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Review

Particulate air pollutants and asthma A paradigm for the role of oxidative stress in PM-induced adverse health effects

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Abstract

Asthma is a chronic inflammatory disease, which involves a variety of different mediators, including reactive oxygen species. There is growing awareness that particulate pollutants act as adjuvants during allergic sensitization and can also induce acute asthma exacerbations. In this communication we review the role of oxidative stress in asthma, with an emphasis on the pro-oxidative effects of diesel exhaust particles and their chemicals in the respiratory tract. We review the biology of oxidative stress, including protective and injurious effects that explain the impact of particulate matter-induced oxidative stress in asthma.
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Keywords: Antioxidant enzyme; Asthma exacerbation; Diesel exhaust particles; Inflammation; Particulate matter; Polycyclic aromatic hydrocarbons; Quinone; Reactive oxygen species; Redox cycling; Stratified oxidative stress response

1. Introduction

Asthma is a chronic inflammatory disease that involves Th2 lymphocytes, IgE-secreting plasma cells, mast cells, eosinophils, neutrophils, mucus-secreting goblet cells, and smooth muscle and endothelial cells. While it is well recognized that proinflammatory cytokines, chemokines, as well as mast cell and eosinophil mediators play a role in the allergic inflammatory process, the key role of reactive oxygen species (ROS[1]) is often overlooked. This disease aspect is receiving more attention with the growing awareness that particulate pollutants, which are potent inducers of oxidative stress, can impact allergic inflammation and induce acute asthma exacerbations. The purpose of this communication is to review the importance of oxidative stress in asthma, with particular emphasis on the role of particulate matter (PM). We will use diesel exhaust particles (DEP) as a PM model, in which the generation of ROS leads to a hierarchical oxidative stress response that includes both cytoprotective as well as cytotoxic effects. We will discuss the role of organic DEP chemicals, including polycyclic aromatic hydrocarbons (PAH) and redox cycling quinones, in ROS generation and will highlight the role of intracellular signaling pathways in the initiation of cellular responses in epithelial cells and macrophages. We will discuss the possibility that a weakened antioxidant defense may determine susceptibility to the adverse health effects of PM, and how

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[1] *Abbreviations:* AHR, airway hyperreactivity; ARE, antioxidant responsive element; CAPs, concentrated ambient particles; DEP, diesel exhaust particles; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione-*S*-transferase; HO-1, heme oxygenase-1; NQO1, NADPH quinone oxidoreductase 1; $O_2^{\cdot-}$, superoxide; $OH^{\cdot}$, hydroxyl radical; PAH, polycyclic aromatic hydrocarbons; PM, particulate matter; ROS, reactive oxygen species; SOD, superoxide dismutase.

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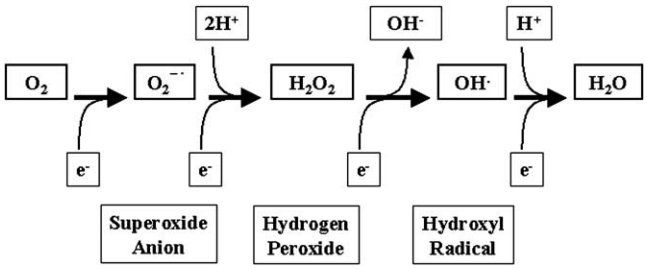

Fig. 1. ROS generation during oxidative phosphorylation. In this process, O_2 receives 4 electrons to form H_2O. Occasional 1-electron additions result in the formation of $O_2^{\cdot-}$, which can be converted to H_2O_2 or $OH^{\cdot}$.

this information could be used to further investigate the role of oxidative stress in asthma.

2. Reactive oxygen species and oxidative stress

The cellular biochemistry of dioxygen (O_2) is earmarked by good and bad sides [1]. The good side includes the numerous enzyme-catalyzed O_2 reactions, which are essential for life and normal cellular function, while the bad side includes the possibility that reactive O_2 species may exert deleterious effects. The major role of O_2 in normal metabolism is oxidative phosphorylation, an event that takes place in the mitochondrion and is responsible for ATP production. Oxidative phosphorylation is dependent on oxygen as an electron acceptor, which under normal coupling conditions requires a four-electron addition to form H_2O (Fig. 1). The addition of a single electron results in the formation of superoxide ($O_2^{\cdot-}$) radical, while the capture of two or three electrons leads to the formation of hydrogen peroxide (H_2O_2), or hydroxyl radical ($OH^{\cdot}$), respectively (Fig. 1). Under normal coupling conditions, these ROS are generated at low frequency. This is fortunate because these oxygen species are very prone to react with proteins, lipids, and DNA, leading to cellular damage [1,2]. Other types of ROS include singlet oxygen ($O^{\cdot}$), reactive anions that contain oxygen atoms (e.g., $OCOO^-$, peroxynitrite), molecules containing oxygen atoms that can produce free radicals (e.g., HOCl), and ozone. "Free radical" refers to molecules with at least one unpaired electron; examples include the $OH^{\cdot}$ radical, $O_2^{\cdot-}$, and NO. Among these, $OH^{\cdot}$ is the most reactive species. Since H_2O_2 has paired electrons it is not considered a free radical, but is included under the rubrick, ROS.

In addition to being produced in mitochondria, $O_2^{\cdot-}$ is generated by cytochrome P450 reductase in the endoplasmic reticulum, reduced NADPH oxidase in the membrane of phagocytic cells, and xanthine oxidase in the cytosol [1]. H_2O_2 is also formed during the dismutation of $O_2^{\cdot-}$ by superoxide dismutatase (SOD) as well as by glycolate oxidase in peroxisomes. $OH^{\cdot}$ radicals are generated by the Fenton reaction, which requires the presence of transition metals (such as iron) and H_2O_2 [1].

To maintain cellular redox equilibrium, the potentially injurious effects of ROS and oxygen radicals are neutralized by a variety of antioxidants. This includes several antioxidant enzymes, which are reviewed in section 8. Under conditions of abundant ROS production, such as may occur during asthma and PM exposure, the antioxidant defenses may be overwhelmed, leading to a state of cellular oxidative stress [3–10]. Oxidative stress is defined as a depletion of reduced glutathione (GSH) in exchange for a rise in oxidized glutathione (GSSG), leading to a drop in the intracellular GSH/GSSG ratio [7]. Cells respond to this disequilibrium by mounting protective or injurious responses. That aspect will be discussed in section 6.

3. The importance of oxidative stress in asthma

Among the participating cell types that play a role in chronic airway inflammation, macrophages, neutrophils, eosinophils, and epithelial cells are capable of ROS generation [8–13] (Table 1). H_2O_2, NO, CO, as well as 8-isoprostane release in expired breath air are noninvasive markers for oxidative stress, which correlate with the extent of airway inflammation in asthmatics [10,14–19] (Table 1). While it may be argued that ROS production is the consequence of airway inflammation, there is good evidence that ROS play an active role in the genesis of pulmonary inflammation [4,20–22]. $O_2^{\cdot-}$ generation has been demonstrated at sites of allergen challenge in the human lung [20]. These studies were duplicated in large animals (e.g., sheep and dogs), where it was demonstrated that oxygen radicals contribute to antigen-induced airway hyperreactivity (AHR) [21–23]. Moreover, oxidative damage to the airway epithelium produces AHR in humans [23]. This is further supported by the demonstration of increased peroxidation [14] and nitrotyrosine products in the lungs of asthmatic subjects [24]. Increased peroxidation products, including 8-iso-PGF2α,

Table 1
Evidence for the role of oxidative stress in asthma

1. Increased $O_2^{\cdot-}$ and H_2O_2 production by MNC, neutrophils, and eosinophils in asthmatic subjects [6–11] correlates with methacholine-induced AHR [8–13,26,28].
2. Increased ROS, NO, CO, 8-isoprostane, and ethane levels are noninvasive markers for oxidative stress/airway inflammation in asthmatics [16–19].
3. $O_2^{\cdot-}$ generated at sites of allergen challenge [20–22].
4. Increased peroxidation and nitrotyrosine products in the lungs of asthmatic subjects [14,24].
5. Oxidative damage to airway epithelial cells correlates with AHR in human and animals [20,22,31].
6. Increased peroxidation products in the urine and blood of asthmatic, e.g., 8-iso-PGF2α [25].
7. Increased extracellular glutathione peroxidase and SOD expression in the lungs of asthmatic subjects [32–34].
8. Decreased nonenzymatic antioxidants (e.g., ascorbate and α-tocopherol) in the lung lining fluid [35].

