6 Particles and Cellular Oxidative and Nitrosative Stress

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6.1 INTRODUCTION

There are three types of reactive oxygen species (ROS): oxygen-containing free radicals, reactive anions containing oxygen atoms, or molecules containing oxygen atoms that can either produce free radicals or are chemically activated by them. Examples are hydroxyl radical ($^{\cdot}OH$), superoxide radical ($^{\cdot}O_2^-$), and hydrogen peroxide (H_2O_2). Similar to ROS, reactive nitrogen species (RNS) can be nitrogen-containing free radicals, reactive anions containing nitrogen atoms, or molecules containing nitrogen atoms that can either produce free radicals or are chemically activated by them. Examples of RNS include nitric oxide ($NO^{\cdot}$) and peroxynitrite ($ONOO^-$). Under normal conditions, an equilibrium exists between ROS and RNS generation, and antioxidant defenses. This equilibrium can be disturbed by a number of factors, many of which are organ, tissue, and/or cell specific. In the lung, inhaled particles can induce an inflammatory response, a component of which is an increase in ROS and RNS production. This increase in ROS/RNS generation can be the result of oxidants being generated from inhaled particles, or from lung phagocytes or epithelial cells, which have been stimulated to produce oxidants. In this review, we describe the sources and mechanisms of particle-induced oxidative stress.

6.2 SOURCES OF CELLULAR ROS

6.2.1 Mitochondria

One source of cellular ROS is the mitochondria. Oxidative phosphorylation is the process by which adenosine-5′-triphosphate (ATP) is formed as electrons are transferred from an electron donor (i.e., nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide (FADH), to the terminal electron acceptor, oxygen, by a series of electron carrying complexes located within the inner mitochondrial membrane. It has been estimated that 2–4% of the oxygen consumed by oxidative phosphorylation produces superoxide as a result of unpaired electrons "leaking" from the electron transport chain (Kirkinezos and Moraes 2001). The most likely sites of superoxide radical formation during oxidative phosphorylation are at complexes I and II of the electron transport chain, because these complexes can exist as semiquinones with unpaired electrons (Ohnishi 1998; Magnitsky et al. 2002; Muller, Crofts, and Kramer 2002). These unpaired electrons can be donated to molecular oxygen, forming superoxide radical.

6.2.2 NADPH Oxidase

Another source of cellular ROS is the "respiratory burst," a term first used in 1933 to describe an increase in oxygen consumption when phagocytic cells were exposed to microorganisms (Balridge and Gerad 1933). Since this initial report, studies have determined that a multi-subunit enzyme complex, called nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, is responsible for the respiratory burst (Patriarca et al. 1971; Suh et al. 1999; De Deken et al. 2000). Active NADPH oxidase is a membrane-bound, five sub-unit complex. At rest, three of these sub-units ($p40^{phox}$, $p47^{phox}$, and $p67^{phox}$) are complexed in the cytosol, while $p22^{phox}$ and $gp91^{phox}$ are membrane bound. Upon stimulation, all subunits are brought together into one macromolecular complex by mechanisms involving phosphoinositide, produced by activated PI3 kinase, and phosphorylation of $p47^{phox}$ by protein kinase C, and activation of mitogen-activated kinases (MAPKs), protein kinase A, and p21-activated kinases (PAK) result in membrane assembly of the active five sub-unit NADPH oxidase (Chen and Castranova 2004). This active NADPH oxidase produces superoxide radical, which in turn can generate other forms of ROS, such as hydrogen peroxide and hydroxyl radical.

6.3 NON-CELLULAR PARTICLE-MEDIATED ROS GENERATION

6.3.1 Silica

As early as 1966, it was proposed that the toxicity of α-quartz (silica) was due to silanol groups (SiOH) on the surface of silica particles acting as hydrogen donors, forming hydrogen bonds with

biological membranes, and disrupting their normal functioning (Nash, Allison, and Harington 1966). Later studies, which examined freshly fractured silica produced by milling or grinding silica, determined that the surface of freshly fractured silica had cleavage planes characterized by the presence of various siloxyl groups (e.g., $Si^{\cdot}$, $SiO^{\cdot}$, Si^{+}, and SiO^{-}) on its surface (Vallyathan et al. 1988; Fubini et al. 1990; Castranova, Dalal, and Vallyathan 1996; Fubini 1998). In an aqueous environment, silica can generate hydrogen peroxide, hydroxyl and superoxide radicals, and singlet oxygen ($^1O_2$) (Vallyathan et al. 1988; Konecny et al. 2001). In addition, there is a positive correlation between the amount of ROS generated and the distribution and quantity of silanol groups on the silica particle surface (Fubini et al. 2001). In cell-free systems, hydroxyl radical generated from silica can interact with membrane lipids, causing lipid peroxidation in proportion to the amount of ROS produced (Dalal, Shi, and Vallyathan 1990; Shi et al. 1994), and also can produce DNA strand breaks (Shi et al. 1994). Electron spin resonance (ESR) has been used to detect siloxyl radicals on the surface of silica particles (Figure 6.1, panels a and b) and also the generation of hydroxyl radical in aqueous medium (Figure 6.1, panels c and d). Furthermore, radical signals produced by freshly ground silica are larger compared to aged silica, which is consistent with freshly fractured silica being more toxic than aged silica (Vallyathan et al. 1988; Vallyathan et al. 1995; Castranova, Dalal, and Vallyathan 1996).

