system. The lung is uniquely susceptible to oxidative stress, as several cell types are continuously exposed to oxygen at atmospheric concentrations, unlike virtually all other cells in the body. The major components of ambient air that contribute to oxidative damage in the lungs include cigarette smoke, exhaust from internal combustion engines (fueled by both diesel and gasoline), and gases such as ozone and nitrogen dioxide.

Antioxidant protective mechanisms in the lungs include the presence of surfactant (from serous and goblet cells), which in turn contains such compounds as reduced glutathione (GSH), α-tocopherol, and ascorbic acid (MacNee and Rahman 2004). The deeper, nonciliated parts of the lung contain free protective cells such as alveolar macrophages, neutrophils, and lymphocytes. Many of the antioxidant protective mechanisms can be upregulated in the presence of oxidative stress; pulmonary disease results when the normal balance between oxidants and antioxidants is lost. Many pulmonary diseases thought to result from such imbalances are hypothesized to do so through inflammatory processes (Reuben et al. 2004). This is especially thought to be the case for smoking-induced diseases, such as pulmonary neoplasia, cardiovascular disease, and chronic obstructive pulmonary disease (COPD).

11.2 Cigarette Smoke

The chemistry of smoke from reference cigarettes has been described in a number of publications (Hoffman and Hoffman 1997; Hoffmann et al. 2001; Rodgman and Green 2003; Roemer 2004; Stabbert 2003), including those that have concentrated in particular on radicals and the role radicals may play in disease (Pryor 1997; Pryor et al. 1998). An integral part of this chemistry is the complicated sequence of processes that occur during the combustion of tobacco. The conditions in the burning cone are highly reducing (temperatures up to 950 °C), as oxygen is consumed by carbonized tobacco to produce heat, carbon monoxide, carbon dioxide, and water (Baker 1999).

Immediately behind the combustion zone is a pyrolysis/distillation zone. Temperatures here are between 200° and 600 °C, still with low concentrations of oxygen (Baker 1999). Aerosol formation occurs as chemicals pass from the burning cone, through the unburned tobacco and (usually) through the cigarette filter.

The smoke that emerges from the cigarette filter is termed mainstream smoke. It is available to smokers, and to machines that mimic human smoking for analytical pur-

poses or for dilution and subsequent distribution to experimental animals. This mainstream smoke is arbitrarily divided into a particulate phase, which does not pass through a Cambridge pad, and a vapor or gas phase, which does. Additional complications include added ingredients (Baker et al. 2004), selective absorption (e.g., phenols by the cellulose acetate fibers used in most cigarette filters), and the use of ventilation holes and additional materials (e.g., activated charcoal) in the filter (Hoffman et al. 2001).

Both particulate and gas phases of cigarette smoke have been reported to contain large numbers (e.g., $10^{15–16}$) of so-called free radicals (Pryor et al. 1998), low-molecular-weight compounds, often carbon- or oxygen-centered radicals. The carbon-centered radicals can react with oxygen to produce other reactive oxygen species (ROS).

Although the concentrations of oxygen are reduced in the burning cone of the cigarette, it is unlikely that this would translate into reduced concentrations in the smoke inhaled by smokers. This is because the puffs taken from the cigarette (puffs usually with volumes of 20–100 ml) are diluted substantially with air at the point of inhalation into the respiratory tract (Bernstein 2004).

Nonsmokers may be exposed to a different kind of smoke, termed environmental tobacco smoke, or ETS (Baker and Proctor 1990). This is the aged and diluted mixture of exhaled smoke from the smoker, with smoke emitted from the burning tip of the cigarette between puffs (the latter being termed sidestream smoke). No filtration is involved in the formation of ETS; because of the dilution that takes place, ETS concentrations are very much smaller than are those of the mainstream smoke taken in by smokers (Jenkins et al. 2000). The free radicals mentioned above for mainstream smoke have also been reported to be present in large numbers in sidestream smoke (Pryor 1997); concentrations in ETS are likely to be very much lower.

11.3 Smoking Machines

A number of designs have been published for smoking machines to expose experimental animals (largely rodents) nose-only to diluted mainstream smoke. The designs have attempted to balance a short path length from the cigarette to the animal, with the ability to expose large numbers of animals. Long path lengths may prevent any short-lived components of the smoke from reaching the experimental animals. A typical range would be short path length, small number of animals (Guerin et al. 1979), medium path length, and medium-large number of animals (Baumgartner and Coggins 1980), and long path length, large number of animals (Henry et al. 1985). Designs involving whole-body exposures (Chen et al. 1992) would appear to have very long path lengths, along with other disadvantages. Long-term inhalation studies with a variety of different animal species have not resulted in useful animal models for human smoking-related disease (Coggins 2002).

Machines for exposing animals to surrogates of ETS (surrogates are required because of the logistical constraint in producing smoke exhaled by smokers) have in general used appropriate aging periods (Ayres et al. 1994; Haussmann et al. 1998a; Teague et al. 1994). In general, responses noted in animals exposed to ETS surrogates have been restricted to localized hyperplasia in the nasal passages (Coggins et al. 1993; Haussmann et al. 1998b; Stinn et al. 2005). By contrast, an inhalation study with mice exposed nose-only to sidestream smoke showed systemic inflammatory responses and accentuated systemic lipid

peroxidation (Zhang et al. 2003). This same group had earlier shown that antioxidant supplementation in healthy, old mice prevented inflammatory responses induced by exposure to sidestream smoke (Zhang et al. 2001). Unfortunately, neither of these two studies made measurements of the amounts of smoke presented—they were almost certainly extremely high.

Although there are reports of measurements of radicals in mainstream smoke (Pryor 1997), there do not appear to any such measurements reported in mainstream smoke presented to experimental animals in smoking machines, or in similar studies using ETS surrogates as the test material.

11.4 Markers of Oxidative Stress in Smoke-Exposed Animals

The National Institute of Environmental Health Sciences (NIEHS) has "taken the lead in initiating the first comparative study for determining which of the available biomarkers of oxidative stress are most specific, sensitive, and selective," using animals treated with carbon tetrachloride (Kadiiska et al. 2000). The original list of biomarkers was α-tocopherol, coenzyme Q, ascorbic acid and uric acid, GHS, and total oxidant capacity (Kadiiska et al. 2000). Very recently, an update was published (Kadiiska et al. 2005), giving a series of analytes for the oxidation products of lipids, proteins, and DNA in different body fluids. For oxidation products in lipids, the following assays are suggested: lipid hydroperoxide, malondialdehyde (MDA), thiobarbituric acid reactive substances (TBARS), and 8-iso-$PGF_{2\alpha}$ (free and esterified) (Kadiiska et al. 2005). For oxidation products of proteins, the list is protein carbonyls, methionine sulfoxidation, and tyrosine products. Finally, for oxidation products of DNA, the list is the Comet assay, leukocyte MDA–DNA adducts, and urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Kadiiska et al. 2005). A variety of different assays is given for different body fluids (Kadiiska et al. 2005).

Very few of the NIEHS list (Kadiiska et al. 2005) have been measured in smoke-exposed animals. The subject of markers of oxidative stress in animals exposed to smoke was included as part of a recent review (van der Vaart et al. 2004), a review which also included studies in humans and which also assessed the effects of smoke on inflammation. A supplemental online report (van der Vaart et al. 2004) lists the various studies examined in the review and lists the various parameters that have been examined.

A total of six studies were included in the review (van der Vaart et al. 2004). The following parameters were reported: 8-OHdG, a DNA oxidation product (Evans et al. 2004) in both lung tissues and bronchoalveolar lavage fluid (BALF), GSH in lung homogenate, BALF and in blood, ascorbic acid in lung homogenate and in BALF, and cysteine in blood and lung tissue (van der Vaart et al. 2004).

Other studies have concentrated on the 8-OHdG endpoint. In a study using whole-body exposures of rats to sidestream smoke, no effect was noted on 8-OHdG concentrations in lung tissue (Arif et al. 2001). This is in contrast to work with both mainstream smoke (Aoshiba et al. 2003a) and ETS (Izzotti et al. 1999), where increased concentrations were noted.

There are some studies that reported an impact of smoke exposure on GSH concentrations. Thus mainstream smoke increased total GSH in BALF (March et al. 2002), with no effect of concurrent treatment with ozone. Work has shown that it is the particulate phase of the smoke that is responsible for the oxidative damage, and that it can be

blocked by vitamin C (Panda et al. 2001). Various antioxidants have also been effective (D'Agostini 2001; De Flora 2003; Izzotti 2001; Sadowska 2005).

A study with only an indirect assessment of oxidative stress examined the effects after smoke exposure of an instilled catalytic antioxidant, manganese (III) *meso*-tetrakis(*N*,*N*'-diethyl-1,3-imidazolium-2-yl) porphyrin (Smith et al. 2002). The catalyst was given by intratracheal instillation to groups of rats exposed to filtered air or cigarette smoke, for up to 8 weeks (6 h/day, 3days/week). Smoke exposures were well characterized and were at conventional concentrations. The experimental treatment significantly reduced the number of cells recovered in BALF, specifically macrophages, neutrophils, and lymphocytes. The authors concluded that the catalysts decreased the adverse effects of smoke exposure. Metalloporphyrins are known to have multiple antioxidant properties, including scavenging superoxide, hydrogen peroxide, peroxynitrite, and lipid peroxyl radicals (Smith et al. 2002).

11.5 COPD

COPD is a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and is associated with an abnormal inflammatory response of the lungs to noxious particles and gases (Pauweis et al. 2001). The "abnormal" or chronic inflammation leads to a narrowing of the small airways (bronchiolitis) and to alveolar wall destruction (Hogg 2002; Snider 2003). The chronic inflammation is characterized by increased numbers of alveolar macrophages, neutrophils, and cytotoxic T lymphocytes (Barnes and Cosio 2004), and the release of multiple inflammatory mediators (lipids, chemokines, cytokines, growth factors) (Barnes 2003, 2004; Rennard 1998). The abnormal inflammatory response may be the key to susceptibility (Agusti et al. 2003). Although many types of inflammatory cells and mediators have been identified in COPD patients, their role in the progression of the disease remains largely unknown (Barnes 2003).

The chronic obstructive bronchitis with mucus hypersecretion may contribute to, but is not necessarily associated with, airflow limitation (Barnes 2003; Cosio-Piqueras and Cosio 2001). *Emphysema* is defined as a condition of the lung characterized by abnormal permanent enlargement of airspaces distal to the terminal bronchiole, accompanied by destruction of the lung parenchyma with or without obvious fibrosis and loss of lung elasticity (Cosio-Piqueras and Cosio 2001; Snider 1992a, b, 2003). Subjects with COPD do not often show emphysema without bronchitis and small airway disease (March et al. 2000).

11.5.1 Epidemiology of COPD

COPD is considered a major health concern, with an overall prevalence in adults estimated at between 4 and 10% in countries where it has been rigorously measured (Halbert et al. 2003). A recent estimate for the incidence of COPD in the United States was given as 16 million people (Mahadeva and Shapiro 2002). The major risk factors for COPD are considered to be cigarette smoking, use of biomass fuels, and air pollution

(Halbert et al. 2003); the population-attributable incidence for cigarette smoking and COPD is about 80–90% (Halbert et al. 2003).

Epidemiological studies have shown that it is mainly susceptible smokers that develop COPD (Siafakas and Tzortzaki 2002).

11.5.2 Basic Pathways Mediating Cigarette-Smoke-Induced COPD

Cigarette smoke exposure has been shown to cause severe oxidative stress in the lung (Aoshiba 2003; MacNee and Rahman 2001, 2004). The oxidants present in cigarette smoke, together with abundant infiltration and activation status of inflammatory cells in the smoker's lung, releasing even more oxygen-based free radicals, may be involved in a proteolytic/antiproteolytic imbalance, leading to tissue destruction (Churg et al. 2003; Seagrave 2000; Seagrave et al. 2004). The incidence of such an imbalance in human populations was the subject of a recent review (deSerres 2003).

A recent study characterized the inflammatory and mucus hypersecretory changes in the lungs of smoke-exposed rats, examining both the role of cytokine-induced neutrophil attractants (CINCs) and a possible mediator of the hypersecretion (Stevenson et al. 2005). The results showed that generation of a neutrophilic/mucus hypersecretory lung phenotype could be produced by just two exposures to smoke, 15 h apart (no details were given on smoke composition). There was a time-dependent increase in the number of CINCs in lung tissue and in lavage fluid over the 24-h period following exposure to smoke. These temporal changes in CINCs mirrored increases in neutrophil infiltration, indicative of a likely role in neutrophil influx, in turn thought to correlate well with matrix destruction (Churg and Wright 2005). The smoke-induced neutrophil infiltration could be inhibited in a dose-related manner (Stevenson et al. 2005).

Recent work has indicated that cigarette smoke is the main etiologic factor, through a mechanism that may involve enhanced proinflammatory gene transcription (Marwick et al. 2004). Other work has indicated that the oxidative stress produced by exposure to cigarette smoke (MacNee and Rahman 2001; Moodie et al. 2004) is a highly relevant factor. The responsiveness of the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway may act as a major determinant of the susceptibility to tobacco-smoke-induced airway disease, by upregulating antioxidant defenses and by decreasing inflammation and alveolar cell apoptosis (Rangasamy et al. 2004).

Oxidative stress has been shown to directly inactivate antiproteinases such as α1-antitrypsin (α1-AT) and secretory leukoprotease inhibitor (SLPI) (Betsuyaku et al. 2002; Cavarra et al. 2001b; Hill et al. 2000), as well as activating matrix metalloproteinases (MMPs) (Belvisi 2003; Selman 2003). Moreover, oxidative stress induces the transcription of many proinflammatory genes controlled by transcription factors such as nuclear factor-κB (NF-κB) (Di Stefano 2002; Moodie et al. 2004). Oxidative stress is also thought to be involved in the accumulation of macrophages in the alveolar interstitial spaces, independent of other proinflammatory stimuli (Kirkham et al. 2003). This latter group has hypothesized that the oxidative stress promotes the macrophage accumulation through the production of reactive carbonyls (particularly acrolein) (Kirkham et al. 2003).

11.5.3 Animal Models of COPD

A number of animal models have been reported that exhibit at least one of the features of the complicated pathology of COPD, such as chronic bronchitis (Nikula and Green 2000) and emphysema (Mahadeva and Shapiro 2002; March et al. 2000; Tarasevicius-Stewart 2004; Wright and Churg 2002). In these models, airspace enlargement has been demonstrated after chronic exposure to mainstream smoke, and also in shorter exposures to high concentrations of smoke. Ideally, such models need to represent the various patterns of alveolar wall destruction that have been reported in humans, as well as host factors that parallel the etiology of the pathological condition. Animal models with genetic predisposition (e.g., an inherent α1-AT deficiency or increased sensitivity to oxidative stress) to develop emphysema are probably the most relevant in mimicking the susceptible human population (deSerres 2003; Kodavanti et al. 1998, 2001). The application of genetic engineering strategies in mice offers a great potential to dissect the pathogenetic pathways of emphysema (Kodavanti 2001; Mahadeva et al. 2002). A few examples of susceptible and genetically engineered models are described below.

Promising susceptible animal models have been described that develop emphysema following whole body exposure to mainstream smoke (Caverra et al. 2001b; Takubo et al. 2002). C57Bl/6J mice, which have a mild deficiency in their antielastase screen, and DBA/2 mice, which are sensitive to oxidants, developed emphysema following 6 months of exposure to cigarette smoke, whereas the mouse strain with normal antielastase screen and nonsensitivity to oxidants (ICR-mouse) did not (Cavarra et al. 2001a). It appears that there are considerable strain differences in the extent of emphysema produced in smoke-exposed mice (Churg et al. 2004; Guerassimov et al. 2004; Obot et al. 2004; Shapiro et al. 2004; Valenca et al. 2004). The situation is complicated by large differences in the degree of detail in characterizing the smoke exposures used to produce emphysema.

The pallid mouse (C57Bl/6J, $pa^{+/+}$), with a severe α1-AT deficiency (DeSanti et al. 1995; Martorana et al. 1993), developed panlobular emphysema after only 4 months of whole-body exposure to cigarette smoke (Cavarra et al. 2001a; Takubo et al. 2002). The pallid mice exhibited features similar to the human situation, including a T-lymphocytic inflammatory response and increased lung compliance (after 6 months of exposure).

The development of spontaneous emphysema has been studied in various transgenic mouse models (Mahadeva and Shapiro 2002). Most of these models have contributed to the knowledge of certain aspects of the development of emphysema, but unfortunately, they have not been challenged by exogenous noxious agents.

Recently, a transgenic mouse model was established that expresses low levels of human α1-AT (Churg et al. 2003), as part of an effort to produce a treatment for cigarette smoke-induced emphysema. The transgenic mice were tolerant to exogenously applied human α1-AT. Mice were exposed to mainstream smoke for up to 6 months; some of them received human α1-AT repeatedly. The latter treatment abolished smoke-induced elevations of neutrophil counts in lavage fluide, as well as the elastin and collagen breakdown products desmosine and hydroxyproline, respectively. Treatment also provided some protection against airspace size. It was concluded that α1-AT therapy reduced the inflammation and partially protects the animals against emphysema.

A murine model deficient for macrophage elastase ($MME^{-/-}$) has been used to shown to be protected against development of mainstream smoke-induced emphysema (Hautamaki et al. 1997). The authors concluded that macrophage elastase is probably sufficient

for the development of emphysema that results from chronic inhalation of mainstream smoke. The role of the macrophage elastase in the smoke-induced inflammation and tissue destruction has been corroborated by elegant studies carried out by Ofulue and coworkers (1998).

Recent work has suggested that a further consideration should be taken when examining the role of inflammation and excessive proteolysis in the pulmonary tissue destruction (Aoshiba et al. 2003b). This work provided evidence that alveolar epithelial apoptosis causes emphysema in C57Bl/6J mice. The authors used a novel protein transfection agent (Chariot) to introduce active caspase-3 into bronchial epithelial cells in vivo. These findings indicate that inflammation, proteolysis, oxidative stress, apoptosis, or cell hemostasis in general are interrelated mechanisms contributing to cigarette smoke-induced emphysema (Tuder et al. 2003).

11.6 Future Models

In many of the studies described relatively minor attention was made to the abnormal inflammatory process mentioned earlier (Siafakas and Tzortzaki 2002; Rangasamy 2004). Future models should provide a tool to understand the exact role of inflammation on the etiology and progression of the disease (Adcock et al. 2005; Reuben et al. 2004; Sadowska et al. 2005).

A prototypic chain of events might be as follows: cigarette smoke exposure → oxidative stress → proinflammatory mediators → inflammation → COPD. Support for this hypothesis is given from a recent paper that showed adverse effects of oxygen supplementation in COPD patients (Carpagnano et al. 2004), in conjunction with increased oxidative stress and with airway inflammation.

Novel designs for cigarette designs, and the potential use of antioxidants and other agents (Adcock et al. 2005; Xu et al. 2004), may be able to break this chain for those people unable to quit smoking.

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Chapter 12

Pulmonary Effects of Cigarette Smoke in Humans

12

Nick H.T. ten Hacken and Dirkje S. Postma

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12.1 Diseases Associated or Caused by Tobacco Smoke

Tobacco use is the leading cause of preventable death in the world today. At present, it is estimated that tobacco kills over 3 million people per year. Based on current trends, the death toll will rise to 10 million deaths per year by the 2020s or 2030s, with 70% of those deaths occurring in developing countries. According to estimates of the World Health Organization, there are approximately 1.1 thousand million smokers in the world, about one third of the global population aged 15 years and over. Globally, approximately 47% of men and 12% of women smoke. In developing countries, available data suggest that 48% of men smoke as do 7% of women, whereas in developed countries, 42% of men and 24% of women smoke. Smoking is an established or probable cause of death from cancers of the oral cavity, larynx, lung, esophagus, bladder, pancreas, renal pelvis, stomach, and cervix. It is also a cause of heart disease, stroke, peripheral vascular disease, chronic obstructive lung diseases (COPD) and other respiratory diseases, and low-birth-weight babies. Finally, smoking is associated with peptic ulcer disease, infertility, unsuccessful pregnancies, and increased infant mortality.

Obviously, the first target to be hit by tobacco smoke is the respiratory tract. This chapter addresses the various abnormalities and diseases that may develop in the lung because of cigarette smoking. Cigarette smoke contains a very high amount of free radicals and carcinogens, which may injure the human lung via various mechanisms. If a smoker is lucky, the lungs remain "healthy." Nevertheless, healthy smokers frequently experience respiratory symptoms and demonstrate signs of underlying inflammation. About 50% of the smokers develop chronic respiratory symptoms belonging to chronic bronchitis, whereas about 15–20% develop full-blown chronic obstructive pulmonary disease, i.e., with airflow limitation. It is generally accepted that asthma is induced by inhalation of allergens in susceptible individuals; however, exposure to cigarette smoke at childhood or adulthood is also associated with a higher prevalence of asthma and asthma-like symptoms. Cigarette smoking is also the single most important risk factor for development of lung cancer; unfortunately, the risk decreases only modestly after smoking cessation. Finally, four smoking-related interstitial lung diseases have been described, prevalence rates being relatively rare as compared with other smoking-in-

duced lung diseases. Therefore, this chapter summarizes the present knowledge of the pulmonary effects of cigarette smoking in humans. Discussed are (1) cigarette smoke, (2) healthy smokers, (3) chronic bronchitis, (4) COPD, (5) asthma, (6) lung cancer, and (7) smoking-related interstitial lung diseases.

When possible, these topics are discussed in the context of cigarette smoking (current/past) and exposure to oxidative stress. In addition, the effect of smoking cessation and of antioxidant therapies is summarized.

12.2 Composition and Inhalation Patterns

Mainstream cigarette smoke is a complex mixture of more than 4,700 chemicals including many potent free radicals and carcinogens (Langen et al. 2003; Pryor and Stone 1993). The less stable gas phase contains 10^{15} free radicals per puff, whereas the more stable tar phase contains 10^{17} radicals per gram. Sidestream cigarette smoke contains more than 10^{15} reactive organic compounds per puff including carbon monoxide, ammonia, *N*-nitrosamines, benzopyrene, ethane, pentane, acrolein, nicotine, isoprene, and acetaldehyde formaldehyde. Studies (Bernstein 2004) with smoking machines (mostly 35-ml puffs, 2-s duration) have shown that particle size of cigarette smoke varies between 0.18 and 0.34 μm. Particle size of cigarette smoke does not differ between the different cigarette types, whether filtered or not filtered, ventilated or not ventilated (Bernstein 2004). In contrast, particle concentration of cigarette smoke is significantly reduced using filters, showing efficiencies between 22 and 94%. Most subjects inhale smoke in two phases (Higenbottam et al. 1980b). The first phase is the mouth phase when smoke is drawn into the mouth. After a pause, the inhalation phase starts when smoke is drawn into the lung. The smoking pattern varies importantly between individuals. The mean (range) number of puffs per cigarette is 11 (8–16), interval between puffs, 28 (18–64) s; duration per cigarette, 351 (232–414) s; duration per puff, 1.9 (1.6–2.4) s; volume per puff, 42.5 (21–66) ml; peak flow of inhalation, 35.5 (28–40) ml/s; and volume of inhalation, 560 (413–918) ml (US Department of Health and Human Services 1988). It has been shown that puff volume determines mainly the concentration of plasma nicotine and expired carbon monoxide; the volume and duration of inhalation does not affect these parameters (Zacny et al. 1987). Smokers who switch to low-tar cigarettes may compensate their relative nicotine lack by increasing puff volume and frequency, inhalation volume, and number of cigarettes per day. Unfortunately, little is known about the effect of breathing pattern on the deposition of cigarette smoke in the human lung. Using a mathematical model, a particle size range of 0.1–0.4 μm is predicted to result in a near-zero bronchial deposition (Nazir et al. 2002) and in a 15–25% total lung deposition fraction. This mathematical model approximates the real-life figures of the particle deposition study of Altshuler et al. (1957), who showed a 26–41% total lung deposition, using particles ranging in size from 0.14–3.2 μm. After being deposited, the free radicals in cigarette smoke may injure the human lungs via various mechanisms: depletion of antioxidants, initiation of redox-cycling reactions, direct damage to lipids-nucleic acids-proteins, enhancement of respiratory burst in macrophages and neutrophils, inactivation of protease inhibitors, inhibition of steroid receptor function, activation of nuclear transcription factors such as nuclear factor (NF)-κB and activated protein (AP)-1, and epigenetic modulation of gene expression.

12.3 Healthy Smokers

12.3.1 Epidemiology and Clinical Presentation

Healthy smokers generally more often experience respiratory symptoms, like cough, phlegm and wheeze, than do nonsmokers (Bjornsson et al. 1994; Brown et al. 1991b; Enright et al. 1994; Jansen et al. 1999; Lundback et al. 1991; Rijcken et al. 1987b; Sherman et al. 1992; Sherrill et al. 1993; Viegi et al. 1988; Vollmer et al. 1989). The prevalence of these symptoms in smokers increases with the number of cigarettes smoked per day (Brown et al. 1991b; Higenbottam et al. 1980a; Jansen et al. 1999; Lindstrom et al. 2001; Silverman et al. 2000) and/or the number of pack years (Enright et al. 1994; Silverman et al. 2000; Viegi et al. 1988). Low-tar cigarettes as compared with normal (high) tar cigarettes have been associated with a lower risk for cough and mucus hypersecretion (Higenbottam et al. 1980a; Schenker et al. 1982), although this is not confirmed in all studies (Rimpela and Teperi 1989; Withey et al. 1992a, b). The prevalence of dyspnea in healthy smokers varies between 2 and 42%, and is higher in those who smoke more than 25 cigarettes per day (Rijcken et al. 1987b; Silverman et al. 2000). Healthy smokers without respiratory symptoms have a higher risk to develop these symptoms later in life than have nonsmokers (Jansen et al. 1999; Krzyzanowski and Lebowitz 1992; Xu et al. 1997). Healthy smokers also have a higher risk for acute upper and lower respiratory infections than have nonsmokers (US Department of Health and Human Services 2004). This has been demonstrated for influenza-like illnesses (Finklea et al. 1969, 1971; Kark and Lebiush 1981; Kark et al. 1982), Legionella (Straus et al. 1996), tuberculosis (Alcaide et al. 1996; Alderson et al. 1985; Buskin et al. 1994; Liu et al. 1998), and community-acquired pneumonia (Almirall et al. 1999a, b; Ferrari et al. 2000; Nuorti et al. 2000; von Hertzen et al. 1998a, b). When current smokers are classified according to the number of cigarettes smoked per day, positive exposure–response relationships have been demonstrated in some but not all studies for influenza (Finklea et al. 1969), tuberculosis (Alcaide et al. 1996; Alderson et al. 1985; Liu et al. 1998), and community-acquired pneumonia (Almirall et al. 1999b; Nuorti et al. 2000). Lower tar content of cigarettes was associated with a lower risk for pneumonia and influenza in one study (Petitti and Friedman 1985)

Above observations are compatible with the finding that cigarette smoking decreases the local cellular defense mechanisms in the human lung. Phytohemagglutinin/concanavalin A-stimulated production by lung lymphocytes was lower in smokers than nonsmokers, and reversed 6 weeks after smoking cessation (Daniele et al. 1977). Lipopolysaccharide (LPS)-stimulated interleukin (IL)-1 production by alveolar macrophages was lower in smokers than in nonsmokers (Yamaguchi et al. 1989). Concentration of IL-1 receptor antagonists in bronchoalveolar lavage (BAL) fluid was significantly lower in chronic smokers than in nonsmokers (Mikuniya et al. 1999). In the same study, LPS stimulated IL-6 production, and IL-1 receptor antagonist concentration was lower in chronic smokers than in nonsmokers (Mikuniya et al. 1999). The spontaneous release of tumor necrosis factor (TNF)-α, IL-1β, IL-8, and macrophage inflammatory protein (MIP)-1α by human alveolar macrophages from smokers is significantly lower than from nonsmokers (Dandrea et al. 1997). Recently, Laan et al. (2004) demonstrated that cigarette smoke suppressed the LPS-stimulated granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-8 production of human bronchial epithelial cells (Beas-2B) via suppression of AP-1 activation. The higher risk for pulmonary infections in smokers as demonstrated in epidemiological studies could thus be because of changes in cellular

function, but also because of other mechanisms like (1) dysregulation of the cytokine network by proinflammatory cytokines (Capelli et al. 1999; Chalmers et al. 2001; Keatings et al. 1996; Linden et al. 1993; Morrison et al. 1998a; Sun et al. 1998); (2) lower nitric oxide (NO) production (Balint et al. 2001; Delen et al. 2000; Montuschi et al. 2001; Persson et al. 1994; Rutgers et al. 1999; Schilling et al. 1994); (3) inhibition of local immunoglobulin (Ig)A function because of decreased expression of epithelial secretory component (Rusznak et al. 2001); (4) skewing of the immune response to a T-helper (Th)-2 phenotype (Burrows et al. 1981; Byron et al. 1994); (5) impaired mucociliary clearance (Janoff et al. 1987); (6) metaplastic changes of the airway epithelium (Sherman 1992); and (7) increased bacterial adherence on mucosal surfaces (Piatti et al. 1997). Indeed, bacterial colonization of the bronchial tree occurs more frequently in healthy smokers than in nonsmokers (Qvarfordt et al. 2000; Soler et al. 1999).