Table 2
Evidence for the role of DEP as an adjuvant for allergic inflammation

A. Animals (mice and rats)
1. ↑ Total IgE and specific IgE [3,40–43].
2. ↑ Th2 cytokines (IL-4, IL-5) and GM-CSF [47–49].
3. ↑ Airway eosinophilic inflammation, goblet cell hyperplasia [41, 42].
4. ↑ AHR and combination with ↑ eosinophilic inflammation [42,43, 50,51].

B. Humans (nasal challenge studies)
1. ↑ Total IgE, specific IgE [44,52,53].
2. ↑ IgE isotype switching in B cells [45,52].
3. ↑ C-C chemokines (RANTES, MCP-3, MIP-1α) [54].
4. ↑ Th2-like cytokine profile and basophils mediated IL-4, 8, histamine release [44,55].
5. ↑ IgE response to a neoallergen, KLH [56].

Table 3
Evidence for the role of ROS and oxidative stress in DEP-induced biological effects

A. In vitro studies
1. Tissue culture macrophage and bronchial epithelial cells generate ROS during exposure to DEP, DEP extracts, ambient PM [57–60].
2. Intracellular GSH depletion in macrophage and epithelial cells during exposure to DEP and DEP extracts [57,59,63]
3. Coincubation of lung microsomes with organic DEP extracts leads to $O_2^{\cdot-}$ production [61,62].

B. In vivo studies (animals)
1. ↑ NO and CO production in mice [64,65].
2. Suppression of proinflammatory effects of DEP by iNOS inhibitors or SOD [65,66].
3. Suppression of the adjuvant effects of DEP with thiol antioxidants [67].
4. In vivo chemiluminescence of the heart and lungs in rat exposed to CAPs [68].

C. In vivo studies (humans)
1. ↑ CO production in normal volunteers [10,73].
2. ↑ Ascorbic acid in nasal cavity lining fluid in normal volunteers [74].

can also be detected in the blood and urine of asthmatics [25]. Neutrophils and mononuclear cells from asthmatic patients generate proportionately more $O_2^{\cdot-}$ and H_2O_2 than cells of matched healthy subjects; this activity correlates with methacholine-induced AHR [8,26–29]. Another group demonstrated increased peroxynitrite formation in association with nitric oxide synthase (iNOS) overexpression in asthmatic airways [14]. NO is elevated in the exhaled air, and is a noninvasive marker for lower airway inflammation in asthmatics [7,14,15]. In addition to NO, there is also an increase in CO, which is positively correlated with elevated eosinophil counts in the sputum of asthmatics [18,30]. The principal source of CO in the lung is heme oxygenase-1 (HO-1), which is induced by oxidative stress and plays a role in catabolizing heme to Fe^{2+}, biliverdin, and CO [31]. The final evidence for the importance of oxidative stress is the change in antioxidant defense pathways in asthmatics. This aspect will be further discussed in section 4. Suffice to mention here that asthmatics have altered levels of antioxidant enzymes in the lung [31–34] (Table 1), as well as a decrease in ascorbate and α-tocopherol levels in lung lining fluid [35].

4. Evidence that PM can elicit asthma exacerbation due to an effect on oxidative stress

There is growing epidemiological evidence that increased cardiorespiratory morbidity and mortality follow a sudden surge in ambient PM levels [36,37]. The acute respiratory events include acute asthma exacerbations, as reflected by increased symptom score as well as increased use of medication and hospitalization [38,39]. In addition to these acute effects, there is evidence that DEP act as an adjuvant for allergic sensitization to common environmental allergens [3,40–56] (Table 2). This includes the enhancement of already existing allergies as well as enhancement of IgE responses to a neoallergen, e.g., keyhole limpet hemocyanin, delivered by nasal challenge in humans [44,56]. This raises the possibility that long-term PM exposures may lead to increased prevalence of asthma and allergic diseases. This is compatible with the increased prevalence of asthma in polluted urban environments [46].

DEP is a model particulate pollutant, which has been used to elucidate the mechanisms by which PM generates adverse health effects [3]. There is accumulating evidence that oxidative stress plays a role in the proinflammatory and adjuvant effects of these particles [57–68] (Table 3). The first line of evidence is that tissue culture macrophages and bronchial epithelial cells, the principal targets of PM in the lung, generate ROS upon the addition of DEP or organic DEP extracts [57–59] (Table 3). Similar observations have been made for ambient PM collected by particle concentrators [60] (Table 3). It was also demonstrated that coincubation of lung microsomes with organic DEP extracts generate $O^{\bullet-}_2$ in an NADPH-dependent fashion [61,62]. Use of the fluorescent dyes hydroethidine and dichlorofluorescein acetate to observe ROS production during flow cytometry, suggests that macrophages and epithelial cells generate different oxidative stress responses [57,59] (Table 4). These

Table 4
Comparison of the DEP-induced oxidative stress response in epithelial cells and macrophages[a]

	Epithelial	Macrophage
Decline in GSH/GSSG ratio	Rapid	Slow
Predominant ROS	$O_2^{\cdot-}$	H_2O_2
HO-1 expression	+++	+++
Mitochondrial perturbation	Early	Late
Apoptosis	Starts at 10 μg/ml	$>$ 50 μg/ml
IL-8 production	+++	+
Protection by *N*-acetylcysteine	−	+++

[a] [57,59].

cell types also exhibit differences in the rates of GSH/GSSG decline, and the cellular response to oxidative stress [59] (Table 4). Generally speaking, bronchial epithelial cells are more prone to develop cytotoxicity than macrophages [57]. While the thiol antioxidant *N*-acetylcysteine could suppress ROS production and oxidative stress effects in macrophages, epithelial cells were not protected [57,59] (Table 4).

The second line of evidence supporting the role for oxidative stress in DEP-induced airway inflammation comes from animal studies [65–69] (Table 3). Intratracheal DEP administration leads to increased polymorphonulear cell infiltration, increased mucus and NO production, as well as increased AHR in mice [47,50,65,66,70,71]. These effects could be suppressed by pretreating the animals with polyethyleneglycol (PEG)-conjugated superoxide dismutase or with the NOS inhibitors, *N*-G-monomethyl L-arginine and aminoguanidine [51,65,66,70]. In addition, our own studies have shown that administration of the thiol antioxidants NAC and bucillamine suppress the adjuvant effects of aerosolized DEP on ovalbumin (OVA)-induced allergic responses in mice [67]. The antioxidant *N*-acetylcysteine abrogated AHR induction by incinerator particles [72]. The most direct evidence that PM induce ROS generation in vivo is the detection of in vivo chemiluminescence (H_2O_2 production) over the lungs and mediastinal fields of rats exposed to concentrated ambient particles (CAPs) [68].