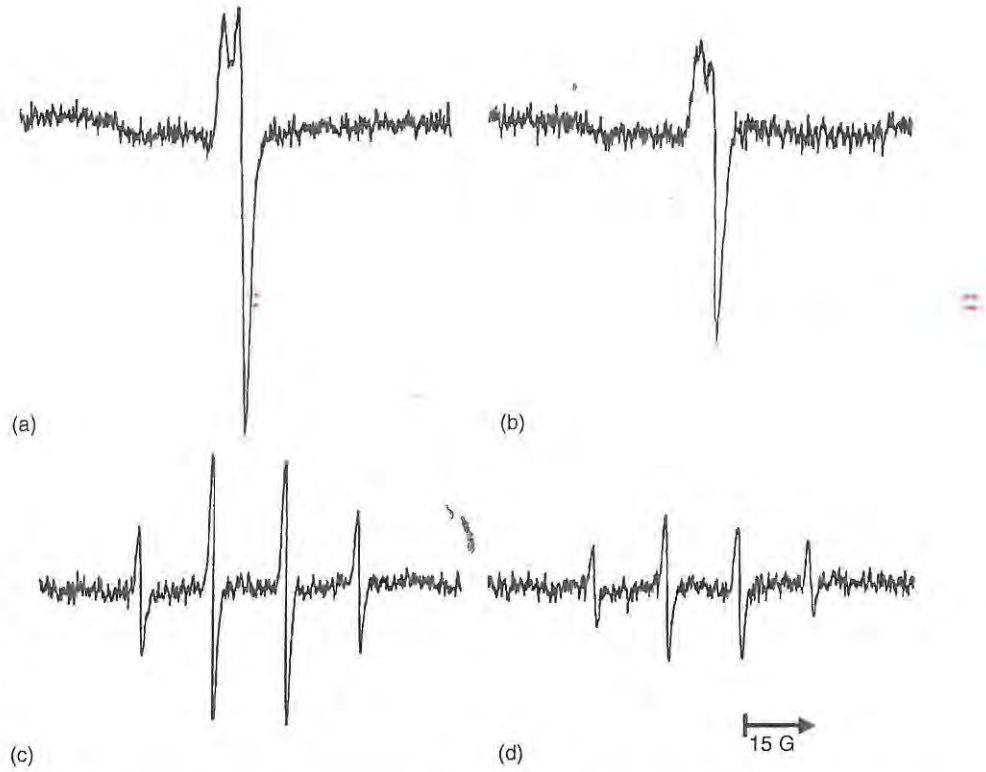

FIGURE 6.1 ESR spectra of freshly fractured and aged silica. Spectra (panel a and panel b) were recorded from 100 mg of dry silica placed in a quartz NMR tube and scanned using the following parameters: receiver gain, 5.02×10^4; time constant, 0.08 s; modulation amplitude, 1 G; scan time, 83 s; number of scans, 5; magnetic field, 3505 ± 50 G. Spectra (panel c and panel d) were recorded 3 min after reaction initiation from a pH 7.4 phosphate buffered saline containing 100 mM DMPO, 10 mM H_2O_2, and the following reactants: (panel c) fresh silica (10 mg/mL); (panel d) aged silica (10 mg/mL). The ESR spectrometer settings were: receiver gain, 6.32×10^4 time constant, 0.04 s; modulation amplitude, 1 G; scan time, 41 s; number of scans, 2; magnetic field, 3490 ± 100 G.

Agents that modify the surface of silica can alter its ability to generate ROS. For example, polyvinylpyridine-*N*-oxide (PVPNO), the organosilane Prosil 28, and aluminum lactate, all decrease non-cellular ROS generation from silica (Wallace et al. 1985; Vallyathan et al. 1991; Mao et al. 1995; Duffin et al. 2001; Knaapen et al. 2002). Iron contamination of silica also affects non-cellular ROS generation. The trace iron contamination of silica may not be soluble iron, but actually iron complexed into the crystal lattice (Donaldson et al. 2001; Fubini et al. 2001). *In vitro*, hydrogen peroxide and trace iron contamination can significantly increase hydroxyl radical production, and this can be inhibited by catalase, suggesting that a Fenton mechanism is responsible for the hydroxyl radical generation (Ghio et al. 1992; Shi et al. 1995). However, the presence of extractable iron is not absolutely required for hydroxyl radical generation, because iron chelation (Fubini et al. 2001) and iron-free or iron-depleted silica (Fenoglio et al. 2001) are still capable of generating $^{\cdot}OH$, albeit at lower levels.

6.3.2 Coal Dust

As determined by ESR, coal dust can produce carbon-centered radicals (Figure 6.2). ESR studies of coal dust samples, obtained from autopsied lymph nodes from asymptomatic miners and patients with Coal Workers' Pneumoconiosis (CWP), determined that coal dust obtained from CWP patients had higher amounts of stable carbon radicals, and the amount of these radicals was related to disease severity (Dalal et al. 1991). In addition, coal dust can generate hydroxyl radical and hydrogen peroxide (Dalal et al. 1995). Coal dust-mediated hydroxyl radical generation is inhibited by deferoxamine and catalase, and is partially inhibited by superoxide dismutase, indicating Fenton chemistry may be responsible for hydroxyl radical generation (Dalal et al. 1995). ESR studies conducted in our laboratory have determined that bituminous coal, which has a high iron contamination, produces more hydroxyl radicals, as measured by ESR, than lignite coal, which has a lower amount of iron in comparison to bituminous coal (data not shown). These determinations add further support to the role of iron in the generation of ROS from coal dust.

6.3.3 Asbestos

All forms of asbestos contain iron, either as a component of their crystalline structure, or as a surface impurity. For example, crocidolite and amosite contain high amounts of iron within their

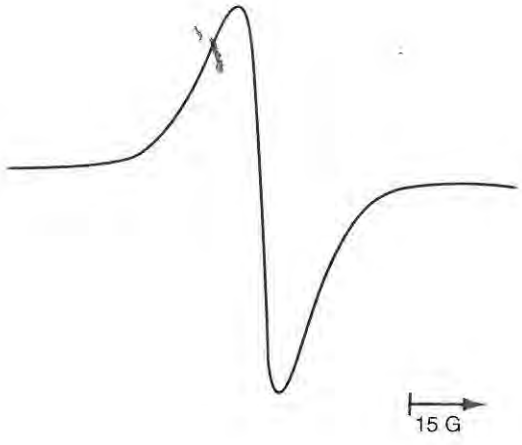

FIGURE 6.2 ESR spectrum of bituminous coal. ESR spectra were recorded from 40 mg of dry bituminous coal placed in a quartz NMR tube and scanned using the following parameters: receiver gain, 5.02×10^4; time constant, 0.08 s; modulation amplitude, 1 G; scan time, 83 s; magnetic field, 3505 ± 50 G.