Active smoking during childhood and adolescence has been investigated in a limited number of studies and consistently demonstrated that lung function growth is slower, prematurely ceases, and begins to decline earlier (US Department of Health and Human Services 2004). In particular, adolescent girls seem to be more vulnerable, as smoking five or more cigarettes per day slowed the increase in forced expiratory volume in 1 second (FEV_1) by 31 ml/year versus 9 ml/year in boys (Gold et al. 1996). Healthy adult smokers have a faster decline in FEV_1 than have nonsmokers (Burchfiel et al. 1995), ranging from –2 to –85 ml/year in smokers versus 0 to –56 ml/year in nonsmokers (Beaty et al. 1984; Bosse et al. 1981; Camilli et al. 1987; Krzyzanowski et al. 1986, 1990; Lange et al. 1989; Pelkonen et al. 2001; Samet and Lange 1996; Sherman et al. 1992; Tager et al. 1988; Tashkin et al. 1984; Taylor et al. 1985b; Townsend et al. 1991; Xu et al. 1992, 1994). This accelerated decline in FEV_1 in healthy adult smokers is related to age, actual cigarette consumption per day, and duration of smoking (Burchfiel et al. 1995; Lange et al. 1989; Xu et al. 1992, 1994), but not clearly to the number of pack years (Buist et al. 1979; Lange et al. 1989; Tashkin et al. 1984; Taylor et al. 1985b; Townsend et al. 1991; Xu et al. 1994). Healthy smokers who are young or who smoke less than 15 cigarettes per day have a similar decline in FEV_1 than have nonsmokers (Lange et al. 1989). Decline in FEV_1 is associated with the presence of respiratory symptoms and bronchial hyperresponsiveness (Bosse et al. 1981; Camilli et al. 1987; Krzyzanowski et al. 1990; Rijcken et al. 1987a, b; Sherrill et al. 1991, 1993). In healthy smokers, the prevalence of airway hyperresponsiveness to methacholine or histamine has found to be higher (Burney et al. 1987; Cerveri et al. 1989; Gerrard et al. 1980; Paoletti et al. 1995; Rijcken et al. 1987b; Sunyer et al. 1997; Taylor et al. 1985b), similar (Brown et al. 1977; Kennedy et al. 1984; Lim et al. 1988; Paoletti et al. 1995; Rijcken et al. 1987b; Sunyer et al. 1997; Xu et al. 1997), and lower (Cockcroft et al. 1983) than in nonsmokers.

Hogg et al. (1968) demonstrated that the increased airway resistance in healthy smokers is because of changes in the peripheral airways. Using the single-breath N_2 washout test, Cosio and coworkers (1978) demonstrated that unevenness of ventilation is associated with structural changes in the small airways of healthy smokers with normal spirometry. In line with this, Verbanck et al. (2004) used the more sophisticated multiple-breath N_2 washout test, and demonstrated in smokers with more than 10 pack years that the earliest manifestations of small airway alterations because of smoking were located around the acinair airway entrance (Fig. 12.1). This is completely in line with the data from Lee et al. (2000), who demonstrated on thin section expiratory computer tomography (CT) scans increased air trapping in asymptomatic individuals with a smoking history of more than 10 pack years (Fig. 12.2). In addition, Berger et al. (2003) demonstrated that increased air trapping on thin-section CT scans was associated with reduced

diameters of the small airways because of inflammation in apparently healthy smokers. Importantly, cigarette smokers have already signs of air trapping and hyperinflation regardless of the presence of functional characteristics of emphysema (Kubo et al. 1999).

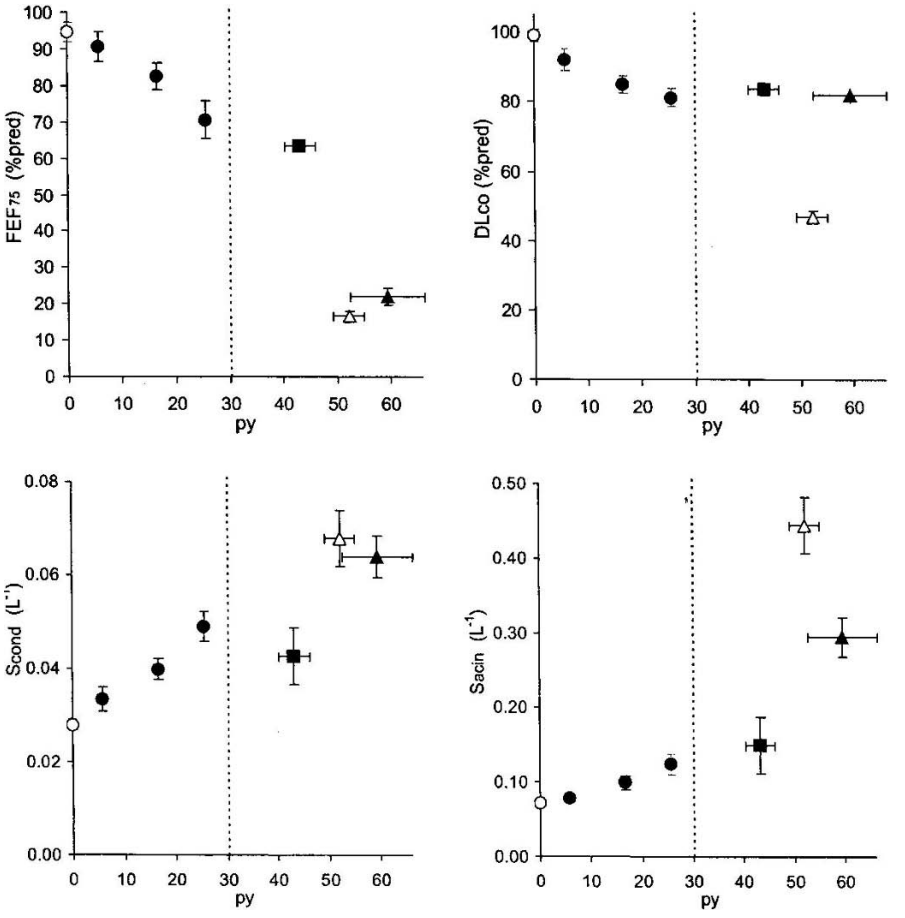

Fig. 12.1 Lung function and ventilation distribution as a function of smoking history for forced expiratory flow between 25 and 75% forced vital capacity (FVC) (FEF_{75}), carbon monoxide diffusing capacity (DL_{CO}), index of acinair conductive ventilation heterogeneity (S_{cond}), and index of acinair ventilation heterogeneity (S_{acin}). *Right* of the *vertical dashed lines* are data representing smokers with a 30-or-more-pack years smoking history classified into three categories: 25 healthy subjects with forced expiratory volume in 1 second (FEV_1)/FVC ≥70% (*solid square*), 26 chronic obstructive lung disease (COPD) subjects with FEV_1/FVC <70%, D_{LCO} ≥60% predicted (*solid triangle*), and 27 COPD subjects with FEV_1/FVC <70%, D_{LCO} <60% predicted and high-resolution computed tomography (CT)-confirmed emphysema (*open triangle*). *Left* of the *vertical dashed line* are data representing 63 never smokers (*open circle*), 27 healthy smokers with <10-pack years smoking history (*solid circle*), 35 healthy smokers with ≥10 and <20 pack years (*solid circle*), and 29 healthy smokers with ≥20 and <30 pack years (*solid circle*). After 10 pack years, healthy smokers demonstrated significantly higher S_{acin} values than the nonsmoking controls ($p<0.05$). After >30 pack years smoking, the COPD patients without emphysema demonstrated higher S_{acin} values than did the healthy smokers ($p<0.05$), and the COPD patients with emphysema demonstrated higher values than did the ones without emphysema ($p<0.05$). (Reproduced with permission from Verbanck et al. 2004)

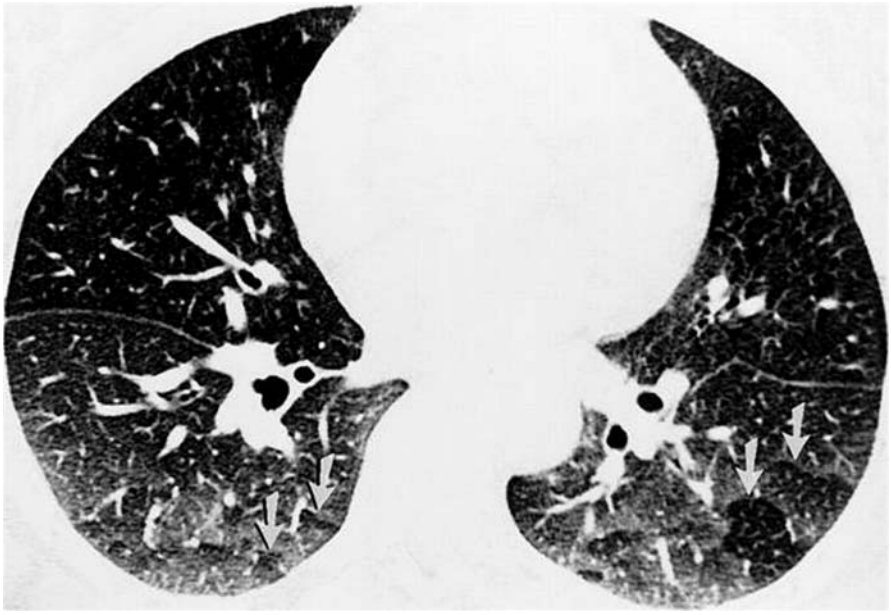

Fig. 12.2 Expiratory transverse, thin-section computer tomography (CT) scan in a 51-year-old man with normal pulmonary function test and without any history of pulmonary disease or symptoms. In contrast to an inspiratory scan at the same level (not shown), this expiratory scan demonstrates air trapping (*arrows*) with a mosaic pattern. (Reproduced with permission from Lee et al. 2000)

12.3.2 The First Steps of Inflammation

Directly after cigarette smoking, increased numbers of neutrophils in BAL fluid have been reported in chronic smokers (Morrison et al. 1998b). The first step in the recruitment of neutrophils toward the airspaces is the sequestration of these cells in the microcirculation of the lung (Macnee and Selby 1993; Macnee et al. 1989), which might be because of decreased deformability and thus stiffening of circulating neutrophils. In vitro and ex vivo experiments have demonstrated such stiffening after exposure to cigarette smoke (Drost et al. 1992, 1993), an effect that was abolished by glutathione (Drost et al. 1992). Once sequestered, cigarette smoke may enhance neutrophil adhesion to the endothelium by upregulating neutrophilic and endothelial adhesion molecules (Patiar et al. 2002). Afterward, the sequestered neutrophils may be attracted by chemotactic components in cigarette smoke, or by chemotactic components (IL-8, leukotriene B_4 [LTB_4]) released from inflammatory and resident cells in lung tissue. NO in cigarette smoke may induce local vasodilatation and plasma exudation, and enhance in this way diapedesis of sequestered neutrophils. Cigarette smoke may activate neutrophils and enhance the formation of reactive oxygen intermediates and release of proteases. Indeed, 1 h after smoking a cigarette, elastase activity was found to be increased in BAL fluid

(Janoff et al. 1983). Consequently, the epithelial integrity may become compromised, leading to a disturbed air space epithelial barrier and allowing in this way the entrance of noxious agents and inflammatory cells. Epithelial permeability has shown to increase immediately after acute exposure to cigarette smoke, compared with nonsmokers and chronic cigarette smokers (Morrison et al. 1999). However, Gil et al. (1995) showed no difference in epithelial permeability between acute and chronic smokers.

12.3.3 Oxidative Stress

Cigarette smoke is a major exogenous source of oxidants containing, e.g., hydroxyl radicals, NO, and hydrogen peroxide. In the lung, these radicals may initiate a number of redox-cycling reactions and give rise to newly formed free radicals. Furthermore, cigarette smoke promotes the recruitment and activation of neutrophils and macrophages, which may act as endogenous sources of free radicals, like superoxide anion and hydrogen peroxide. The only direct method to measure excessive free radical production is electron spin resonance. Using this technique, Otha et al. demonstrated in the lungs of smokers darkly pigmented areas of high electron spin resonance containing heme iron, nonheme iron, and carbon-centered radicals (Fahn et al. 1998; Ohta et al. 1985). Indirect measures of oxidative stress (by determining antioxidant status, tissue damage, or physiological effects) provide overwhelming evidence that cigarette smoking induces oxidative stress inside the human lung (Macnee 2001b; Rahman and Macnee 1996; Repine et al. 1997). Interpretation of these studies has to be done carefully, taking into account conditions of acute, acute on chronic, and chronic cigarette smoke exposure. The following summary includes indirect measures in the lung, BAL fluid, or exhaled air.

One study investigated markers of oxidative stress in BAL fluid from healthy smokers abstaining from smoking for 12 h (chronic smoking) and 1 h after smoking two cigarettes (acute smoking) (Morrison et al. 1999). The superoxide release from PMA-stimulated and unstimulated BAL fluid leukocytes was increased both after acute and chronic smoking. Other studies have also demonstrated increased amounts of superoxide anion in alveolar macrophages of chronic smokers (Hoidal et al. 1981; Nakashima et al. 1987; Schaberg et al. 1992a).

After acute cigarette smoking, the Trolox-equivalent antioxidant capacity (TEAC) in serum was lower, and in BAL fluid higher, than values in chronic smoking (Morrison et al. 1999). In line, after acute cigarette smoking, lower-than-normal serum glutathione levels have been demonstrated, suggesting temporary depletion. In contrast, glutathione in epithelial lining fluid is increased in chronic smokers as compared with nonsmokers (Cantin et al. 1987; Morrison et al. 1999; Rahman et al. 2000), suggesting a compensatory upregulation of the glutathione system. This is associated with an increased expression of γ-glutamylcysteine synthetase mRNA in the lungs (Rahman et al. 2000). In contrast, Harju et al. (2002) found decreased protein γ-glutamylcysteine synthetase immunoreactivity in the lungs of smokers. An explanation for this discrepancy is not available.

Hydrogen peroxide in exhaled breath condensate of healthy smokers increased about 25% 30 min after smoking only one cigarette (Guatura et al. 2000). The same study showed that current smokers had similar hydrogen peroxide concentrations as nonsmokers at baseline. Contradictory results were reported about exhaled NO immediately after cigarette smoking. No differences were observed at 15 (Kharitonov et al. 1995), 30

(Balint et al. 2001), and 90 min (Balint et al. 2001) after smoking. One study reported an increase in NO at 1 and 10 min after smoking a single cigarette (Chambers et al. 1998), whereas another study reported a decrease after 5 min (Kharitonov et al. 1995). Breath condensate levels of nitrate increased 30 min after cigarette smoking, but nitrite and nitrotyrosine levels in this study did not change (Balint et al. 2001). Exhaled NO in *chronic* smokers is consistently lower than in nonsmokers (Balint et al. 2001; Delen et al. 2000; Montuschi et al. 2001; Persson et al. 1994; Rutgers et al. 1999; Schilling et al. 1994). Recently, Comhair et al. (2000) showed that cigarette smoke did not upregulate inducible NO synthase (*iNOS*) mRNA expression in human epithelial cells and alveolar macrophages. Maestrelli (2001) demonstrated that heme oxygenase (HO)-1 is induced in the alveolar spaces of smokers, probably leading to increased carbon monoxide in exhaled air. Indeed, carbon monoxide in exhaled breath condensate of healthy smokers is higher than in healthy nonsmokers (Carpagnano et al. 2003)

8-Isoprostane concentrations in exhaled breath condensate of healthy smokers increased about 50% 15 min after inhaling cigarette smoke (Montuschi et al. 2000). In the same study, it was shown that current smokers had about 2.2-fold higher 8-isoprostane concentrations than had nonsmokers. In addition, ethane (Paredi et al. 2000a) and pentane (Euler et al. 1996) concentrations in exhaled breath condensate were increased. Higher levels of carcinogen–DNA adducts have been demonstrated in lung tissue of smokers, which have been associated with increased iron burden in the lower respiratory tract (Phillips et al. 1988; Thompson et al. 1991).

12.3.4 Inflammation and Pathological Changes

It is generally accepted that long-term cigarette smoking elicits an inflammatory reaction involving the entire tracheobronchial tree.

12.3.4.1 Changes in Sputum and BAL Fluid

More inflammatory cells have been demonstrated in sputum and BAL fluid of healthy smokers, suggesting more inflammation both in the central and peripheral airways (Balzano et al. 1999; Chalmers et al. 2001; Costabel et al. 1986, 1992; Dippolito et al. 2001; Keatings et al. 1996; Koyama et al. 1991; Kuschner et al. 1996; Linden et al. 1993; Morrison et al. 1998a; Schaberg et al. 1992b; Sun et al. 1998). In addition, macrophages in BAL fluid of healthy smokers seemed to be activated, because they express more CD11, CD18, and have increased chemotactic activity (Holt 1987; Schaberg et al. 1992b). When analyzing subsets of lymphocytes in BAL fluid from healthy smokers, the percentage of $CD4^+$ cells was lower, whereas the percentage of $CD8^+$ cells was higher, resulting in a lower CD4/CD8 ratio in healthy smokers than in nonsmokers (Costabel et al. 1986, 1992). Proinflammatory cytokines like TNF-α, IL-6, and chemokines like monocyte-chemoattractactic protein-1 and IL-8 were elevated in sputum supernatant or BAL fluid of healthy smokers (Capelli et al. 1999; Chalmers et al. 2001; Keatings et al. 1996; Linden et al. 1993; Morrison et al. 1998a; Sun et al. 1998). One study demonstrated that healthy smokers have higher IL-6 and LTB_4 concentrations in exhaled breath condensate

than have nonsmoking controls (Carpagnano et al. 2003). In addition, IL-6 correlated positively with the number of cigarettes smoked per day and negatively with FEV_1 %predicted.

12.3.4.2 Mucosal Changes in the Airways

In the submucosa of central airways, the density of neutrophils, eosinophils, CD3, CD4, and CD8 immunopositive cells was similar between healthy smokers and nonsmokers. Only the number of macrophages in the epithelium and the number of CD8 immunopositive cells near the pulmonary arteries in the peripheral airways were higher in healthy smokers (Fournier et al. 1989; Lams et al. 2000; Saetta et al. 1999, 2000; Wallace et al. 1992). Pathological changes because of smoking have been reported mostly in older subjects; however, already at a young age smoking leads to changes. Niewoehner et al. (1974) demonstrated an inflammatory cell infiltrate consisting of mononuclear cells and clusters of pigmented macrophages in the wall of the respiratory bronchioles from young smokers (aged 25 years) who suddenly died outside the hospital setting. These lesions were present in the absence of noteworthy tissue destruction and fibrosis, suggesting that such changes are largely reversible. Alternatively, it will take longstanding smoking before such changes occur. Cigarette smokers older than 40 years showed increased goblet cell metaplasia and smooth muscle hypertrophy in the small airways, and inflammation in the walls of bronchioles and respiratory bronchioles (Cosio et al. 1980). A pathological score on the degree of inflammatory cell infiltrate, squamous cell metaplasia of the airway epithelium and airway fibrosis in the small airways was associated with unevenness of ventilation in smokers, although they showed normal spirometry (Cosio et al. 1980). In the central airways, no differences in pathological changes between young healthy smokers and nonsmokers were reported (Sobonya and Kleinerman 1972). However, at an older age, the central airways showed higher numbers of goblet cells and inflammatory cells (Wright et al. 1988), smooth muscle hypertrophy, and a lower number of alveolar attachments (Cosio et al. 1980; Saetta et al. 1985). Thus, smoking-induced changes seem to start in the peripheral airways and to involve the larger airways later in life. Most likely, in the large airways, the mucus layer is protective to a certain extent. These findings are in line with the observed radiological changes that were present without overt lung function changes in healthy smokers, as described above.

A higher occurrence of basal cell hyperplasia, stratification, goblet cell hyperplasia, basement membrane thickening, and increased nucleus/cytoplasma (NC) ratio was found in bronchial biopsies of 31 healthy smokers (age 37 years, 25 pack years) than in 53 nonsmokers (Fligiel et al. 1997). In addition, a higher occurrence of DNA ploidy and higher immunohistochemical expression of epidermal growth factor (EGF) receptor have been demonstrated in the bronchial epithelium of smokers (Barsky et al. 1998), suggesting that smokers are at increased risk for the subsequent development of lung cancer. Recently, O'Donnell et al. (2004) demonstrated in bronchial biopsies from long-term current smokers a higher expression of EGF receptor (including ErbB3) and MUC5AC expression (Fig. 12.3) than in nonsmokers. The group of current smokers in this study consisted of healthy smokers, chronic bronchitis (Global Initiative for Chronic Obstructive Lung Disease [GOLD] stage 0) and COPD (GOLD stage I and II) patients, but demonstrated no differences between subgroups in this respect. Current smoking

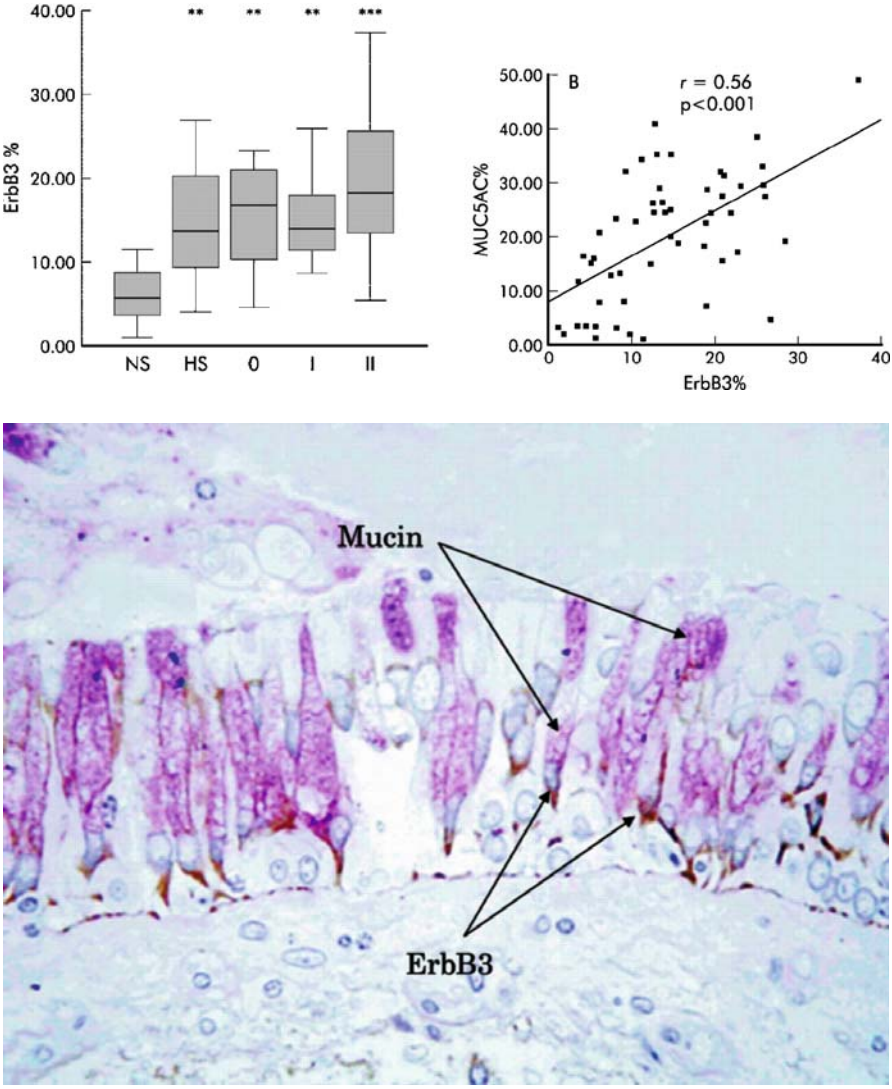

Fig. 12.3 *Left panel* demonstrates the ErbB3 receptor expression in bronchial epithelium of nonsmokers vs current smokers without or with chronic obstructive pulmonary disease (COPD). The *box* and *whisker plots* show median, range and interquartile ranges of the percentages of the epithelial area staining with the antibody. The percentage area expressing ErbB3 was significantly increased in all smoker groups, compared with nonsmokers. *NS* Nonsmokers (n=10), *HS* healthy smokers (n=11), *0* stage 0 COPD (n=17), *I* stage I COPD (n=9), *II* stage II COPD (n=14). *p<0.05 vs NS, **p<0.01 vs NS, ***p<0.001 vs NS. *Right panel* is a *scatter plot* showing the correlation between MUC5AC and ErbB3 expression in bronchial epithelium in all subjects (smokers and nonsmokers). *Lower panel* demonstrates colocalization of ErbB3 and mucin in goblet cells in a bronchial biopsy specimen from a smoker with COPD. The *photomicrograph* shows epithelial cells with intracellular mucin stained *pink* with periodic acid-Schiff (PAS) and membrane–bound ErbB3 stained *brown* with 3,3-diaminobenzidine (DAB). Magnification ×40. (Reproduced with permission from O'Donnell et al. 2004)

(number of cigarettes per day) correlated weakly but significantly with ErbB3 expression (r=0.37, p<0.05). Neutrophil numbers in sputum, epithelium, and submucosa were not associated with MUC5AC and EGF receptor expression. Interestingly, MUC5AC expression was significantly associated with ErbB3 expression, suggesting that cigarette smoking promotes epithelial goblet cell hyperplasia via activation of ErbB3 receptors. This study did not find a higher expression of ErbB2, although ErbB2 expression has been associated with malignant transformations (Selvaggi et al. 2002), whereas premalignant changes have been demonstrated in healthy smokers (Fligiel et al. 1997). Unfortunately, this study did not include ex-smokers, thus we are not informed about the possible reversibility of these changes.

12.3.5 Smoking Cessation

Healthy subjects who quit smoking show lower prevalence of cough, phlegm, and wheeze than do healthy smokers. The prevalence in ex-smokers was 5–21%, 5–30%, and 1–19% respectively, and in smokers 10–40%, 10–40%, and 7–32% (Bjornsson et al. 1994; Brown et al. 1991b; Enright et al. 1994; Lundback et al. 1991; Rijcken et al. 1987b; Sherman et al. 1992; Sherrill et al. 1993; Sparrow et al. 1987; Viegi et al. 1988). Most of these symptoms decreased within 1–2 months after smoking cessation (Barbee et al. 1991; Buczko et al. 1984; Buist et al. 1976; Comstock et al. 1970; Israel et al. 1988; Krzyzanowski et al. 1993; Lange et al. 1990b; Peterson et al. 1968; Tashkin et al. 1984; Wilhelmsen 1967). The prevalence of cough and wheeze in healthy smokers decreased to the level of nonsmokers, whereas the prevalence of phlegm remained slightly higher (Comstock et al. 1970; Tashkin et al. 1984). Not only did symptoms decrease on smoking cessation, but they were also less likely to develop if healthy smokers quit smoking than when they continued to smoke (Krzyzanowski et al. 1993; Lange et al. 1990b). The effect of smoking cessation on dyspnea in healthy smokers is controversial. Three studies showed no difference in the prevalence of dyspnea after smoking cessation (Israel et al. 1988; Krzyzanowski et al. 1993; Tashkin et al. 1984). In these studies, dyspnea was defined as the feeling of "shortness of breath" or "having to stop for a breath while walking up a slight hill" or "walking with other people of the same age on level ground." Also, the incidence of dyspnea was similar in those who quit and continued smoking: 17 and 16%, respectively (Krzyzanowski et al. 1993). In contrast, another study suggested that 5-years smoking cessation led to an increase in dyspnea when hurrying on the level or walking up a slight hill (41–52%) (Comstock et al. 1970).

12.4 Chronic Bronchitis

12.4.1 Epidemiology and Clinical Presentation

Chronic bronchitis has been clinically defined by the American Thoracic Society (1995) as "the production of sputum on most days for at least 3 months in at least 2 consecutive years when another cause of chronic cough has been excluded." Chronic bronchitis has been classified into simple bronchitis, chronic or recurrent mucopurulent bronchitis, and obstructive bronchitis. Recently, GOLD emphasized that chronic cough and sputum

production may precede the development of airflow limitation. Consequently, subjects with chronic cough and sputum production, but with still normal spirometry, were classified as being "at risk" of COPD (GOLD stage 0). Chronic smoking in almost every person leads to the universally called smokers cough (and phlegm); however, awareness of these symptoms varies enormously between persons. In smoking patients with chronic bronchitis the prevalence of wheeze, a respiratory symptom not included in the definition of chronic bronchitis, is significantly higher (39%) than in healthy nonsmokers (12%) (Ekberg-Jansson et al. 2001). In contrast, smokers with chronic bronchitis hardly experience dyspnea (Ekberg-Jansson et al. 2001). There are insufficient data as to whether smoking constitutes an independent risk factor for acute respiratory infections in chronic bronchitis (US Department of Health and Human Services 2004).