To date, no direct evidence for ROS production has been provided in human PM exposures. It has been demonstrated, however, that experimental DEP exposures result in increased CO production [10,18,73]. CO is a catalytic HO-1 product that serves as a sensitive oxidative stress marker [31]. Controlled exposure in an exposure chamber confirmed the link between DEP and oxidative stress in humans [73]. These authors demonstrated that exposure to DEP leads to airway inflammation, as determined by increased neutrophils and myeloperoxidase in the sputum, in parallel with increased CO in the exhaled air [73]. Proteins and lipid peroxidation markers have also been documented in the blood of humans exposed to PM [25,74].

In addition to adjuvant effects, PM exposures induce acute asthma exacerbations independent of their effects on allergic sensitization [75]. DEP induce increased AHR in naive mice in the absence of allergen [50,51]. It has also been demonstrated that DEP alone can induce increased AHR in asthmatic individuals taking inhalant steroids [76]. While these effects may be related to DEP effects on allergic sensitization, the particles and their components may also directly contribute to increased AHR [43,77]. One possible mechanism is NO generation, as evidenced by the ability of NOS inhibitors to interfere with DEP-induced AHR in mice [65]. Shedding of airway epithelial cells is another possibility, based on the ability of DEP to induce acute epithelial damage in vivo and in vitro [59,78,79]. DEP also induce the expression of genes involved in airway remodeling and fibrogenesis [80].

5. How does DEP generate oxidative stress? Is it the particles or the chemicals?

DEP contain a carbonaceous core, which is coated by hundreds of organic chemicals and transition metals [81,82]. A key question is whether the particles or the chemicals are responsible for the generation of oxidative stress. Not only do intact DEP induce ROS production, but it has also been demonstrated that methanol extracts made from these particles induce ROS production in macrophages and epithelial cells [57] (Table 4). Moreover, organic DEP extracts induce $O_2^{\cdot-}$ production in lung microsomes [61,62]. Since the extracted particle residue does not induce ROS production, this suggests that the particle core is inert [83]. However, it is important to keep in mind that inert ultrafine particles (section 7) have been shown to exert biological effects independent of their chemical composition [84]. One possibility is that these particles penetrate subcellular targets, such as mitochondria [85] (section 7). Our current view is that both the particles and the chemicals are important, because the particles act a carrier for the chemicals and may also provide a reaction surface on which redox cycling chemistry can take place.

To investigate the role of organic chemical compounds, dichloromethane extracts of DEP were applied to silica gel columns [85,86]. Following elution of these columns with increasing polar solvents, three major fractions, designated aliphatic, aromatic and polar, were obtained [86,87]. While the aliphatic fraction was unable to induce oxidative stress, the aromatic and the polar fractions were able to decrease the cellular GSH/GSSG ratio in parallel with HO-1 expression [88]. Chemical analysis revealed that the aromatic fractions were enriched for polycyclic aromatic hydrocarbons (PAH), while the polar fractions were enriched for quinones [88]. The chemical structures of representative PAH and quinones are shown in Fig. 2A. Quinones and PAHs are relevant organic chemical groups that induce oxidative stress and electrophilic chemistry in the lung [3,61,89]. PAHs are converted to quinones via biotransformation involving cytochrome P450 1A1, expoxide hydrolase, and dihydrodiol dehydrogenase, and are relevant toxicological agents in themselves [90]. A role for PAH is supported by the excellent correlation between the PAH content of fine and ultrafine particles and their ability to induce oxidative stress in macrophages [60,84]. Moreover, there is excellent correlation between the PAH content of ultrafine particles and their ability to engage in redox cycling reactions in vitro (see section 7). While DEP induce cytochrome P450 1A1 expression in bronchial epithelial cells [91] and in rodents [92–94], it is not clear from human studies whether this enzyme plays an essential role in the adverse health effects of PM.

Quinones act as catalysts to produce ROS and may be key compounds in PM toxicity along with transition metals [89,90]. Redox cycling quinones undergo one-electron reductions by NADPH cytochrome P450 reductase to form

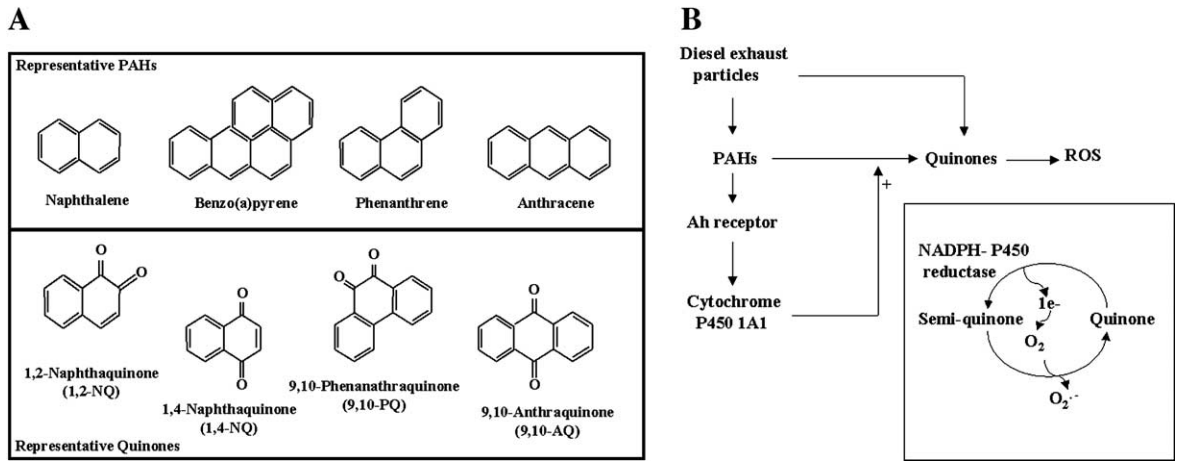

Fig. 2. Redox cycling organic compounds. (A) Representative PAH and quinone compounds. (B) PAH can be converted to quinones through the action of cytochrome P450 1A1 in the cells. This cytochrome is expressed via the aryl hydrocarbon (Ah) receptor. One-electron reduction of quinones results in the formation of semiquinones, which can be recycled to a quinone. In the process, the electron is donated to molecular O_2, leading to the formation of $O_2^{\cdot-}$.

semiquinones [89] (Fig. 2B). These semiquinones can be recycled to the original quinones, leading to the formation of $O_2^{\cdot-}$ (Fig. 2B). Not only are quinones byproducts of diesel fuel combustion, but they can also be formed by enzymatic conversion of PAH in lung tissue [61]. It is relevant, therefore, that the addition of an organic DEP extract to a lung microsomal preparation induce $O_2^{\cdot-}$ production in a cytochrome P450 reductase-dependent fashion [61]. Moreover, $O_2^{\cdot-}$ generation by this extract was suppressed by chemical derivatization of quinones [61]. Indirect support for the role of quinones comes from the demonstration that polar chemical groups fractionated from DEP induce oxidative stress in macrophages and epithelial cells [59]. Whether these compounds play a biologically relevant role in the adverse health effects of PM in humans will require further study. Large-scale efforts are currently under way in the Southern California Particulate Center and Supersite (SCPCS) at UCLA to determine whether there is a correlation between ambient quinone/PAH levels and the prevalence of asthma in the community [95]. This issue is complicated by the fact that these semivolatile substances partition between the particle and gaseous phases, and that this exchange is influenced by the number of polycyclic rings, environmental temperature, and seasonal effects [60,96]. In addition to participating in ROS production, quinones are electrophiles that can induce covalent modification of proteins and DNA strands [89]. This leads to irreversible damage in tissues.

