

Thiol Antioxidants Inhibit the Adjuvant Effects of Aerosolized Diesel Exhaust Particles in a Murine Model for Ovalbumin Sensitization¹

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Although several epidemiological studies indicate a correlation between exposure to ambient particulate matter and adverse health effects in humans, there is still a fundamental lack of understanding of the mechanisms involved. We set out to test the hypothesis that reactive oxygen species are involved in the adjuvant effects of diesel exhaust particles (DEP) in a murine OVA sensitization model. First, we tested six different antioxidants, *N*-acetylcysteine (NAC), bucillamine (BUC), silibinin, luteolin, trolox (vitamin E), and ascorbic acid, for their ability to interfere in DEP-mediated oxidative stress *in vitro*. Of the six agents tested, only the thiol antioxidants, BUC and NAC, were effective at preventing a decrease in intracellular reduced glutathione:glutathione disulfide ratios, protecting cells from protein and lipid oxidation, and preventing heme oxygenase 1 expression. Therefore, we selected the thiol antioxidants for testing in the murine OVA inhalation sensitization model. Our data demonstrate that NAC and BUC effectively inhibited the adjuvant effects of DEP in the induction of OVA-specific IgE and IgG1 production. Furthermore, NAC and BUC prevented the generation of lipid peroxidation and protein oxidation in the lungs of OVA- plus DEP-exposed animals. These findings indicate that NAC and BUC are capable of preventing the adjuvant effects of inhaled DEP and suggest that oxidative stress is a key mechanistic component in the adjuvant effect of DEP. Antioxidant treatment strategies may therefore serve to alleviate allergic inflammation and may provide a rational basis for treating the contribution of particulate matter to asthmatic disease. *The Journal of Immunology*, 2002, 168: 2560–2567.

While several epidemiological studies have shown a positive relationship between exposure to ambient particulate matter (PM)⁴ and adverse health effects in humans, there is still a fundamental lack of understanding of the biological mechanisms and the particle components involved in these effects (1–3). An important advance in this area has been the

discovery that diesel exhaust particles (DEP), used as a model air pollutant, exert adjuvant effects on the response of humans and mice to inhaled or instilled allergens (4–12). This adjuvant effect includes increased Ag-specific IgE production, skewing of the immune response to Th2 cytokine production, increased eosinophils in the bronchoalveolar lavage (BAL) fluid, and increased airway hyperreactivity (4–12). This model allows for an orderly dissection of the mechanisms of DEP action.

Inhaled or intratracheally instilled DEP generate reactive oxygen species (ROS) in the lungs of exposed mice (13–16). Moreover, incubation of lung microsomes with organic DEP extracts leads to superoxide (O_2^-) generation in a NADPH-cytochrome P450 reductase-dependent fashion (17). The ability of the DEP extract to generate O_2^- could be suppressed by $NaBH_4$, an agent that reduces and inactivates oxygenated polycyclic aromatic hydrocarbons (oxy-PAH), including quinones (17). ROS are responsible for protein oxidation, lipid peroxidation, and DNA damage in target cells such as macrophages and epithelial cells (18–22). Additional attempts to defend against oxidative tissue damage leads to a depletion of cellular glutathione reserves and a drop in the cellular reduced glutathione (GSH):glutathione disulfide (GSSG) ratio. This state of oxidative stress incites further cellular responses, such as the induction of heme oxygenase 1 (HO-1) expression, the production of proinflammatory cytokines, and cellular apoptosis (4, 18, 23–26). These responses are dependent on the activation of the mitogen-activated protein kinase and NF- κ B signaling cascades as well as activation of the antioxidant response element (18, 27–29).

We believe that the generation of oxidative stress is key to understanding the biological effects of PM and therefore provides an important target for reversing the adverse effects of PM in the lung.

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⁴ Abbreviations used in this paper: PM, particulate matter; BAL, bronchoalveolar lavage; BUC, bucillamine; DEP, diesel exhaust particle; DNP, dinitrophenyl hydrazine; GSH, reduced glutathione; GSSG, glutathione disulfide; HO-1, heme oxygenase-1; MBP, major basic protein; NAC, *N*-acetylcysteine; oxy-PAH, oxygenated polycyclic aromatic hydrocarbon; PT, permeability transition; ROS, reactive oxygen species.