Epidemiological studies on the prevalence of chronic nonobstructive bronchitis may have problems in recognizing this condition because of the use of self-administrated questionnaires, without the use of spirometry to rule out COPD. Considering the methodological differences between studies, the prevalence of chronic bronchitis in general population studies varies between 0.7 and 14% (Cerveri et al. 2001; Cetinkaya et al. 2000; Collins 1997; Dutu and Paun 1998; Huchon et al. 2002; Liard et al. 1980; Lundback et al. 1993; Menezes et al. 1994; von Hertzen et al. 2000), which strongly depends on smoking habits. Recently, the European Community Respiratory Health Survey reported on the prevalence of chronic bronchitis and smoking habits in 18,966 young adults across 16 European countries (Cerveri et al. 2001). The median (range) prevalence of chronic bronchitis was 2.6% (0.7–9.7%) and of current smoking, 40% (20–57%). Current smoking was the major risk factor for chronic bronchitis, especially in males. In males the odds ratio was 3.51 (confidence interval [CI] = 2.21–5.32) in 1–14 pack-years smokers, which increased to 17.32 (9.97–30.11) in ≥45 pack-years smokers. Comparable odds ratios for current smoking were demonstrated in 1,053 subjects, aged ≥40 years, living in a southern area of Brazil (Menezes et al. 1994). Interestingly, smokers using filter cigarettes showed the lowest risk. In a study of 10,359 subjects (2,801 current smokers), aged 40–59 years and living in Scotland, daily cigarette consumption and number of pack years smoked was the most important risk factor for chronic bronchitis symptoms (Brown et al. 1991a). This study demonstrated that cigarette tar content was a small but significant risk factor for chronic bronchitis in females only.

12.4.2 Inflammation and Pathological Changes

Hyperplasia of goblet cells together with an increase in glands size and increased numbers of inflammatory cells have been reported in the central and peripheral airways (Mullen et al. 1985; Spurzem et al. 1991). In addition, the percentage of abnormal bronchial cilia is increased (Trevisani et al. 1992). Inhalation of cigarette smoke may paralyze the cilia and stimulate irritant receptors in the bronchial wall. This may give rise to an increased mucus production, unfortunately without being cleared effectively. A nonspecific inflammation has been demonstrated in chronic bronchitis with higher numbers of macrophages, neutrophils, eosinophils, and mast cells in BAL fluid as compared with healthy smokers and nonsmokers. In addition, the number of CD8 immunopositive lymphocytes was increased (Sun et al. 1998). In bronchial biopsies, a higher protein expression of IL-4 positive cells has been demonstrated in the submucosal airways from chronic bronchitis patients as compared with normal healthy controls (Mueller et al.

1996). In line with this, Zhu et al. (2001) demonstrated in a semiquantitative way strong IL-4 and IL-5 mRNA expression in the submucosal glands and in the subepithelium of smokers with chronic bronchitis. Recently, a higher IL-4 and IL-13 submucosal immunoreactivity was demonstrated in a quantitative way in smoking chronic bronchitis patients as compared with smoking healthy controls (Miotto et al. 2003). The most abundant sources of these Th-2 type cytokines were CD4- and CD8-immunopositive lymphocytes. Immunoreactivity of the Th-1 type cytokine interferon-γ did not differ between chronic bronchitis patients and healthy smokers in this study. In particular, IL-13 might play a supportive role in the mucus hypersecretion of chronic bronchitis patients, e.g., via the activation of the EGF receptor cascade as discussed above.

12.4.3 Smoking Cessation

Longitudinal data about the effects of smoking cessation in patients with chronic bronchitis are scarce. Friedman and Siegelaub (1980) showed in almost all persons with chronic bronchitis who quit smoking that chronic cough had disappeared 1.5 years later. Decline in FEV_1 in smokers with chronic bronchitis who quit smoking decreased, compared with those who continued smoking (Comstock et al. 1970). Mullen and coworkers (1987) compared the large and small airways of current and ex-smokers. In the central airways, goblet cell hyperplasia was lower in ex-smokers than in current smokers. In contrast, inflammation near the glands was higher in ex-smokers as compared with current smokers (Mullen et al. 1987). In the peripheral airways, goblet cell hyperplasia was similar in ex- and current smokers. Again, inflammation in ex-smokers was higher than in current smokers. In contrast, macrophages in the airway lumen were lower in ex-smokers than in current smokers with chronic bronchitis (Mullen et al. 1987). Taken together, the above studies suggest that smoking cessation decreases respiratory symptoms and the underlying mucus producing elements in the central airways. However, the apparent increases in airway inflammation in the walls of the central and peripheral airways are hard to explain.

12.4.4 Antioxidative Therapy

Only one study did investigate the effect of antioxidant therapy in patients with non-obstructive chronic bronchitis. This open study in 1,392 patients in general practice did show that *N*-acetylcysteine (600 mg per day) after 2 months treatment effectively changed the viscosity and character of sputum, with resultant ease of expectoration and cough severity. There was a notable improvement in associated abnormal physical signs such as the presence of rhonchi, crepitations, and symptoms including dyspnea at rest (Tattersall et al. 1983). Three meta-analyses have been published on the clinical efficacy of *N*-acetylcysteine in chronic bronchitis patients (Grandjean et al. 2000; Poole and Black 2001; Stey et al. 2000). Because they included also obstructive chronic bronchitis patients, they are presented in the following section.

12.5 COPD

12.5.1 Epidemiology and Clinical Presentation

COPD is defined by the GOLD standard as "a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases" (www.goldcopd.com). The major environmental factors are tobacco smoke, occupational dusts and chemicals, and indoor/outdoor pollution. The risk to develop chronic airflow limitation because of tobacco smoke increases with increasing pack years, and after >10 pack years, 15–20% of all smokers have developed full-blown COPD. This means that the inflammatory response to tobacco smoke is probably different between susceptible and nonsusceptible individuals; however, at this time we have no clue about the underlying genetic factors that modify an individual's risk. A large national survey conducted in the United States between 1988 and 1994 demonstrated that airflow limitation (FEV_1/forced vital capacity [FVC] <70%) in white males was present in 14.2% of the current smokers, 6.9% of ex-smokers, and 3.3% of never smokers (National Center for Health Statistics 1995). Among white females, the prevalence was 13.6, 6.8, and 3.1% respectively. Prevalence of physician-diagnosed COPD in the United Kingdom between 1990 and 1997 demonstrated that men reached a plateau of 1.6% in the mid 1990s, while the women rose continuously from 0.8 to 1.36% (Soriano et al. 2000). A formal analysis on past smoking behavior was not performed in this study, but the trends (comparable to lung cancer) may fit with changed smoking habits in the past. Worldwide the prevalence of COPD in 1990 was estimated to be 0.9% in all men, and 0.7% in all women (Murray and Lopez 1996). Across countries, the prevalence varies between 0.3 and 3%, and strongly relates to tobacco consumption.

Progressive airflow limitation is one of the hallmarks of COPD. Smoking COPD patients have a decline in FEV_1 of more than 60 ml/year and sometimes even more than 100 ml/year (Campbell et al. 1985; Postma and Sluiter 1989). This decline is strongly related to current and the number of pack years smoking (Postma et al. 1986; Scanlon et al. 2000). Decline in FEV_1 is not related to use of filter-tipped cigarettes (containing on average 23 mg tar) or unfiltered cigarettes (containing on average 35 mg tar) (Lange et al. 1992). Increased airway hyperresponsiveness frequently occurs both in smoking and nonsmoking patients with COPD (Tashkin et al. 1992; Yan et al. 1985). A large prospective cohort study in mild-to-moderately severe COPD patients demonstrated that 67% had increased airway hyperresponsiveness to methacholine, being partially dependent on FEV_1 (Tashkin et al. 1992). The severity of airway hyperresponsiveness was not related to current or past smoking in males, but there was a positive association between increased airway hyperresponsiveness and the number of pack years in females. In smoking patients with severe COPD, the small airways showed increased numbers of leukocytes, which correlated with reduced expiratory flow, lung hyperinflation, carbon monoxide diffusion impairment, and radiological emphysema (Turato et al. 2002). Verbanck et al. (2004), using the multiple breath N_2 washout test, demonstrated that COPD patients with a smoking history of more than 30 pack years have a disproportional larger unevenness of ventilation originating from the small airways (S_{cond}), irrespective of the presence of emphysema (Fig. 12.1).

Chronic cough and phlegm are frequently reported symptoms in COPD. Therefore, in the past the American Thoracic Society based its definition of COPD partly on the

presence of at least 3 months' cough and phlegm during at least 2 consecutive years. In the new mondial GOLD standard (www.goldcopd.com), the presence of these symptoms is not obligatory any more. One population-based study reported cough and phlegm in 84% of the patients with mild disease, and 68% of the patients with severe disease (von Hertzen et al. 2000). Dyspnea is more prevalent in patients with severe COPD (80%) than in patients with mild (33%) or moderate (53%) COPD (von Hertzen et al. 2000). COPD is associated with a greater risk of acute respiratory infections (Monto and Ross 1977, 1978). The effects of current smoking on this risk are strongest in men with chronic bronchitis symptoms and show a positive exposure–response relationship. Many studies have demonstrated a higher prevalence of potentially pathogenic bacteria isolated from sputa of patients with an exacerbation. The impact of current smoking on this prevalence, however, is not formally investigated. One study demonstrated similar prevalence of positive cultures for gram-negative bacilli in former and current smokers (23 vs 32%), whereas another study demonstrated an increased risk of current smoking for quantitative sputum cultures yielding *Haemophilus influenza* (odds ratio = 8.16, CI = 1.9–43). The prevalence of positive serology for respiratory viral infections was higher in COPD patients than in healthy individuals (74 vs 48%) (Omenaas et al. 1996). Finally, a population-based study demonstrated that cigarette smoking was a strong independent risk factor for invasive pneumococcal infection in COPD patients (Nuorti et al. 2000).

12.5.2 Inflammation and Pathological Changes

Inflammatory and pathological changes in smokers with established COPD have received much attention in the last two decades as summarized in a number of excellent reviews (Cosio and Guerassimov 1999; Hogg 2001; Jeffery 1991, 1998, 2001; Saetta et al. 2001). Briefly, the peripheral airways show inflammatory wall infiltration, fibrosis, smooth muscle hypertrophy, goblet cell metaplasia, and luminal occlusion because of mucus plugging. Central airway walls show increased infiltration with macrophages and T lymphocytes (especially $CD8^+$ cells), whereas the airway lumen contains a neutrophilic inflammation. In sputum, the anti-inflammatory cytokine IL-10 is decreased, whereas the proinflammatory cytokines IL-8 and TNF-α are increased. The adhesion molecules E-selectin and intercellular adhesion molecule (ICAM)-1 were upregulated on the bronchial epithelium and on submucosal vessels, suggesting a role in the recruitment of neutrophils. The lung parenchyma also shows inflammation, especially $CD8^+$ T lymphocytes. Even the pulmonary arteries are involved in the inflammatory process in established COPD, showing endothelial dysfunction, intimal thickening, medial thickening, and adventitial inflammation (especially with $CD8^+$ cells). Some patient may show signs of emphysema, which is defined anatomically as a permanent destructive enlargement of airspaces distal to the terminal bronchioles, without fibrosis. COPD patients can develop centriacinar emphysema (mainly in upper lobes) or panacinar emphysema (mainly in lower lobes). Centriacinar emphysema is the most common form in smokers. The destruction is associated with the presence of an inflammatory process in the alveolar walls, consisting predominantly of $CD8^+$ T cells and correlating with reduced airflow limitation (Saetta et al. 1999). Apparently, cytotoxic $CD8^+$ T cells infiltrate the complete lung and seem to play a causal role in the development of COPD (Saetta et al. 2001).

An imbalance between proteases and antiproteases because of cigarette smoking has been postulated as one important factor in its etiology of emphysema. Among the vari-

ous proteases and antiproteases that have been proposed to affect the extracellular matrix, there is now increasing evidence that matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of MMP [TIMPs]) play an important role. Patients with emphysema have increased MMP-9 expression and production by alveolar macrophages (Finlay et al. 1997a) and have elevated levels in BAL fluid (Finlay et al. 1997b). Furthermore, increased MMP-2, MMP-9, and TIMP-1 levels have been demonstrated in sputum of COPD patients (Beeh et al. 2003; Cataldo et al. 2000; Vignola et al. 1998). One study showed that FEV_1 %predicted correlated negatively with MMP-9 expression in lung tissue protein extracts from subjects undergoing lung surgery because of (suspected) malignancy (Kang et al. 2003). Moreover, FEV_1 %predicted correlated negatively with the MMP-9/TIMP-1 molar ratio. In contrast, a sputum study demonstrated that FEV_1 %predicted correlated positively with this ratio (Vignola et al. 1998). These cross-sectional studies cannot elucidate whether there is a causal relationship between smoking and development of COPD. Supportive evidence stems from the observation that alveolar macrophages from healthy cigarette smokers produce more MMP-9 and TIMP-1 than healthy nonsmokers (Lim et al. 2000). In addition, MMP-9 expression and the MMP-9/TIMP-1 ratio in lung tissue from subjects with and without airway obstruction correlate positively with the amount of past and current cigarette smoking (Kang et al. 2003). At this time, it is not clear how cigarette smoking exactly induces the functional imbalance between proteases and antiproteases. Interestingly, many oxidants present in smoke itself, or those generated by inflammatory cells may inactivate proteinase inhibitors and stimulate the release and activation of proteases (Seagrave 2000).

Comparable to nonobstructive chronic bronchitis, increased numbers of neutrophils together with goblet cell hyperplasia have been demonstrated in the bronchial epithelium of smokers with COPD (Jeffery 1991; Saetta et al. 2000). In addition, neutrophils are increased in the bronchial glands of these subjects and may support mucus hypersecretion via the release of neutrophil elastase (Saetta et al. 1997). However, increased $CD8^+$ cells, mast cells, and macrophages have also been demonstrated in the airway epithelium. Similar to smoking healthy controls and smoking chronic bronchitis patients, higher MUC5AC and EGF receptor (ErbB1 and ErbB3) expression have been demonstrated in the epithelium of smoking COPD patients (GOLD stages II–III) as compared with nonsmoking healthy controls (Fig. 12.3) (O'Donnell et al. 2004). Neutrophil numbers in sputum from COPD stage III patients were also higher in this study, and they correlated negatively with FEV_1 levels, but not with the expression of mucins and ErbB receptors. Obviously, more studies are needed to understand better the exact role of smoking-induced airway inflammation and mucus hypersecretion in COPD. Apparently, the EGF receptor cascade does not play a role in the development of airway obstruction.

12.5.3 Oxidative Stress

Several reviews have summarized the studies on the presence and consequences of oxidative stress in the lungs of smokers with COPD (Dekhuijzen 2004; Macnee 2000, 2001a; Macnee and Rahman 1996, 1999; Repine et al. 1997). Evidence for local oxidative stress has been found in sputum, BAL fluid, exhaled air, and exhaled breath condensates of patients with COPD.

Hydrogen peroxide production seems to be elevated in COPD patients. Measured in exhaled breath hydrogen peroxide is higher in patients with stable COPD as compared

with healthy controls (Dekhuijzen et al. 1996; Nowak et al. 1999), and even more so during exacerbations (Dekhuijzen et al. 1996). Current smokers have shown similarly increased hydrogen peroxide levels as ex-smokers or never smokers with COPD (Nowak et al. 1998, 1999). In addition, no correlation was found between hydrogen peroxide and daily cigarette consumption or cumulative cigarette consumption in current smokers or ex-smokers with COPD (Nowak et al. 1998). One study showed that smoking patients with COPD (GOLD stage >0) had significantly higher hydrogen peroxide levels in exhaled breath condensate than in smoking patients with chronic bronchitis (GOLD stage 0) (Kostikas et al. 2003). Moreover, hydrogen peroxide levels correlated significantly with FEV_1 ($r = -0.83$, $p<0.0001$), Medical Research Council (MRC) dyspnea score ($r = 0.68$, $p<0.0001$), and percentage of neutrophils in induced sputum ($r = 0.83$, $p<0.0001$) in the COPD group (Kostikas et al. 2003). Apparently, the level of hydrogen peroxide is determined by endogenous hydrogen peroxide production and not by cigarette smoking.

Exhaled NO has been reported to be elevated (Corradi et al. 1999; Maziak et al. 1998) and to be normal (Ichinose et al. 2000; Rutgers et al. 1998, 1999) in stable COPD patients. Despite normal exhaled NO levels, Ichinose et al. (2000) demonstrated higher numbers of iNOS-positive and nitrotyrosine-positive cells in induced sputum in nonsmoking COPD patients than in healthy controls. In addition, there was a negative correlation between the number of nitrotyrosine-immunopositive cells and the degree of airway obstruction ($r = -68$, $p<0.05$). Kanazawa et al. demonstrated that nitrite and nitrate levels in induced sputum were significantly higher in patients with stable COPD than in normal healthy controls. Peroxynitrite inhibitory activity in induced sputum was significantly lower in patients with COPD than in normal controls. In addition, there was a negative correlation between peroxynitrite inhibitory activity and FEV_1 %predicted (r=0.539, p=0.004) and the percentage of neutrophils ($r = -0.754$, $p<0.001$). In contrast, Corradi et al. (2003a) demonstrated in exhaled breath condensate of COPD patients that the amount of nitrate is not higher than in condensate of nonsmoking controls. In the study of Rutgers et al. (1999), exhaled NO was also not increased, but it correlated positively with percentage sputum eosinophils (r=0.65, p=0.009), suggesting that patients with less stable COPD have more airway inflammation as reflected by exhaled NO. Carbon monoxide also has shown to be elevated in exhaled breath of COPD patients (Choi and Alam 1996).

Lipid peroxidation because of oxidative stress can be demonstrated in various ways in COPD. Increased immunostaining expression for adducts of the lipid peroxidation product 4-hydroxy-2-nonenal (4-HNE) have been demonstrated in the bronchi and alveoli of smokers with and without COPD (Rahman et al. 2002). Levels of 4-HNE adducts in alveolar and airway epithelium correlated negatively with FEV_1 in this study, suggesting a role for 4-HNE in the development of irreversible airway obstruction in COPD. Aldehydes (malondialdehyde, hexanal, heptanal) were higher in exhaled breath condensate of COPD patients than of nonsmoking controls; however, only malondialdehyde was higher than in condensate of smoking controls (Corradi et al. 2003b). Thiobarbituric acid-reactive substances (TBARs) in exhaled breath condensate have shown to be higher in smokers and ex-smokers with stable COPD than in healthy subjects who had never smoked (Nowak et al. 1999), without significant correlation with actual or cumulative cigarette consumption. TBARs plasma levels increased significantly in patients with COPD exacerbations as compared with healthy nonsmokers (Rahman et al. 1997). Exhaled ethane in stable COPD patients was higher than in healthy controls, and correlated with FEV_1 ($r = -0.67$, $p<0.05$) (Paredi et al. 2000b). 8-Isoprostanes because of peroxidation of arachidonic acid can be measured in exhaled breath and urine. Patients

with stable COPD demonstrated higher exhaled breath condensate levels than healthy controls (Kostikas et al. 2003; Montuschi et al. 2000); whereas ex-smokers had similar levels as current smokers (Montuschi et al. 2000). One study demonstrated increased 8-isoprostane levels in exhaled breath condensate during exacerbation, a decrease 2 weeks after treatment, and normalization after 2 months (Biernacki et al. 2003). In urine, F_2-isoprostanes were elevated in patients with stable COPD (Pratico et al. 1998); levels further increased during exacerbations (Pratico et al. 1998). This elevation in urine was independent of age, sex, COPD duration, or smoking history.

We conclude in line with a number of reviews that there is overwhelming evidence that the lungs of COPD patients suffer from oxidative stress. Interestingly, the level of oxidative stress in ex-smokers seems to be similar as in current smokers, suggesting that an endogenous source is responsible for persistence of oxidative tress. This is in line with the observation that there is ongoing inflammation after smoking cessation.

12.5.4 Smoking Cessation

The Lung Healthy Study is a large prospective cohort study on smoking cessation in 5,887 mild-to-moderate COPD patients. One year after successful quitting the proportion of subjects that reported at the start of the study respiratory symptoms (cough, phlegm, wheeze, or dyspnea) reduced to approximately 20%, and this was maintained in the next 4 years of follow-up. Moreover, smoking cessation was associated with a lower risk to develop new respiratory symptoms: about 5% in sustained quitters versus 25% in continuous smokers (Kanner et al. 1999; Pride 2001). A cross-sectional population study suggested that dyspnea improves in mild-to-moderate COPD patients who were ex-smokers, but persists in severe COPD patients who quitted smoking (von Hertzen et al. 2000). A large population study with a mean follow-up of 14 years demonstrated that the risk of hospital admission because of COPD was approximately 40% lower in those who quitted smoking (Godtfredsen et al. 2002). In line, the Lung Health Study demonstrated that smoking cessation reduced the frequency of self-reported lower respiratory illnesses resulting in physician visits (Kanner et al. 2001).

Cross-sectional studies show that increased airway hyperresponsiveness to histamine and methacholine is not different between smokers and ex-smokers with COPD (Oosterhoff et al. 1993; Postma et al. 1988). In contrast, increased airway hyperresponsiveness to adenosine 5'-monophosphate is present in many smoking individuals with COPD, whereas it is less severe or absent in ex-smoking COPD patients (Pesci et al. 1994). A longitudinal study in 16 patients with mild-to-moderate COPD showed that both airway hyperresponsiveness to methacholine and adenosine 5'-monophosphate improved significantly after 1 year smoking cessation, though with 1.6 doubling doses with methacholine and 2.1 with AMP (Willemse et al. 2004b). The Lung Health Study showed that both persistent smokers and sustained quitters over a 5-year period had more severe airway hyperresponsiveness to methacholine than at baseline (Wise et al. 2003). However, airway hyperresponsiveness in quitters was over 3-fold less severe than in sustained smokers and closely associated with decline in FEV_1. Sustained quitters had a less rapid decline in FEV_1 over 5 years than persistent smokers: –34 ml/year and –63 ml/year, respectively (Anthonisen et al. 1994, 1997; Murray et al. 1998; Scanlon et al. 2000). During the first year after smoking cessation, FEV_1 improved by 57 ml in quitters, whereas it fell with –32 ml in persistent smokers (Anthonisen et al. 1994). After 11 years'

follow-up, the decline in FEV_1 in quitters was –30 ml/year for men and –22 ml/year for women, values for persistent smokers being –66 and –54 ml/year, respectively (Fig. 12.4) (Anthonisen et al. 2002). Heavy smokers showed larger declines in FEV_1 than light smokers, and these heavy smokers had greater improvements after smoking cessation than light smokers (Scanlon et al. 2000). If symptoms, hyperresponsiveness, and decline in FEV_1 all improve after smoking cessation, one would expect that local inflammation improves also. Unfortunately, the few available sputum studies do not confirm this (Willemse et al. 2004a). Similar levels of IL-6, IL-8, myeloperoxidase, and eosinophil cation protein were demonstrated in sputum from ex- and current smokers (Bhowmik et al. 1998; Yamamoto et al. 1997). A longitudinal study even demonstrated an increase in the percentage of neutrophils and IL-8 levels after smoking cessation, suggesting deterioration of airway inflammation (Willemse et al. 2004b). The few available histological studies provide contradictory results. In the peripheral airway walls, squamous metaplasia, inflammatory cell density, fibrosis, and muscle hypertrophy are similar in ex- and current smokers with mild COPD (Wright et al. 1983). In contrast, goblet cell hypertrophy tended to be lower in ex-smokers (Wright et al. 1983), which may explain improvement in cough and sputum production with smoking cessation. In the central airways, Pesci et al. (1994) showed that ex-smokers tended to have lower numbers of mast cells in the epithelium and lamina propria than current smokers with mild-to-moderately severe COPD. As adenosine 5'-monophosphate triggers mast cells to release the bronchoconstrictive agent histamine, this finding might explain why hyperresponsiveness to AMP decreases after smoking cessation (Willemse et al. 2004b). De Boer et al. (2000) found no differences in expression of IL-8, monocyte chemotactic protein (MCP)-1 and its receptor, CCR2, in the central airways from current and ex-smokers with moderate-to-severe COPD (de Boer et al. 2000). In line, Turato et al. (1995) found no differences in expression of a number of inflammatory cells, cytokines, and adhesion molecules. However, Lapperre et al. (2003) found a higher number of submucosal $CD3^+$ and $CD4^+$ T cells in central airway biopsies from ex-smokers than from current smokers with COPD. The latter study is in line with earlier described sputum findings (Willemse et al. 2004b), suggesting that central airway inflammation increases immediately after smoking cessation. Together, these data suggest that some components of airway inflammation improve, whereas most components persist or even deteriorate after smoking cessation. However, this might reflect a beneficial healing process more than a detrimental effect.

12.5.3 Antioxidative Treatment

N-Acetylcysteine is the only commercially available antioxidative agent that can be prescribed in patients with COPD. The presently available experimental and clinical data on the antioxidative effects of *N*-acetylcysteine in COPD have recently been reviewed (Dekhuijzen 2004).

In a randomized 1-year study after 9 and 12 months treatment with *N*-acetylcysteine (600 mg/day), exhaled hydrogen peroxide was 2.3-fold less than with placebo treatment (Kasielski and Nowak 2001). In this study, no significant effects of *N*-acetylcysteine administration were noted on exhaled levels of TBARs or on serum levels of TBARs and lipid peroxides. After administration of a higher dose of 1,200 mg *N*-acetylcysteine per day, the concentrations of hydrogen peroxide in exhaled breath condensate reduced within a 1-month treatment (De Benedetto et al. 2000).

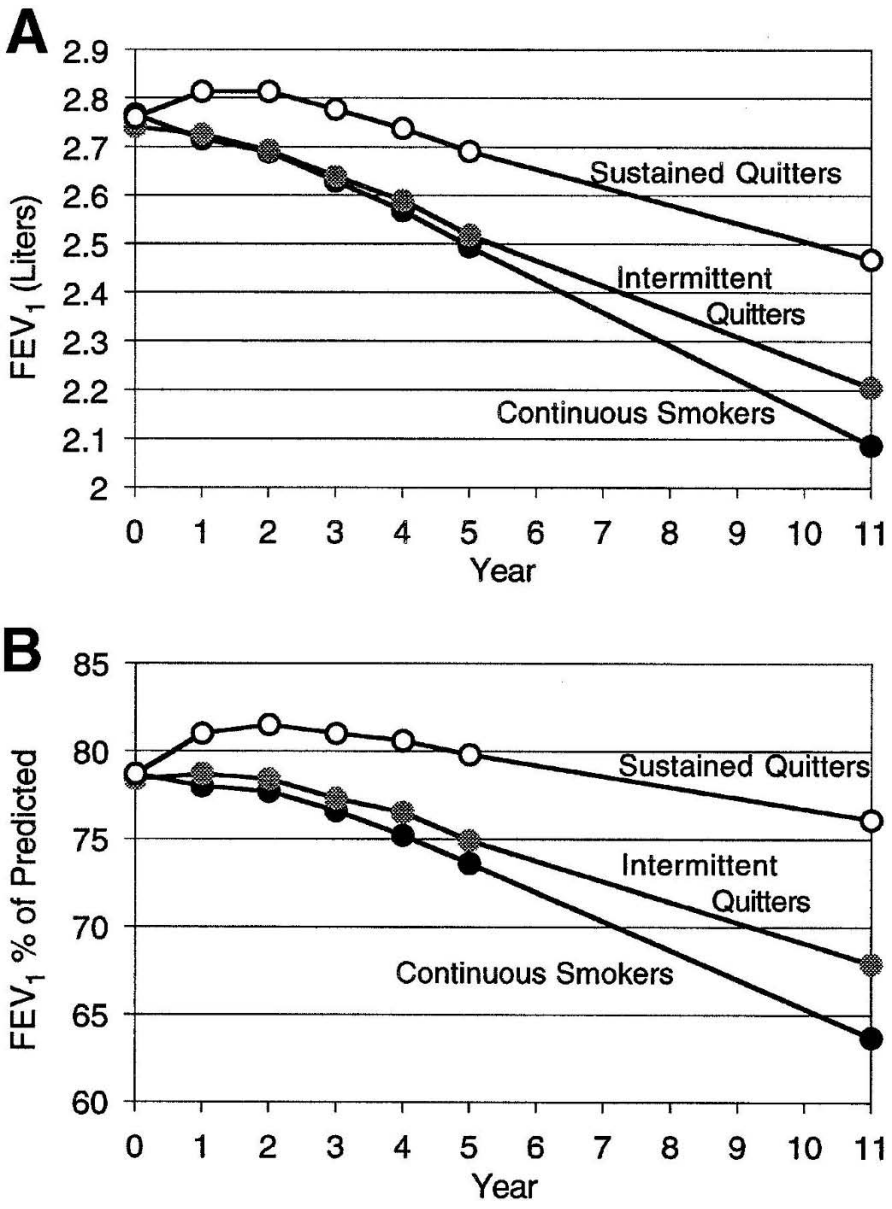

Fig. 12.4 Loss of lung function over the years of the Lung Health Study among 722 sustained quitters (*white circles*), 2,369 continuing smokers (*black circles*), and 1,054 intermittent smokers (*gray circles*). Average values of post bronchodilator forced expiratory volume in 1 second (FEV_1) are shown expressed in absolute terms in **a**, and as a percentage of predicted normal value in **b**. (Reproduced with permission from Anthonisen et al. 2002)

Only a few randomized controlled studies are presently available on the effects of *N*-acetylcysteine on clinical outcome variables in COPD patients. In a small (n=2×25) placebo-controlled study *N*-acetylcysteine did not improve recovery of FEV_1, oxygen saturation, or length of hospital stay in COPD patients admitted to the hospital because of an exacerbation (Black et al. 2004). However, the risk of rehospitalization within 1 year after hospitalization was reduced by approximately 30% if COPD patients (>55 years) used prophylactic *N*-acetylcysteine (Gerrits et al. 2003). In addition, in this post hoc analysis the relative risk significantly decreased with increasing average daily dose of *N*-acetylcysteine. In a systematic review, 286 of 466 (61.4%) chronic bronchitis patients who received *N*-acetylcysteine reported improvements of respiratory symptoms versus 160 of 462 (34.6%) patients who received placebo. Of 723 actively treated patients, 351 (48.5%) did not have an exacerbation, versus 229 of 733 (31.2%) placebo-treated patients (Stey et al. 2000). The percentage of patients (per randomized controlled trial) who were smokers or ex-smokers ranged from 72 to 100%. These findings are in line with two previous meta-analyses (Grandjean et al. 2000; Poole and Black 2001), but interpretation is difficult, as many studies included both obstructive and nonobstructive patients. We have to await the definite results of a large prospective placebo-controlled study that included only COPD patients (BRONCUS trial: Bronchitis Randomized on NAC Cost-Utility Study) (Decramer et al. 2001). This 3-year study included 523 COPD patients with moderate-to-severe disease, 46% being current smokers and 54% ex-smokers. This study will also provide data about the possible effect of *N*-acetylcysteine administration on decline of lung function in COPD.