It is important to emphasize that DEP contain hundreds of chemicals, and that PAH and quinones are merely two of the chemical groups that we have focused on in formulating our hypotheses [85,97]. It is quite possible that other redox cycling chemicals are involved in ROS generation, and that PAH and quinones merely serve as a proxy for other biochemically relevant pro-oxidative compounds. In this regard, it is also important to keep the role of transition metals (e.g., Fe, Ni, Cu, Co, and Cr) in mind, since these may play a role in ROS generation through the Fenton and Haber-Weiss reactions [98–100]. It is a formal possibility that transition metals may synergize with organic PM components in ROS generation [101].

The presence of endotoxin is another possible explanation for the biological effects of PM, including their ability to induce airway hyperreactivity (AHR) and cytokine release (TNFα, MIP-2, and IL-6) in rat and human alveolar macrophages [102,103]. Moreover, it has been demonstrated that the antioxidant *N*-acetylcysteine can protect against LPS-induced AHR [103]. Whether this effect is related to the ligation of the toll-like receptor 4 (TLR4) remains to be determined.

6. The pathways by which incremental levels of oxidative stress induce a hierarchy of biological effects

An important role of cellular homeostasis is maintenance of the balance between ongoing ROS generation and antioxidant defense. If at any stage ROS production overwhelms the antioxidant protection, this can result in oxidative stress [4]. Oxidative stress is a biological emergency, which elicits a range of cellular responses. This can vary from protective to injurious effects, depending on the level of oxidative stress. Using DEP as a model pollutant, we have proposed a three-tiered oxidative stress model [60,97] (Fig. 3). The first and most sensitive responses are the induction of antioxidant and phase 2 drug metabolizing enzymes (Fig. 3). HO-1 is an example of an antioxidant enzyme, which through heme catabolism is able to generate bilirubin, a potent antioxidant [31,88] (Figs. 3 and 4). An example of a phase 2 enzyme is NADPH quinone-oxidoreductase (NQO1), which converts redox cycling quinones to less toxic hydroxyl derivatives [89,104]. Both HO-1 and NQO1 are induced by the transcription factor Nrf-2, which operates on the antioxidant response element (ARE) in the promoter of these genes [105,106]. We envis-

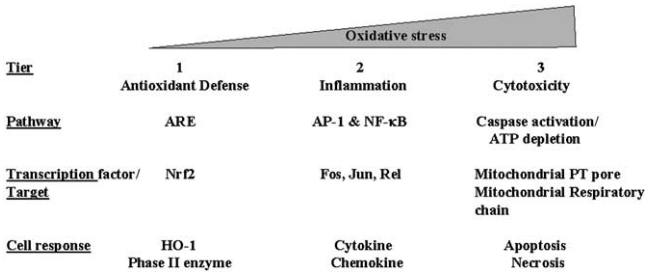

Fig. 3. Hierarchical oxidative stress model in response to DEP exposure. At a lower level of oxidative stress (tier 1), antioxidant enzymes are induced to restore cellular redox homeostasis. At an intermediate level of oxidative stress (tier 2), activation of MAPK and NF-κB cascade induces proinflammatory responses. At a high level of oxidative stress (tier 3), perturbation of the mitochondrial permeability transition pore and disruption of electron transfer result in cellular apoptosis or necrosis.

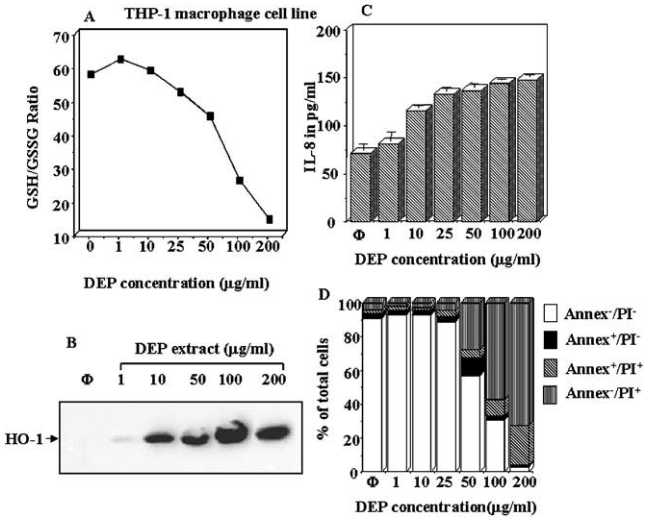

Fig. 4. Evidence for the hierarchical oxidative stress model in a macrophage cell line. Oxidative stress was induced by adding crude organic DEP extracts to the THP-1 cell culture medium. Four parameters including GSH/GSSG ratio, HO-1 expression, IL-8 production, and apoptosis were used to monitor different tiers of oxidative stress. (A) Dose-dependent decrease of GSH/GSSG ratio in THP-1 cells. The cells were treated with DEP extract for 8 h at indicated concentrations before being used for GSH/GSSG analysis. (B) Dose-dependent induction of HO-1 protein by DEP. THP-1 cells were stimulated with the DEP extract at indicated concentrations for 7 h before cellular protein extraction and SDS-polyacrilamide gel electrophoresis. (C) Dose-dependent increase in IL-8. THP-1 cells were treated with the DEP extract for 16 h before culture medium collections for IL-8 analysis. (D) Dose-dependent increase in cellular apoptosis. THP-1 cells were exposed to the DEP extract at indicated concentrations for 18 h. Apoptosis was analysed by flow cytometry using dual annexin V/PI staining. Low concentrations of DEP extract (1–10 μg/ml) induced HO-1 expression while the GSH/GSSG ratio remained normal. A higher concentration (10–50 μg/ml) resulted in an inflammatory response as determined by increased IL-8 production. Cytotoxicity occurred at DEP concentrations $>$ 50 μg/ml. [Copyright 2002 from Use of a Stratified Oxidative Stress Model to Study the Biological Effects of Ambient Concentrated and Diesel Exhaust Particulate Matter, by Ning Li et al. Reproduced by permission of Taylor & Francis, Inc., http://www.routledge-ny.com.]

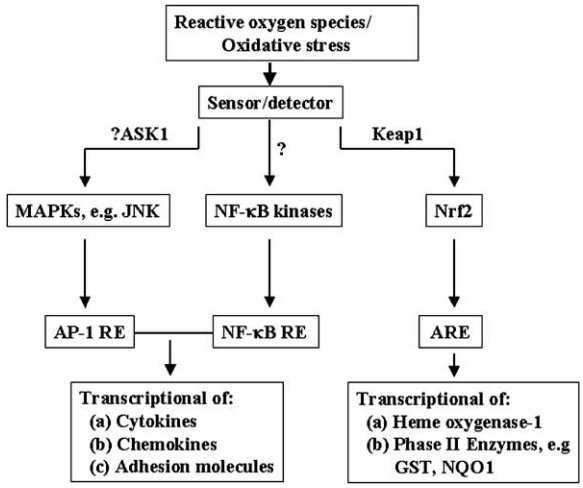

Fig. 5. Cellular sensors and signaling pathways involved in oxidative stress. ROS generation and/or oxidative stress is detected by cellular sensors. The possible candidates for the sensors include ASK1 for the AP-1 pathway and Keap-1 for the ARE pathway. These afferent components activate the MAP kinase cascade and, in the case of Keap1, lead to the release of the transcription factor, Nrf2, to the nucleus. The sensor for the NF-κB cascade is unknown, but ultimately leads to the phosphorylation and degradation of I-κBα, thereby releasing the attached Rel protein to the nucleus. Binding of AP-1 and/or NF-κB transcription factors to their respective DNA binding sites eventually leads to the production of cytokines, chemokines, and adhesion molecules. These products exert proinflammatory effects. The binding of Nrf2 to the ARE results in the expression of HO-1 (antioxidative) and phase II (detoxifying) enzymes. These products are cytoprotective.

age that the failure of these antioxidant and detoxification mechanisms to curb the level of oxidative stress will lead to more damaging responses (Figs. 3 and 4). This includes the initiation of inflammation (tier 2) and the activation of programmed cell death (tier 3).