While there is good evidence that ROS production follows the induction of airway inflammation, it is possible that ROS may also be involved in initiating this inflammation (30–34). In this regard it is known that O_2^- generation occurs at the site of allergen challenge in the human lung (30). These studies were reproduced in large animals, where it was demonstrated that oxygen radicals contribute to Ag-induced airway hyperreactivity (35, 36). In addition, neutrophils and mononuclear cells generate proportionately more O_2^- and H_2O_2 in the lungs of asthmatics compared with healthy controls (32, 34). ROS generation also correlates with increased airway hyperreactivity in asthmatic lungs (31, 33). These findings imply that antioxidants may be effective for treating select aspects of allergen sensitization as well as ongoing allergic inflammation following sensitization.

In this study we use a murine model to test the principle that antioxidants can block DEP-enhanced allergic sensitization. Among the different classes of antioxidants, it is not clear whether radical scavengers, lipid-soluble chain terminators, inducers of glutathione synthesis, or covalent modifiers of redox cycling chemicals are the most effective for inhibiting the pro-oxidative effect of DEP chemicals. For this reason we tested different classes of antioxidants for their abilities to interfere in the generation of oxidative stress *in vitro*. Among six different agents tested, only thiol antioxidants were effective in preventing a decrease in GSH:GSSG ratios *in vitro* and were therefore used for the *in vivo* studies. Our data demonstrate that *N*-acetylcysteine (NAC) and butylamine (BUC) effectively prevented the enhancement of OVA-specific IgE and IgG1 production in animals cochallenged by DEP inhalation. Moreover, the same agents also decreased the generation of lipid peroxides and carbonyl proteins in the lungs of OVA-plus DEP-exposed animals. These data indicate that thiol antioxidants may be effective for reversing the adjuvant effects of PM in the lung.

Materials and Methods

Reagents

DMEM, penicillin-streptomycin mixture, L-glutamine, and FBS were purchased from Life Technologies (Rockville, MD). NAC, ascorbic acid, reduced and oxidized glutathione (GSH and GSSG), reduced β -NADPH, and glutathione reductase were obtained from Sigma-Aldrich (St. Louis, MO). Luteolin, silibinin, and trolox (water-soluble vitamin E) were purchased from Calbiochem (San Diego, CA). BUC was a gift from Keystone Biomedical (Los Angeles, CA). Anti-HO-1 mAb was purchased from Stressgen (Victoria, Canada). Rabbit anti-mouse Ab, swine anti-rabbit Ab, and avidin-biotin complex were purchased from DAKO (Carpinteria, CA). Rabbit anti-major basic protein (anti-MBP) Abs were provided by Dr. J. Lee (Mayo Clinic, Scottsdale, AZ). ECL reagents were obtained from Pierce (Rockford, IL). Chicken egg OVA (Sigma-Aldrich) was prepared in physiological saline. DEP were a gift from Dr. M. Sagai (National Institute for Environmental Studies, Tsukuba, Japan). These particles were generated by a light-duty, four-cylinder diesel engine (4JB1 type; Isuzu Automobile, Japan) using standard diesel fuel as previously described (16, 26).

Cell culture and stimulation

RAW 264.7 cells were cultured in DMEM containing penicillin/streptomycin and 10% FBS. DEP extracts were prepared as previously described (24–26). Briefly, 100 mg DEP was suspended in 25 ml methanol and sonicated for 20 min. The DEP/methanol suspension was centrifuged at 2000 rpm for 10 min at 4°C. The methanol supernatant was transferred to a preweighed polypropylene tube and dried under nitrogen gas. Dried DEP extracts were resuspended in DMSO at 100 μ g/ μ l and stored at –20°C in the dark. The NAC stock solution (1 M) was made in HEPES buffer before dilution in culture medium to a final concentration of 20 mM. BUC was prepared in H_2O at a stock concentration of 25 mM (37, 38) and used at a final concentration of 5 mM. Luteolin, silibinin, and trolox were made in DMSO at final concentrations of 50 μ g/ml, 25 μ g/ml, and 1 μ M, respectively. Ascorbic acid stock (100 mM) was made in PBS and used at a final concentration of 100 μ M. Cells were plated at 2×10^6 /well in six-well plates containing 2 ml medium, and stimulations were conducted in a total

volume of 3 ml. Controls were treated with DMSO at a final concentration of 0.1%. All cell cultures were maintained at 37°C in a humidified incubator supplemented with 5% CO_2 .