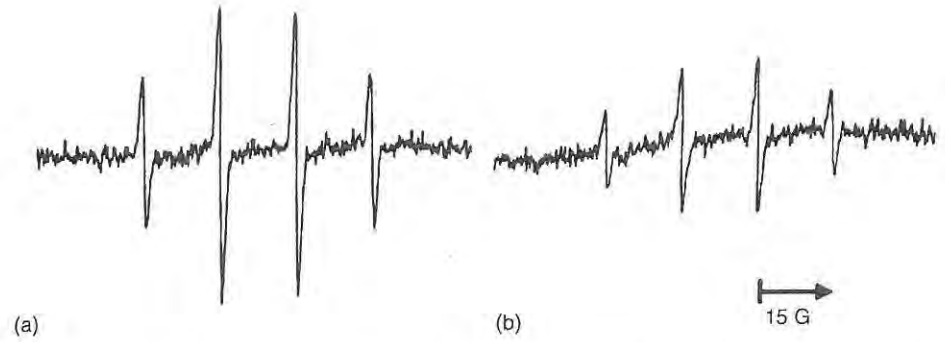

FIGURE 6.3 ESR spectra of crocidolite and chrysotile asbestos. ESR spectra were recorded 3 min after reaction initiation from a pH 7.4 phosphate buffered saline containing 100 mM DMPO, 10 mM H_2O_2, and the following reactants: (panel a) crocidolite (10 mg/mL); (panel b) chrysotile (10 mg/mL). The ESR spectrometer settings were: receiver gain, 6.32×10^4; time constant, 0.04 s; modulation amplitude, 1 G; scan time, 41 s; number of scans, 2; magnetic field, 3490 ± 100 G.

crystal lattice, whereas chrysotile contains trace iron as a contaminant (Harrington 1965; Timbrell 1970; Zussman 1978; Hodgson 1979; Pooley 1981; DeWaele and Adams 1988). The chemical properties of asbestos, especially their iron content, made it likely that they may cause the formation of hydroxyl radicals through iron-catalyzed reactions. This hypothesis was confirmed in a study which reported that chrysotile, amosite, and crocidolite asbestos all generate hydroxyl radical, detected by ESR spectroscopy, in the presence of hydrogen peroxide (Weitzman and Graceffa 1984). As seen in Figure 6.3, hydroxyl radical generation from both crocidolite (panel a) and chrysotile (panel b) is easily detected using ESR, with crodidolite producing a larger signal in comparison to chrysotile. This relates to the fact that Fe is part of the crocidolite crystal structure, whereas Fe is a contaminate of chrysotile. The pivotal role of iron was further established using the iron chelator deferoxamine. Deferoxamine inhibited asbestos-induced $^{\cdot}OH$ radical generation when it was added to the incubation mixture, or when the asbestos was pretreated with desferrioxamine, then washed to remove the extractable iron (Weitzman and Graceffa 1984). Lastly, fibers coated with a passivating material which resisted dissolution, making the iron inaccessible to react with oxygen, exhibited little ability to generate ROS. When the passivating material was removed by grinding or chemical reduction, the asbestos fibers were able to generate ROS (Pezerat et al. 1989).

6.3.4 Other Particles

Residual oil fly ash (ROFA) is a particulate pollutant produced by the combustion of fossil fuels, and is composed of soluble and insoluble metals. In one study (Antonini et al. 2004), ROFA (ROFA-total) was resuspedend in phosphate buffered saline (PBS) for 24 h, and then the particle-free supernatant (ROFA-sol) sample was separated from the insoluble component (ROFA-insol). Elemental analysis of the ROFA-total sample found it to contain greater amounts of Fe and other transition metals than ROFA-insol sample. ESR studies obtained a spectrum representative of hydroxyl radical when each of the samples was treated with H_2O_2 (Figure 6.4). The response was much stronger for the ROFA-total than the ROFA-insol sample, which correlated with the higher amounts of Fe and other transition metals in the ROFA-total sample compared to ROFA-insol sample. This association was further supported by the observation that deferoxamine significantly reduced hydroxyl radical signal from ROFA (Antonini et al. 2004).

Welding is another source of particulates that can generate ROS. Arc welding joins pieces of metal that have been made liquid by the heat produced as electricity passes from one conductor to another. The extremely high temperatures ($>4{,}000°C$) of this process heat both the base metal

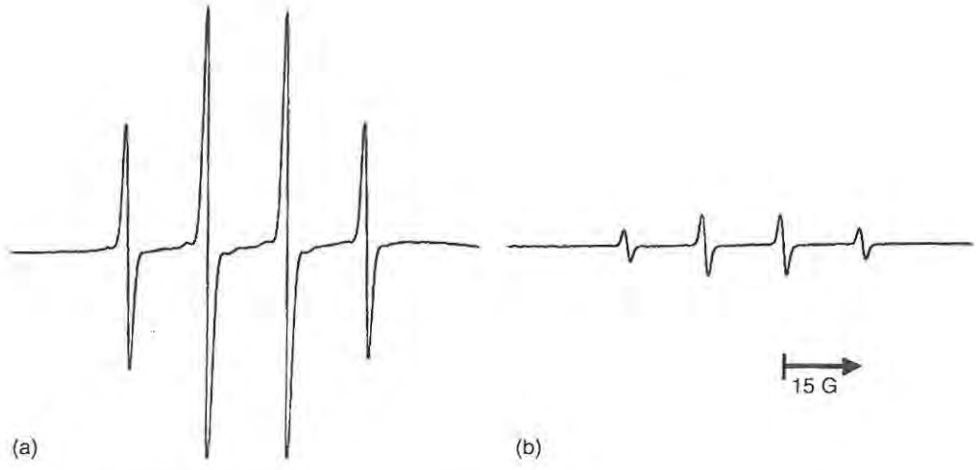

FIGURE 6.4 ESR spectra of ROFA-total and ROFA-insol. ESR spectra were recorded 3 min after reaction initiation from a pH 7.4 phosphate buffered saline containing 100 mM DMPO, 10 mM H_2O_2, and the following reactants: (panel a) ROFA-total (10 mg/mL); (panel b) ROFA-insol (10 mg/mL). The ESR spectrometer settings were: receiver gain, 6.32×10^4; time constant, 0.04 s; modulation amplitude, 1 G; scan time, 41 s; number of scans, 2; magnetic field, 3490 ± 100 G.

pieces to be joined and a consumable electrode fed into the weld. Fumes are formed by the evaporation of the metals, primarily at the tip of the electrode. The metal vapors are oxidized on contact with the air and form small particulates of different complexes of metal oxides. The fumes produced by welding can vary greatly. For example, welding fumes collected from manual metal arc (MMA) welding using a stainless steel (SS) electrode contains soluble metals, in particular chromium. In contrast, welding fume from gas metal arc (GMA) with a SS electrode, or GMA with a mild steel (MS) electrode produces low levels of soluble metals (Taylor et al. 2003). ESR was used to assess the ability of the fumes to produce free radicals in cell-free systems, and only MMA–SS fume produced a spectra characteristic of hydroxyl radical. Furthermore, when the total MMA–SS was compared with its insol fraction, the soluble metals in total MMA–SS were found to be most responsible for the production of hydroxyl radicals (Taylor et al. 2003). The ability of welding fumes to produce ROS decays with time after collection, and is highest in freshly generated welding fume, as measured by dichlorofluorescein fluorescence (Antonini et al. 1998).