While a lot needs to be learned about the pathways through which oxidative stress induce proinflammatory effects, evidence has been provided that redox cycling chemicals, including PAH, quinones, crude DEP extracts, and polar DEP fractions, can induce MAP kinase and NF-κB activation [106,107] (Fig. 5). These signaling cascades play important roles in the transcriptional activation of cytokines, chemokines, and adhesion molecules that impact PM-induced airway inflammation [83,97] (Fig. 5). These include the production of IL-4, IL-5, IL-8, IL-10, IL-13, RANTES, MIP-1α, MCP-3, GM-CSF, TNF-α, ICAM-1, and VCAM-1 [108]. These proinflammatory products act in a synergistic fashion to induce Th2 responses [108]. This may explain the basis for the adjuvant effects of DEP [3] (Fig. 6). In addition, the generation of oxidative stress in antigen-presenting cells (APCs) could enhance their ability to activate T-helper lymphocytes (Fig. 6). This could involve induction of CD80 and CD86 expression [3], as well as improved antigen presentation by the APCs (Fig. 7). Since the contact between the APC and T-helper lymphocytes takes place in focal deposition sites in the respiratory

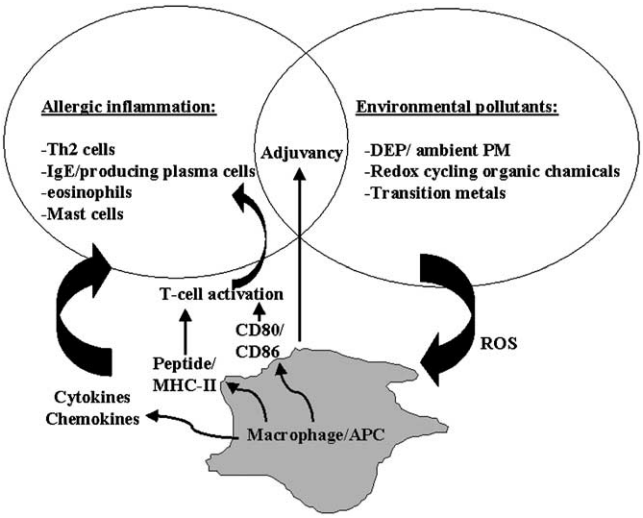

Fig. 6. Schematic to explain the molecular basis for the adjuvant effect of DEP at the level of the antigen-presenting cell (APC). Oxidative stress induces cytokines and chemokines that contribute to allergic inflammation through effects on T cells, B cells, and eosinophils. In addition, oxidative stress assists in the expression of costimulatory receptors (CD80, CD86) as well as enhancing antigen processing. This increases antigen-specific T-helper 2 cells responsiveness. These interactions likely only require a limited mucosal area to lead to allergen-specific IgE production. Once sensitized to an allergen, re-exposure may lead to widespread allergic inflammation in the bronchial mucosa.

tract, it is possible that only limited mucosal areas may be required for the adjuvant effect of PM. In section 9 we will discuss the concept of "hot spots of particle deposition" to explain how a limited particle dose delivered to a small mucosal area may provide the threshold of oxidative stress that is required for an adjuvant effect.

While it is clear from in vitro studies using epithelial cells and macrophages that intact particles as well as extracted DEP extracts induce apoptosis or apoptosis-necrosis, the biological significance of this event is unknown [57,109]. One possibility is that cytotoxic damage to bronchial epithelial cells may lead to shedding and AHR. This may explain acute asthma exacerbations following a sudden rise in ambient PM levels [38,39,70]. This hypothesis needs to be proven. Whatever the clinical significance of programmed cell death may be, in vitro studies have shown that the mitochondrion is an important target for toxic DEP chemicals [97,109]. Mitochondria are intimately linked to apoptosis through their ability to release cytochrome c and APAF-1 [110]. These molecules, in turn, lead to caspase 9 activation. Organic DEP chemicals also disrupt the mitochondrial transmembrane potential, interfering with one-electronic transfers in the inner membrane [109]. This leads to $O_2^{\cdot-}$ generation and uncoupling of oxidative phosphorylation. Not only will this exacerbate oxidative stress, but interference in ATP production may lead to cellular necrosis in addition to apoptosis (Figs. 3, 4, and 8). In section 7, we will discuss recent observations that ultrafine particles target and lodge in mitochondria [85].

7. Evidence that ambient particulate matter collected in a polluted urban environment induces oxidative stress

The aerodynamic diameters of ambient air particles vary from 0.005 to 100 μm. Three different types of ambient particles, as defined by size, are characterized in Table 5. An important advance in PM research has been the development of the Versatile Aerosol Concentration Enrichment Systems (VACES), which can collect highly concentrated ambient particles (CAPs) of various sizes to study their chemical composition, biological potency, and capacity to induce oxidative stress [60,111]. Taking advantage of this technique, investigators in the SCPCS have conducted studies to identify the relative toxicity of coarse, fine, and ultrafine particles in the Los Angeles basin [85]. We demonstrated that the biological activity of the different CAPs sizes is determined by their content of redox cycling chemicals [85] (Table 5). A strong correlation exists between the particulate content of redox cycling chemicals and their capability to induce HO-1 expression and glutathione depletion [85] (Table 5). While coarse CAPs contained mostly crustal elements, ultrafines contained significantly more organic carbons and PAH than coarse or fine particles [60,84]. Ultrafines were also more active in an in vitro assay that measures the redox cycling capacity of ambient particles [85,112]. This assay is premised on the interaction of redox cycling quinones (Q) with dithiothreitol (DTT):

$$\text{quinone} + \text{DTT} \rightarrow \text{semi-Q} + \text{DTT-thiyl} \quad (1)$$

$$\text{quinone} + \text{DTT-thiyl} \rightarrow \text{semi-Q} + \text{DTT-disulfide} \quad (2)$$

$$2\ \text{semiquinones} + 2\ O_2 \rightarrow 2\ \text{quinones} + 2\ O_2^{\cdot-} \quad (3)$$

$$\text{DTT} + 2\ O_2 \rightarrow \text{DTT-disulfide} + 2\ O_2^{\cdot-}\ \text{(net)}$$

In the presence of quinones, 1 mol of DTT plus 2 mol of O_2 generates 1 mol of DTT-disulfide plus 2 $O_2^{\cdot-}$. The loss of DTT can be followed by its reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). This assay provides a convenient means of comparing the pro-oxidative activity of ambient samples collected in an urban environment [85].