HO-1 Western blot

The cells were harvested by scraping and lysed as previously described (26). One hundred micrograms of total lysate protein was electrophoresed on SDS-polyacrylamide gels before transferal to polyvinylidene difluoride membranes. The blots were sequentially overlaid with anti-HO-1 mAb at 0.3 μ g/ml and rabbit anti-mouse Ab conjugated to HRP according to the manufacturer's instructions. All blots were developed with the ECL reagent according to the manufacturer's instructions.

Determination of cellular GSH:GSSG ratios

Total glutathione (GSH plus 50% GSSG) and GSSG were measured using recycling assays involving the reaction of 5,5'-dithio-bis(2-nitrobenzoic acid) and glutathione reductase (39, 40). Briefly, cells were lysed and deproteinized in 3% 5-sulfosalicylic acid. Whole-cell lysates were then cleared by centrifugation at 4°C at 14,000 rpm in an Eppendorf centrifuge. The supernatant was used for the measurement of total and oxidized glutathione. Total glutathione in each sample was calculated from a GSH standard curve prepared in 5-sulfosalicylic acid. For the GSSG assay, 100 μ l supernatant was incubated with 2 μ l 2-vinylpyridine and 6 μ l triethanolamine for 60 min on ice. GSSG standards were treated in the same way as samples. The amount of GSSG in the samples was calculated from the GSSG standard curve. The amount of reduced GSH was calculated by subtracting the amount of GSSG from that of total glutathione.

Determination of carbonyl protein content

Lung tissue or cells were homogenized in a buffer (1/5 dilution) containing 10 mM HEPES, 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4 , 0.6 mM $MgSO_4$, and 1.1 mM EDTA. The buffer also contained Tween 20 (5 mg/l), butylated hydroxytoluene (1 μ M), and the protease inhibitors 0.5 μ g/ml leupeptin, 0.7 μ g/ml pepstatin, 40 μ g/ml PMSF, and 0.5 μ g/ml aprotinin to prevent proteolysis of oxidized proteins during preparation. The homogenates were centrifuged at 20,000 $\times g$ for 20 min. Supernatants were used for carbonyl protein determinations.

Protein carbonyl groups were determined as previously described (41). Briefly, supernatant fractions were divided into two equal aliquots containing ~1–2 mg protein each, precipitated with 10% TCA, and centrifuged at 2,000 $\times g$ for 10 min. One pellet was treated with 2.5 M HCl, and the other was treated with an equal volume of 10 mM dinitrophenyl hydrazine (DNPH) in 2.5 M HCl at room temperature for 1 h. Samples were reprecipitated with 10% TCA and subsequently extracted with ethanol and ethyl acetate (1/1, v/v) until virtually colorless (this indicates complete removal of unreacted and lipid-bound DNPH). The pellets were dissolved in 6 M guanidine HCl with 20 mM potassium phosphate buffer, pH 2.3, and left for 10 min at 37°C with general vortex mixing. The difference in absorbance between DNPH-treated cultures and the HCl control was determined at 370 nm. Data were expressed as nanomoles of carbonyl groups per milligram of protein using the molar extinction coefficient of 21,000 for DNPH derivatives.

Determination of lipid hydroperoxides

Lipid hydroperoxides were determined as previously described (42). Briefly, aliquots of the sample were added to 10 vol working reagent containing 25 mM ferrous ammonium sulfate, 2.5 M H_2SO_4 , 4 mM butylated hydroxytoluene, and 125 μ M xylene orange in methanol. Solutions were mixed well and incubated at room temperature for 15–20 min. The absorbance at 560–600 (560 optimal) was read using a spectrophotometer. Data were expressed as micromoles of lipid peroxide per milligram of protein using the molar extinction coefficient of 43,000 for hydroperoxides.