Wood smoke, produced by the combustion of wood, has been identified as a source of particles that can generate free radicals (Leonard et al. 2000). Wood smoke particulate, collected on a filter, has been determined by ESR to have carbon-centered radicals based on the spectral line shape and position. These carbon-centered radicals are relatively stable, with a half-life of several days, depending on environmental conditions. In addition to carbon centered radicals, filters treated with H_2O_2 exhibited an ESR spectra indicative of hydroxyl radical generation. This generation of hydroxyl radicals was associated with the ability of wood smoke to cause DNA damage and induce lipid peroxidation, nuclear factor kappa B (NF-κB) activation, and tumor necrosis factor-alpha (TNF-α) production in macrophages (Leonard et al. 2000).

6.4 PARTICLE-MEDIATED CELLULAR ROS GENERATION

6.4.1 Silica

In vitro silica exposure has been shown to significantly increase alveolar macrophage (AM) intracellular superoxide radical and hydrogen peroxide levels in comparison to controls

(Zeidler et al. 2003). Silica exposure also has been shown to activate NADPH oxidase, resulting in increased oxygen consumption and extracellular secretion of superoxide and hydrogen peroxide from AMs (Castranova, Pailes, and Li 1990), polymorphonuclear leukocytes (PMNs) (Kang et al. 1991), and alveolar type II cells (Kanj, Kang, and Castranova 2005).

Extensive data exist regarding ROS production by lung pneumocytes after *in vivo* quartz exposure. Exposure of rats to silica results in potentiation of particle-stimulated ROS and RNS production in harvested AMs ex vivo. AMs isolated from silica-exposed animals have increased hydrogen peroxide production (Castranova 1994). Chemiluminescence, which is an indicator of ROS production, has also been shown to be increased in silica-exposed rats (Castranova et al. 1985; Porter et al. 2002a), and exposure to freshly fractured silica stimulates AM chemiluminescence to an even greater extent than aged silica (Castranova et al. 1996; Porter et al. 2002a).

The impact of trace iron contamination on toxicity *in vivo* is unclear. In one study, silica with surface associated iron caused greater pulmonary inflammation in comparison to iron-free silica (Ghio et al. 1992). However, another study which compared the amount of iron, ROS generation, and toxicity between different silica samples, found that silica-induced toxicity and iron contamination were not correlated (Donaldson et al. 2001).

Similar to the animal study results, human pneumocytes isolated from silica-exposed subjects exhibit increased ROS production. Specifically, human AMs, obtained from patients with silicosis, a disease caused by inhalation of silica, have increased production of superoxide (Rom et al. 1987; Wallaert et al. 1990), hydrogen peroxide (Rom et al. 1987), and AM chemiluminescence (Goodman et al. 1992; Castranova et al. 1998), in comparison to healthy controls.

6.4.2 Coal Dust

AMs obtained from rats 24 h after intratracheal (IT) instillation of coal dust have significantly increased ROS generation, as measured by zymosan-stimulated chemiluminescence (Blackford et al. 1997). In another study, rats exposed by inhalation to 2 mg/m^3 coal dust (6 h/day) for two years also had significantly increased chemiluminescence (Castranova et al. 1985). AMs obtained from human patients with CWP have increased AM chemiluminescence (Goodman et al. 1992; Castranova et al. 1998) and superoxide production (Rom et al. 1987; Wallaert et al. 1990) in comparison to healthy controls.

6.4.3 Asbestos

Oxidant release, specifically hydrogen peroxide and superoxide, have been determined to occur after *in vitro* exposure of alveolar and peritoneal macrophages to asbestos (Donaldson et al. 1985; Hansen and Mossman 1987; Petruska et al. 1990). Comparison of the ability long and short crocidolite fibers to stimulate the release of hydrogen peroxide and induce cytotoxicity found no differences (Goodglick and Kane 1990). However, with respect to superoxide production, fibrous asbestos (length:diameter ratio greater than 3:1) caused a significant increase in superoxide release from rat AMs in comparison to non-fibrous dusts, suggesting the geometry of the particles does effect superoxide generation (Hansen and Mossman 1987). In an earlier study (Goodglick and Kane 1986), mouse peritoneal macrophages exposed to crocidolite asbestos *in vitro* were found to release ROS and experience increased cytotoxicity. This crocidolite-induced cytotoxicity was prevented by incubation in a hypoxic environment, by addition of superoxide dismutases (SOD) and catalase, or if the crocidolite fibers were pretreated with deferoxamine, suggesting that oxygen and asbestos-associated iron play a role in asbestos-induced ROS and cytotoxicity.

In vivo exposure to asbestos has been shown to enhance the capacity of lung inflammatory cells to release oxidants. Bronchoalveolar lavage (BAL) cells obtained from sheep exposed to chrysotile asbestos did not have an increased basal level of superoxide production, but did release significantly

higher amounts when stimulated with phorbol myristate acetate, in compassion to BAL cells obtained from controls (Cantin, Dubois, and Begin 1988). Chemiluminescence, measured from peritoneal macrophages obtained from asbestos-exposed and control mice, demonstrated that chemiluminescence was higher for asbestos-exposed mice (Donaldson and Cullen 1984). ROS production from human AMs, obtained from patients with asbestosis, a disease linked to asbestos exposure, and healthy controls, has also been studied. AMs obtained from asbestosis patients had increased release of superoxide and hydrogen peroxide, in comparison to healthy controls (Rom et al. 1987). Thus, the *in vitro* data and animal studies are consistent with the results obtained from humans, suggesting a role of asbestos-induced ROS in disease initiation and progression.