12.6 Asthma

12.6.1 Smoking and Induction of Asthma

Epidemiological studies suggest that both active (mainstream) and passive (sidestream) exposure to tobacco smoke may contribute to induction of asthma at childhood as well as adulthood. In utero exposure to maternal smoking was associated with a higher incidence and an earlier onset of asthma (Agabiti et al. 1999; Weitzman et al. 1990). Smoking 20 cigarettes or more during pregnancy was associated with an odds ratio of 2.1 for childhood asthma at age 0–5 years, as compared with nonsmoking (Weitzman et al. 1990). Postnatal maternal smoking was positively associated with the prevalence of childhood asthma at age 7–13 years (Soyseth et al. 1995). A large review article including 37 studies concluded that there is compelling evidence that parental smoking induces asthma at childhood (odds ratio = 1.75–2.25) (Cook and Strachan 1999). Hu et al. (1997) demonstrated in young adults a dose–response relationship with the number of cigarettes smoked and the number of parents who smoked. At older ages, passive smoking seemed to increase the risk of asthma only slightly (Coultas 1998; Greer et al. 1993; Leuenberger et al. 1994; Wang et al. 1999).

There are contradictory results about the role of active (mainstream) smoking. Rasmussen et al. (2000) demonstrated in a longitudinal study of 271 asymptomatic adolescents an independent contribution of smoking to the development of respiratory symptoms (odds ratio = 2.1). In cross-sectional studies the risk of asthma was either increased (Arif et al. 2003; Backer et al. 2002; Flodin et al. 1995; Kim et al. 2002; Kiviloog et al. 1974; Piipari et al. 2004) or similar (Hjern et al. 2001; Lebowitz 1977; Pilotto et al. 1999;

Senthilselvan et al. 1993; Walraven et al. 2001) compared with never smokers. Contradictory results were also reported in longitudinal studies, demonstrating an increased (Plaschke et al. 2000; Toren and Hermansson 1999); equal (Vesterinen et al. 1988); or decreased risk (Troisi et al. 1995) in current smokers. Piipari et al. (2004) investigated in a prospective case control study the associations between new adult asthma (n=521) and smoking habits. The risk to develop asthma was slightly but significantly higher among current smokers (odds ratio = 1.33) and among ex-smokers (odds ratio = 1.49), compared with never smokers. Among current smokers, the risk increased up to 14 cigarettes per day, but was normal in heavy smokers. A similar trend was detected for cumulative lifetime smoking. Females in this study were at higher risk than males, both in current (odds ratio = 2.43) and ex-smokers (odds ratio = 2.38). In a large review, the Surgeon General's report concluded recently that available studies provide inconsistent findings on the causal relationship between active smoking and the induction of asthma (US Department of Health and Human Services 2004). Inconsistent findings may be caused by not fully controlling for known risk factors for asthma in childhood and adolescence studies, and by different definitions of asthma, different study designs, recall bias, and healthy smoker bias in adult-onset studies (US Department of Health and Human Services 2004).

12.6.2 Smoking and Clinical Severity of Asthma

Cigarette smoking has without doubt far-fetching detrimental effects on the outcome of asthma. In a cross-sectional study of 225 asthmatics, aged 20–54 years, actual smoking was strongly associated with bothersome asthma symptoms that affect daily life activities (Althuis et al. 1999). In a large European epidemiological study (the EGEA Study), active current smoking was associated with asthma severity (Siroux et al. 2000). Cigarette smoking in asthma was associated with increased wheeze (Althuis et al. 1999; Siroux et al. 2000); increased sputum production (Lange et al. 1990b); acute bronchoconstriction (Higenbottam et al. 1980c; Jensen et al. 2000); greater need for rescue medication (Gallefoss and Bakke 2003); unresponsiveness to steroids (Chalmers et al. 2002; Chaudhuri et al. 2003; Kerstjens et al. 1993; Pedersen et al. 1996; Thomson and Spears 2005); accelerated decline in lung function (Almind et al. 1992; Apostol et al. 2002; James et al. 2005; Lange et al. 1998); signs of hyperinflation and emphysema (Lynch et al. 1993; Mitsunobu et al. 2004); greater risk for invasive pneumococcal pneumonia (Nuorti et al. 2000); higher number of hospital admissions (Prescott et al. 1997; Sippel et al. 1999); and higher number of life-threatening asthma attacks (LeSon and Gershwin 1996; Mitchell et al. 2002; Ryan et al. 1991). The Surgeon General concluded that there is sufficient evidence to infer a causal relationship between active smoking and poor asthma control (US Department of Health and Human Services 2004).

There are several reports suggesting that cigarette smoking decreases the responsiveness to inhaled glucocorticosteroids in asthma (Thomson and Spears 2005). Kerstjens et al. (1993) showed that smokers on treatment with inhaled beclomethasone (800 μg/day) had a 383-ml smaller improvement in FEV_1 after 3 months' treatment than nonsmokers did. Pedersen et al. (1996) showed that smoking asthmatics did not improve on treatment with high doses of budesonide (1,600 μg/day) with respect to FEV_1, PC_{20} histamine, serum eosinophil cation protein (ECP), and eosinophil protein X (EPX), bronchodilator use, whereas nonsmoking asthmatics did improve significantly. In line with this,

Chalmers et al. (2002) showed that smoking asthmatics did not improve on treatment with fluticasone propionate (1,000 μg/day) with respect to morning PEF, FEV_1, PC_{20} methacholine, and sputum eosinophilia, in contrast to nonsmoking asthmatics. The detrimental effects of smoking on hyperresponsiveness can not solely be explained by decreased deposition of inhaled corticosteroids in these studies, as smoking has also shown to affect hyperresponsiveness after oral corticosteroid administration (Fig. 12.5) (Chaudhuri et al. 2003). The effect of smoking on corticosteroid responsiveness seems

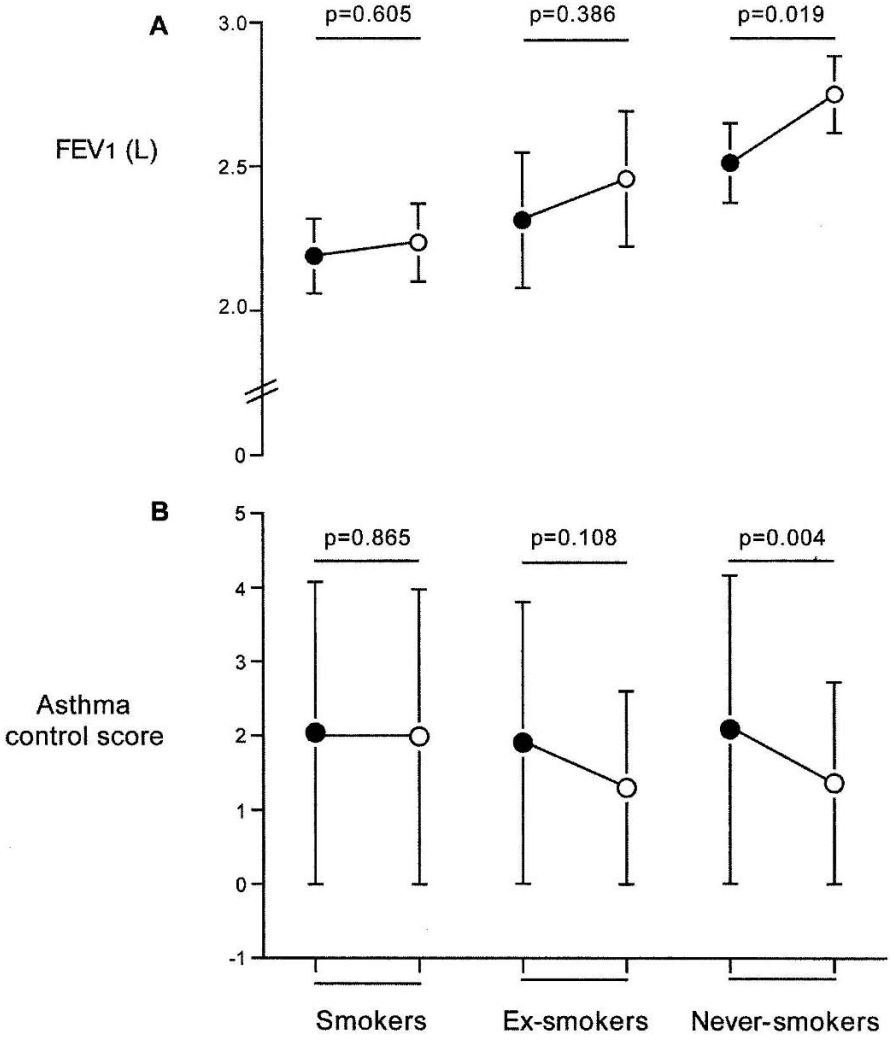

Fig. 12.5 Mean difference (95% confidence interval) after placebo (*solid circle*) and after prednisolone, 40 mg/day for 2 weeks, in smokers with asthma, ex-smokers with asthma, and never smokers with asthma for **a** change in forced volume in one second (FEV_1), and **b** asthma control score. A reduction in the asthma control score implies an improvement. (Reproduced with permission from Chaudhuri et al. 2003)

partially reversible, as ex-smokers in contrast to current smokers show improved morning PEF values after oral corticosteroid treatment (Chaudhuri et al. 2003). The underlying mechanisms of smoking-induced steroid unresponsiveness in asthma are not clear. Possible mechanisms are changed corticosteroid pharmacokinetics, interaction with corticosteroid and β-2 adrenergic receptors, increased airway neutrophil and $CD8^+$ lymphocyte numbers, reduced airway eosinophil numbers, changed cytokine and mediator levels, overexpression of glucocorticoid receptor-β, reduced expression of glucocorticoid receptor-α, overexpression of proinflammatory transcription factors, and reduced histone deacetylase activity (Thomson et al. 2004).

12.6.3 Smoking and Inflammation

Limited data in humans are available with respect to effects of cigarette smoking on airways inflammation and systemic inflammation in asthmatics. Chalmers et al. (2001) demonstrated that asthmatic smokers had higher sputum cell counts, neutrophil numbers, and IL-8 concentrations than did asthmatic nonsmokers. Furthermore, a lower lung function (FEV_1 %predicted) was present in smoking asthmatics, which was related to higher levels of sputum IL-8 and percent neutrophils, whereas higher levels of sputum IL-8 were related to higher numbers of pack years smoking and higher percentages sputum neutrophils. In peripheral blood, the leucocytes from asthmatic smokers produced higher LTB_4 levels than from nonasthmatic smokers (Mitsunobu et al. 2004). In contrast, circulating MPO concentrations in peripheral blood of asthmatic smokers were similar to those of asthmatic nonsmokers (Pedersen et al. 1996).

Sputum eosinophils and ECP levels in the study of Chalmers et al. were lower in asthmatics smokers than in asthmatic nonsmokers. This is in line with the results of studies on circulating blood cells (Halonen et al. 1982; Jensen et al. 1998; Taylor et al. 1985a). Together, these findings suggest that cigarette smoking may alter the balance of Th-1/Th-2 cytokine production; however, we can only speculate about the underlying mechanisms. A reduction in eosinophil numbers may be because of exogenous Nitric Oxide in cigarette smoke, which may increase apoptosis of activated eosinophils (Zhang et al. 2003). On the other hand, a reduced endogenous NO production was demonstrated in mild steroid-naïve asthmatic smokers as compared with asthmatic nonsmokers (Verleden et al. 1999). Interestingly, Melgert et al. (2004) demonstrated in ovalbumine-sensitized mice that short-term exposure to cigarette smoke significantly reduced airway obstruction 24 h after ovalbumine challenge, accompanied by reduced eosinophil numbers in lung lavage fluid and lung tissue. This might have been because of carbon monoxide in cigarette smoke.

Taken together, cigarette smoking seems to both increase and decrease aspects of airway inflammation. Obviously, histological studies are needed to assess whether the airways of smoking asthmatics predominantly show airway pathology characteristic for asthma or COPD, or a combination of these two diseases.

12.6.4 Smoking Cessation

Little data are available about the impact of smoking cessation in asthma. One small cross-sectional study reported less cough and sputum production in ex-smokers, com-

pared with current smokers (Higenbottam et al. 1980c). However, another study not designed for this purpose reported similar asthma-symptom scores in current smokers and ex-smokers (Chaudhuri et al. 2003). In a small longitudinal study, 14 asthmatic patients managed to stop for 24 h, and 7 for 1 week. Twenty-four hours after smoking cessation, airway obstruction improved significantly; after 7 days, there was a further improvement (Fennerty et al. 1987). Seven days after smoking cessation, bronchial responsiveness to histamine improved, and four of the seven subjects recorded an improvement in symptoms. In another longitudinal study, blood samples were collected in 160 asthmatics who quit smoking, and in 30 continuing smokers (Jensen et al. 1998). In particular, the asthmatic subjects with decreased lung function and heavy smoking showed significant increases in blood eosinophil counts after smoking cessation. Gotfretson et al. (2001) demonstrated in a large epidemiological cohort study that ex-smokers had a higher incidence of self-reported asthma than did never smokers (Godtfredsen et al. 2001). The risk to develop self-reported asthma in ex-smokers, compared with never smokers was also increased in two small clinical studies (Hillerdahl and Rylander 1984; Troisi et al. 1995).

12.7 Lung Cancer

12.7.1 Epidemiology

Lung cancer is one of the leading causes of death from neoplastic disease in most developed countries. About 87% of all lung cancer cases are caused by cigarette smoking (American Cancer Society 2001). Diseases such as lung cancer were rare before the widespread use of tobacco; however, after the 1940s, lung cancer was increasing at alarming rates (Auerbach et al. 1957; Cornfield et al. 1959). In the United States, the incidence of lung cancer peaked in 1990 and since has fallen (Cole and Rodu 1996). In Europe, the incidence of lung cancer in men in Denmark, Finland, Germany, Italy, The Netherlands, Switzerland, and the United Kingdom decreased since the 1980s; however, the age-adjusted rate for men in other European countries increased at least until the 1990s. In women, the peak in incidence had not been reached in the 1990s (Janssen-Heijnen and Coebergh 2003). A meta-analysis including 48 studies published between 1970 and 1999 provided additional evidence for a causal relationship between smoking and all histological subtypes of lung cancer (Khuder 2001). The association was stronger with squamous cell carcinoma and small cell carcinoma than was the association with large cell cancer and adenocarcinoma. Combined odd ratios for heavy smoking (>30 cig/day) ranged from 4.1 (CI = 3.16–5.31) for adenocarcinoma to 18.3 (CI = 9.26–36.4) for small cell lung cancer (Khuder 2001).

The ratio of adenocarcinoma and squamous cell carcinoma in the United States was about 1:18 in the 1950s, whereas it was 1:1.2–1.4 in the 1990s. This shift to a higher proportion of adenocarcinoma has also been demonstrated in Europe (Janssen-Heijnen and Coebergh 2003) and other parts of the world (Janssen-Heijnen and Coebergh 2001). The shift to low-tar filter cigarettes during the 1960s and 1970s is the most likely cause (Wynder and Muscat 1995). Ironically, low-tar cigarette smokers may compensate for their low-nicotine delivery by inhaling deeper, in this way exposing the peripheral parts of the lung to increased amounts of carcinogens. In addition, a more intense smoking pattern may increase the generation of *N*-nitrosamines in smoke 3-fold (Wynder and Muscat

1995). An increase in the nitrate content of cigarette tobacco blends may be another explanation for the observed shift in subtypes (Wynder and Muscat 1995). Because there is an overwhelming body of evidence linking cigarette smoking to lung cancer, smoking cessation might be the most effective way to decrease risk of lung cancer. Indeed, smoking cessation reduces the risk to develop lung cancer; however, 15 years after smoking cessation the risk is still higher as compared with never smokers (Halpern et al. 1993). The relative benefits of smoking cessation in this respect appear to be larger for persons with shorter smoking histories (Halpern et al. 1993; Sobue et al. 1993). This is also reflected by the mortality rates of 34,439 male British doctors who have been followed for 50 years (Doll et al. 2004). Continuous smokers born between 1900 and 1930 died, on average, 10 years younger than lifelong nonsmokers (Fig. 12.6). Smoking cessation at age 60, 50, 40, or 30 years gained respectively about 6, 9, 9, or 10 years of life expectancy. Excess mortality associated with cigarette smoking involved vascular, respiratory, and neoplastic diseases; 1,052 of them died because of lung cancer. Age-standardized lung cancer mortality rates (per 100 men per year) of current cigarette smokers depended on the number of cigarettes smoked per day; mortality rates of light (<15/day), moderate (15–24/day), and heavy (>24/day) smokers being 1.31, 2.33, and 4.17 respectively. Lung cancer mortality rates of lifelong nonsmokers, current smokers, and former smokers were 0.17, 0.68, and 2.49, respectively. Those who stopped at age 55–64 were at lower risk to die of lung cancer than continuous smokers, but still were at higher risk than lifelong nonsmokers. Stopping at earlier ages protected even more, but until about age 40 there was still some excess risk to develop lung cancer at older ages. Evidently, smoking cessation at an early age is the most effective way to decrease lung cancer risk; however, many smokers are not able to quit smoking, and those who successfully quit still have a higher risk of lung cancer.

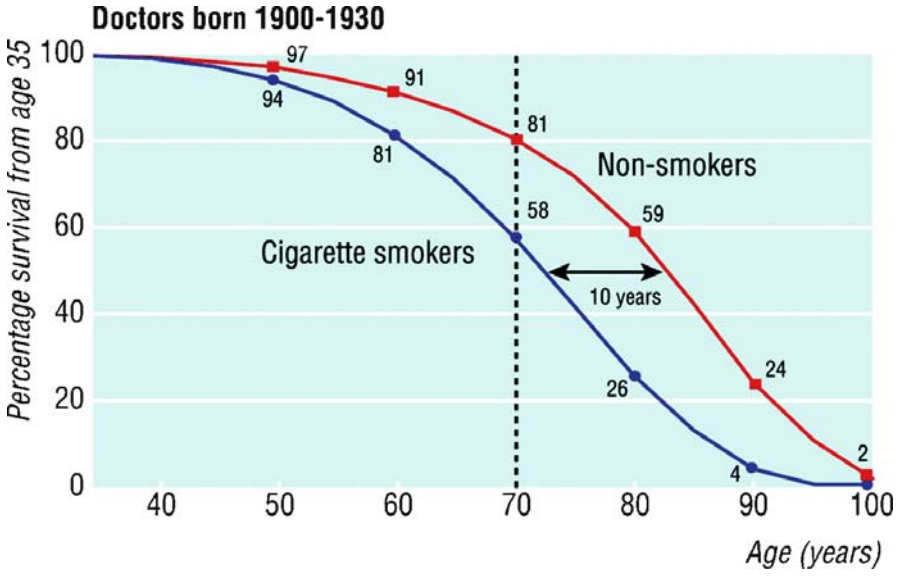

Fig. 12.6 Survival from age 35 for continuing cigarette smokers and life-long nonsmokers among UK male doctors born between 1900 and 1930, with percentage alive at each decade of age. (Reproduced with permission from Doll et al. 2004)

12.7.2 Mutagens, Carcinogens, and Molecular Changes in the Lung

Cigarette smoking results in the exposure to more than 60 established mutagens and carcinogens (Hoffmann and Hoffmann 1997). The strongest carcinogens are polycyclic aromatic hydrocarbons (PAHs) and *N*-nitrosamines. Metabolic detoxification of these agents may lead to excretion; however, sometimes reactive intermediates are formed, which may result in the formation of covalently bound DNA products (DNA adducts). PAHs are formed from the incomplete pyrolysis of tobacco leaves, and many types are present as a complex mixture in tobacco smoke (Lodovici et al. 1998; Seto et al. 1993). Parent PAHs and PAH-related DNA adducts have been demonstrated in the human lung (Lodovici et al. 1998; Seto et al. 1993). *N*-Nitrosamines are formed at higher burning temperatures of the cigarette, e.g., in case smokers inhale faster to compensate for low nicotine contents. *N*-Nitrosamines undergo metabolic activation by cytochrome P450s in the lung and may form three different classes of DNA adducts (Hecht 1999a). They include methylation of different nucleotides (Hecht 1999a), oxidative DNA damage (Hecht 1999a), and bulky DNA adducts (Hecht 1999b). *N*-Nitrosamines adducts have also been demonstrated in human lung tissue. Once DNA adducts are present in the lung cellular DNA repair processes may remove adducts and return the DNA to its normal structure. However, some adducts may escape repair and cause persistent miscoding. Such mutations can activate oncogens (like *RAS*), or inactivate tumor suppressor genes (like *p53*), leading to self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, tissue invasion and metastasis, sustained angiogenesis, and limitless replicative potential (Hanahan and Weinberg 2000).

Theoretically, cigarette smoking may cause lung cancer also through hypermethylation of promoter regions of tumor suppressor genes. In bronchial brush samples from ex-smokers, hypermethylation of the promoter region of multiple genes including *GSTP1*, *p16* (*INK4a*) tumor suppressor gene, and O^6-methylguanine DNA methyltransferase (*MGMT*) DNA repair gene has been demonstrated (Soria et al. 2002). This has also been demonstrated in sputum and bronchial brush samples of current smokers and subjects at high risk for developing lung cancer (Belinsky et al. 2002). The degree of aberrant *p16* and *MGMT* methylation was investigated in patients with premalignant lesions, carcinoma in situ lesions, and squamous cell carcinomas of the lung (Herman et al. 1996). The frequency of aberrant p16 methylation in sputum increased during disease progression from basal cell hyperplasia (17%), to squamous metaplasia (24%), to carcinoma in situ (50%), to squamous cell carcinoma (80%). Palmisano et al. (2000) demonstrated aberrant methylation of the *p16* (and *MGMT*) promoter regions in sputum DNA from all patients with squamous cell carcinoma at the time of clinical diagnosis, and in sputum samples up to 3 years before diagnosis. Assessment of p16 hypermethylation therefore has been advocated as a useful biomarker to detect lung cancer in an early phase in high-risk patients (heavy smokers). Hypermethylation of promoter regions in a susceptible gene because of cigarette smoke is an epigenetic mechanism demonstrating gene–environmental interaction.

12.7.3 Lung Cancer and COPD

Airway obstruction because of COPD has been associated with increased risk of lung cancer, independent of smoking history. This has been demonstrated both in cross-sectional (Kishi et al. 2002; Tockman et al. 1987) and longitudinal studies (Lange et al. 1990c; Mannino et al. 2003a; Nomura et al. 1991; Skillrud et al. 1986), and one study demonstrated it specifically in COPD patients with a more rapid decline of FEV_1 (Islam and Schottenfeld 1994). Both in current and past smokers the relative risk of lung cancer, when adjusted for age and pack years smoked, ranged from 2 to 6, depending on the degree of airflow obstruction (Lange et al. 1990a; Mannino et al. 2003b; Nomura et al. 1991; Tockman et al. 1987). An explanation for the higher risk of lung cancer in COPD patients might be the decreased mucociliary clearance and pooling of inhaled carcinogens in the central airways. Important in this respect is that Nomura (1991) found only an association between COPD and lung cancer for centrally located lung tumors. Another explanation might be a common etiological factor such as the inability of the lung to dispose free radicals and oxidants in cigarette smoke, leading to oxidative stress. Recently, Papi et al. (2004) demonstrated in patients with resectable non–small cell carcinoma lung cancer (NSCLC) that COPD increased the risk for the squamous cell histological subtype by more than four times. In accordance with previous studies, they also demonstrated that chronic bronchitis (without airflow obstruction) increased the risk for the adenocarcinoma subtype by more than four times. The authors speculated that the latter might be because of a more peripheral distribution of tobacco smoke, causing activation of cell signaling pathways (e.g., via the EGF receptor) and inducing increased mucus, producing elements together with adenocarcinoma differentiation of preneoplastic lesions of the bronchial/bronchiolar epithelium.

12.7.4 Chemoprevention

Chemoprevention has been advocated as an alternative for smoking cessation. *Chemoprevention* is defined as the use of agents to prevent, inhibit, or reverse the process of carcinogenesis (Bertram et al. 1987). Two major categories of compounds have been investigated. The first group consists of naturally occurring dietary micronutrients and their synthetic analogues. The second group is composed of synthetic agents like nonsteroidal anti-inflammatory drugs (NSAIDs) and difluoromethyl ornithine. Such agents have been tested in three settings: primary prevention (in healthy subjects being at high risk because of smoking), secondary prevention (in subjects with premalignant lesions), and tertiary prevention (in previously treated lung cancer patients). No randomized trials, in human, in any setting, have shown evidence for efficacy of any agents tested, including α-tocopherol, β-carotene, selenium, retinyl palmitate, isotretinoin, and *N*-acetylcysteine (Cohen and Khuri 2004; Goodman 2002; Hecht 2002; Kelley and McCrory 2003; Lippman and Spitz 2001). Many of these agents might act as scavengers for cigarette smoke-induced free radicals. For example, *N*-acetylcysteine, 600 mg/day, as a potent glutathione donor, and retinyl palmitate were investigated in a randomized controlled trial of 2,592 patients who previously had been treated for head/neck (60%) or lung cancer (40%) (van Zandwijk et al. 2000). Of these patients, 93.5% had smoked, and 25% were still smoking. After a median follow-up of 49 months, no beneficial effect of

retinyl palmitate and/or *N*-acetylcysteine on second primary cancer incidence could be demonstrated. Although the results of the above-described studies all are negative, there still is hope for a future role of chemoprevention in lung cancer. A better understanding of human uptake of tobacco carcinogens and of individual differences in the metabolic activation and detoxification of carcinogens may help to effectively tailor chemoprevention, using promising new agents that are still in a preclinical phase (Hecht 2002).

12.8 Smoking-Related Interstitial Lung Diseases

Four interstitial lung disorders have been linked to cigarette smoking; the clinical, radiological, and histopathological features have been summarized in a concise review recently (Ryu et al. 2001).

Desquamative interstitial pneumonia is characterized by the presence of increased numbers of pigmented macrophages evenly dispersed within the alveolar spaces (Katzenstein and Myers 1998). Honeycombing is minimal, and the overall architecture is maintained in most cases. Ninety percent of the patients smoke or have smoked; however, there is also an association with systemic disorders or infections, as well as exposures to environmental agents and drugs. Untreated, about two thirds of patients show clinical worsening. Patients treated with smoking cessation and steroids generally have a good prognosis and may recover completely.

Respiratory bronchiolitis-associated interstitial lung disease is characterized by the presence of pigmented macrophages and mild interstitial inflammatory changes centering around respiratory bronchioles and neighboring alveoli. This clinicopathological entity is seen almost exclusively in current and former cigarette smokers (Myers et al. 1987) and extends the normally occurring respiratory bronchiolitis because of smoking. Patients with respiratory bronchiolitis-associated interstitial lung disease have a good prognosis and show complete recovery, especially with smoking cessation.

Pulmonary Langerhans cell histiocytosis is characterized by nodular sclerosing lesions containing Langerhans cells, accompanied by mixed cellular infiltrates (Travis et al. 1993). The isolated pulmonary form of Langerhans cell histiocytosis in adults occurs almost exclusively in cigarette smokers. In addition, in young healthy individuals, the accumulation of Langerhans cells on the epithelial surface of the respiratory tract is strongly associated with cigarette smoking (Casolaro et al. 1988). Radiological improvement and even complete resolution after smoking cessation has been described in two case reports (Mogulkoc et al. 1999; Von Essen et al. 1990).

Idiopathic pulmonary fibrosis or cryptogenic fibrosing alveolitis is defined as a specific form of chronic fibrosing interstitial pneumonia limited to the lung and associated with the histological appearance of usual interstitial pneumonia on surgical lung biopsy. The morphological findings range from a normal appearance in early cases to diffuse honeycombing in the later stages. The prevalence of current or former cigarette smoking varies between 41 and 83%. Baumgartner et al. (1997) found a history of smoking to be associated with a lightly increased risk to develop idiopathic pulmonary fibrosis (odds ratio = 1.6). Unfortunately, smoking cessation does not affect the poor prognosis. Although *N*-acetylcysteine administration intravenously or orally has led to increased glutathione levels in BAL fluid and epithelial lining fluid of idiopathic pulmonary fibrosis (IPF) patients (Meyer et al. 1994, 1995), its clinical efficacy has not been demonstrated in long-term studies.

We conclude that cigarette smoking has been implicated in the development of the four interstitial lung diseases described above. Nevertheless, the exact role of cigarette smoking still needs to be defined; the relatively low prevalence of interstitial lung disease makes epidemiologic studies difficult (Flaherty and Martinez 2004).