Murine inhalation exposure protocol to test the adjuvant effect of DEP and the effect of *i.p.* antioxidants

Six- to 8-wk-old female BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in filter-topped cages under standard laboratory conditions (room temperature, $23 \pm 2^\circ C$; 60% relative humidity; 12-h light, 12-h dark cycle) and maintained on autoclaved food and acidified water. In the first experiment, aimed at establishing the inhalation-sensitization model, we used a saline- and OVA-only control together with six DEP treatment groups (Table I). Each group was comprised of six mice. Mice were placed in a gas anesthetizing box and exposed to 200, 600, and 2000 μ g/ m^3 DEP for 1 h daily for 10 days. OVA-only animals received 1% aerosolized OVA daily for 20 min, while

Table I. Demonstration of the adjuvant effect of DEP in a murine model^a

	OVA-IgE (U/ml)	OVA-IgG1 (ug/ml)	Total IgE (ng/ml)
Saline	0 ± 0	1.3 ± 0.4	846 ± 214
OVA	4.7 ± 3.0	18.2 ± 7.8	758 ± 313
DEP (200 μg/m ³)	0 ± 0	0.8 ± 0.4	948 ± 245
DEP (600 μg/m ³)	0 ± 0	0.9 ± 0.6	879 ± 221
DEP (2000 μg/m ³)	0 ± 0	0.7 ± 0.5	1050 ± 271
OVA+DEP (200 μg/m ³)	8.7 ± 8.9	42.5 ± 15.7 ^b	1105 ± 218
OVA+DEP (600 μg/m ³)	19.2 ± 7.5 ^c	59.4 ± 16.1 ^d	1216 ± 349
OVA+DEP (2000 μg/m ³)	22.5 ± 9.7 ^d	68.8 ± 21.5 ^d	1314 ± 187 ^b

^a BALB/c mice (six per treatment group) were exposed to DEP and/or OVA by inhalation for 10 days. Mouse serum was analyzed using ELISA. Values are expressed in the units indicated in the table ± SD.

^b Values of $p < 0.05$ (OVA+DEP vs OVA).

^c Values of $p < 0.01$ (OVA+DEP vs OVA).

^d Values of $p < 0.001$ (OVA+DEP vs OVA).

DEP plus OVA animals received the same amounts of DEP as described above for 1 h, followed by 1% OVA for 20 min, daily for 10 days. Nebulization was performed with a Schuco 2000 (Allied Health Care Products, St. Louis, MO) with a flow rate of 6 l/min at the nebulizer cup, yielding particles in the 0.5- to 4-μm size range. DEP and OVA were both dissolved in 0.9% saline solution; DEP was sonicated for 10 min on ice before use. In the second experiment looking at the effects of thiol antioxidants, there were seven animal groups, each containing six mice per group. In the DEP plus OVA treatment group, animals were exposed to 2000 μg/ml DEP for 1 h, followed by a 20-min exposure to 1% OVA daily for 10 days. Test animals were treated with 20 mg/kg BUC or 320 mg/kg NAC i.p. immediately before the inhalation exposure. These animals were also compared with mice receiving OVA only, with the same drugs administered i.p. Blood was collected by periorbital bleeding on day 0 (before any chemical exposure) and again 24 h after the last exposure by cardiac puncture. The trachea was cannulated, the lungs were lavaged three times with 1 ml sterile saline, and the fluid was harvested by gentle aspiration.

Determination of OVA-specific IgE and IgG1

Serum samples were analyzed for OVA-specific IgE and IgG1 using an ELISA. Briefly, an IgE capture Ab (rat anti-mouse; BioSource International, Camarillo, CA) along with biotinylated OVA (OVA from Sigma-Aldrich; biotinylation kit from Calbiochem) were used to analyze blood samples for IgE. Because there is no commercially available standard reference for OVA-IgE, we used a reference serum from mice that were repeatedly exposed to OVA as previously described by us (43). For serum analysis of IgG1, wells were coated with 2 mg/ml OVA and exposed to serum samples, and a biotin-conjugated rat anti-mouse IgG1 mAb (BD PharMingen, San Diego, CA) was used for detection. The OVA-IgG1 standard used to quantitate the OVA-IgG1 was a monoclonal anti-chicken egg albumin from Sigma-Aldrich.