6.4.4 Other Particles

In vitro exposure of RAW 264.7 macrophages to lead chromate ($PbCrO_4$) particles has been reported to cause a respiratory burst and increase hydrogen peroxide production by 7-fold. This ROS production has been associated with activation of NF-κB and activator protein-1 (AP-1) (Leonard et al. 2004).

In vivo exposure of rats to a variety of environmentally or occupationally relevant particles has been reported to potentiate the production of ROS by AM, as measured by stimulant-induced chemiluminescence (Table 6.1). In general, the potency of a particle to stimulate ROS production has been associated with its inflammatory potential. For example, MMA/SS electrode welding fume has been shown to generate more ROS than GMA/MS or SS welding fumes and cause greater lung damage (BAL fluid LDH and albumin) and oxidant injury (lung lipid peroxidation), respectively (Taylor et al. 2003). In addition, oxidant stress was reported in welders as increased serum isoprostane and antioxidant levels, with the degree of oxidant stress being associated with years of welding in a shipyard (Han et al. 2005).

TABLE 6.1
Stimulation of ROS Production by Alveolar Macrophages Harvested from Particle Exposed Rats

Particle	Exposure	Chemiluminescence (Increase From Control)	Reference
Diesel exhaust	IT (5 mg/kg BW); 3 days post	2.3-Fold zymosan-stimulated	Yang et al. (2001)
Carbon black	IT (5 mg/kg BW); 3 days post	2.3-Fold zymosan-stimulated	Yang et al. (2001)
Titanium dioxide	IT (5 mg/100 g BW); 1 day post	3.0-Fold zymosan-stimulated	Blackford et al. (1997)
Carbonyl iron	IT (5 mg/100 g BW); 1 days post	1.6-Fold zymosan-stimulated	Blackford et al. (1997)
Residual oil fly ash (ROFA)	IT (1 mg/100 g BW); 1 days post	9–11-Fold PMA-stimulated	Antonini et al. (2004), Lewis et al. (2003)
Residual oil fly ash (ROFA)	IT (2 mg/rat); 1 days post	3.0-Fold zymosan-stimulated	Nurkiewicz et al. (2004)
Welding fume (manual metal arc/stainless sleet electrode)	IT (5 mg/rat BW); 3 days post	2.5-Fold zymosan-stimulated	Antonini et al. (2004)

6.5 CELLULAR RNS GENERATION

NO synthase catalyzes the formation of NO˙ using L-arginine as a substrate. Three isoforms of NO synthase, two of which are constitutively expressed and one which is inducible, have been described. The inducible isoform of nitric oxide synthase (NOS), commonly referred to as inducible nitric oxide synthase (iNOS or NOS2), is the isoform important with respect to particle-induced toxicity because its expression can be induced in various pneumocytes by particle exposure.

NO˙ is a free radical, but despite this, it is not particularly toxic (Beckman and Koppenol 1996). However, the conditions that stimulate pneumocyte NO˙ production from iNOS (i.e., particle exposure), also stimulate ROS production by many of these same cells. One of these forms of ROS, superoxide, can react in a rapid isostoichiometric reaction with NO˙, forming peroxynitrite in a near-diffusion-limited reaction (Beckman and Koppenol 1996). Peroxynitrite is a potent oxidant, reacting with and disrupting the normal functions of proteins via nitrosation of tyrosine residues (Beckman 1996; Beckman and Koppenol 1996; van der Vliet and Cross 2000), and has also been associated with enhanced lipid peroxidation and DNA damage (Rubbo et al. 1994; Eiserich, Patel, and O'Donnell 1998; Hofseth et al. 2003).

6.6 PARTICLE-MEDIATED CELLULAR RNS GENERATION

6.6.1 Silica

The mouse macrophage cell line, IC-21, when exposed to silica *in vitro*, has a 12-fold increase in NO˙ production at 4 h post-exposure (Srivastava et al. 2002). In contrast, rat primary AMs exposed to silica *in vitro* do not exhibit increased production of NO˙ (Huffman, Judy, and Castranova 1998; Kanj, Kang, and Castranova 2005). However, naïve primary rat AM, when cultured in media previously conditioned by BAL cells obtained from silica-exposed rats, do produce NO˙ in response to *in vitro* silica exposure, suggesting that extracellular mediators are critical to the induction of iNOS (Huffman, Judy, and Castranova 1998). Neither primary alveolar type II cells, nor the rat type II cell line RLE-6TN, releases NO˙ after *in vitro* exposure to silica (Kanj, Kang, and Castranova 2005).

Many studies have been conducted that report that *in vivo* silica exposure results in increased NO˙ production from various lung cells. Silica administered by IT instillation to rats results in 3-fold increase in mRNA for iNOS and a 5-fold increase in NO˙ production from BAL cells 24 h after exposure (Blackford et al. 1994; Huffman, Judy, and Castranova 1998). A silica time course inhalation study reported that the BAL fluid level of NO products, nitrite and nitrate (NO_x), was elevated 1.8-fold after 10 days of exposure, while NO-dependent chemiluminescence was elevated 15-fold at this exposure time, and these levels remained relatively constant throughout the first 41 days of exposure (Porter et al. 2002b). Continued exposure after 41 days inhalation resulted in a rapid rise in NO˙ production (i.e., BAL fluid NO_x levels increased 22-fold and NO-dependent chemiluminescence 151-fold) after 116 days of silica exposure (Porter et al. 2002b). Immunohistochemical evidence of iNOS induction in AMs and alveolar type II epithelial cells suggested these cells were the source of the NO˙ production (Porter et al. 2002b). There was a temporal and spatial relationship between induction of NO˙ production and pulmonary inflammation, in this study. Consistent with these observations was the determination that iNOS expression was induced in AMs in response to silica inhalation and that silica- induced pathology was significantly decreased in iNOS knockout mice (Srivastava et al. 2002).