To explain the biological potency and ability of different size particles to induce ROS production, it is important to know where these particles localize in the cell. A possible subcellular target for the PM is the mitochondrion, as demonstrated by the ability of DEP and organic DEP extracts to induce structural and functional damage in this organelle [59,85,109]. Using electron microscopy, we have recently demonstrated that different CAP's sizes localize in different cellular locations [85] (Fig. 7). While coarse particles were

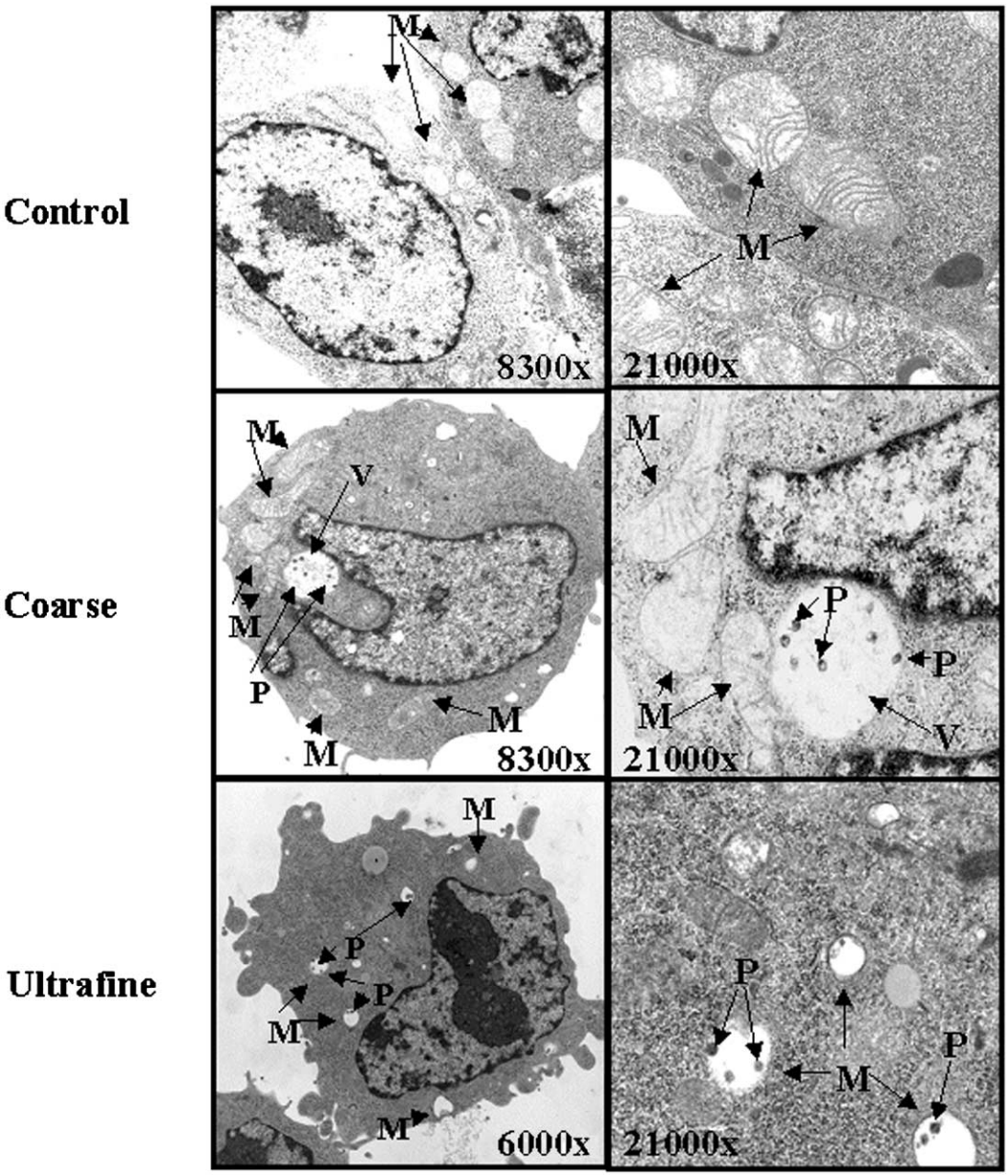

Fig. 7. Electron microscopy (EM) showing select subcellular localization of ultrafine and coarse particles. Coarse (2.5–10 μm) and ultrafine (<0.15 μm) CAPs were collected in an aqueous medium in Claremont, CA, using the VACES [84]. RAW264.7 cells were incubated with these particles for 16 h before fixation. EM was performed as previously described [84]. While coarse CAPs are localized in large vacuoles (phagocomes?) and do not damage mitochondria, ultrafine particles often induce structural damage as demonstrated by the disappearance of cristae. In addition, ultrafine particles appear to lodge inside damaged mitchondria. M, mitochondria; P, particles; V, vacuoles.

seen in large cytoplasmic vacuoles in macrophages, the ultrafines appear to localize inside damaged mitochondria [85]. The same mitochondrial effects were also observed in human bronchial epithelial cells treated with ultrafines [85]. The capacity of CAPs to damage mitochondria is directly related to their PAH content, redox cycling potential, and ability to induce HO-1 expression [85]. The mitochondrion may also be an important site of ROS production by DEP chemicals [109]. The availability of the VACES and the DTT assay are important tools to assess PM toxicity in polluted areas.

8. Evidence for the importance of antioxidant defense polymorphisms in asthma susceptibility

The deleterious effects of ROS are controlled by an elaborate antioxidant defense system that operates intracellularly, in bronchial lining fluid and in the blood (Table 6;[1,113–121]). Some antioxidants are ingested while others are synthesized in the human body, including enzymes, proteins, and low molecular weight scavengers (Fig. 8). Antioxidant proteins are superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione reductase

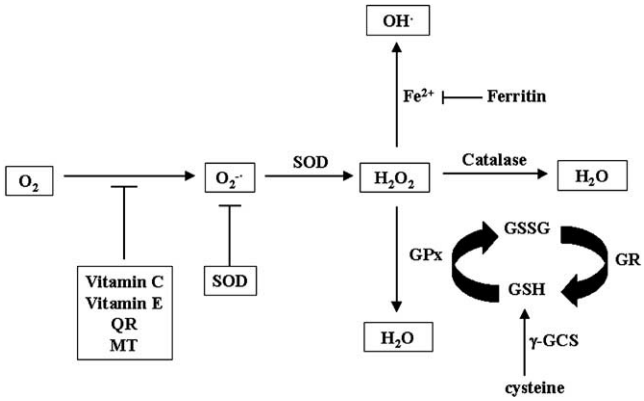

Fig. 8. Cellular antioxidant defense mechanisms. The cellular antioxidant defense system consists of antioxidant enzymes, metal binding proteins, and low molecular weight antioxidants (Table 6). While superoxide dismutase (SOD) catalyzes $O_2^{\cdot-}$ dismutation to H_2O_2, catalase catalyzes the decomposition of H_2O_2 to H_2O. Glutathione peroxide (GPx) reduces hydroperoxides by oxidizing GSH to GSSG. Ferritin, a nonenzyme protein, plays an important role in preventing the formation of highly toxic $OH^{\cdot}$ by preventing the Fenton reaction, whereas other antioxidants, such as vitamins C and E, quinone reductase (QR), and metallothionein (MT) can effectively block the generation of $O_2^{\cdot-}$ from redox cycling compounds.

(GR), thiol-specific antioxidants, metallothionein (MT), other metal-binding proteins (e.g., ferritin), heme oxygenase-1, urate, GSH, and ubiquinol [1]. The antioxidant enzymes act coordinately to convert ROS to less toxic species and play an important role in protecting the lung against ROS (Fig. 8). Three SOD isozymes have been identified in mammalian cells, namely Mn-SOD, Cu/Zn-SOD, and extracellular SOD (ECSOD), which is also a Cu/Zn-containing enzyme [113]. Although these isozymes differ in their metal cofactors and cellular location, they all share the function of converting $O_2^{\cdot-}$ to H_2O_2. Catalase, a heme-containing protein, catalyzes the decomposition of H_2O_2 to H_2O [1]. GPx reduces lipid and nonlipid hydroperoxides, such as H_2O_2, by oxidizing two moles of reduced glutathione (GSH) to one mole of glutathione disulfide (GSSG)

Table 5
Contrasting features of coarse, fine, and ultrafine particles[a]

Parameters	Particle mode		
	Coarse (PM_{10})	Fine ($PM_{2.5}$)	Ultrafine
Size	2.5–10 μm	2.5–0.15 μm	<0.15 μm
Organic carbon content	+	++	+++
Elemental carbon content	+	++	+++
Metals as % of total elements	+++	++	+
PAH content	+	+	+++
Redox activity (DTT assay)	+	++	+++
HO-1 induction	+	++	+++
GSH depletion	+	+++	+++
Mitochondrial damage	None	Some	Extensive

[a] [85].