Lung histology and immunocytochemistry

Lungs were expanded with 10% buffered formalin phosphate before excision and sectioning. These lung sections were stained and analyzed using H&E, Southgate's mucicarmine, and Alcian blue according to standard histology techniques (44). For conducting immunohistochemistry for MBP, tissue sections were deparaffinized in xylene and eventually rehydrated in PBS. Endogenous peroxidase activity was quenched by 0.5% H₂O₂ in methanol. After pepsin digestion, nonspecific binding was blocked using 1% normal goat serum. The sections were then incubated with rabbit anti-MBP (1/1000) Ab at 4°C for 16 h, followed by incubation with goat anti-rabbit (1/500) Ab for 30 min. Staining was conducted using the Vectastain Elite ABC and diaminobenzidine substrate kits (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. The slides were finally counterstained with 0.5% methyl green.

Results

Thiol antioxidants prevent the oxidative stress effects of organic DEP extracts in vitro

We and others have previously shown that organic DEP extracts induce oxidative stress through ROS production in pulmonary alveolar macrophages and macrophage cell lines (24–26). To determine whether this effect is reversed by antioxidants, we used different classes of antioxidants to determine their effects on the

cellular GSH:GSSG ratio. The murine macrophage cell line, RAW 264.7, exposed to 50 μg/ml DEP extract for 5 h showed a 3-fold reduction in the cellular GSH:GSSG ratio (Fig. 1). This is in agreement with previous data showing potent oxidative stress effects by DEP chemicals (45). While the addition of the thiol antioxidants, NAC and BUC, prevented a drop in the GSH:GSSG ratio, neither the flavanoid antioxidants (silibinin and luteolin) nor the naturally occurring antioxidants, trolox (vitamin E) or ascorbic acid, had any protective effect on GSH/GSSG levels (Fig. 1) (37, 38, 46). This suggests that the sulfhydryl groups are critical for antioxidant protective effects against DEP chemicals.

Oxidative stress leads to covalent modification of cellular lipids and proteins, which can be conveniently assessed by lipid peroxidation and protein oxidation assays (41, 42). RAW 264.7 cells treated with 50 μg/ml extract for 5 h showed a 1.8-fold increase in lipid peroxide levels compared with control cells ($p < 0.001$; Fig. 2A). Interestingly, all the antioxidants, including nonthiol agents, were effective in depressing lipid peroxidation ($p < 0.001$; Fig. 2A), suggesting effective cellular uptake. We also assessed carbonyl groups as a measure of protein ε-amino oxidation. RAW 264.7 cells treated with the same extract showed a 15-fold increase

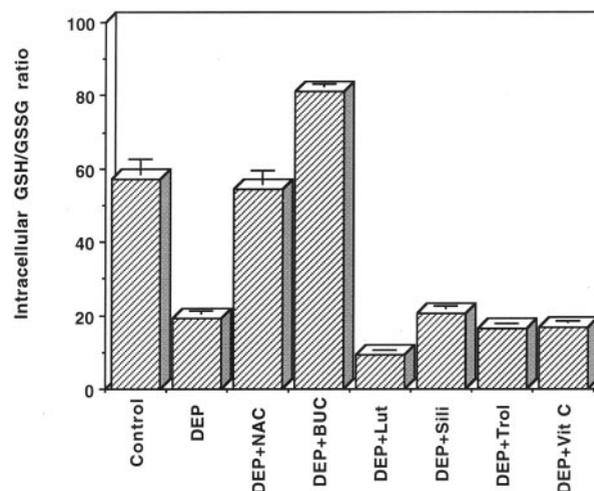


FIGURE 1. In vitro exposure to an organic DEP extract induces a decrease in the GSH:GSSG ratio in RAW 264.7 cells. Cells were treated with 50 μg/ml DEP extract in the presence or the absence of the following antioxidants for 5 h: NAC, 20 mM; BUC, 5 mM; luteolin (Lut), 50 μg/ml; silibinin (sili), 25 μg/ml; trolox (water-soluble vitamin E), 1 μM; and vitamin C (Vit C), 100 μM. Cell lysates were used to measure the cellular GSH:GSSG ratios in duplicate, as described in *Materials and Methods*. Values are the mean ± SEM. This experiment was reproduced once, with similar results.