12.9 Summary

Many studies have shown the detrimental role of smoking on development and progression of pulmonary diseases such as chronic bronchitis, COPD, asthma, lung cancer, and some interstitial lung diseases. The role of oxidative stress is in this respect is variable, clearly established in COPD, and either less explicitly present or not reported because lack of studies in the other diseases.

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Chapter 13

Smoking and Oxidative Stress: Vascular Damage

13

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13.1 Introduction

Chronic smoking is one of the important risk factors for the development of atherosclerosis. Although the precise mechanisms underlying smoking-induced atherosclerosis remain unclear, it became evident that oxidants delivered by the tar and by the gas phase become deposited in the lung but are also delivered directly to the plasma and the vasculature, thereby activating superoxide producing enzymes within the vascular wall (Heitzer et al. 2000). In addition, there is a marked activation of neutrophils, monocytes, platelets and T cells, all of which will significantly enhance the damage to the vasculature. Endothelial dysfunction, which has been demonstrated to be present in active but also passive smokers, is markedly improved by the antioxidant vitamin C, pointing to a crucial role of reactive oxygen species (ROS) in mediating this phenomenon (Heitzer et al. 1996). Because oxidative stress has been shown to be associated with an increased cardiovascular event rate including death, myocardial infarction, stroke and coronary revascularization procedures, the therapeutic strategy should include treatment forms, which effectively reduce ROS-induced damage to the vascular wall (Heitzer et al. 2001). This chapter reviews experimental and clinical studies addressing the mechanisms underlying smoking-induced vascular damage, with a special focus on oxidative stress.

13.2 Active and Passive Smoking and the Epidemiology of Cardiovascular Disease

Smoking has been shown to cause about 140,000 deaths per year from cardiovascular disease in the United States (for detailed review, see Burns 2003). Smoking as a cardiovascular risk factor exponentially enhances the detrimental effects of other risk factor such as diabetes, hypertension, and hypercholesterolemia. Importantly, in other countries than the United States, where the blood pressure is less well controlled, numbers concerning the cardiovascular risk in smokers may be substantially higher. It has been shown that smoking increases the risk of cardiovascular events during surgery performed for noncardiac reasons, and it also increases ischemic episodes after coronary bypass surgery. In addition, smoking increases the risk of reocclusion after myocardial

infarction. Nonsmokers exposed to environmental smoke are at 25–30% increased risk for cardiovascular morbidity and mortality, and over 35,000 deaths per year have been attributed to ischemic heart disease caused by second-hand smoke (Law and Wald 2003).

Endothelial dysfunction represents one of the early stages of atherosclerosis. In the next paragraphs, we focus on the mechanisms by which the endothelium regulates vascular tone and which mechanisms lead to increased oxidative stress within vascular tissue, thereby leading to endothelial dysfunction.

13.3 Endothelium and Vascular Tone

The endothelium, a single-layered continuous cell sheet lining the luminal vessel wall separates the intravascular (blood) from the interstitial compartment and the vascular smooth muscle. Based on cell count (6×10^{13}), mass (1.5 kg), and surface area (1,000 m^2) the endothelium is an autonomous organ. Though for long time regarded as a passive barrier for blood cells and macrosolutes, this view completely changed with the discovery of endothelial autacoids like prostacyclin (PGI_2) (Moncada et al. 1978) and nitric oxide, NO· (Palmer et al. 1987), as well as with the discovery of integrins and other surface signals (Stupack and Cheresh 2004). It is now evident that the endothelium is not only at the crossbridges of communication between blood and tissue cells, but actively controls this process and the function of surrounding cells by a plethora of signaling routes. One of the prominent communication lines is established by the so-called L-arginine-NO·-cyclic GMP pathway (Busse and Fleming 1998). This signaling cascade starts with endothelial NO· synthase (eNOS, NOSIII), which generates NO· and L-citrulline from L-arginine and O_2 in response to receptor-dependent agonists (bradykinin, acetylcholine, ATP) and physicochemical stimuli (shear, stretch) (Fleming and Busse 2003). Reducing equivalents are provided by NADPH, and electrons are passed via a flavin chain to the catalytic center, the enzyme's heme iron. The first step of the normal NOS reaction is a classical monooxygenation, which consumes 1 mol of NADPH and O_2. Molecular oxygen bound to the iron is activated and split to accomplish a hydroxylation of the substrate, the guanidino-nitrogen of L-arginine, forming N^G-hydroxy-L-arginine. The second step is an atypical monooxygenation, which consumes 1 mol O_2 and 0.5 mol NADPH. It is a three-electron oxidation of N^G-hydroxy-L-arginine to afford the final products NO· and L-citrulline. In order to guarantee this reaction path, the enzyme has to be homodimeric and the cofactor tetrahydrobiopterin must be present. For details, the reader is referred to excellent reviews on this topic (Alderton et al. 2001; Ghosh and Salerno 2003).

13.4 ROS and Endothelial Dysfunction

The endothelium-derived relaxing factor, previously identified as nitric oxide (NO·) (Palmer et al. 1987) or a closely related compound (Myers et al. 1990), has potent antiatherosclerotic properties. NO· released from endothelial cells works in concert with prostacyclin to inhibit platelet aggregation (Radomski et al. 1987), it inhibits the attachment of neutrophils to endothelial cells and the expression of adhesion molecules. NO· in high concentrations inhibits the proliferation of smooth muscle cells (Garg and Has-

sid 1989). Therefore, under all conditions, where an absolute or relative NO· deficit is encountered, the process of atherosclerosis is being initiated or accelerated. The half-life of NO· and therefore its biological activity is decisively determined by oxygen-derived free radicals such as superoxide (Gryglewski et al 1986). Superoxide rapidly reacts with NO· to form the highly reactive intermediate peroxynitrite ($ONOO^-$) (Beckman 1996). The rapid bimolecular reaction between NO· and superoxide yielding peroxynitrite (rate constant: $5–10\times10^9$ $M^{-1}s^{-1}$) is about three to four times faster than the dismutation of superoxide by the superoxide dismutase. Therefore, peroxynitrite formation represents a major potential pathway of NO· reactivity pending on the rates of tissue superoxide production. Peroxynitrite in high concentrations is cytotoxic and may cause oxidative damage to proteins, lipids, and DNA (Beckman 1996). Recent studies also indicate that peroxynitrite may have deleterious effects on activity and function of the prostacyclin synthase (Zou and Ullrich 1996) and the endothelial NOS (Zou et al. 2002). Other ROS such as the dismutation product of superoxide, hydrogen peroxide, and the hypochlorous acid released by activated neutrophils, are not free radicals, but have a powerful oxidizing capacity, which will further contribute to oxidative stress within vascular tissue. In addition, myeloperoxidase (MPO) secreted by neutrophils has been demonstrated to be a potent producer of HOCl and to have NO· consuming activity (Baldus et al. 2001, 2002, 2003).

13.4.1 Endothelial Dysfunction and Cardiovascular Risk Factors

It is well known that in the presence of cardiovascular risk factors endothelial dysfunction is frequently encountered. This has been shown for chronic smokers (Heitzer et al. 1996), patients with increased low-density lipoprotein (LDL) levels (Vita et al. 1990), patients with type I diabetes (Johnstone et al. 1993) and type 2 diabetes (Nitenberg et al. 1993), for hypertensive patients (Treasure et al. 1993), and for patients with metabolic syndrome (Deedwania 2003). There are several potential abnormalities that could account for reductions in endothelium-dependent vascular relaxation including changes in the activity and/or expression of the endothelial NOS, decreased sensitivity of vascular smooth muscle cells to NO·, or increased degradation of NO· via its interaction with ROS such as superoxide. The NO··degradation concept is the most attractive one because in the presence of cardiovascular risk factors endothelial dysfunction is established and even more importantly, it is markedly improved by the acute administration of the antioxidant vitamin C (Duffy et al. 2001; Heitzer et al. 1996; Levine et al. 1996; Ting et al. 1996). Superoxide and/or peroxynitrite have/has also been shown to act further downstream by oxidative inactivation of soluble guanylyl cyclase (sGC) as well as activation of cGMP/cGMP-dependent protein kinase (cGK-I) (for review see Munzel et al. 2003).

13.4.2 Endothelial Dysfunction and Prognosis

Recent studies have demonstrated that endothelial dysfunction of the coronary as well as peripheral arteries has prognostic implications. This has been shown for patients with coronary artery disease (Heitzer et al. 2001; Schachinger et al. 2000), arterial hyperten-

sion (Perticone et al. 2001), peripheral vascular disease (Gokce et al. 2002, 2003), and chronic congestive heart failure (Fischer et al. 2005). Importantly, Heitzer et al. (2001) have shown that the degree of oxidative stress within vascular tissue assessed by acute vitamin C challenges is an independent predictor of future vascular events such as death because of myocardial infarction, stroke, and coronary revascularization.

13.5 Constituents of Cigarette Smoke and Oxidative Stress

In general, cigarette smoke can be divided into two phases (Ambrose and Barua 2004). The tar or particulate phase is defined as material that is trapped when the smoke stream is passed through the Cambridge glass-fiber filter that retains 99.9% of all particulate material with a size >0.1μm. The gas phase is therefore the material that passes the filter. The tar phase of cigarette smoke contains 10^{17} radicals per gram, and the gas phase contains $>10^{15}$ free radicals per puff (Smith and Fischer 2001). The radicals associated with the tar phase are long lived (hours to months), whereas the radicals associated with the gas phase have a shorter life span (seconds) (Ambrose and Barua 2004).

Cigarette smoke that is drawn through the tobacco into an active smoker's mouth is known as mainstream smoke. Sidestream smoke is the smoke emitted from the burning end of a cigarette. Mainstream smoke is composed of 8% tar and 92% gaseous phase (Ambrose and Barua 2004). Environmental tobacco smoke results from the combination of sidestream smoke (85%) from smokers and a small fraction of exhaled mainstream smoke (15%) from smokers. Sidestream smoke contains a relatively higher concentration of the toxic gaseous component than mainstream cigarette smoke.

Of all the known constituents, nicotine is the addictive substance of cigarette smoke (Powell 1998).

13.6 Smoking and Oxidative Stress Parameters

The radicals in the gas and tar phase will not only produce oxidative stress when deposited in the lung, but will also cause oxidative damage to the vasculature remote from the lung. There are several possibilities to quantify smoking induced oxidative stress. Spin trapping as well as chemiluminescence techniques have been used (Munzel et al. 2002). In addition, fingerprinting methods that largely detect oxidatively modified biomolecules, such as lipid peroxides, have been used to detect oxidative stress in living organisms. Markers for lipid peroxidation include malondialdehyde and the gold standard for detection of oxidative stress such as isoprostanes (Meagher and Fitzgerald 2000). Isoprostane levels in blood and urine can be increased in a dose-dependent fashion, and they decrease immediately during quitting of smoking, although complete normalization of isoprostane levels may require almost 1 year (Morrow et al. 1995). Markers of protein oxidation include assays for nitrotyrosine, which can be used as a footprint for increased peroxynitrite formation (Beckman 1996).

Measurements of antioxidant capacity can also be performed. The total peroxyl trapping antioxidant parameter is the most popular one to detect if there is a depletion of antioxidants because of increased oxidative stress (Ghiselli et al. 1995).

13.7 Active Smoking and Endothelial Dysfunction

Endothelial dysfunction represents the first detectable manifestation of atherosclerotic disease. Direct evidence that smoking could result in endothelial injury was provided by morphological observations on the umbilical arteries from smoking mothers. Animals exposed to cigarette smoke have been shown to have marked morphological alterations that were accompanied by reductions in prostacyclin production (Pittilo et al. 1982). Later, endothelial dysfunction has been demonstrated for large coronary arteries in response to intracoronary acetylcholine and flow dependent dilation (Nitenberg et al. 1993; Zeiher et al. 1995) and peripheral conductance (Celermajer et al. 1993) and resistance vessels (Heitzer et al. 1996). Importantly, in patients with angiographically normal coronary arteries, intracoronary infusion of acetylcholine has been shown to cause potent coronary artery constriction, compatible with severe endothelial dysfunction (Nitenberg et al. 1993). Previously, we could demonstrate that risk factors such as smoking or hypercholesterolemia reduced endothelium-dependent vasodilation to a similar degree. When both risk factors were present, however, endothelium dependent dilation was strikingly reduced (Fig. 13.1a; Heitzer et al. 1996). α-Receptor blockade was not able to improve endothelium-dependent vasodilation, indicating that increased sympathetic tone did not contribute to this phenomenon (Heitzer et al. 1996). In contrast, acute treatment with vitamin C was able to improve endothelial function in peripheral conductance (Raitakari et al. 2000) and resistance vessels (Fig. 13.1b; Heitzer et al. 1996)

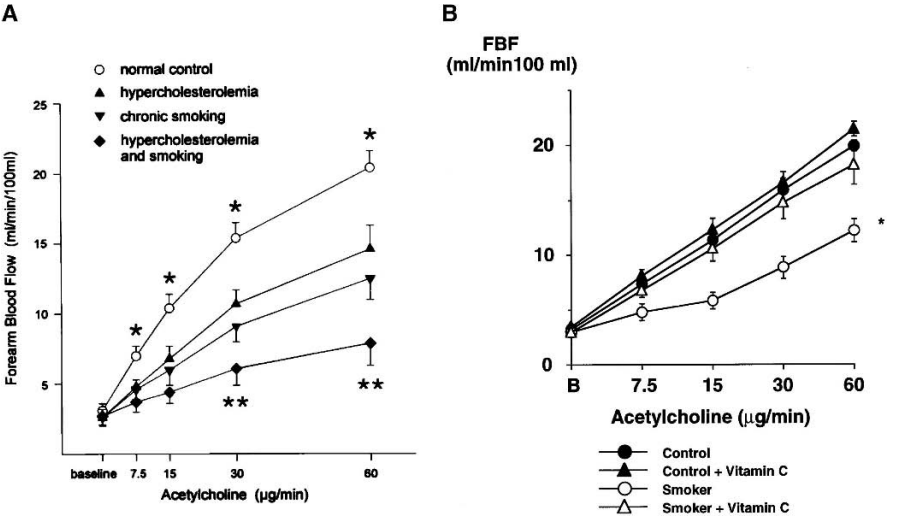

Fig. 13.1 The figure summarizes the effects of cardiovascular risk factors on endothelial function as assessed by forearm plethysmography and the effects of vitamin C on endothelial dysfunction in chronic smokers. **a** Increased low-density lipoprotein (LDL) (mean LDL 236±10 mg/dl) as well as a history of chronic smoking (mean 46±3 pack years) caused a significant degree of endothelial dysfunction on its own. When patients had both hypercholesterolemia as well as a history of chronic smoking, the endothelial function was drastically impaired. **b** Intra-arterial infusion of vitamin C almost completely normalized endothelial dysfunction in chronic smokers, pointing to a crucial role of reactive oxygen species in mediating this phenomenon

as well as in the coronary artery (Schindler et al. 2000), suggesting that increased concentrations of ROS mediate this phenomenon to a large part. Using the technique of flow-dependent dilation of the brachial artery, Neunteufl et al. (2002) recently demonstrated that nicotine also contributes to endothelial dysfunction in chronic smokers, but to a lesser extent than smoking a cigarette of the same nicotine yield.

There has been a debate as to whether endothelial dysfunction occurs solely in response to chronic or also in response to acute smoking. Papamichael et al. (2004) showed that endothelial dysfunction was established in response to acute smoking in the brachial artery. The authors also provided insight into the mechanisms underlying this phenomenon, because red wine's antioxidants were able to correct endothelial dysfunction (Papamichael et al. 2004), indicating that, as observed in chronic smokers, ROS markedly contribute to acute smoking-induced vascular damage as well.

13.8 Passive Smoking and Endothelial Dysfunction

Passive smoking has been demonstrated to be associated with a strikingly reduced coronary flow velocity reserve in young adults (Otsuka et al. 2001). In this study, the authors speculated that endothelial dysfunction mainly accounts for this phenomenon (Otsuka et al. 2001). Indeed, abnormal endothelium-dependent vasodilation in response to passive smoking has been demonstrated in several investigations studying endothelial function in the coronary and forearm circulation (Celermajer et al. 1996; Sumida et al. 1998). Importantly, in some of these studies, there was no significant difference in the response seen in passive versus active smokers, even though cotinine levels in the passive were well below those attained in active smokers (Celermajer et al. 1996). Similar to studies with active smokers, increased oxidative stress as indicated by reduced total antioxidative capacity and increased peroxide levels has been established (Kosecik et al. 2005). Interestingly, chronic treatment with L-arginine slows down atherosclerosis and improves endothelial dysfunction in animals exposed to secondhand smoke, suggesting that a dysfunction in eNOS contributes to this phenomenon (Hutchison et al. 1997).

Despite clear evidence that active and passive smoking have negative effects on vascular function, it remains to be established, however, whether constituents of the cigarette smoke have direct toxic effects to the vasculature and/or whether these components may activate vascular superoxide producing enzymes that may further increase the free radical burden to the vasculature in a positive feedback fashion.

13.9 Evidence for Direct Toxic Effects of Smoke-Derived Free Radicals

The mechanisms underlying smoking related vascular dysfunction are unclear. Cigarette smoke contains more than 4,000 known components, and few components have been characterized in isolation.

Carbon monoxide (CO) represents one of these components, but the precise role in mediating vascular disease remains unclear. Carboxyhemoglobin levels average about 5%, but may reach levels of 10% in heavy smokers. CO exposure increases the number

and complexity of arrhythmias in patients with coronary artery disease (CAD) (Sheps et al. 1990) and also impairs left ventricular function during exercise (Sheps et al. 1990).

Polycyclic aromatic hydrocarbons (PAHs) are found in the tar fraction and have been shown to accelerate atherosclerosis in experimental animal models. Weekly injections of PAHs increase atherosclerotic plaque development in the aortas in cockerels (Penn and Snyder 1988). Likewise, inhalation of butadiene, a vapor component of cigarette smoke, increases atherosclerotic plaques in the same animal model (Penn et al. 1996), whereas the tar fraction does not.

Nicotine is so far the most studied component. It is a sympathomimetic compound that releases catecholamines both locally from neurons and systemically from the adrenal cortex. When given acutely, nicotine accounts for smoking induced-increases in heart rate, blood pressure, and cardiac output, but the precise contribution to the atherosclerotic process remains unclear. The dose of nicotine absorbed from each cigarette is about 1–2 mg. Nicotine exposure has been shown to decrease, not to change or to increase NO· bioavailability. In addition, it increases the rate of copper-induced LDL oxidation in vitro (Gouaze et al. 1998), and high concentrations of nicotine have been shown to accelerate atherogenesis in mice (Heeschen et al. 2001). Nicotine also activates dendritic cells, facilitating their ability to mediate T-cell activation and cytokine release, which also may be relevant to atherogenesis (Aicher et al. 2003). Although nicotine replacement therapy may facilitate reduced exposure to carcinogens in cigarette smoke, the long-term cardiovascular consequences of such a strategy remain to be elucidated.

13.10 Smoking-Related Activation of Superoxide-Producing Enzymes

13.10.1 eNOS

In most situations where endothelial dysfunction because of increased oxidative stress is encountered, the expression of the eNOS has been shown to be paradoxically increased rather than decreased (Guzik et al. 2002; Hink et al. 2001, Laursen et al. 2001; Vaziri et al. 1998). This has also been demonstrated in human umbilical vein endothelial cells (HUVECs) exposed to serum from chronic smokers (Barua et al. 2001, 2003). The mechanisms underlying increased expression of eNOS are likely to be secondary to increased endothelial levels of hydrogen peroxide, which has been shown to increase the expression at the transcriptional and translational level (Drummond et al. 2000). In addition in cultured HUVECs, the increase in eNOS expression in response to serum from chronic smokers was prevented by catalase, which metabolizes hydrogen peroxide to water and oxygen (Barua et al. 2003). The demonstration of endothelial dysfunction in the presence of increased expression of eNOS indicates that the capacity of the enzyme to produce NO· may be limited. Very intriguing are observations that the eNOS itself can be a superoxide source, thereby causing endothelial dysfunction. It has become clear from studies with the purified enzyme that eNOS may become "uncoupled," e.g., in the absence of the NOS substrate L-arginine or the cofactor tetrahydrobiopterin (BH_4). In such uncoupled state, electrons normally flowing from the reductase domain of one subunit to the oxygenase domain of the other subunit are diverted to molecular oxygen rather than to L-arginine (Vasquez-Vivar et al. 1998; Xia and Zweier 1997), resulting in the production of superoxide rather than NO· (Fig. 13.2). For proper function of NOS,

BH_4 seems to be essential in several ways. BH_4 stabilizes the NOS dimer, facilitates its formation, and protects NOS against proteolysis (Panda et al. 2002; Stuehr et al. 2004). It also increases the affinity of NOS for L-arginine, and affects the spin state of the heme iron, the heme redox potential, and the oxygen binding. Most importantly, however, BH_4 plays a decisive role for oxygen activation and the time-critical delivery of one electron and proton to the Fe^{II}–O_2 intermediate, which converts to an iron-oxo species and releases H_2O in the catalytic cycle of NOS (Mansuy et al. 2004). BH_4 provides the second electron in the first monooxygenation reaction, which hydroxylates L-arginine to *N*-hydroxy-arginine. Rapid kinetics analysis by freeze-quench electron paramagnetic resonance (EPR) revealed the transient formation of a $BH_4^{+}\cdot$ cation radical during this reaction, which rapidly splits off a proton to form a $BH_3\cdot$ radical. The $BH_3\cdot$ radical is reduced by one electron and proton delivered by the reductase domain to BH_4, which participates in a second oxygen activation step, leading to the final products NO· and L-citrulline. In the absence of BH_4, the Fe^{II}–O_2-intermediate decays to form superoxide and Fe^{III}. Dihydrobiopterin (BH_2) and other derivatives such as sepiapterin can bind to NOS, but cannot support NO· formation (for review, see Mansuy et al. 2004). Therefore,

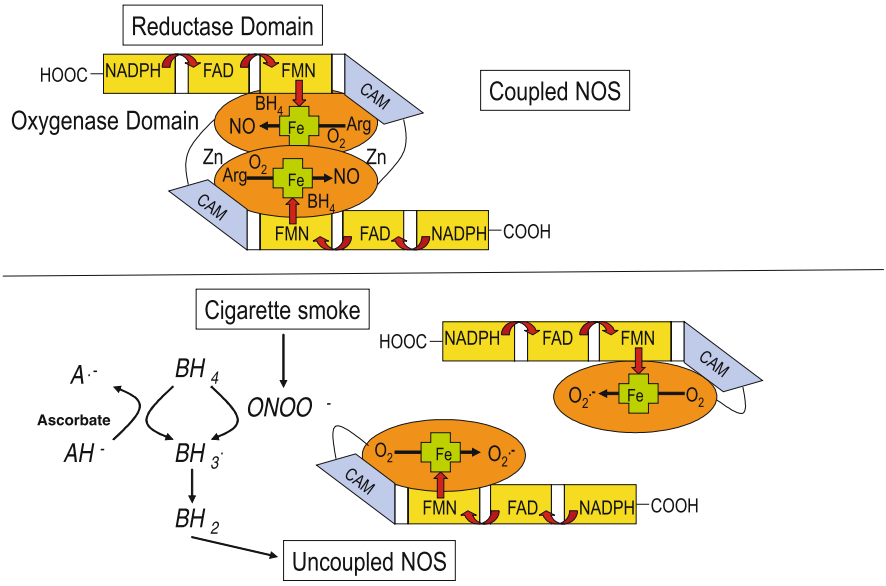

Fig. 13.2 Scheme depicting the mechanisms underlying endothelial nitric oxide (NO) synthase (eNOS) uncoupling in chronic smokers. Under normal conditions, the electron flow starts from NADPH to flavins FAD and FMN of the reductase domain, which delivers the electrons to the iron of the heme (*oxygenase domain*) and to the trihydrobiopterin radical (*BH_3·*) generated as an intermediate in the catalytic cycle. Tetrahydrobiopterin (*BH_4*) seems to be essential to donate an electron and proton to versatile intermediates in the reaction cycle of L-arginine/O_2 to L-citrulline/NO·. Thus, when BH_4 is present in sufficiently high concentrations, eNOS exists as a dimer and couples its heme and O_2 reduction to NO· synthesis. In the absence of BH_4, e.g., because of peroxynitrite-induced oxidation of BH_4 to the $BH_3\cdot$ radical in the setting of chronic smoking, the "uncoupling reaction" is initiated, which means that significant $O_2^{-}\cdot$ production by eNOS may occur by the heme-catalyzed O_2 reduction. Acute treatment with vitamin C may improve endothelial dysfunction by reducing the $BH_3\cdot$ radical to BH_4 rather than by directly scavenging superoxide anions

limited availability of BH_4 for NOS will inevitably result in increased superoxide formation at the expense of NO· formation, i.e., it will uncouple NOS.

What are the mechanisms leading to BH_4 depletion? In vitro studies proposed that native LDL (Pritchard et al. 1995) and, even more pronounced, oxidized LDL (Vergnani et al. 2000), are able to stimulate endothelial superoxide production, and that this phenomenon is inhibited by the NOS inhibitor L-NAME, pointing to a specific role of eNOS in superoxide production. Hypercholesterolemia has also been shown to increase vascular formation of superoxide via activation of the NAD(P)H oxidase (Warnholtz et al. 1999) and/or xanthine oxidase (Ohara et al. 1993). Superoxide derived from both enzyme sources may lead to increased formation of peroxynitrite (Laursen et al. 2001; White et al. 1994). Peroxynitrite in turn rapidly oxidizes the active NOS cofactor BH_4 to inactive molecules such as BH_2 (Laursen et al. 2001; Milstien and Katusic 1999). Accordingly, treatment of HUVECs exposed to serum from smokers with BH_4 ex vivo markedly increased eNOS activity and NO· production, pointing to a recoupling of eNOS (Barua et al. 2003)

Recently, we were able to demonstrate that eNOS uncoupling may represent an important mechanism contributing to endothelial dysfunction in chronic smokers in vivo (Heitzer et al. 2000). As mentioned before, cigarette smoke contains free radicals such as NO· and O_2^-·, which may react with each other to form the strong prooxidant $ONOO^-$. Moreover, autooxidation of polyhydroxyaromatic compounds such as catechol and 1,4-hydroquinone present in cigarette tar (particulate phase) has been demonstrated to induce superoxide production in lung tissue, which in turn could react with NO· from the gas phase of cigarette smoke to form $ONOO^-$. $ONOO^-$ has been associated with increased oxidative reactions (Yoshie and Ohshima 1997) and DNA damage (Radi et al. 1991), and it may cause a reduction of plasma antioxidants as well. Nitration of tyrosine residues of proteins leads to the production of 3-nitrotyrosine, which may be considered as a marker of $ONOO^-$-dependent oxidative damage (Szabo et al. 1996). Interestingly, in plasma from chronic smokers, increased nitrotyrosine levels have been established (Petruzzelli et al. 1997). In addition, in vitro studies with saphenous veins from chronic smokers showed improved endothelium-dependent relaxation after preincubation with BH_4 (Pieper and Siebeneich 1997). All these observations would imply that BH_4 oxidation rather than intracellular BH_4 depletion may induce eNOS dysfunction, which in turn may be a source of altered endothelium-dependent vasorelaxation in chronic smokers. We therefore tested whether BH_4 could improve basal and stimulated NOS· activity in chronic smokers by measuring endothelium-dependent vasomotion using forearm plethysmography, an approach to reflect endothelial function of forearm resistance vessels. We also compared the antioxidant effects of the pteridine tetrahydroneopterin (NH_4) with BH_4 effects in vitro and in vivo to determine whether BH_4 induced improvements in forearm blood flow in chronic smokers are secondary to its effects as a cofactor on a dysfunctional eNOS or because of its nonspecific antioxidant effects. The results demonstrated that in chronic smokers, administration of the endothelial eNOS cofactor BH_4 improves both basal and stimulated NO·-mediated vasodilation (Fig. 13.3). Importantly, the improvement was observed in response to BH_4 and not to NH_4, indicating that a recoupling of eNOS and not a nonspecific antioxidant property of these reduced pteridines was responsible for this phenomenon (Fig. 13.4).