Increased NO˙ production has also been reported in humans with silica-induced lung disease. Specifically, iNOS mRNA levels and NO˙ production from BAL cells were determined from a silica-exposed coal miner with an abnormal chest x-ray, a silica-exposed coal miner with a normal chest x-ray, and an unexposed control. iNOS mRNA from BAL cells isolated from the two coal miners demonstrated that both were higher than the unexposed control, and that the miner with the abnormal chest x-ray had more iNOS mRNA than that from the miner with a normal chest x-ray

(Castranova et al. 1998). AM NO˙ production was measured by NO-dependent chemiluminescence, and in comparison to the unexposed control, the coal miners with normal and abnormal chest x-rays had 15- and 31-fold higher NO-dependent chemiluminescence, respectively (Castranova et al. 1998).

6.6.2 Coal Dust

Rat BAL cells, obtained 24 h after IT instillation of coal dust, express iNOS and have increased NO˙ production, measured as NO-dependent chemiluminescence, in comparison to saline-exposed controls (Blackford et al. 1997).

6.6.3 Asbestos

Exposure of rat AMs and the mouse peritoneal monocyte-macrophage cell line, RAW 264.7, to crocidolite induces activation of the iNOS promoter gene and transcription of iNOS mRNA (Quinlan et al. 1998). The mouse AM cell line MH-S, when exposed to crocidolite, exhibited a 4-fold increase in NOS activity and NO˙ production at 24 h post-exposure (Aldieri et al. 2001). Increased mRNA levels for iNOS and NO˙ production have also been reported for A549 cells, a human alveolar type II cell line, in response to asbestos (Chao, Park, and Aust 1996). *In vitro* exposure of rat AMs to asbestos fibers results in a significant increase in NO˙ production 48 h after exposure, with chrysotile being a more potent stimulant than crocidolite on an equal mass basis (Thomas et al. 1994).

IT instillation of rats with asbestos has been shown to increase NOS activity of lung tissue 48 h post-IT exposure (Iguchi, Kojo, and Ikeda 1996). In mice, IT instillation of crocidolite causes induction of iNOS mRNA in lung tissue and increased immunohistochemical staining for iNOS protein and nitrotyrosine residues in bronchial epithelial cells, alveolar epithelial cells, and AMs (Dorger et al. 2002a). In rats, 24 h after IT instillation of crocidolite, increased iNOS mRNA and protein have been observed in lung tissue, as well as positive staining for iNOS and nitrotyrosine in AMs and alveolar epithelial cells (Dorger et al. 2002b). Inhalation exposure of rats to crocidolite or chrysotile asbestos results in a more than 2-fold increase in NO˙ production from AMs, and was temporally correlated with pulmonary inflammation (Quinlan et al. 1998).

6.6.4 Other Particles

Exposure of rats by IT instillation to fine titanium dioxide or carbonyl iron significantly increased NO-dependent chemiluminescence from harvested AMs 24 h post-exposure (Blackford et al. 1997). However, as with inflammatory potency, these nuisance dusts were significantly less stimulatory than silica or coal dust. Intratracheal instillation of rats with diesel exhaust particles or ultrafine carbon black also caused a small (2-fold increase) but significant increase in NO production by AMs (Yang et al. 2001). Exposure of rats to MMA/SS electrode welding fumes resulted in a 3-fold increase in nitrate/nitrite levels in bronchoalveolar lavage fluid three days after IT instillation (Antonini et al. 2004). Additionally, iNOS protein was found by immunohistochemical staining of the lung to be associated anatomically with areas of welding fume-induced inflammation. A recent study also reported that NO-dependent chemiluminescence from AMs was elevated 6.8-fold 24 h after IT instillation of ROFA (Nurkiewicz et al. 2004).

6.7 PARTICLE-INDUCED ACTIVATION OF NUCLEAR FACTOR-κB

6.7.1 ROS and RNS Regulation of Nuclear Factor-κB

NF-κB is a transcription factor found in many different cell types, and functions in the molecular signaling between the cytoplasm and nucleus. In resting cells, NF-κB is retained in the cytoplasm in

an inactive form by binding to inhibitory proteins IκBα and IκBβ. Upon activation by extracellular stimuli, these inhibitory proteins dissociate from the complex, and NF-κB translocates to the nucleus in an activated form. Once in the nucleus, activated NF-κB binds to specific binding sequences found in the promoter region of many genes, contributing to the regulation of these genes. Specifically, with respect to particles and cellular oxidative stress, NF-κB regulates a variety of genes involved in inflammatory or acute phase responses, including several proinflammatory or fibrogenic cytokines (i.e., IL-1, IL-2, IL-6, and TNF-α).

It is well established by numerous studies, reviewed elsewhere, that cellular oxidative stress induced by ROS can cause activation of NF-κB (Kabe et al. 2005). In contrast, the effect of $NO^{\cdot}$ on NF-κB activation is controversial. Studies using the RAW 264.7 cells have indicated that exogenous $NO^{\cdot}$ inhibits NF-κB activation (Chen et al. 1995), whereas a later study demonstrated that exogenous $NO^{\cdot}$ can stimulate NF-κB activation (Kang et al. 2000a). The contradictory results of these studies may reflect differences in $NO^{\cdot}$ concentrations, the duration of $NO^{\cdot}$ exposure, and/or the basal activity of macrophages (Kang et al. 2000a), since these variables have been shown to alter the effect of $NO^{\cdot}$ on NF-κB activation in other studies (Umansky et al. 1998; Diaz-Cazorla, Perez-Sala, and Lamas 1999).

6.7.2 Particle-Induced Activation of NF-κB

Particle-induced activation of NF-κB has been demonstrated in *in vitro* and *in vivo* studies. Exposure of RAW 264.7 cells to silica *in vitro* results in activation of NF-κB (Chen et al. 1995). Furthermore, NF-κB activation is stimulated in rat primary AM exposed to silica *in vitro*, and ROS scavengers and iNOS inhibitors reduced this NF-κB activation (Kang et al. 2000a; Kang, Lee, and Castranova 2000b). BAL cells isolated from rats after IT instillation of silica demonstrated NF-κB activation, which was decreased by pre-treatment with the anti-inflammatory agent dexamethasone (Sacks et al. 1998). In a silica inhalation study, progressive increase in NF-κB activation during a 116-day time course study was demonstrated (Porter et al. 2002c). Crocidolite fibers have also been shown to activate NF-κB in hamster tracheal epithelia cells (Janssen et al. 1995a). Fiberglass has been reported to activate NF-κB in cultured AMs (Ye et al. 1999). This NF-κB activation, and the resultant TNF-α production, were shown to depend on fiber length and be inhibited by antioxidant treatment. Exposure of macrophages to lead chromate particles has also been reported to activate NF-κB in a ROS-dependent manner (Leonard et al. 2004).