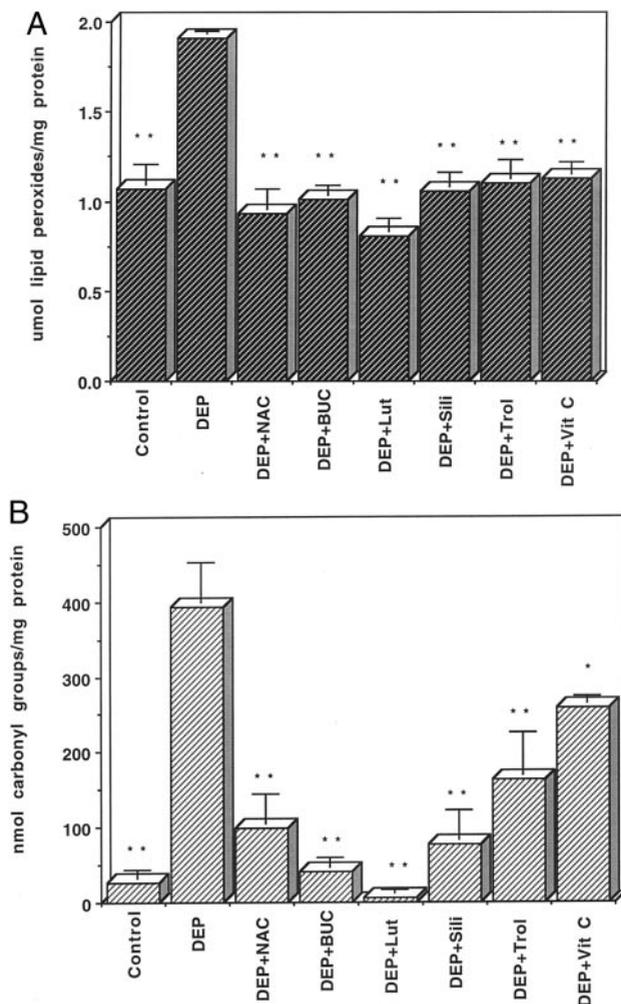


FIGURE 2. Effects of various antioxidants on lipid and protein oxidation in RAW 264.7 cells. RAW 264.7 cells were exposed to 50 $\mu\text{g}/\text{ml}$ DEP with or without the indicated antioxidants for 5 h. Cell lysates from triplicate cellular aliquots were used to measure carbonyl protein content (A) and lipid hydroperoxides (B) as described in *Materials and Methods*. Values are the mean \pm SEM. *, $p < 0.05$; **, $p < 0.001$ (statistically different compared with DEP).

in carbonyl groups compared with controls ($p < 0.001$; Fig. 2B). Similar to the effect on lipid peroxides, thiol and nonthiol antioxidants suppressed protein oxidation ($p < 0.001$). However, the thiols and flavanoids were more potent than trolox and vitamin C (Fig. 2B).

Although Fig. 2 shows that all antioxidants tested exert antioxidant effects in RAW 264.7 cells, the data in Fig. 1 suggest that these drugs are not equivalent in their effects on the intracellular glutathione ratio. Therefore, these drugs may not exert equivalent effects in vivo. For that reason we compared antioxidant interference with HO-1 expression in vitro (26). HO-1 is a very sensitive oxidative stress protein that is involved in cytoprotective effects against redox-cycling chemicals, including DEP quinones (47–49). HO-1 expression involves a cascade of events that include transcriptional activation of several antioxidant response elements in the promoter of that gene (47, 49). RAW 264.7 cells treated with 50 $\mu\text{g}/\text{ml}$ of the DEP extract were used to compare the effects of different antioxidants on HO-1 expression (Fig. 3). While both thiol agents effectively suppressed HO-1 expression, as determined by immunoblotting (Fig. 3, lanes 3 and 4), silibinin, vitamin E, and vitamin C had no inhibitory effect (Fig. 3, lanes 6–8).