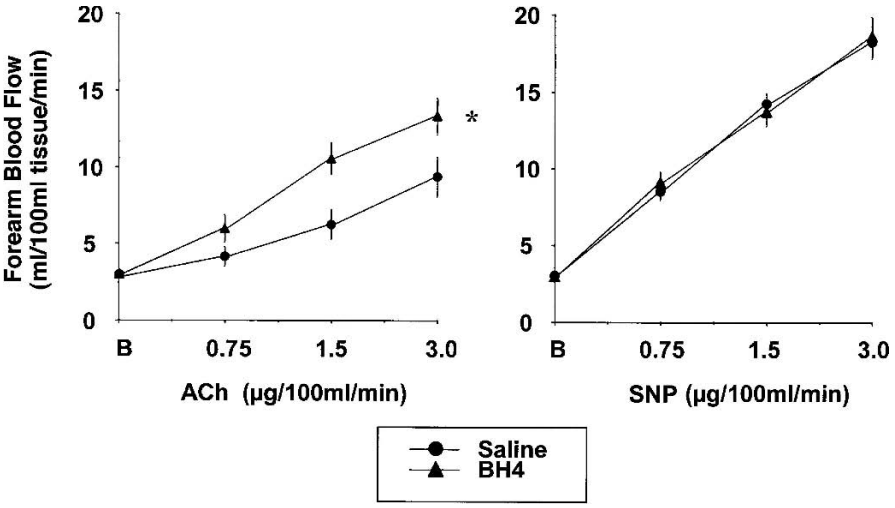

Fig. 13.3 Effects of the nitric oxide synthase (eNOS) cofactor tetrahydrobiopterin (BH_4) on endothelial dysfunction of chronic smokers. Intra-arterial infusion markedly enhanced acetylcholine-induced forearm blood flow, in chronic smokers while having no effect in healthy control subjects, indicating that eNOS uncoupling, e.g., because of BH_4 deficiency, may significantly contribute to endothelial dysfunction in chronic smokers

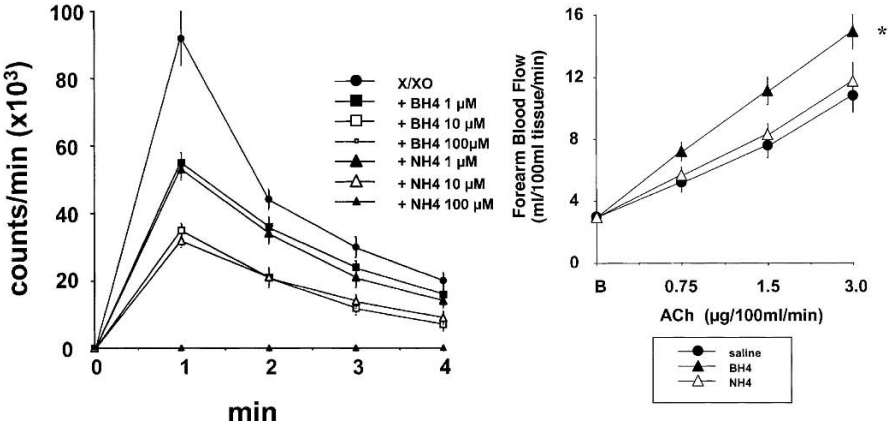

Fig. 13.4 (*left*) Effects of tetrahydrobiopterin and tetrahydroneopterin on the lucigenin-enhanced chemiluminescence signal in response to the xanthine/xanthine oxidase reaction in the presence and absence of tetrahydrobiopterin (*BH_4*) and tetrahydroneopterin (*NH_4*). Both reduced pteridines decreased the chemiluminescence signal to a similar degree with equimolar concentrations, but BH_4 only was able to improve the acetylcholine (*ACh*) dose–response relationship (*right*), suggesting that recoupling of eNOS rather than nonspecific antioxidant properties of reduced pteridines accounts for these beneficial effects

13.10.2 NAD(P)H Oxidase

NAD(P)H-oxidase is a superoxide producing enzyme, which was first characterized in neutrophils (Bastian and Hibbs 1994). We know that a similar enzyme exists also in endothelial and smooth muscle cells as well as in the adventitia. The activity of the enzyme in endothelial as well as smooth muscle cells is increased on stimulation with angiotensin II (Griendling et al. 2000). The stimulatory effects of angiotensin II on the activity of this enzyme would suggest that in the presence of an activated renin angiotensin system (local or circulating), vascular dysfunction because of increased vascular superoxide production is likely to be expected. Experimental hypercholesterolemia has been shown to be associated with an activation of the NAD(P)H-oxidase (Warnholtz et al. 1999). There is a close association with endothelial dysfunction and clinical risk factors, and the activity of this enzyme in human saphenous veins in patients with coronary artery disease (Guzik et al. 2000). At present, no data exist demonstrating that constituents of cigarette smoke are able to increase the activity and/or the expression of the vascular, nonphagocytic NADPH oxidase. With respect to inflammatory cells, acute smoking increases the oxidative burst reflecting NADPH oxidase activity, a phenomenon that is completely corrected by a 3-week abstinence from smoking (Sorensen et al. 2004).

13.10.3 Xanthine Oxidase

Xanthine oxido-reductase catalyzes the sequential hydroxylation of hypoxanthine to yield xanthine and uric acid. The enzyme can exist in two forms that differ primarily in their oxidizing substrate specificity. The dehydrogenase form preferentially utilizes NAD^+ as an electron acceptor but is also able to donate electrons to molecular oxygen. By proteolytic breakdown as well as thiol oxidation, xanthine dehydrogenase from mammalian sources can be converted to the oxidase (XO) form that readily donates electrons to molecular oxygen, thereby producing superoxide and hydrogen peroxide, but does not reduce NAD^+. Oxypurinol, an inhibitor of xanthine oxidoreductase, has been shown to reduce superoxide production and to improve endothelium-dependent vascular relaxations to acetylcholine in vessels from hyperlipidemic animals (Ohara et al. 1993). In vitro, incubation of cultured pulmonary endothelial cells with tobacco smoke condensate (TSC) significantly upregulated XO activity after 24 h of exposure. Further TSC treatment upregulated the XO mRNA expression and the *XO* promoter activity. Actinomycin was able to block the upregulation, suggesting that TSC upregulates XO activity at the transcriptional level (Kayyali et al. 2003). In agreement with these in vitro findings, treatment of smokers with the xanthine oxidase inhibitor oxypurinol was able to improve endothelial dysfunction at the conductance or resistance vessel level markedly (Guthikonda et al. 2003, 2004), providing indirect evidence for a role of XO in mediating at least in part this phenomenon.

13.11 Smoking and Inflammation

Chronic smoking leads to a strong inflammatory response, e.g., marked increases in leukocyte count as well as increases in C-reactive protein (CRP), interleukin-6, and tumor necrosis factor-α have been observed (Jensen et al. 1998; Tracy et al. 1997; Tuut and Hense 2001). In addition, increased levels of circulating soluble vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), and E-selectin, and simultaneously, decreased levels of NO· have been demonstrated in chronic smokers (Mazzone et al. 2001). Exposure of HUVECs to cigarette smoke condensate (CSC) markedly increased adherence of monocytes to the endothelium (Kalra et al. 1994) as a consequence of increased expression of adhesion molecules and also increased transmigration of monocytes to the subendothelial space (Shen et al. 1996). Monocytes isolated from smokers have increased expression of the integrin CD11b/CD18, which further augments the adhesiveness of monocytes to HUVECs in culture (Weber et al. 1996). In addition, exposing human monocytes and HUVECs to smoker serum markedly increases the expression of ICAM-1 (Adams et al. 1997), a phenomenon which is reversed by L-arginine but not by vitamin C treatment.

Nicotine may also contribute to the inflammatory process by acting as a chemotactic agent for neutrophil migration (Totti et al. 1984). Nicotine also causes leukocyte rolling and adhesion within the microcirculation as a parameter for enhanced leukocyte-endothelium interaction (Yong et al. 1997).

13.12 Smoking and Lipid Profile

There are several reports demonstrating that the oxidation of LDL may trigger many proatherogenic properties in vivo. In general, smokers have higher total cholesterol, triglycerides, and LDL levels, and high-density lipoprotein (HDL) levels are lower (Craig et al. 1989). Hypercholesterolemia per se is itself associated with increased oxidative stress as indicated by increased isoprostane levels (Davi et al. 1997), that are drastically increased by chronic smoking as well (Morrow 2005). Interestingly, the triglyceride/HDL abnormalities have been recently related to insulin resistance, all of which may represent a link between cigarette smoking and cardiovascular disease (Reaven and Tsao 2003). Smoking also increases oxidative modification of LDL in animal models (Yamaguchi et al. 2001) and in humans (Heitzer et al. 1996). We have shown that lipid peroxidation and antibody titers to oxLDL are markedly increased in smokers (Heitzer et al. 1996). It is also reported that exposure of LDL to CSC caused a modification of LDL, which was then rapidly taken up by macrophages to form foam cells in culture (Yokode et al. 1988). Exposure of human plasma to the gas phase of cigarette smoke caused oxidative modification of LDL (Frei et al. 1991). Further, HUVECs isolated from smokers caused significantly stronger oxidative modification of LDL than HUVECs from nonsmokers (Pech-Amsellem et al. 1996). Cigarette smoke also increases the plasma activity of paraoxonase, an enzyme that protects against LDL oxidation. In addition, injection of CSC accelerated atherosclerosis in a hyperlipidemic rabbit model, which was at least in part mediated through oxidative modification of LDL (Yamaguchi et al. 2000). Importantly, the susceptibility of LDL to oxidation decreases immediately in subjects in response to smoking cessation.

13.13 Smoking and Thrombosis

Platelet activation and subsequent focal myocardial ischemia has been implicated in sudden cardiac death, which is disproportionally increased in chronic smokers (Castelli et al. 1981). Platelet turnover is accelerated in chronic smokers and urinary thromboxane excretion—a marker of platelet activation—is increased in smokers (Nowak et al. 1987). Interestingly, this phenomenon is prevented by switching to nicotine replacement therapy. Importantly, platelets also contain eNOS, which, may become uncoupled thereby contributing to vascular dysfunction by producing large amounts of superoxide. Indeed, platelets from smokers produce less NO· (Ichiki et al. 1996), a phenomenon, which is partly corrected by L-arginine (Ichiki et al. 1996), and by vitamin C (Takajo et al. 2001). Recent data also indicate effects of smoking on thrombohemostatic factors such as tissue factor and tissue factor pathway inhibitor (TFPI-1) and on fibrinolytic factors such as NO·, tissue plasminogen activator (t-PA), and plasminogen activator inhibitor 1 (PAI-1) (Barua et al. 2002). Incubation of cultured endothelial cells (HUVECs) with serum from chronic smokers was associated with a marked decrease in the t-PA/PAI-1 ratio, and the TFPI-1 levels were lower in smokers. Basal as well as stimulated NO· were also reduced (Barua et al. 2002).

13.14 Effects of Smoking on Antioxidant Levels

Numerous studies have demonstrated decreased plasma levels of vitamins in smokers as compared with nonsmokers. In particular, vitamin C levels in smokers decrease markedly in response to not only chronic, but also to acute smoking. This remains significant when plasma levels were corrected for the decreased vitamin intake of smokers (Ma et al. 2000)

13.15 Cessation of Smoking and Oxidative Stress

As mentioned above, chronic smoking is associated with increased platelet aggregation and production of isoprostanes, lipid peroxides reflecting oxidative stress. Cessation of smoking for about 2 weeks markedly ameliorates enhanced platelet aggregability and also normalizes excretion of isoprostanes. In addition, the redox balance within platelets was restored by increasing the reduced form of reduced glutathione (GSH) and by decreasing the oxidized form of GHS (GSSG) (Morita et al. 2005).

13.16 Treatment of Smoking-Induced Endothelial Dysfunction

13.16.1 Vitamin C

In 1996, we were able to demonstrate that endothelial dysfunction of the forearm (Heitzer et al. 1996) and the coronary artery (Schindler et al. 2000) in chronic smokers is markedly improved by acute intra-arterial or intravenous administration of vitamin C. Similar has been observed in response to acute treatment with 2 g of vitamin C when taken orally (Raitakari et al. 2000). These findings clearly indicate that oxidative stress is a major player in causing endothelial dysfunction. The question is whether these beneficial effects are sustained during chronic therapy. This topic was addressed in a study by Raitakari et al (2000). The authors tested endothelial function in chronic smokers (10 pack years) in response to acute vitamin C treatment (2g orally) and in response to 8 weeks treatment with 1 g/day. Although vitamin C had a quite marked effect when given acutely on endothelial dysfunction, these beneficial effects disappeared completely during the long-term treatment period despite markedly increased plasma vitamin C levels (Raitakari et al. 2000). Based on the low rate constant for reaction of vitamin C and superoxide, this observation suggests that the vitamin C dose given was likely too low in order to scavenge superoxide within the endothelial cell. At higher doses, however, side effects and even prooxidant effects may come into play questioning this kind of treatment.

13.16.2 Vitamin E

A wealth of previous experimental and epidemiological data suggested that excess LDL oxidation was, in part, responsible for the development of atherosclerosis. Since vitamin E inhibits LDL oxidation ex vivo, it seemed logical that one strategy to reduce oxidative stress would include the administration of antioxidants, such as vitamin E. There is considerable risk with such an approach if the antioxidant is not active against all relevant oxidants. Although vitamin E effectively scavenges lipid peroxyl radicals, it has little activity against other oxidants such as superoxide, peroxynitrite, and hypochlorous acid that have also been implicated in atherosclerosis. Another risk with an antioxidant strategy relates to putative cellular compartments or microdomains in the vascular wall that do not contain appreciable amounts of the antioxidant. The sum total of these effects would be continued oxidation even in the presence of the antioxidant. Experimental evidence from both animals and patients suggests that lipid peroxidation does proceed in the vascular wall even in the presence of vitamin E (Upston et al. 1999). Attempts to increase the effectiveness of vitamin E with higher doses have generally met with worsening atherosclerosis and vascular function (Keaney et al. 1994), perhaps because of the well-described prooxidant activity of vitamin E (Bowry et al. 1992). Thus, a single-agent antioxidant strategy may not completely reduce vascular oxidative stress and may leave other important processes, such as smooth muscle proliferation and impaired vascular function untouched. Treatment of chronic smokers (23 pack years) with vitamin E (600 IU/day) for 4 weeks has been shown to improve acute and chronic endothelial dysfunction (flow-mediated vasodilation [FMD]) in chronic smokers. In another study, Heitzer

et al. (1999) have shown that chronic smokers with increased oxLDL levels only had benefit with respect to endothelial dysfunction from a long-term vitamin E therapy (544 IU/day, 4 months' treatment period). Despite these beneficial effects in this small trial, recent results from a meta-analysis as well as data from the HOPE-TOO Trial (Lonn et al. 2005) indicate that long-term treatment with vitamin E might endanger people. Chronic use in a concentration of 400 IE/day markedly increased mortality and also the incidence of left heart failure as well as hospitalization because of heart failure, suggesting that treatment with vitamin E should be avoided.

13.16.3 Folic Acid and Tetrahydrobiopterin

Interesting is the observation that treatment with folic acid is able to improve endothelial dysfunction in chronic smokers (Doshi et al. 2001; Mangoni et al. 2002). As mentioned above, one of the important superoxide producing enzymes is an uncoupled eNOS because of an intracellular BH_4 deficiency. Recent work from Ton Rabelink's group clearly indicated that folic acid improves endothelial dysfunction by preventing this uncoupling phenomenon (Verhaar et al. 2002). Mechanisms discussed are a chemical stabilization of BH_4, preventing it from being degraded, antioxidant effects of folic acid itself, or a direct effect on eNOS (Verhaar et al. 2002). These findings clearly indicate the therapeutic potential of folic acid in treating smoking-induced vascular dysfunction. Another option is treatment with the NOS cofactor BH_4 itself. As pointed out before, acute treatment with intra-arterially applied BH_4 markedly improved endothelial dysfunction in the forearm of chronic smokers in response to acetylcholine and serotonin (Heitzer et al. 2000). Recent studies indicate that oral treatment with sapropterin hydrochloride, an active analogue of BH_4, markedly improved vascular NO· bioactivity, further pointing to an uncoupled NOS in chronic smokers (Ueda et al. 2000).

13.16.4 Angiotensin-Converting Enzyme Inhibitor

Angiotensin-converting enzyme (ACE) inhibitors have been shown to improve vascular function because of stimulatory effects on vascular NO· production as a consequence of the inhibition of the kinase II, which is responsible for the breakdown of bradykinin. Bradykinin in turn stimulates the release of NO·, the endothelium-derived hyperpolarizing factor as well as prostacyclin, all of which will contribute to an improvement of endothelial dysfunction (Griendling and Ushio-Fukai 2000). In addition, ACE inhibitors reduce oxidative stress within vascular tissue by blocking the ACE enzyme, which will lead to a marked reduction in circulating angiotensin II levels. Angiotensin II in turn has been shown to have potent stimulatory effects on vascular superoxide production, proliferation of cells but also on the inflammatory status of the vasculature. Butler et al. (2001) have shown that the ACE inhibitor lisinopril in a concentration of 20 mg for 8 weeks markedly improved forearm blood flow responses on intra-arterially administered acetylcholine, whereas the endothelium-independent responses to sodium nitroprusside remained unchanged. In addition, the vasoconstrictor responses to the NOS inhibitor L-NMMA were enhanced in response to lisinopril treatment. These findings clearly indicate that treatment with an ACE inhibitor is able to improve endothelial

dysfunction in chronic smokers because of enhanced basal as well as stimulated NO· production.

13.16.5 Statins

Statins have been shown to have important pleiotropic effects, which include an up-regulation of vascular eNOS (Laufs et al. 1998) and therefore NO· production, and also a decrease in vascular superoxide in particular because of an inhibition of the activity and expression of important superoxide producing enzymes (Wassmann et al. 2002). Recently, Beckman et al. (2004) demonstrated that treatment of chronic smokers with atorvastatin markedly improved endothelial function, independent of changes in LDL. Endothelium-dependent FMD improved from 8.0 to 10.5%. Because the degree of endothelial function has been shown to have prognostic importance, these findings implicate that statin treatment should be initiated when endothelial dysfunction is encountered in chronic smokers.

13.16.6 Nebivolol

Nebivolol is a third-class β-receptor blocking agent with vasodilating properties, which are largely mediated by releasing NO· from the endothelium. Nebivolol also has potent antioxidant (Troost et al. 2000) properties, which may favorably influence endothelial function in animal models (Mollnau et al. 2003) and in patients with cardiovascular risk factors (Tzemos et al. 2001). Importantly, nebivolol markedly inhibits formation of reactive species in inflammatory cells such as neutrophils and macrophages (Mollnau et al. 2003), which have been shown to produce larger amounts of ROS in chronic smokers. Recently Vyssoulis et al. (2004) demonstrated that treatment of chronic smokers with nebivolol, but not treatment with celiprolol or carvedilol, was able to reduce fibrinogen, PAI-1, and homocysteine. The authors conclude that the smoking status should be an important determinant of antihypertensive treatment choice.

13.17 Summary

Smoking-induced vascular damage is likely to be secondary to the generation of ROS. There is a growing body of evidence that cigarette smoke causes damage via direct delivery of ROS, but also indirectly via delivery of ROS formed by superoxide producing enzymes within the vascular wall, all of which contribute to the acceleration of the atherosclerotic process. Oxidative stress within the blood and within vascular tissue triggers abnormalities with respect to lipid metabolism and the coagulation cascade, which will further contribute to endothelial dysfunction and vascular damage in a positive feedback fashion (Fig. 13.5). The results of treatment of smokers with classical antioxidants such as vitamin C and vitamin E are very disappointing, which may be related to the slow rate constant between the compounds and ROS and the prooxidant effects observed in response to chronic treatment. In contrast, substances that are able to stimulate NO·

production and that simultaneously have inhibitory effects on vascular superoxide production such as statins, ACE inhibitors, or angiotensin I receptor blockers may represent compounds, which will be suited to reduce the consequences of chronic smoking to the vascular wall.

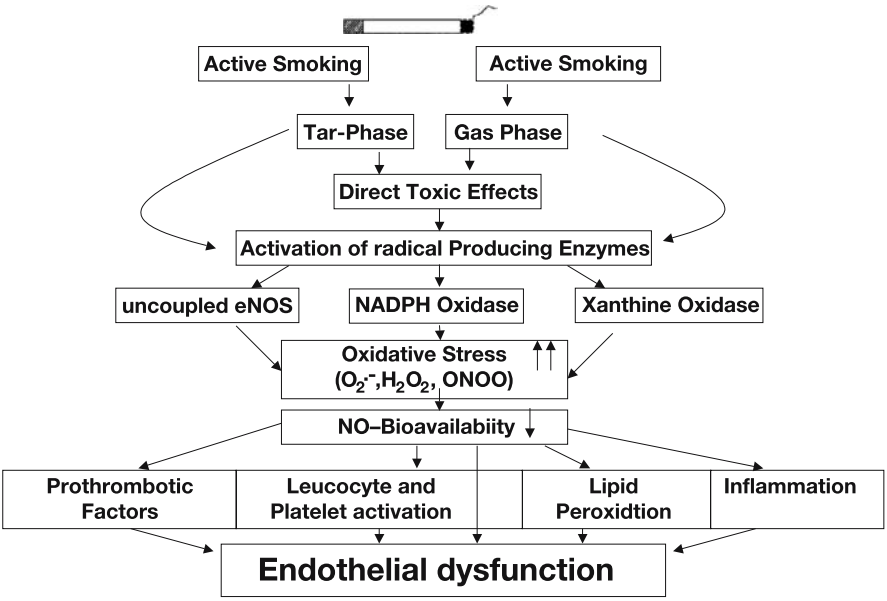

Fig. 13.5 Mechanisms causing endothelial dysfunction in active and passive smokers. Active and passive smoking has toxic effects via contact of lung tissue with the tar and gas phase of the cigarette smoke, respectively. The toxic effects of cigarette smoke may be further enhanced via activation of superoxide-producing enzymes within vascular tissue and therefore remote from the lung such as the NADPH oxidase, xanthine oxidase, and by uncoupling nitric oxide (*NO*) synthase (eNOS). Increased oxidative stress reduces vascular NO· bioavailability, which in turn triggers events that cause endothelial activation/dysfunction by platelet and thrombocyte activation, lipid peroxidation, inflammation, and by creating a prothrombotic environment

13.18 Conclusions

Epidemiological studies indicate that chronic smoking is associated with a marked incidence of cardiovascular morbidity and mortality. Experimental as well as clinical studies have demonstrated that not only active, but also passive exposure to cigarette smoke promotes the phenomenon of endothelial dysfunction. Acute administration of antioxidants markedly improves endothelial dysfunction, pointing to an involvement of reactive oxygen species in mediating this phenomenon. The principal source of free radicals is the cigarette smoke and secondary oxygen-derived free radicals produced from enzymes located within the vessel wall. Cigarette smoke has also strong proinflammatory and procoagulatory effects, all of which contribute to vascular dysfunction as well. Results from small studies suggest that substances that are able to restore eNOS function such as folic acid, and tetrahydrobiopterin as well as established substances such as ACE inhibi-

tors and statins markedly improve smoking-induced endothelial dysfunction. However, despite potentially effective pharmacological treatment options, cessation of smoking remains the most recommended and most cost-effective treatment of smoking induced oxidative stress and endothelial dysfunction.

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Chapter 14

Nrf2: a Transcription Factor that Modifies Susceptibility to Cigarette Smoke-Induced Pulmonary Oxidative Stress and Emphysema

14

Shyam Biswal and Thomas W. Kensler

Contents

14.1 Introduction

Pulmonary emphysema is a major manifestation of chronic obstructive pulmonary disease (COPD), which affects more than 16 million Americans (Blanchard 2003). COPD, which is caused primarily by cigarette smoking, is likely to become the third largest cause of death worldwide by 2020 (Viegi et al. 2001). Oxidative stress caused by chronic exposure of lungs to cigarette smoke contributes to the pathogenesis of emphysema and COPD. The capacity of the lungs to respond to this stress is an important determinant of their relative resistance or susceptibility to COPD. A battery of protective genes can be induced in a rapid and highly coordinated response to oxidants through activation of redox–sensitive transcription factors. The coordinated induction of these genes is fundamental to maintaining antioxidant and other cytoprotective functions against free radicals and electrophiles that arise from exposure to cigarette smoke. Multiple redox-sensitive transcription factors, such as activator protein 1, activator protein 2, nuclear factor kappa B (NF-κB), redox factor 1, signal transducer and activator of transcription, peroxisome proliferation activator receptor, p53, and aryl hydrocarbon receptor, are activated in lungs in response to oxidative stress. This chapter focuses on a relatively newly described basic leucine zipper transcription factor named nuclear factor erythroid 2-related factor 2 (Nrf2). Studies in knockout mice in particular are establishing Nrf2 as an important modifier of pulmonary oxidative stress, inflammation and emphysema.

14.2 Nrf2 as a Key Regulator of Antioxidative and Other Cytoprotective Genes

The capacity to detoxify environmental toxicants, such as may be found in tobacco smoke, is driven in part by the levels of expression of genes that enhance the conjugation and elimination of reactive intermediates by what have been classically termed phase II enzymes. A common regulatory element in the 5' flanking regions of many of these cytoprotective phase II genes has been defined and is termed the antioxidant-response element (ARE). A thorough characterization of the 5' upstream region of the murine NAD(P)H:quinone oxidoreductase (*nqo1*) gene has defined a 24-bp region spanning nucleotides –444 to –421 as the ARE consensus sequence (5'-gagTcA C aGTgAGt C ggCAaaatt-3') (Nioi et al. 2003). Several years ago, Nrf2 was recognized as the major transcription factor binding to AREs. As shown in Fig. 14.1, Nrf2 is an essential regulator of the coordinated expression of ARE-regulated genes. Nrf2 belongs to the cap 'n' collar (CNC) family of bZip transcription factors and primarily acts through formation of heterodimers with one of several small Maf proteins. Major insight into the contribution of Nrf2 in the regulation of phase II genes was provided by Itoh et al. (1997), who showed that disruption of *nrf2* in mice largely abrogated both the basal and inducible expression of prototypical phase II enzymes such as glutathione *S*-transferases (GSTs) and NQO1. As is discussed later in this chapter, genome-wide screening, using wild-type and *nrf2* knockout comparisons in several tissues, has now defined over 200 genes, composing a dynamic, highly coordinated, and diverse mammalian defense system against electrophilic and oxidative stresses. Indeed, *nrf2*-deficient mice are highly susceptible to environmental stresses in many forms because of the impaired expression of these cytoprotective genes.

An actin-binding cytoplasmic protein termed Keap1 represses the transcriptional activation of Nrf2-regluated genes (Itoh et al. 1999). Under basal conditions, Nrf2 is tethered to Keap1, which through interaction with components of ubiquitin ligase, leads to the degradation of Nrf2 by proteasomes (Itoh et al. 2003). Thus, transcription of Nrf2 target genes remains low. Conversely, expression of Nrf2 target genes is very high in *keap1*-disrupted mice (Wakabayashi et al. 2003). Following oxidative stress or exposure to pharmacological enzyme inducers, Nrf2 dissociates from Keap1, triggering the nuclear accumulation of Nrf2 and the transcriptional activation of its target genes (Chan et al. 2001; Cho et al. 2002; Kwak et al. 2002; Rangasamy et al. 2004). In this way, inducible expression of Nrf2-regulated genes can be targeted through the use of small molecules such as dithiolethiones and isothiocyanates (Kwak et al. 2002; Thimmulappa et al. 2002). Such agents are currently being evaluated for use in protection against carcinogenesis, neurodegenerative diseases, and other pathologies associated with inflammation and oxidative stress, including COPD. Keap1 is rich in cysteine residues, and it is currently thought that some of these sulfhydryl residues enable Keap1 to serve as a primary sensor molecule for oxidative stress and response to small molecule inducers (Dinkova-Kostova et al. 2002; Wakabayashi et al. 2004)

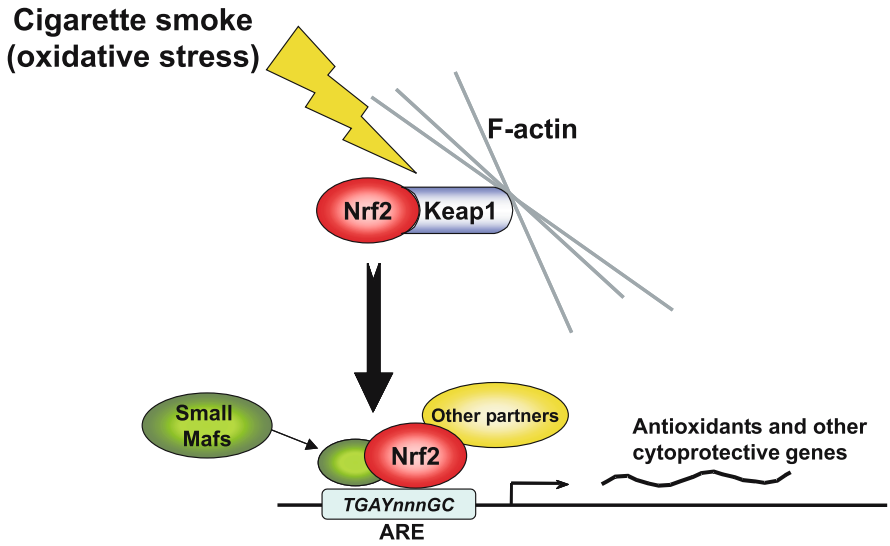

Fig. 14.1 Pathway for nuclear factor erythroid 2-related factor 2 (*Nrf2*)-mediated transcriptional regulation of antioxidant and cytoprotective genes following exposure to cigarette smoke

14.3 Emphysema Results from the Interplay of Oxidative Stress and Inflammation

Pulmonary emphysema is a complex disease characterized by abnormal inflammation, air space enlargement, and the loss of alveolar structures. Permanent destruction of peripheral air spaces distal to terminal bronchioles is the hallmark of emphysema (Hogg 2004). Imbalances in levels of expression of proteinases and antiproteinases as well as in

levels of oxidants and antioxidants resulting from chronic insult with cigarette smoke contribute to the development of emphysema. There is accumulation of inflammatory cells such as macrophages and neutrophils in bronchioles and alveoli (Keatings et al. 1996). Greater numbers of neutrophils have been found in the bronchiolar lavage fluid (BALF) of COPD patients; however, there are relatively small increases in neutrophils in the lung parenchyma (Finkelstein et al. 1995). Neutrophils secrete serine proteases such as neutrophil elastase, cathepsin G, and matrix metalloproteinases (MMP)-8 and MMP-9. These and other proteases may cause alveolar destruction. Macrophages are the predominant inflammatory cells in the lower airways, and their number correlates with disease progression (Shapiro 1999). There is a several-fold increase in the number of macrophages in airways, lung parenchyma, and BALF of patients with COPD. Matrix metalloproteinases (specifically the macrophage elastase MMP-12) play a central role, as documented by the complete resistance of macrophage *mmp12* knockout mice to smoke induced-emphysema (Hautamaki et al. 1997). Mice defective in activation of latent transforming growth factor-β (TGF-β) develop emphysema because TGF-β suppresses the production of MMP-12 by macrophages and increases the tissue inhibitor of MMP (TIMP-1) (Morris et al. 2003). Moreover, pulmonary overexpression of interleukin 13 (IL-13) in transgenic mice results in MMP- and cathepsin-dependent emphysema (Zheng et al. 2000). More recently, MMP-12 has been shown to degrade α1-antitrypsin, and neutrophil elastase degrades TIMP-1. Thus, macrophages and neutrophils clearly alter the proteinase/antiproteinase balance (Shapiro et al. 2003). Neutrophil elastase is also involved in the conversion of proMMP-12 to active MMP-12 (Shapiro et al. 2003). Thus, crosstalk between neutrophils and macrophages enhances the inflammatory processes in mice following exposure to cigarette smoke.