Table 6
Major antioxidants in different biological compartments

Biological compartment	Type	Antioxidants
Epithelial lining fluid	Low molecular weight	Vitamin C [115]
		Urate [115]
		Vitamin E [115]
	Thiol	GSH [115]
	Metal binding protein	Ceruloplasmin [116]
Blood	Low molecular weight	Vitamin C [1]
		Urate [1]
		Vitamin E [1]
	Thiol	GSH [1]
		Thioredoxin [117,118]
	Enzyme	EC-SOD [113]
	Metal binding protein	Ceruloplasmin [120]
		Ferritin [1]
	Other	Bilirubin [119]
Intracellular	Enzyme	Catalase [1]
		HO-1 [1]
		GPx [1]
		GR [1]
		Glutathione *S*-transferase (GST) [121]
		SOD (Mn-SOD, Cu/Zn-SOD) [1]
		Thioredoxin reductase [117]
	Thiol	GSH [1]
		Thioredoxin [117]
	Metal binding protein	Ferritin [1]
		Metallothionein [1]

[1,114]. Ferritin is a nonenzymatic protein that plays an important role in antioxidant defense through its ability to block the Fe^{2+}-catalyzed Fenton reaction, thereby reducing the formation of the $OH^{\cdot}$ radical. The most important nonprotein antioxidant is GSH, which provides major redox buffering capacity to oxidatively stressed cells [114] (Fig. 8). Since GPx requires GSH to detoxify peroxides, a very high concentration of GSH is routinely maintained in mammalian cells [114]. Additional protection is provided by dietary antioxidants such as vitamins C and E (Fig. 8). We have already discussed that asthmatics show a decrease in ascorbate and α-tocopherol levels in the lung lining fluid [35] (Table 1). Major antioxidants in different biological compartments are summarized in Table 6 [1,113,115–121].

Several studies conducted in asthmatic patients have shown changes in antioxidant protection. SOD activity is generally decreased in asthmatics, irrespective of whether the measurements are made in erythrocytes [122], bronchial epithelial cells [123,124], or lung lining fluid [32]. The major change in SOD activity is in Cu/Zn-SOD [123] and ECSOD [125]. This loss in activity may be due to oxidative inactivation of SOD [123]. In addition to its important role in intracellular antioxidant defense, glutathione peroxidase (GPx) and GSH in the asthmatic lung play important roles in protecting the extracellular surface of the epithelial cells [32]. Decreased GSH levels have been reported in adults

Table 7
Altered antioxidant defense mechanisms in asthma[a]

1. Altered lung antioxidant status in patients with asthma, including ↓ SOD activity during antigen-induced asthmatic responses.
2. Increased lung but decreased blood glutathione peroxidase activity in asthmatics.
3. Association of GST genotypes/polymorphisms with asthma, including occupational asthma.
   a. GST-M1 null genotype: asthma risk ↑ 3.5-fold.
   b. GST-M1 + GST-T1 genotype: asthma risk ↑ 4-fold.
   c. GST-P1 (Val^{105}/Val^{105}) 6-fold lower risk of asthma compared to Ile^{105}/Ile^{105}.
   d. GST-P1 (Val^{105}/Val^{105}) protects vs. TDI-induced asthma.
   e. ↑ Risk of asthma and wheezing in children with the GST-M1/null phenotype after exposure to tobacco smoke in utero.

[a] [126–134].

and children with asthma, while red blood cell GPx activity was shown to be decreased in pediatric asthma [33].

There is growing evidence that genes involved in xenobiotic detoxification and antioxidant defense could serve as susceptibility genes for asthma pathogenesis [32,33,35,126–134] (Table 7). Glutathione-*S*-transferase (GST) is an enzyme that is involved in the detoxification of environmental chemicals, including redox cycling components in tobacco smoke and PM. Individuals who are homozygous for the GST M1 (null) genotype are totally lacking in GST activity, and have been shown to have an increased risk for asthma development [130]. This includes an increased risk of wheezing in children with the M1 genotype who are exposed to tobacco smoke in utero [134]. In contrast, homozygous expression of the GST P1 (Val) genotype confers a protective effect on asthma, and has also been shown to protect against toluene di-isocyanate-induced (occupational) asthma [130–133]. Based on these findings, we posit that genetic polymorphisms in antioxidant defense genes play a role in the susceptibility to adverse PM effects, including PM-induced asthma. In addition to GST, the HO-1 gene exhibits a number of polymorphisms that are based on poly-$(GT)_n$ repeats in its promoter [135]. The number of $(GT)_n$ repeats determines the inducibility of this gene, such that Japanese male smokers with a short poly-$(GT)_n$ polymorphism and a poorly inducible gene have a statistically higher incidence of emphysema than smokers with a long poly-$(GT)_n$ repeat and a better inducible gene [135]. We predict that similar and related polymorphisms in phase II drug metabolizing genes may determine PM susceptibility.

9. Reconciliation of in vitro and vivo PM dosimetry

The data in favor of an in vitro hierarchical response to DEP are summarized in Fig. 3. A frequently asked question is how experimental in vitro DEP concentrations (1–100 μg/ml) relate to real-life PM exposures. To probe that question, it was necessary to reconcile the in vivo PM exposures, measured in micrograms per cubic meter ($\mu g/m^3$), with the tissue culture concentrations of DEP chemicals, measured in micrograms per milliliter (μg/ml). To find a common basis for comparison, we converted the in vivo and in vitro doses to micrograms (μg) of particulate matter per unit of surface area. Table 8 demonstrates how the in vitro DEP doses were converted to micrograms per square centimeter ($\mu g/cm^2$), using the DEP chemical dose, particle weight, the volume of the tissue culture medium, and surface area of the culture vessel. The biological relevant tissue culture concentration of DEP ranges from 0.2 to 20 $\mu g/cm^2$.

We also performed an in vivo dosimetric evaluation of total PM (TPM) and $PM_{2.5}$ deposition in an exposed adult. The exposure site was in Rubidoux, California. This represents a highly polluted area in Southern California. The in vivo assessment is premised on the fact that a fraction of inhaled airborne PM will deposit on respiratory surfaces [1,136–143]. We considered the deposited particles only, because the exhaled particles do not exert biological effects. The probability that inhaled particles will be deposited depends on particle size, breathing pattern (e.g., oral vs. nasal breathing, or resting vs. exercise conditions), and the different macroscopic regions of the respiratory tract [137,140,141]. These regions are divided into the nasopharyngeal (NPR), tracheobronchial (TBR), and alveolar (AVR) regions. Well-established particle dosimetry models

Table 8
Conversion of in vitro DEP doses to particle dose/unit surface area

DEP extract dose[a]	Equivalent particle dose[a]	Oxidative stress level[a]
1–10 μg/ml	1.4–14 μg/ml	Low
10–50 μg/ml	14–71 μg/ml	Intermediate
15–100 μg/ml	71–143 μg/ml	High
Calculations		
Tissue culture dish diameter	= 3 cm	
Culture surface area	= 7.1 cm^2	
Biological dose-response range (extract)	= 1–100 μg/ml	
Biological dose-response range (particles)	= 1.4–143 μg/ml	
Extract dose/cm^2	= 0.14–14.1 $\mu g/cm^2$	
Particle dose/cm^2	= 0.2–20 $\mu g/cm^2$	

[a] Refers to dose-response studies in macrophages [60]. Epithelial cells are more sensitive and respond to lower chemical doses ([59]; Table 4).