6.7.3 Particle-Induced Activation of AP-1

AP-1 is a transcription factor composed of homodimers and/or heterodimers of Jun (*c*-Jun, Jun B, and Jun D) and Fos (*c*-Fos, Fos B, Fra-1, Fra-2, and FosB2) gene families (Angel and Karin 1991). It interacts with DNA sequences known as TPA response elements, or AP-1 sites that govern inflammation, proliferation, and apoptosis. AP-1 activation is controlled by MAPK (Bernstein et al. 1994). ROS have been shown to act as MAPK activators.

Crystalline silica has been reported to induce the phosphorylation of MAPK, specifically p38 and extracellular signal regulated protein kinases (ERK1 and ERK 2), in an epidermal cell line (Ding et al. 1999). This MAPK activation was associated with activation of AP-1 in cultured cells as well as in AP-1 luciferase reporter transgenic mice. This silica-induced AP-1 activation was mediated through ROS (Ding et al. 2001). Asbestos has been shown to elevate expression of *c*-Fos and *c*-Jun in mesothelial cells (Janssen, Heintz, and Mossman 1995b). Asbestos activated ERKs in cell culture systems via an ROS-dependent mechanism (Ding et al. 1999; Buder-Hoffmann et al. 2001). This asbestos-induced MAPK activation resulted in activation of AP-1 both *in vitro* and in lung and bronchiolar tissue from AP-1 luciferase transgenic mice (Ding et al. 1999). ERK and p38 MAPK and AP-1 activation have also been demonstrated in macrophages in response to

in vitro treatment with glass fibers (Ye et al. 2001). Likewise, lead chromate particles have been shown to activate AP-1 in macrophages via an ROS-dependent mechanisms (Leonard et al. 2004).

6.8 PARTICLE-INDUCED APOPTOSIS

The molecular mechanisms regulating apoptosis have been reviewed elsewhere (Granville et al. 1998; Green and Reed 1998), but it is clear that ROS generation and/or alteration in cellular redox state may stimulate apoptosis. Since many particulates induce ROS and RNS, and thus disturb the cellular redox status, they might be expected to induce apoptosis.

In the normal rat lung, apoptosis is very low, but asbestos exposure causes a significant increase in apoptosis in bronchiolar and alveolar epithelial cells (Mossman and Churg 1998). ROS has been suggested to play a role in asbestos-induced apoptosis, because asbestos-induced apoptosis can be ameliorated by exogenous antioxidants, catalase, superoxide dismutase, and deferoxamine (BéruBé et al. 1996; Broaddus et al. 1996). Asbestos-induced apoptosis is observed in human cell systems, as well as in rats; indeed chrysotile and crocidolite have been reported to cause apoptosis in human AMs 48 h after exposure (Hamilton, Iyer, and Holian 1996).

Apoptosis has been observed in BAL cells isolated from rats 10 days after IT instillation of silica (Leigh et al. 1997). At two months post-IT exposure, apoptotic cells were present in granulomatous lesions (Leigh et al. 1997). A silica inhalation time course study, conducted using rats, also reported that apoptotic cells were located in the airspaces and increased significantly in number as silica lung burden increased during a 116-day exposure (Porter et al. 2002b). *In vitro* exposure of human AMs to crystalline silica for 6 h or 24 h induces apoptosis, whereas exposures to amorphous silica or titanium dioxide did not; these observations suggest the human AM apoptotic response appears to be specific to crystalline silica (Iyer et al. 1996). Incubation of human AMs with a caspase inhibitor (Z-VAD-FMK) prevented silica-induced apoptosis, indicating silica induces apoptosis via activation of the caspase system (Iyer et al. 1996).

Generation of ROS appears to play a role in silica-induced apoptosis. The role of ROS was established in an *in vitro* study using silica exposed rat AMs. The determination that ROS generation preceded caspase activation and subsequent apoptosis suggested ROS generation was an initiating step (Shen et al. 2001). The antioxidant ebselen prevented silica-induced apoptosis in this *in vitro* system, thus adding support to the role of ROS in initiating apoptosis.

Another mechanism that may participate in silica-induced apoptosis is RNS generation. The mouse macrophage cell line IC21, when exposed to crystalline silica *in vitro*, exhibited increased apoptosis (Srivastava et al. 2002). However, silica-induced apoptosis can be prevented by the NOS inhibitor N(G)-nitro-L-arginine-methyl ester, suggesting that $NO^{\cdot}$ also contributes to the initiation of apoptosis in silica-exposed AMs (Srivastava et al. 2002). To confirm this observation *in vivo*, wild-type and iNOS knockout mice were exposed to either air (controls) or silica by inhalation exposure (Srivastava et al. 2002). Silica-exposed wild-type mice had much more apoptosis in comparison air-exposed wild-type mice, indicating silica did induce apoptosis. Comparison of the silica-exposed wild-type mice and iNOS knockout mice demonstrated that iNOS knockout mice had much less apoptosis. These results indicate that silica induces apoptosis, and $NO^{\cdot}$ participated in initiating apoptosis induced by silica exposure.

The role of apoptosis in the pathogenesis of silicosis is not without controversy. Since apoptosis, unlike necrosis, is cell death without the release of pro-inflammatory stimuli and apoptotic bodies are rapidly phagocytized, it is considered to play a role in the resolution of inflammation (Savill et al. 1993). However, free apoptotic bodies have been observed in rat lungs after a threshold burden of silica was achieved. Evidence indicated that such free apoptotic bodies induce production of TNF-α and TGF-β, causing inflammation, and lead to pulmonary fibrosis (Wang et al. 2003).