The oxidative burden in the lungs of smokers has been estimated to be very high—10^{14} free radicals per puff (Church and Pryor 1985). The potent oxidants in cigarette smoke include superoxide anion (O_2^-), nitric oxide (NO·) and, through their interaction, the even more reactive peroxynitrite ($ONOO^-$) (Pryor and Stone 1993). Semiquinones and benzoquinones present in the cigarette tar along with superoxide radicals lead to production of reactive hydroxyl radicals (·OH) and hydrogen peroxide (H_2O_2) (Zang et al. 1995). Iron in the fluid of the epithelial lining and in tar causes production of ·OH by the Fenton reaction. Some of these reactive oxygen species cause lipid peroxidation, leading to destruction of membrane lipids (Rahman et al. 1996, 2002). Markers of oxidative stress (e.g., hydrogen peroxide and the end products of lipid peroxidation such as ethane, pentane, and 8-isoprostane) are elevated in the breath and serum of patients with COPD (Horvath et al. 2001). Oxidative stress also enhances inflammation and inactivates critical antiproteinase inhibitors such as α1-antitrypsin (Macnee and Rahman 2001). There is recent evidence that apoptosis of alveolar septal cells contributes to human emphysema (Yokohori et al. 2004) and is required for experimental emphysema caused by inhibition of the vascular endothelial growth factor (VEGF) receptor, a model where oxidative stress has been shown also to be involved (Tuder et al. 2003).

Evidence of the interplay between inflammation and oxidative stress in abetting lung destruction is highlighted by measures of significant release of reactive oxygen species from macrophages and neutrophils in smokers (Rahman and Macnee 1996; Rahman et al. 1996). The oxidants of cigarette smoke can activate alveolar macrophages to produce reactive oxygen species as well as several inflammatory mediators that attract neutrophils, macrophages, and other inflammatory cells into lungs. Inflammatory mediators such as interleukin-8 and tumor necrosis factor-α (TNF-α), whose levels are increased in BALF obtained from patients with COPD (Barnes 2000), are regulated by the proin-

flammatory redox-sensitive transcription factor NF-κB (Rahman et al. 2002, Rahman and Macnee 1998). Cigarette smoke has been shown to activate NF-κB in the lungs of mice (Churg et al. 2003). This activation leads to production of inflammatory mediators, such as macrophage chemoattractant protein 1 and macrophage inflammatory protein 2, which in turn attract macrophages and neutrophils, respectively. The initial increase in macrophages has been postulated to convert pro-TNF-α from macrophages to active TNF-α (Churg et al. 2003). TNF-α then activates endothelial cells with adhesion of neutrophils to the endothelial cells and subsequent entry of neutrophils into the lungs (Churg et al. 2003). Thus, the oxidant/antioxidant imbalance may crosstalk with NF-κB activation to aid in recruitment of inflammatory cells, thereby creating or exacerbating the proteinase/antiproteinase imbalance.

The net result of the interplay between oxidative stress and inflammation is increased release of proteases from activated inflammatory cells that then override the homeostatic control of antiproteinases and initiate the proteolysis of lungs. Further, the increased oxidant burden from the activated macrophages and neutrophils directly contribute to destruction of lung tissue by overwhelming the actions of protective antioxidants.

14.4 Differences in Susceptibility to Cigarette Smoke-Induced COPD

Only 10–20% of heavy cigarette smokers develop COPD (Fletcher and Peto 1977). Susceptibility to COPD is likely to be determined by multiple genes and their interaction with environmental factors. Twin studies examining lung function in monozygotic and dizygotic twins have indicated that 50–80% of variability in lung function has a genetic basis (Mcclearn et al. 1994, Redline et al. 1987, 1989). Modifier genes that regulate the oxidant/antioxidant and proteinase/antiproteinase balances and their interactions are candidates to contribute to susceptibility towards COPD in smokers. Other than congenital deficiencies of α1-antitypsin, which contribute to less than 1–2% of all COPD cases, the genes that determine susceptibility to emphysema are unknown (Sandford and Pare 2000; Sandford et al. 2001). Hence, it is important to elucidate genes and pathways that act as "risk modifiers" for emphysema. Recently, it has been shown that the development of emphysema in response to chronic cigarette smoke exposure in mice is strain dependent (Cavarra et al. 2001; Guerassimov et al. 2004). Factors that have emerged to play a role in susceptibility to emphysema in different mouse strains are α1-antitypsin and sensitivity to oxidants (Cavarra et al. 2001). The ICR strain has been shown to be resistant to cigarette smoke induced-emphysema, whereas the C57BL/6J and the DBA 2/J strains have been shown to be sensitive. These sensitive strains have significantly lower levels of antioxidant defenses. Thus, genetic factors, including Nrf2, that lead to lower antioxidant capacity of lungs may be determinants of susceptibility.

14.5 Nrf2 as a Modifier Gene for Pulmonary Oxidative Stress, Inflammation, and Emphysema

Numerous studies have demonstrated that the susceptibility of the lung to oxidative injury depends largely on the upregulation of protective antioxidant systems (Macnee

2001; Macnee and Rahman 2001). Critical host factors that protect the lungs against oxidative stress may either directly determine susceptibility to alveolar tissue destruction in emphysema or act as modifiers of risk by affecting the intensity of inflammation associated with chronic cigarette smoke inhalation. We have recently reported that disruption of the *nrf2* gene in a genetic background of (emphysema resistant) ICR mice led to early onset, severe emphysema with chronic exposure to cigarette smoke (Rangasamy et al. 2004). Emphysema in *nrf2*-deficient mice exposed to cigarette smoke for 6 months was associated with pronounced bronchoalveolar inflammation (mostly macrophages), enhanced oxidative stress, and apoptosis of alveolar septal cells (Rangasamy et al. 2004).

14.5.1 Nrf2 Protects against Emphysema

As assessed by computer-assisted morphometry, there was a dramatic increase in alveolar destruction in the lungs of *nrf2*-disrupted mice when compared to wild-type ICR mice after 6 months of exposure to cigarette smoke (Fig. 14.2). Both the alveolar diameter and mean linear intercept were significantly higher in smoke exposed *nrf2*-disrupted mice. Alveolar enlargement was detected in the lungs of *nrf2* knockout mice as early as after 3 months of exposure to smoke. The intrinsic resistance of *nrf2* wild-type ICR mice to cigarette smoke-induced pulmonary emphysema was evident from the modest increase (<10%) in the mean linear intercept and alveolar diameter, even after long-term exposure cigarette smoke. Thus, in this model, Nrf2 is critical for resistance to cigarette smoke-induced emphysema. It is noteworthy that *nrf2* knockout mice have also been shown to exhibit enhanced susceptibility to pulmonary damage from hyperoxia (Cho et al. 2002) and chemically induced lung injury (Chan and Kan 1999).

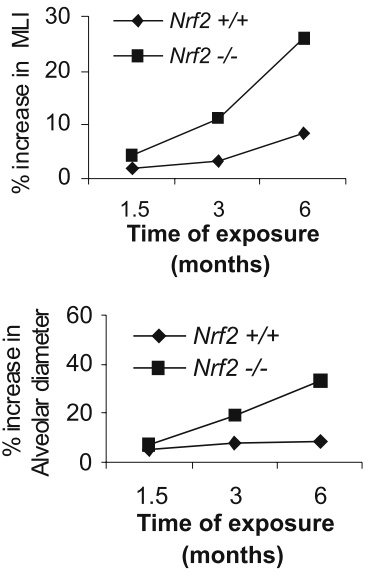

Fig. 14.2 Increased emphysema in nuclear factor erythroid 2-related factor 2 (*nrf2*)-deficient mice. The $nrf2^{+/+}$ and $nrf2^{-/-}$ mice (n=6) were exposed for 1.5–6 months of cigarette smoke. Alveolar diameter and mean linear intercept were measured by computed-assisted morphometry as described in Sect. 14.5.1

14.5.2 Nrf2 Protects against Cigarette Smoke Pulmonary Oxidative Stress and Apoptosis

Consistent with a central role for Nrf2 in the regulation of antioxidant defenses, levels of the oxidative DNA damage product 8-oxo-dG are markedly elevated in the lungs of *nrf2* knockout compared with wild-type mice, following chronic exposure to cigarette smoke. Chronic cigarette smoke also caused increased apoptosis of type II and endothelial cells in the lungs of *nrf2* knockout mice, as evidenced by terminal transferase dUTP nick end labeling (TUNEL) assay and caspase 3 immunostaining (Rangasamy et al. 2004). The presence of enhanced apoptosis in the lungs of *nrf2*-disrupted mice might be related to oxidative stress, inflammation, or excessive lung proteolysis. Oxidative stress and apoptosis are part of a mutually interactive feedback loop in VEGF receptor blockade-induced emphysema (Tuder et al. 2003). Furthermore, reactive oxygen and nitrogen species can modify and inactivate prosurvival cell signaling molecules and cause apoptosis. Inflammation and protease/antiprotease imbalance may also promote apoptosis by means of activated T lymphocytes, which are increased in COPD and seem to correlate with the degree of emphysema (Finkelstein et al. 1995; Majo et al. 2001) and unopposed leukocyte elastase, gelatinase, or collagenase activity. The relation of oxidative stress and apoptosis of lung cells with emphysema is emerging. Apoptosis is required for emphysema caused by VEGF receptor inhibition and is sufficient to cause emphysema (Kasahara et al. 2000; Tang et al. 2004; Tuder et al. 2003), as demonstrated in mice instilled intrabronchially with active caspase 3 (Aoshiba et al. 2003). Recent studies with lung samples derived from emphysema patients have indicated that there is increased apoptosis of alveolar cells (Yokohori et al. 2004). Our studies indicate that Nrf2 could be protecting against emphysema in part by decreasing oxidative stress and the resulting apoptosis of alveolar cells.

14.5.3 Nrf2 Protects against Pulmonary Inflammation

Proteinase/antiproteinase imbalance has been accepted as the most important factor leading to the destruction of the lung during emphysema. Prior and concomitant to airspace enlargement caused by exposure to cigarette smoke, there was increased infiltration of inflammatory cells, predominantly macrophages. These recruited macrophages may have contributed to the alveolar injury through the activity of their elastolytic enzymes, particularly MMP-12. A decrease in the activity and levels of antiproteases may follow the enhanced oxidative stress in our model, and thus contribute to protease/antiprotease imbalance. Studies with hyperoxia and bleomycin as stresses have also shown that Nrf2 protects against pulmonary inflammation. Even though the exact mechanism by which Nrf2 inhibits pulmonary inflammation remains unclear, at least in the cigarette smoke model, the net effect of the Nrf2-regulated pathway is to decrease oxidative stress in the lungs.

14.5.4 Activation of Nrf2 by Cigarette Smoke

Cigarette smoke contains several strong electrophiles such as acrolein, which has been shown to activate Nrf2 in lung cell lines in vitro (Tirumalai et al. 2002). In addition to electrophiles, free radicals and quinones found in smoke can activate Nrf2. Oxidative stresses can also activate several kinases in the lung, which may indirectly contribute to activation of Nrf2. Exposure of ICR mice to acute cigarette smoke (5 h) caused activation of Nrf2 in the lungs, as evident from a gel shift analysis showing increased binding of nuclear proteins to an oligonucleotide containing the ARE sequence (Rangasamy et al. 2004). Increased nuclear accumulation of Nrf2 is critical for the transcriptional activation of ARE-responsive genes. Understanding the targets of Nrf2 should provide insights into how this Keap1-Nrf2-ARE pathway confers protection against oxidative stress and inflammation, and hence, modifies host responses to chronic exposure to cigarette smoke.

14.5.5 Transcriptional Induction of Pulmonary Nrf2 Target Genes after Cigarette Smoke Induction

Comparison of global gene expression profiles in naïve and cigarette smoke-treated wild-type and *nrf2* knockout mice has greatly facilitated the identification of target genes that are directly or indirectly regulated by this transcription factor. Genes differentially expressed in wild-type compared to *nrf2* knockout mice can be assumed to be Nrf2-regulated. Using a microarray approach, we have recently reported that activation of Nrf2 in the lung in response to cigarette smoke leads to transcriptional induction of many different antioxidant and xenobiotic detoxication genes that can attenuate the formation or counteract the damage produced by a broad spectrum of reactive oxygen and reactive nitrogen species (Fig. 14.3) (Rangasamy et al. 2004).

Nrf2, in response to cigarette smoke, regulates genes involved in two major redox systems, the glutathione (GSH) and thioredoxin (Trx) systems. In response to activation of Nrf2 by cigarette smoke, the enzymes involved in GSH synthesis (γ-GCS catalytic and regulatory subunits), members of the GST family, glutathione reductase (GSR), glutathione peroxidases (GPx2, GPx3) and genes that constitute the thioredoxin system (*TrxR* and *Prx1*) were all induced in the lungs of *nrf2* wild-type mice. The members of these redox systems interact with various transducers and effector molecules to bring about antioxidant-specific responses. The regeneration of reduced Trx and GSH by TrxR and GSR, respectively, utilizes NADPH as a reducing equivalent generated by glucose-6-phosphate dehydrogenase (G6PDH) and phosphogluconate dehydrogenase (PGDH), both of which are also induced only in the lungs of wild-type mice. Prx1 and GPx reduce hydroperoxides by utilizing two electrons provided by Trx and GSH, respectively. In addition, GPx and peroxiredoxins have been shown to play a potential role in protection against peroxynitrite, the potent oxidant generated from the reaction of superoxide and nitrous oxide of cigarette smoke. The oxidized forms of GPx and peroxiredoxins are reduced back to their functional forms by Trx. These results suggest important interdependence between the thioredoxin and GSH redox systems and the NADPH generating system.

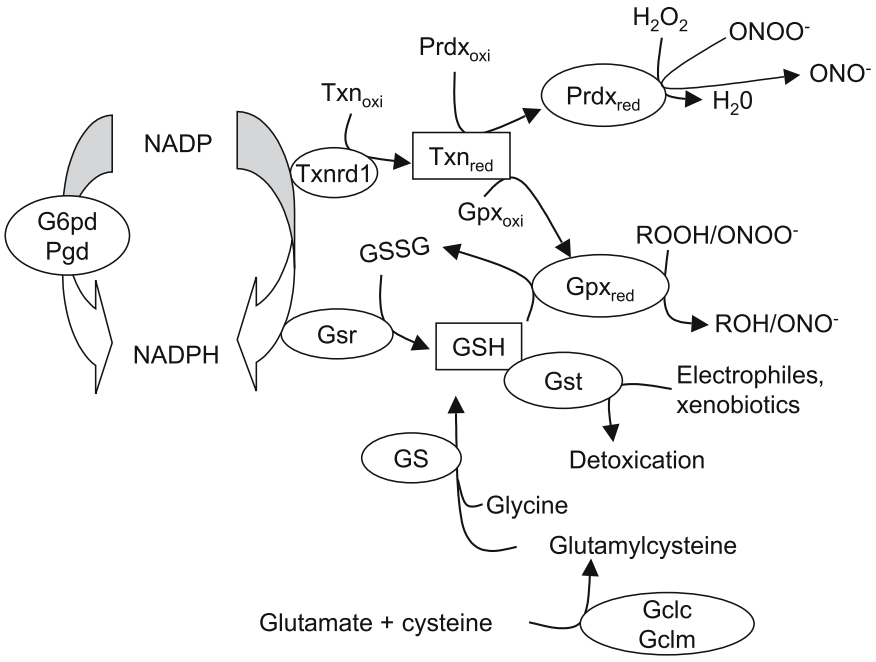

Fig. 14.3 Nuclear factor erythroid 2-related factor 2 (Nrf2)-regulated pulmonary antioxidant pathways

Several GSTs as well as UDP-glucuronosyl transferase (UGT) and NQO1 were selectively induced only in *nrf2* wild-type mice in response to cigarette smoke. Various isoforms of GSTs and UGTs play important roles in the detoxification of tobacco smoke carcinogens such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, benzo[*a*]pyrene, and other polycyclic aromatic hydrocarbons that act as electrophiles and cause DNA damage and cytotoxicity. NQO1 blocks redox cycling of polyaromatic hydrocarbons and benzoquinones present in cigarette smoke, thereby reducing the levels of reactive oxygen species and, presumably, oxidative stress and its consequences such as formation of 8-oxo-dG. Various enzymes, including aldehyde dehydrogenase and aldo-keto reductase, which are involved in the detoxification of reactive aldehydes such as acetaldehyde and acrolein, were selectively induced in the lungs of cigarette smoke-exposed *nrf2* wild-type mice. Heme oxygenase 1 (HO-1), a critical enzyme involved in protection against oxidant-mediated cellular injury, as well as the iron sequestering protein, ferritin light chain 1, which prevents uncontrolled surges in the intracellular free concentration of the highly reactive, yet poorly soluble, ferric iron, were induced only in the lungs of cigarette smoke-exposed wild-type mice. Reduction of ferric ion by superoxide can generate reactive hydroxyl radicals via the Fenton reaction. Superoxide dismutase 3, the major extracellular antioxidant enzyme in the lung that attenuates reactive oxygen-mediated lung cell injury and inflammation, is also selectively upregulated in response to cigarette smoke. Other Nrf2-regulated genes included ubiquitin C, a protein involved in the degradation of oxidized proteins, the DNA damage repair protein GADD45G, and

lung structural proteins, such as tropoelastin and procollagen type IV, alpha 2, and endomucin 1, sequestosome 1, MafF, and HIF-1α-related factor. One or more AREs in the upstream regions of most of these differentially expressed genes was located, indicating the likelihood of a direct role for Nrf2 in their transcriptional induction (Rangasamy et al. 2004)

14.6 Conclusions

Although oxidative stress, which originates directly from components of cigarette smoke and indirectly via infiltrating inflammatory cells, has been suspected to be involved in the etiopathogenesis of emphysema, there has been limited experimental evidence to support this hypothesis. Genetic manipulation of the Nrf2 pathway in mice has provided a model that indicates that oxidative stress because of cigarette smoke may be a central player in the development of emphysema. Oxidative stress regulates the intensity of alveolar inflammation, the extent of alveolar cell apoptosis, and ultimately, the rate of onset and severity of emphysema. There is now a clear experimental link between

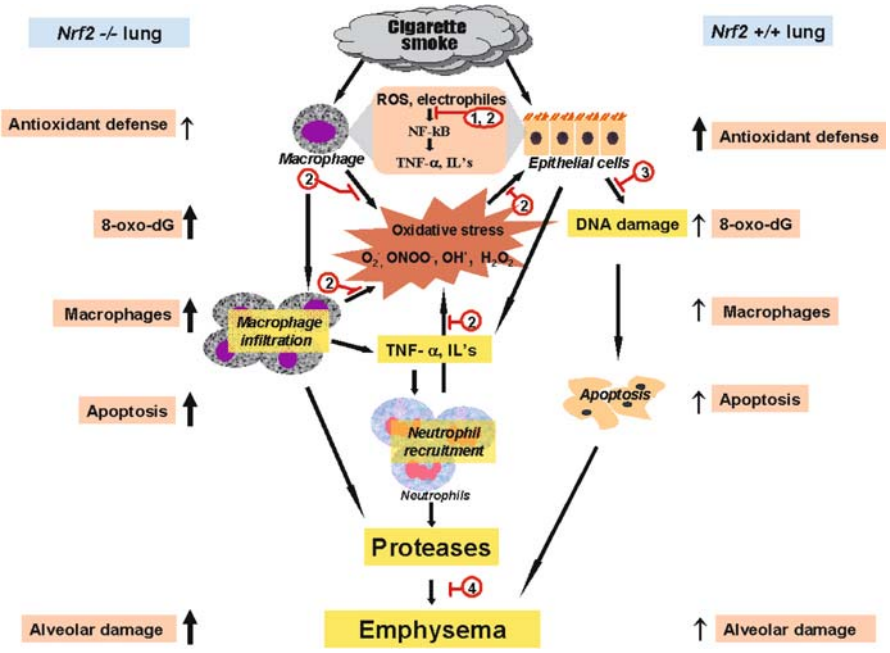

Fig. 14.4 Schematic indicating the increased susceptibility of lungs of nuclear factor erythroid 2-related factor 2 (*nrf2*)-deficient mice to emphysema and probable sites at which Nrf2 targets can block the progression of emphysema. *1* *S*-transferase (GST), UDP-glucuronosyl transferase (UGT), NAD(P)H: quinone oxidoreductase (NQO1), carboxyl esterase, aldo-keto reductase, antidiuretic hormone (ADH), aldehyde dehydrogenase (ALDH); *2* glutathione peroxidase (GPx), glutathione reductase (GSR), superoxide dismutase 3 (SOD3), pregnane X receptor reductase (PXR), thioredoxin reductase (TXR), glucose-6-phosphate dehydrogenase (G6PDH); *3* DNA damage repair protein GADD45; and *4* antioxidants may decrease inactivation of antiproteinases that inhibit proteases. ▲ High, ▼ Less *3*

excessive oxidative stress, increased apoptosis, inflammation, and worsened emphysema with the functional status of the Keap1-Nrf2-ARE signal transduction pathway. Nrf2 is activated in response to cigarette smoke in the lungs of ICR wild-type mice, leading to transcriptional induction of target genes that provides resistance against the development of emphysema. Conversely, a lack of a responsive Nrf2 pathway confers susceptibility to severe emphysema following exposure to cigarette smoke in the *nrf2* knockout counterpart of this model. As summarized in Fig. 14.4, Nrf2 is a critical transcription factor that determines susceptibility to lung inflammation, oxidative stress, and alveolar cell apoptosis caused by chronic exposure to cigarette smoke. The identification of Nrf2 as a determinant of susceptibility may have wide implications to tobacco smoke-related lung diseases and may serve as a target for interventions that seek to retard or block pulmonary diseases where oxidative stress and inflammation play important roles.

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Chapter 15

Tobacco Smoke and Skin Aging

15

Akimichi Morita

Contents

15.1 Epidemiological Study

As early as 1971, Daniell (1971) found that tobacco smoking has a deleterious effect on the skin, and smoker's wrinkles are typical clinical features of smokers. A recent epidemiological study has clearly shown that tobacco smoking is one of the numerous factors contributing to premature skin aging, which is dependent on age, sex, pigmentation, sun exposure history, alcohol consumption, and other factors (Ernster et al. 1995; Frances 1998; Grady and Ernster 1992; Kadunce et al. 1991). In a further cross-section study, sun exposure, pack years of smoking history, and potential confounding variables were assessed by questionnaire. Facial wrinkles were quantified using the Daniell score. Logistic statistic analysis of the data revealed that age, pack year, and sun exposure independently contributed to facial wrinkle formation (Yin et al. 2001a). In this survey, age (OR = 7.5, 95% CI = 1.87–30.16), pack year (OR = 5.8, 95% CI = 1.72–19.87), and sun exposure (OR = 2.65, 95% CI = 1.0–7.0) were independently contributing to the facial wrinkles, as estimated by a logistic regression analysis model. Using silicone rubber replicas combined with computerized image processing, an objective measurement of skin's topography, the association between wrinkle formation and tobacco smoking was investigated. Sixty-three volunteers were enrolled by assessing their skin replicas and in an attempt to elucidate the association between tobacco smoking and wrinkles (Yin et al. 2001b). The replica analysis showed that the depth (R_z) and variance (R_v) of furrows (R_v) in subjects with smoking history ≥35 pack years were significantly higher than nonsmokers ($p<0.05$). The lines of furrows (R_l) in subjects with smoking history were significantly lower than nonsmokers ($p<0.05$) (Yin et al. 2000a; 2001b).

Tobacco smoking, which is regarded as another an important environmental factor, can potentially cause "tobacco wrinkles" (Daniell 1971), although chronic exposure of skin to ultraviolet (UV) radiation results in marked alterations in the structure and composition of the epidermis and dermis, i.e., photoaging (Fisher et al. 1999; Grether-Beck 1997; Wenk et al. 2001). In a recent study, tobacco smoking per se or when smoking combined with UV exposure were strong predictors of skin aging (Leung and Harvey 2002).

15.2 Molecular Mechanisms of Tobacco Smoke-Induced Skin Aging

Tobacco smoking probably exerts its deleterious effects on skin directly through its irritant components on the epidermis and indirectly on the dermis via the blood circulation (Frances 1998; Lofroth 1989). The decreased stratum corneum moisture of the face contributes to facial wrinkling because of the direct toxicity of the smoke. Pursing the lips during smoking with contraction of facial muscles and squinting because of the irritating of smoke may cause the formation of wrinkling around the mouth and in the crow's foot area (Smith and Fenske 1996). The changes in the dermis of macromolecular metabolism have been brought into focus as a major factor leading to skin aging (Uitto et al. 1989). Specifically, it has been demonstrated that accumulation of elastosis material is accompanied by degradation of matrix protein, which is mediated by matrix metalloproteinases (MMPs) in skin aging. The molecular alteration in the dermis includes the

decrease of collagen synthesis, induction of MMPs, abnormal accumulation of elastic fibers, and proteoglycans (Fisher and Voorhees 1998; Shuster 2001; Yin et al. 2000b).

15.2.1 Effects of Tobacco Smoke on Skin Models In Vitro

The biosynthesis of new collagen was decreased significantly by tobacco smoke extracts in cultured skin fibroblasts (Yin et al. 2006). The studies also showed that the production of both procollagen types I and III, the precursors of collagen, were significantly decreased from the supernatant of cultured fibroblast treated with tobacco smoke extracts, using Western blot analysis (Yin et al. 2006). This result indicated that the final production of collagen secreted into the medium as reduced, regardless of the rate of collagen synthesis in the cell tested in ^{3}H-proline incorporation.

Although elastic fibers account for only 2–4% of extracellular matrix, these provide elasticity and resilience to normal skin. Tobacco smoke extracts induced the significant increase in tropoelastin mRNA in cultured skin fibroblasts. Accumulation of abnormal elastic material (termed solar elastosis) is the prominent histopathologic alterations in photoaged skin (Montagna et al. 1989; Tsuji 1987). Boyd et al. (1999) reported that tobacco smoking could facilitate smoke's elastosis of the subjects with an average of 42 pack years of tobacco smoking. In an in vitro study using cultured skin fibroblasts, tobacco smoke extracts induced elevation of tropoelastin. This might be attributed to premature skin aging.

The expressions of *MMP-1* and *MMP-3* mRNA, extracellular matrix (ECM)-associated members of the MMPs gene family, were induced in cultured skin fibroblast stimulated with tobacco smoke extracts in a dose-dependent manner (Yin et al. 2000b). These results support the concept that MMPs are primary mediators of connective tissue damage in skin exposed to tobacco smoke extracts and of the premature skin aging. In addition, expression of *TIMP-1* and *TIMP-3* remained unchanged (Yin et al. 2000b). By inducing the expressions of *MMP-1* and *MMP-3*, but not the induction of tissue inhibitor of MMPs, tobacco smoke extracts could alter their ratio in favor of the induction of MMPs and appears to result in a more degradative environment that produces loss of cutaneous collagen (Yin et al. 2000b). In addition, MMPs comprise a family of degradative enzymes, which are responsible for the degradation of extracellular matrix components such as native collagen, elastin fibers, and various proteoglycans. MMP-3 and MMP-7 may play a key role in the degradation of elastin and proteoglycans (Saarialho-Kere et al. 1999). MMP-7 was increased in fibroblasts induced by tobacco smoke extract.

15.2.3 Effect of Tobacco Smoke In Vivo

In a clinical study, significant higher levels of MMP-1 mRNA were observed in the buttock skin of smokers, compared with nonsmokers, using quantitative real-time PCR (Lahmann et al. 2001). The elevated enzyme should lead to the degradation of collagen, elastic fibers, and proteoglycans. Therefore, the observations in dermal connective tissue induced by the treatments of tobacco suggested an imbalance between the biosynthesis and degradation, with less repair capacity on the face of the ongoing degradation, which

leads to loss of collagen and elastic fibers, manifesting clinically as aging appearance of skin.