[138,139] can be used to predict size-dependent particle deposition in these macroscopic regions. A shortfall of these models is that they do not take into account variables that may specify a higher rate of particle deposition in susceptible human subjects. These variables include: (1) nonhomogeneous airflow due to airway obstruction, as occurs in asthma and COPD; (2) higher rates of PM deposition at airway bifurcation points; and (3) a high efficiency of particle deposition due to variations in body size, airway anatomy, and particle clearance mechanisms. Normal variations in airflow and airway anatomy can lead to as much as a 2.5-fold increase in average particle deposition, while increased particle deposition at airway bifurcation points may create an enhancement of >100-fold [136,144]. A high-risk individual may therefore be a person with a condition that produces uneven ventilation, who is breathing nasally, is physically active, has clearance stasis (e.g., as a result of a respiratory tract infection), and whose airways are more efficient than the average in promoting particle deposition (Fig. 9). When all of these factors are considered, the predicted surface dose at these so-called hot spots of deposition may be surprisingly high (Fig. 9).

Fig. 9 shows the reconciliation between in vitro and in vivo doses. It outlines in vivo calculations for a high-risk individual exposed to ambient PM levels of 79 $\mu g/m^3$ over a 24-h period in Rubidoux. Column 1 shows TPM deposition per square centimeter (cm^2) in each of the anatomical areas over a 24-h time period. Column 5 shows how those values were converted to $PM_{2.5}$ deposition per cm^2. When further corrections were made for the individual variations in airway anatomy, nasal breathing, deposition at bifurcation points, and uneven airflow due to asthma, these values translate to 204, 2.3, and 0.05 $\mu g/cm^2$ in the NPR, TBR, and AVR, respectively (Fig. 9). These data show that it is possible to achieve the in vitro dose range of 0.2–20 $\mu g/cm^2$ (Table 8) that is required to induce biological effects.

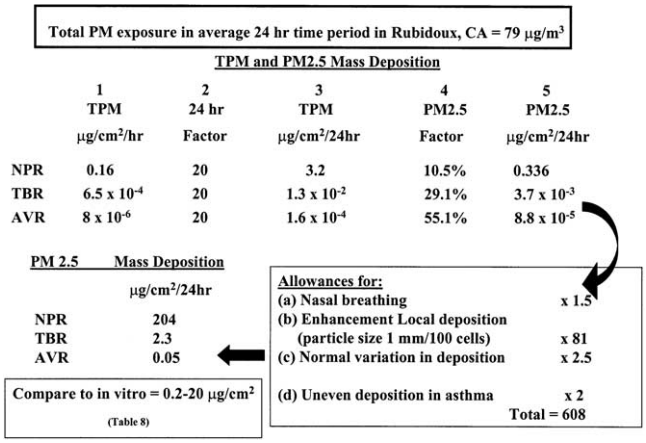

Fig. 9. Reconciliation of in vitro DEP dose-response effects to in vivo PM dosimetry. Twenty-four-hour, 15-min averaged, detailed size distribution measurements for particles with aerodynamic diameter 0.014–20 μm were performed in Rubidoux between June and September 2001. Particle measurements were made with the Particle Instrumentation Unit (PIU), using a TSI scanning mobility particle size spectrometer (SMPS) and combined with TSI aerodynamic particle sizer (APS). Using the average size distribution data from the SMPS and APS, deposition was calculated for each particle size over the diameter range 14 nm to 20 μm, and integrated to get total fractional deposition for each of the three anatomic regions of the respiratory tract. Column 1 shows the TPM mass deposition per unit surface area calculated according to the volume (M^3) of inhaled ambient air. This was accomplished by using the following surface areas for the nasopharyngeal (NPR), tracheobroncial (TBR), and alveolar regions (AVR): 296 cm^2, 3,725 cm^2, and 705,000 cm^2 [145]. The data in column 1 were converted to 24-h TPM deposition (column 3), using 20-m^3 air exchange by an active adult over a 24-h time period (column 3). These data were converted to $PM_{2.5}$ deposition (column 5), using the correction factor in column 4. The data in column 5 were adapted for high-risk individuals as shown in the enclosed box in Fig. 9. These allowances include corrections for nasal breathing (1.5-fold), nonhomogeneous airflow in asthmatic airways (2-fold), anatomical variations enhancing deposition efficiency (2.5-fold), and increased particle deposition at bifurcation points in the airway (81-fold). The latter value was derived from the work of Balashazy and Hofmann [136], who calculated that for 1-μm particles impacting a bifurcation area of 0.1 × 0.1 mm, particle desposition is enhanced by a factor of 81-fold. Such an area will have 200 to 400 cells, which is approximately the same cell density as in the tissue culture dish.

10. Implications of the oxidative stress hypothesis for the diagnosis and treatment of asthma

It should be clear from the foregoing that use of an oxidative stress model has important implications for the mechanisms by which PM impact allergic airway disease. While the epidemiology of PM exposures has received a lot of attention, we lack an understanding of toxic PM components and their mechanisms of action in the lung. The introduction of the oxidative stress model makes it possible to formulate testable hypotheses to explore these questions. A more complete understanding of the principles of toxicity and the disease mechanisms should allow us to implement the appropriate regulatory procedures. Currently, the Environmental Protection Agency is using a $PM_{2.5}$ mass standard to regulate particulate exposures. While this strategy has been successful in improving air quality [146], a mass standard does not consider the impact of ultrafine particles, which lack appreciable mass. Since our preliminary data indicate that ultrafine particles have a higher content of redox cycling chemicals [85], it will be important to determine whether their ability to generate ROS render them more toxic than $PM_{2.5}$ and PM_{10}. Because it is difficult to directly study ROS generation in humans, it will be necessary to develop suitable clinical markers for oxidative stress. Although we have outlined possible candidates that can be used for this purpose in Table 1, these may turn out to be insensitive in field studies. To find more sensitive markers, we are using the strengths of proteomics to identify oxidative stress markers in vitro and in vivo. These studies are being conducted on tissue culture cells, animal and human exposure models, and are premised on the principle that newly induced oxidative stress proteins are suppressible by thiol antioxidants, and may also be identified by carbonyl

and nitrotyrosine modifications [67]. If such markers can be established, it should also help to identify susceptible human subsets in smaller study populations. The ability to detect in vivo oxidative stress markers will also assist in therapy development to interfere with the adverse health effects of PM, including asthma exacerbations. In this regard, we have demonstrated that the thiol antioxidants *N*-acetylcysteine and bucillamine can be used to interfere with the adjuvant effects of DEP in a murine asthma model [67].

Finally, it should be mentioned that a PM contribution to oxidative stress is not the only means by which air pollutants may contribute to asthma exacerbation. Much of what has been said about the role of the particles and their chemicals is also applicable to ozone, a well-known inducer of oxidative stress in the respiratory tract [147,148]. Moreover, it is known that ozone can induce asthma exacerbations [149,150]. It is also possible that ozone may synergize with PM in their proinflammatory and pro-oxidative effects in the respiratory tract [151].

Acknowledgments

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