6.9 ANTIOXIDANT DEFENSES AND PARTICULATE EXPOSURE

6.9.1 Antioxidant Defenses

The respiratory system has numerous nonenzymatic and enzymatic antioxidant defense systems which are present both in intracellular and extracellular compartments. These systems will be discussed here briefly, but have been extensively reviewed elsewhere (Heffner and Kensler 1989). Enzymatic defense mechanisms include SOD, catalase, glutathione peroxidase, and heme oxygenase. SOD catalyzes the reaction of superoxide to hydrogen peroxide. There are three types of SODs in the lung: extracellular superoxide dismutases (ECSOD), Mn-SOD, and Cu,Zn-SOD. These enzymes are located in the epithelial lining fluid and interstitial space in the mitochondria, and in the cytosol and peroxisomes, respectively. Catalase and glutathione peroxidase participate in scavenging hydrogen peroxide. Catalase detoxifies hydrogen peroxide by converting it to oxygen and water, and is located primarily in peroxisomes. Glutathione peroxidase is part of the glutathione oxidation–reduction system, which also includes glutathione reductase and glucose-6-phosphate dehydrogenase. The enzyme heme oxygenase is also believed to be an important cell-associated antioxidant in the lung and other tissues. The primary function of this enzyme is the degradation of heme-containing proteins, which is thought to prevent iron-mediated hydroxyl radical production. The epithelial lining and interstitial fluid, representing the extracellular compartment of the lung, contains many low-molecular-weight antioxidants. These include α-tocopherol (vitamin E), uric acid, glutathione, and ascorbic acid, all of which function by scavenging free radicals.

6.9.2 Particulate Exposure Induces Antioxidant Defenses

As previously discussed in this chapter, particulate exposure results in increased ROS and RNS production, and this in turn contributes to particulate-induced oxidative stress. Antioxidant defenses can respond to particulate-induced cellular oxidative stress in an attempt to limit the oxidative damage.

The effect of *in vitro* particulate exposure on glutathione levels is unclear. In one study, *in vitro* exposure of rat AMs to silica or crocidolite resulted in an increase in glutathione production and release (Boehme, Maples, and Henderson 1992), whereas asbestos exposure of rat pleural mesothelial cells caused a depletion of total cellular glutathione (Janssen, Heintz, and Mossman 1995b).

Effects of particulate exposure on enzymatic antioxidant defenses have been more consistent. Crocidolite- and chrysotile-exposed hamster tracheal epithelial cells have increased SOD activity (Mossman, Marsh, and Shatos 1986). This up-regulation of SOD appears to be a specific response, because non-pathogenic glass fibers did not cause increased SOD activity in tracheal epithelial cells (Shatos et al. 1987). Small increases in heme oxygenase and Mn-SOD activities have been reported for crocidolite- and chrysotile-exposed human mesothelial cells and human adult lung fibroblasts (Janssen et al. 1994). This response also appears to be particle-specific, because expression of MnSOD or heme oxygenase did not occur after exposure to polystyrene beads or riebeckite, a nonfibrous analog of crocidolite (Janssen et al. 1994).

In vivo studies in rodents have suggested that SOD is produced in proportion to the oxidant stress present in the lung, and this has been suggested to be a defensive response (Vallyathan et al. 1995). This hypothesis was supported by data obtained from a rat silica inhalation time course study. In this study, BAL fluid SOD increased steadily throughout the 116 days of exposure, and paralleled increases in lung lipid peroxidation levels, a marker of oxidative stress (Porter et al. 2002b). Asbestos exposure has also been shown to alter enzymatic antioxidant levels. Specifically, rats exposed to asbestos have increased mRNA and enzymatic activity for Mn-SOD, Cu,Zn-SOD, and glutathione peroxidase (Janssen et al. 1992).

Changes in antioxidant defenses in response to particle-exposure have also been determined to occur in humans. PMA-induced ROS production and also increased SOD and Mn-SOD levels in AMs obtained from subjects with CWP (Voisin et al. 1985). Coal miners with early stage CWP

have decreased total glutathione levels in erythrocytes, but levels are increased in those with more severe fibrosis, suggesting an up-regulation of glutathione-dependent enzymes in late stage CWP (Engelen et al. 1990; Perrin-Nadif et al. 1998). A longitudinal study, which evaluated disease progression and antioxidant status, reported that SOD activity increased with progression of CWP (Schins, Keman, and Borm 1997). In addition, this study found that individuals with increased levels of nonenzymatic antioxidants had less risk, while individuals with elevated enzymatic antioxidants were at higher risk of developing CWP (Schins, Keman, and Borm 1997). Another study (Vallyathan et al. 2000) reported that bronchoalveolar lavage levels of catalase, superoxide dismutase, and glutathione peroxidase were unchanged in asymptomatic coal miners, but increased in miners with simple CWP. This induction in antioxidant levels was associated with oxidative stress (lipid peroxidation) in these symptomatic miners. Lastly, elevated serum antioxidant levels have been associated with oxidant stress (i.e., increased isoprostanes), in shipyard welders (Han et al. 2005).

6.10 SUMMARY

Oxidant stress has been suggested as a causative factor in the initiation and progression of pneumoconiosis (Castranova 2000). Under normal physiological conditions, an equilibrium exists between ROS and RNS generation and antioxidant defenses. However, the inhalation of particles can induce oxidant stress in the lung, disrupting the equilibrium existing between ROS and RNS generation and antioxidant defenses. Specifically, at high particle burdens, the oxidant–antioxidant balance shifts to an excess of oxidant production, causing oxidant injury and initiating the disease process.

As reviewed in this chapter, particle exposure can induce oxidant stress by two distinct mechanisms: (1) noncellular particle-mediated ROS generation, and (2) particle-mediated cellular ROS and RNS generation. Non-cellular ROS generation results from, or is enhanced by, Fenton-like reaction(s) involving iron or other transition metals. Particle-mediated cellular ROS and RNS generation results from the stimulation of lung cells, in particular AMs, PMNs, and type II cells. These cells produce ROS via NADPH oxidase, as well as from the mitochondrial electron transport chain. In addition, particle-exposure can induce the expression of the enzyme iNOS, which results in these cells producing nitric oxide, a form of RNS. Both ROS and RNS can induce radical-specific cell damage, such as lipid peroxidation and nitration of tyrosine residues in proteins. Additionally, oxidant stress activates transcription factors (i.e., NF-κB and AP-1), which contribute to the transcriptional regulation of genes for inflammatory chemokines and cytokines, as well as growth and fibrogenic factors. Oxidant stress also can induce apoptosis, which at high dust burdens may reach levels which exceed the ability of AMs to clear apoptotic bodies. Free apoptotic bodies may further enhance particle-induced inflammatory and fibrotic processes.

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