Although staining of skin specimen and biochemical analysis of photodamaged skin demonstrated increased glycosaminoglycan content of sun-damaged skin, the underlying molecular pathogenesis remains unclear. Versican, the large chondroitin sulfate (CS) proteoglycan, has been identified in the dermis in association with elastic fibers, which contain a hyaluronic acid-binding domain. The core protein has been postulated to play a role in molecular interactions and specifically, to facilitate the binding of these macromolecules to other matrix components or cytokines such as transforming growth factor (TGF) (Fisher et al. 1989). Decorin, a small CS proteoglycan, has been shown to codistribute with collagen fibers and postulated to function in cell recognition, possible by connecting extracellular matrix components and cell surface glycoproteins (Zimmermann and Ruoslahti 1989). Targeted disruption of decorin synthesis in mice resulted in a significant reduction in the tensile strength of skin (Danielson et al. 1997). There was a decrease in the proportion of large chondroitin sulfate proteoglycan (versican) and a concomitant increase in the proportion of small dermatan sulfate proteoglycan (decorin) as a function of age as reported by Carrino et al. (2000). Ito et al. (2001) also observed that versican was stained strongly in young rats and faintly in old rats. On the other hand, decorin was faintly stained in the young rats and distinctly stained in the old rats. There were several reports concerning the changes of proteoglycans on photoaging, especially UVB irradiation (Bernstein et al. 1995; Margelin et al. 1993). The analysis of new synthesized proteoglycans showed a marked increase after UVB radiation in mice (Margelin et al. 1993). Versican and decorin immunostaining increased in photoaged tissue samples, accompanied by similar alterations in gene expression (Bernstein et al. 1995). Tobacco smoke extracts decreased both versican protein and mRNA levels in cultured akin fibroblasts. However, tobacco smoke extract exposure resulted in a significant increase of decorin. These results have a similar to those observed in photoaging.

15.3. Some Molecular Mechanisms and Protective Factors

Based on experimental evidence, a working model for UVA damage skin was proposed in which UV irradiation gene expression was mediated via the generation of singlet oxygen through a pathway involving activation of transcription factor AP-2 (Grether-Beck 1997). In order to define whether the reactive oxygen species (ROS) were involved in upregulation of MMPs induced by tobacco, sodium azide (NaN_3), L-ascorbic acid, and vitamin E, which are potent quenchers of singlet oxygen and other ROS, were employed. NaN_3, L-ascorbic acid, and vitamin E abrogated the induction of MMPs after exposure of fibroblast to tobacco smoke extract. Among the antioxidant reagents, L-ascorbic acid most obviously diminished the increase in MMP-1 expression level on exposure of fibroblasts to tobacco smoke extracts (Yin et al. 2000b). This points at that ROS were most probable responsible for the enhanced induction of MMPs by tobacco smoke extract.

The transforming growth factor-β1 (TGF-β1) is a multifunctional cytokine that regulates cell proliferation and differentiation, tissue remodeling, and repair (Massague 1998). TGF-β1 is a potent growth inhibitor in the epidermis, playing an important role in maintenance of tissue homeostasis. In the dermis, however, TGF-β1 acts as a positive growth factor, inducing the synthesis of extracellular matrix proteins. TGF-β signals

through a heteromeric complex of type I/II TGF-β receptors, which initiate signal transduction (Kadin 1994; Piek 1999). A recent report showed that UV irradiation can cause downregulation of TGF-β type II receptor mRNA and protein, and induction of Smad7 mRNA and protein in human skin (Quan et al. 2001).

Tobacco smoke extracts induced the latent form TGF-β, not the active form, assayed by enzyme-linked immunosorbent assay (ELISA), in the supernatants of cultured skin fibroblasts (Yin et al. 2003). The induction of endogenous TGF-β1 from tobacco-exposed cells contributes to the intracellular defense capacity. Fibroblasts responses to TGF-β1 are mediated through its active form binding to the cell surface receptor. Tobacco smoke extracts blocked cellular responsiveness to TGF-β1 through the induction of nonfunctional latent form and downregulation of TGF-β1 receptor (Yin et al. 2003). Exogenous addition of TGF-β1 might be useful to stimulate the collagen production or to protect against the deleterious effects of tobacco smoke.

15.4. Conclusions

Tobacco smoke contains numerous compounds, with at least 3,800 constituents (Batsch et al. 1993). Just which constituents that contributed to the damage of connective tissue are still unclear. The tobacco-induced skin aging provides a tool for studying the effects of smoking. Also, detailed knowledge may provide a motivation to stop smoking, especially among those who are more concerned about their appearances than the potential internal damage associated with smoking.

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Chapter 16

Cigarette Smoke and Oxidative DNA Modification

16

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Contents

16.1. Introduction

In this chapter, we give an overview of the nature of oxidative DNA modification, which factors are of importance for oxidation and repair of DNA, how to analyze the lesions and avoid pitfalls of oxidation, how to set up experiments, and how to interpret such experiments. We end up with an evaluation about the degree to which we can associate oxidative DNA modifications with cancer, particularly in relation to cigarette smoking.

In 1992, Leandersson and Tagesson (1992) showed that cigarette smoke increased DNA damage in cultured human lung cells, and at the same time our group showed that cigarette smoking induced increased DNA modification in humans (Loft et al. 1992), measured by urinary excretion of 8-hydroxy-2'-deoxyguanosine (8oxodG); this lesion is the most examined prototype of oxidative DNA modification. Later we showed that smoking cessation reduced DNA modification. Hiroshi Kasai's group (1997) showed that cigarette smoking induces an increase in 8-hydroxydeoxyguanosine in a central site of the lung. Taken together, these findings provide strong evidence that cigarette smoking induces oxidative stress to DNA in the form of increased oxidative base modification.

The area of oxidative DNA modification has particularly been promoted by the pioneers in this area: Hiroshi Kasai, who was the first to report the 8-hydroxy-2'-deoxyguanosine modification in 1984 (Kasai and Nishimura 1984), based on studies on mutagens in heated glucose, and Robert Floyd, who reported that this lesion could be measured by high-performance liquid chromatography (HPLC) with electrochemical detection (Floyd et al. 1986). In an excellent review in 1997, Hiroshi Kasai listed most of the reports about findings in different organs and diseases (Kasai 1997).

Bruce Ames promoted the relationship between oxidative DNA modification and aging, and also the relationship to the antioxidant intake (Ames 1989a, b; Ames et al. 1993), and hypothesized that micronutrient deficiency was a major cause of cancer (Ames 2001).

16.2 Nature and Extent of Oxidative DNA Modifications in DNA

The elucidation of the chemical nature of DNA oxidation was done by the pioneering work of Miral Dizdaroglu (Dizdaroglu 1985, 1991, 1993, 1994; Dizdaroglu and Bergtold 1986; Dizdaroglu et al. 2002) and Jean Cadet (Cadet and Treoule 1978; Cadet et al. 1986, 2003, 2005), preceded by the first report of the 8oxodG modification in 1984 (Kasai and Nishimura 1984).

Oxidation can modify DNA in several positions, in the purine/pyrimidine moiety as well as in the sugar moiety in almost any part of the molecule. After the initial oxidative modification, further rearrangements/changes can occur. For example, the initial modification of guanine can form the C8-OH radical of guanine, which can undergo ring opening, forming the Fapy (2,6-diamino-4-hydroxy-5-formamidopyrimidine) or forming the 8-oxo form dependent on the redox conditions in the reaction mixture or in cells. In total, the reported number of different possible modifications to DNA from oxidation is close to 100; however, only a few of these have been demonstrated in the in vivo situation (Cadet et al. 1997, 2003; Dizdaroglu 1991,1994; Guetens et al. 2002; Schram 1998).

With regard to oxidative modifications because of cigarette smoke, there are no reports of specific oxidative adducts, rather the reports unanimously indicate an increase in preexisting modifications. On the other hand, the literature is mainly focused on the 8oxodG lesion, so it is not known for certain if tobacco smoke gives a specific pattern of oxidation. The reported levels of 8oxodG in nuclear DNA vary over a vide range but may be about 1–10 per million dGs, as expected because 8oxodG is repaired very fast (Asami et al. 1996, 1997; Halliwell 2002; Spencer et al. 1996). In comparison, the levels of polycyclic aromatic hydrocarbon-derived adducts are about 1–10 per 100 million nucleotides (Farmer and Shuker 1999; Godschalk et al. 2002), i.e., about 100 times less frequent. However, as pointed out in an excellent review of smoking-related DNA adducts in a variety of human tissues (Phillips 2002), the half-life of polycyclic aromatic hydrocarbon-derived adducts in the lung is about 1–2 years. In animals exposed to oxidative stress the levels of, e.g., liver 8oxodG returns to normal values within 24 h (Deng et al. 1998), indicating an elimination half-life of some hours. In cellular systems, elimination half-lives of various types of oxidative damage range from minutes to hours. Some studies suggest that oxidized purines are eliminated faster than oxidized pyrimidines (Spencer et al. 1996); other investigators report the reverse (Jaruga and Dizdaroglu 1996). Injected 8oxodG is eliminated with at half-life of few hours into urine (Loft et al. 1995), and after smoking cessation, urinary excretion of 8oxodG decreases within weeks (Prieme et al. 1998a).

Exposure to environmental tobacco smoke in the workplace resulted in a 63% increase in white blood cell DNA 8oxodG levels. The same study included a nonrandomized and noncontrolled intervention showing that the high 8oxodG levels were mitigated by antioxidant supplementation (Howard et al. 1998b). In another study on occupational exposure of metal fume and residual oil fly ash, urinary excretion of 8oxodG was higher in nonsmokers than in smokers (Mukherjee et al. 2004) at the beginning of the work week, but after 2 days of work, the excretion rates were identical.

Taken together, it appears that oxidative lesions are much more frequent than polycyclic aromatic hydrocarbon (PAH)-derived adducts, i.e., with several orders of magnitude, and that the repair of the PAH adducts occurs with a much slower half-life. In this situation, it is very difficult to infer which of the adducts are most important for carcinogenesis, because all the adducts/modifications show mutagenic properties; rather, such information should rely on the predictive values of the lesions in prospective studies, e.g., case-control or cohort studies. Such studies are not presently published (reviewed by Halliwell 2002).

With the use of the Comet assay, a long list of studies has shown increased DNA modification in lymphocytes from smokers compared with nonsmokers (Einhaus et al. 1994; Holz et al. 1993; Lam et al. 2002; Park and Kang 2004; Piperakis et al. 1998; Poli et al. 1999; Welch et al. 1999; Zhu et al. 1999), in lung, stomach, and liver of mice exposed to cigarette smoke (Tsuda et al. 2000) and in oocyte-related cumulus cells (Sinko et al. 2005). On the other hand, there are also reports of no differences between smokers and nonsmokers (Hoffmann and Speit 2005; Speit et al. 2003; Wojewodzka et al. 1999), even after taking genetic polymorphisms in the glutathione *S*-transferase mu (*GSTM1*), cytochrome P450 1A1 (*CYP1A1*), xeroderma pigmentosum group D (*XPD*), X-ray repair cross complementing group 1 (*XRCC1*), and X-ray repair cross complementing group 3 (*XRCC3*) genes into account (Hoffmann et al. 2005). Regarding environmental tobacco smoke exposure, there are reports showing increased DNA strand breaks as measured by the Comet assay (Collier et al. 2005; Wolz et al. 2002).

Most animal studies have focused on target tissue of interest in relation to cigarette

smoke-related cancers, whereas human studies mainly use peripheral lymphocytes as a surrogate tissue. However, one study focused on the DNA modification in placenta and found that 8oxodG increased in smokers as well as after exposure to environmental tobacco smoke (Daube et al. 1997). In mice exposed to acute side stream tobacco smoke, heart and lung levels of 8oxodG increased (Howard et al. 1998a). These findings indicate that cigarette smoke also leads to a more general oxidative stress that just to the DNA of the most directly exposed organ, i.e., the lungs.

16.3 Urinary Excretion of Oxidatively Modified DNA Components

Simple back-of-the-envelope calculation from an estimated average of 2,500 hits to bases in each cell's genome per 24 h gives the result that it would take 1 year to oxidize 1% of the genome if the lesions were not repaired (Poulsen et al. 1998), or about 50% at the age of 50. The excretion of such lesions into urine has been proposed to reflect the oxidative stress to DNA and its precursors (Poulsen et al. 2000, 2003).

In 1992, we showed that smokers excreted on average (big overlapping ranges) about 50% more 8oxodG than nonsmokers (Loft et al. 1992), and later we showed that smokers randomized to smoking cessation decreased their 8oxodG excretion as compared with smokers randomized to continued smoking (Loft et al. 1992; Prieme et al. 1998a). A subsequent study showed that smokers also excreted more of the corresponding base 8oxoG (Suzuki et al. 1995), a finding that could not be repeated in a later study (Harman et al. 2003), and the Poulsen laboratory did not find a difference in 8oxoG excretion between smokers and nonsmokers (unpublished observations). These finding are in agreement with the experimental findings that rats exposed to cigarette smoke for 30 days increased the content of 8oxodG and decreased glutathione levels in all tissues analyszd (Park et al. 1998) and that L-buthionine sulfoximine (BSO) treatment, which depletes glutathione levels, led to a further increase in liver and lung 8oxodG levels. Together with our findings, there is a clear indication that tobacco smoke induces oxidative stress in the lungs but also in other tissues, if not all tissues. In lung cancer patients, the 8oxodG antibody-based assay indicated higher excretion of 8oxodG (Erhola et al. 1997).

Also, urinary 5-(hydroxymethyl)uracil has been reported increased in smokers (Bianchini et al. 1998); however, only when given per excreted creatinine, and only an increase of about 10%. The corresponding nucleoside was also measured in this study, but levels were close to the detection limit. Interestingly, the urinary excretion of 5-(hydroxymethyl)uracil appears higher than other oxidative modifications such as 8oxodG and thymine glycol. A later study from the same group did not find a difference in 5-(hydroxymethyl)uracil excretion, but this time they found a 16% higher excretion of 8oxodG in smokers versus nonsmokers (Pourcelot et al. 1999). A more recent study measured three nucleic acid oxidation products (Harman et al. 2003). In a reasonably sized study, no difference between smokers, ex-smokers, and never smokers could be found with regard to excretion of 8oxodG and the corresponding base 8oxoG, whereas the 5-(hydroxymethyl)uracil was found increased just as in the study mentioned above, however, with a 55% increase. Interestingly, the excretion of the modified base 8oxoG did not differ between never smokers and smokers. In the Malmö Diet and Cancer Cohort (Wallstrom et al. 2003), plasma autoantibodies against 5-hydroxymethyl-2'-deoxyuridine were higher in the smokers lacking glutathione *S*-transferase M1 activity and in

the persons with high alcohol consumption. In urban bus drivers, the urinary 8oxodG excretion rate was higher than in rural bus drivers, regardless of exposure to environmental tobacco smoke and smoking (Loft et al. 1999).

16.4 Analysis of Oxidative DNA Lesions

The analysis of oxidative DNA lesions falls in two distinct categories, specific chemical analysis and unspecific analysis.

16.4.1 Specific Chemical Analysis

This type of analysis is based on a combination of chromatographic separation systems coupled with more or less specific detections systems. A very comprehensive and detailed review of the subject has been published recently (Guetens et al. 2002), and also several more selected reviews are available (Cadet et al. 2004; Cooke et al. 2003; Evans et al. 2004; Halliwell and Whiteman 2004; Jaruga et al. 2001; Loft and Poulsen 1999; Poulsen et al. 2000; Ravanat et al. 1999) including in the forthcoming fourth edition of *Free Radicals in Biology and Medicine, 2006*, by Halliwell and Gutteridge (Oxford University Press).

Choosing a system of analysis depends on a variety of factors. Is the system is to be used for determination of a single or several base oxidation products, and is it to be used to measure in tissue extracts or for urine determinations? Naturally, resources such a price and infrastructure have to be considered.

If a single lesion such as 8oxodG is to be measured in tissue extracts, the system of choice is HPLC with electrochemical detection. This is the most widely used system, and it provides a high sensitivity and specificity. From a cost point of view it is also the most favorable system, and it does not require the special skill that, e.g., mass spectrometry does. HPLC with electrochemical detection can also be used to measure 8-hydroxyadenine, 5-hydroxycytosine, 5-hydroxyuracil and the corresponding 2'-deoxyribonucleosides (Guetens et al. 2002). There are, however, very few reports on simultaneously measured modifications except from the lab of Miral Dizdaroglu by gas chromatography/mass spectrometry (GC/MS), and in human samples such reports are scarce.

If a single lesion such as 8oxodG is to be measured in urine, HPLC with electrochemical detection is also a choice. It should be noted, however, that urinary measurement is a tricky business and only few laboratories have been able to analyze large series of samples. Whereas analysis on tissue extracts is done by a relatively uncomplicated system, i.e., single column with straight or gradient elution, analysis of urine requires separation on a multicolumn system with reverse phase and cation exchange columns combined with selection of relevant fractions (Kasai 2003, 2005; Loft et al. 1993) or reverse phase combined with a proprietary carbon column (Bogdanov et al. 1999). Lin et al. (2004) have reported a GC/MS method that can be used for analysis of 8oxodG in urine.

When multiple lesions are to be measured, the prevailing method used is chromatography combined with MS. Initially, GC combined with MS was extensively used. For analysis of tissue extracts, derivatization at high temperature has been used to make the modified bases volatile and thereby suitable for GC. This can introduce artificial oxida-

tion of nonmodified bases. Since nonmodified bases are in concentrations that are about a million times higher than the oxidized, even a minor artificial oxidation can produce erroneous results. For this reason GC with MS, if used, requires modifications of the derivatization process to reduce or eliminate such artifacts, or one can utilize the progress in the coupling between liquid chromatography (LC) and MS and thereby avoid such cumbersome procedures. It is also possible to use GC/MS if the samples are preseparated by liquid chromatography (Gackowski et al. 2003; Rozalski et al. 2004, 2005).

LC coupled with MS (LC-MS) (Dizdaroglu et al. 2001, 2002; Jaruga et al. 2001) or with tandem MS (LC-MS/MS) can be used to measure multiple oxidative modifications (Harman et al. 2003; Ravanat et al. 1998, 1999; Weimann et al. 2001, 2002), in urine as well as in tissue samples.

Whereas much emphasis has been put on the chromatography and detection systems, early investigations did not put much emphasis on the tissue sample preparation. In late 1997, a workshop was held in Scotland, where the problems in the analysis were discussed (Collins et al. 1997) and later compilation of reported values of 8oxodG showed a 5,000-fold range in nuclear estimates and a 60,000-fold range in mitochondrial DNA. It became clear subsequently that this was not correct and that a large part of the variation was because methodological problems and artificial oxidation. This prompted a group to set up a European Union Framework 5-sponsored project, where the problems were identified and a standard protocol for tissue sample preparation established (Collins et al. 2002a, b, 2004; Gedik and Collins 2004; Lunec 1998; Riis and European Standards Committee on Oxidative DNA damage [ESCODD] 2002. Also, US researchers took initiative to optimize the quality of assays for the 8oxodG modification (Huang et al. 2001).

It is beyond the scope of this chapter to detail the quality control and optimization of the analysis of 8oxodG, alone or together with other modifications. Caution should be taken when reading the scientific literature on this subject, because results might be because artifacts and poor methodology rather than to real biological events. Particularly, results where the levels are high should be regarded with skepticism, i.e., levels for 8oxodG that are substantially higher than the levels of 1–10 per million dGs found by ESCODD. This applies to analysis of tissue extract levels, i.e., nuclear or mitochondrial DNA extracts. In urine measurements, the level of dG (Weimann et al. 2002) is very low, and artificial oxidation is not a problem even if using GC (Lin et al. 2004).

16.4.2 Nonspecific Analysis

16.4.2.1 The Comet Assay

The most commonly used method is single-cell gel electrophoresis, or the Comet assay. This method uses single cells on a glass slide and is based on the charge on DNA subjected to an electrophoretic field followed by DNA staining. If there is a conformational change or strand breaks in the DNA, this part will move faster in the electrophoretic field than unmodified DNA. When conditions are adjusted properly, intact cells will appear as round, stained nuclei and with increasing DNA modification, the nucleus will shrink as the DNA migrates in the field and a comet-like picture will appear. There are several ways of expressing the results, but a method where, e.g., 100 cells are scored on a damage

scale up to 5 and total cumulative scores for 100 cells as a semiquantitative measure of damage/modification gives reproducible results (Collins 2004; Collins et al. 2002b).

It is evident that strand breaks or conformational DNA changes do not equal oxidative damage, and consequently, the Comet assay cannot be taken as a specific marker for oxidative damage. In addition, the Comet assay may underestimate damage/modification if clustered, say, e.g., that all oxidation occurs on all guanosines in a segment of DNA. The method can be made more specific by the use of DNA repair enzymes that will nick DNA at sites with a certain modification, and measuring with and without incubation with such enzymes, e.g., 8-oxoguanine DNA *N*-glycosylase (hOGG1) (Collins 2005). But this assumes that these enzymes have access to all base lesions (unlikely in chromatin) and thus may tend to underestimate.

16.4.2.2 Alkaline Elution

Alkaline elution is a technique where DNA is eluted through filters. If DNA is fragmented, it will elute earlier because of small molecular size, compared with nonfragmented DNA. Such methodology has been applied to oxidative damage (Osterod et al. 2001; Pflaum et al. 1997), and the same argument about specificity as for the Comet assay can be done. We have only been able to find a single report relating to tobacco smoke and oxidative DNA modification using this method. Human lung cells exposed to smoke in buffered saline showed strand breaks that were abolished by catalase (Fielding et al. 1989; Mukherjee et al. 2004).

16.4.2.3 Immunological-Based Methods

Several commercial enzyme-linked immunosorbent assay (ELISA)-based assays have been marketed, and some investigators have produced similar assays. As of today there has not been an assay developed based on immunological methods that has shown sufficient specificity. Testing out an assay, Poulsen et al. found both lack of specificity and sensitivity (Prieme et al. 1996), and recently we tested a newer commercially available assay on different HPLC fractions of urine samples and found that several of the eluted fractions other than 8oxodG reacted in the ELISA kit (unpublished data). Comparison between an ELISA method and HPLC-electrochemical detection (ECD) showed that for quantification HPLC, clean up was necessary (Shimoi et al. 2002). Nevertheless, this paper has been quoted for a demonstration that there is agreement between the HPLC-ECD and ELISA measurements. We have doubt about what the ELISA kit measures besides 8oxdG. It has been suggested that oligonucleotides in urine containing 8oxodG are comeasured by ELISA; however, we demonstrated that such oligonucleotides do not exist in urine at measurable concentrations. We believe that it is very difficult to make an antibody that is sufficiently specific for detection of 8oxodG because of other unknown substances in urine. Consequently, data based on such ELISA methods should be interpreted with caution. Antibodies can be very useful for upconcentrating samples.

16.5 Planning In Vivo Experiments to Investigate Oxidative DNA Lesions: Design Considerations

According to the design used scientific evidence can be graded for quality (Concato et al. 2000) into five groups:

- Grade 1: Evidence obtained from at least one properly randomized, controlled trial
- Grade 2-1: Evidence obtained from well-designed controlled trials without randomization
- Grade 2-2: Evidence obtained from well-designed cohort or case–control analytical studies, preferably from more than one centre or research group
- Grade 2-3: Evidence obtained from multiple time series with or without the intervention. Dramatic results in uncontrolled experiments (such as the results of the introduction of penicillin treatment in the 1940s) could also be regarded as this type of evidence.
- Grade 3: Opinions of respected authorities, based on clinical experience; descriptive studies and case reports; or reports of expert committees

Taking starting point in the studies cited in Sects. 16.4.2.2 and 16.4.2.3, it is evident that the studies belong to grade 2-2 or lower. The only study with quality grade 1 is that of Prieme et al. (1998b), who used a design where smokers were randomized to continued smoking, later followed by a smoking cessation program, or immediately entering a smoking cessation program. This design is the ethically acceptable alternative to randomize nonsmokers to smoking or nonsmoking. Evidence from comparing cases and controls most often overestimates the effects (Kunz and Oxman 1998), and indeed, when we compare our cohort study (Loft et al. 1992) with the randomized intervention study (Prieme et al. 1998b), we find a 2- to 3-fold difference in the effect of smoking.

Although we have not performed a complete survey of studies on oxidative DNA markers and smoking with regard to quality, it is clear that most researchers use a design that is inferior regarding quality of design.

Regarding the total evidence available, however, it is quite clear, that the single grade 1 quality design and several grade 2-2 and grade 3 quality studies performed provides strong evidence that tobacco smoking induces oxidative modification to DNA

16.6 Some Aspects of DNA Repair of Oxidative Modifications

Whereas focus has been on the modification to DNA, it is becoming increasingly clear that DNA repair processes may have equal importance. Repair of oxidative modification in DNA is extensive, and individual differences in DNA repair are proposed to be important for development of cancer and premature aging (Hoeijmakers 2001a, b).

There are a large number of enzyme systems that can recognize oxidative DNA modifications and start a multistep process of repair that seems important for the modulation of oxidative mutagenesis and carcinogenesis (Nohmi et al. 2005).

The human homologue of the MutT protein (hMTH1) enzyme hydrolyses 8oxodGTP and prevent its incorporation into DNA. In lung cancer tissue, the activity of hMTH1 is lower than in normal lung tissue (Speina et al. 2005), and expression, i.e., mRNA levels, in lung cancer cells parallels cellular levels of 8oxodG (Kennedy et al. 1998).

hOGG1 is the initial step in recognition and incision of the 8oxodG lesion and in a case control study, the activity of hOGG1 was lower in peripheral lymphocytes from lung cancer patients than in those from controls (Paz-Elizur et al. 2003). hOGG1 shows a single nucleotide polymorphism (SNP), Ser326Cys, that gives relative risk (RR) of 5.8 in women and 2.0 in men for developing lung cancer in smokers with occupational exposure to smoky coal (Lan et al. 2004), as also observed inanother study where an odds ratio (OR) of 2.1 was found (Le Marchand et al. 2002). Lung tumors with loss of heterozygosity at loci associated with *hOGG1* and the glutathione peroxidase 1 (*GXP1*) genes had about double levels of 8oxodG in nuclear DNA (Hardie et al. 2000).

The xeroderma pigmentosa type a (XPA) protein is involved in nucleotide excision repair and a SNP A-G in the 5' non–coding region of the *XPA* gene and one or two G alleles is associated with a reduced lung cancer risk from smoking (Wu et al. 2003).

In a recent study where several polymorphisms was studied simultaneously, including the repair genes *XRCC1* (one polymorphism) and *ERCC2* (two polymorphisms), their interaction with smoking was studied, and the most striking finding was that the adjusted ORs for lung cancer of individuals carrying five or six variant alleles was 0.3, whereas it was 5.2 for the wild-type alleles/nonsmokers (Zhou et al. 2003).

The *XRCC1* has a SNP, Arg194Trp, and this allele also seems to lower the risk of lung cancer, RR 0.4 in cases with high serum retinol values (Ratnasinghe et al. 2003); however, a SNP, Arg399Gln, gave an OR of 1.4 for smokers developing bladder cancer (Kelsey et al. 2004).

DNA ligases also plays a role in DNA repair, but a SNP (A to C) in exon 6 of DNA ligase I (*LIG1*), seems not related to a changed risk of lung cancer (Shen et al. 2002).

16.7 How Important Are Oxidative DNA Lesions for the Causal Relation between Cigarette Smoke and Development of Cancer and Other Diseases?

The bulk of evidence indicates that tobacco smoking and exposure to environmental smoke lead to increased levels of oxidative modifications in DNA and to increased excretion of repair products into urine. As argued above, urinary excretion does not reflect repair but the total "stress-burden," i.e., the rate of oxidation of DNA and its precursors, and therefore smoking cessation reduces this oxidative stress to DNA. The lesions have been shown to be promutagenic, and it can therefore be concluded that tobacco smoking leads to increased promutagenic oxidative lesions in DNA, as discussed by Halliwell (2002).

The quality of the evidence is strong, and some of the studies demonstrating the oxidative stress to DNA in humans are of the highest grade of scientific evidence, as detailed above.

The subsequent question is: How important is this increased oxidative stress in the development of tobacco related diseases? In this aspect, oxidative DNA modification by mechanistic implications relates mostly to cancer development. Several facts are important to realize. (1) The oxidative DNA modifications are not specific for tobacco exposure; rather, tobacco smoke exposure increases preexisting oxidative modifications. (2) Tobacco smoke contains many chemicals that may modify DNA and form premutagenic lesions, or other types of disease-relevant DNA modifications. (3) The locations within

DNA of the oxidative modifications are not known, and location may be very important in disease processes. (4) The relative contribution of oxidative DNA modifications to the overall number of mutational events in DNA is not known, and the number of mutational spots in DNA from oxidative modification may represent a large or small fraction of other mutations/modifications and therefore also a minor or large contribution to disease. (5) Other endogenous and/or exogenous factors may be important for development of cancer.

Based on the data available as of today, it is clear that oxidative stress to DNA can be an important initial part of the pathogenesis of tobacco-related cancer development as well as in the later stages after malignant clones has been formed. Whereas there is a clear biological plausibility that oxidative stress could be important, it is important to make quantitative estimations of the importance of oxidative stress among all other biological possible mechanisms. One way of doing this is from trials or epidemiological studies where the relative risk of cancer risk from measures of, e.g., high or low oxidative stress to DNA are estimated; such studies are presently not available, but are under way. Even if such data reveal a low cancer risk from oxidative stress, this will not rule out its importance. It could very well be that oxidative stress to DNA in combination with other factors may be very important. Such factors could be DNA repair activity, inflammatory response, baseline DNA damage in nonsmokers, and so forth. Clearly, there is continued need for considering oxidative stress to DNA in the conquest for deciphering the mechanisms of tobacco-related diseases, particularly cancer.

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