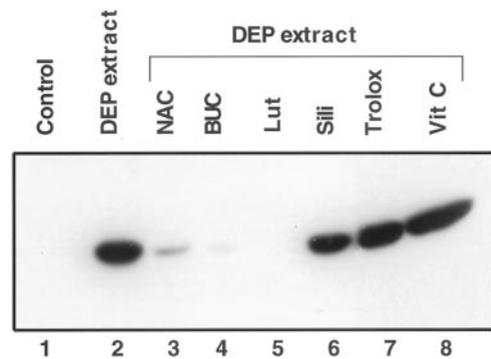


FIGURE 3. Autoradiogram showing the effects of various antioxidants on HO-1 protein expression in RAW 264.7 cells. Cells were treated with 50 $\mu\text{g}/\text{ml}$ DEP extract for 5 h in the presence or the absence of the various antioxidants as described in Fig. 1. Immunoblotting of cell lysates was performed as previously described (26). The results in this experiment were confirmed in additional experiments in which two or more of these antioxidants were used concurrently to attempt to inhibit HO-1 expression.

Interestingly, luteolin suppressed HO-1 expression (Fig. 3, lane 5), which agrees with its in vitro suppressive effects on protein oxidation (Fig. 2B).

The above data clearly indicate that the thiol agents are the only antioxidants that replenished glutathione levels and suppressed HO-1 expression. However, we show that antioxidants in general could abrogate the generation of oxidative stress markers such as lipid peroxides and oxidized proteins.

Thiol antioxidants interfere in the adjuvant effect of inhaled DEP in an OVA sensitization model

Because we are pursuing the hypothesis that DEP-induced proinflammatory and proallergic effects are mediated through the generation of oxidative stress, we tested the thiol antioxidants in an OVA inhalation-sensitization model in BALB/c mice. In establishing this model, mice were sequentially exposed to a range of aerosolized DEP concentrations plus OVA daily for 10 days. Controls consisted of mice receiving daily saline, OVA alone, or DEP alone for the same duration. While animals exposed to aerosolized OVA alone showed a small increase in OVA-specific IgE, animals treated with DEP alone failed to induce a response (Table I). In contrast, animals receiving OVA plus DEP showed a dose-dependent increase in OVA-specific IgE, which became statistically significant at a DEP dose of 600 $\mu\text{g}/\text{m}^3$ ($p < 0.01$; Table I). Because IgG1 isotype switching accompanies rearrangement of the ϵ -chain locus in the mouse, we also assessed OVA-specific IgG1 levels in the same experiment. The data show a statistically significant increase in OVA-specific IgG1 in animals receiving OVA plus DEP, with the DEP effect starting at 200 $\mu\text{g}/\text{m}^3$ ($p < 0.05$; Table I). These changes were accompanied by a small, but statistically significant, increase in total IgE levels in animals exposed to OVA plus 2000 $\mu\text{g}/\text{m}^3$ DEP ($p < 0.05$; Table I).

In contrast to the serological evidence of sensitization, there were no morphological changes or evidence of gross airway inflammation in lung sections from all eight groups stained with H&E. BAL fluid showed a small, but statistically insignificant, increase in neutrophil numbers in animals treated with DEP doses of 600 $\mu\text{g}/\text{m}^3$ ($11.0 \pm 3.4 \times 10^3/\text{ml}$) and 2000 $\mu\text{g}/\text{m}^3$ ($12.1 \pm 2.8 \times 10^3/\text{ml}$) compared with the saline control ($7.8 \pm 1.8 \times 10^3/\text{ml}$). Addition of OVA made no difference to the neutrophil response at 600 $\mu\text{g}/\text{m}^3$ DEP ($11.1 \pm 3.2 \times 10^3/\text{ml}$) but did increase the neutrophil count to $16.1 \pm 3.9 \times 10^3/\text{ml}$ ($p < 0.001$) when combined with 2000 $\mu\text{g}/\text{m}^3$ DEP. There was no significant

change in BAL eosinophil numbers, which generally remained at <2% of the total BAL cell count in all groups tested (data not shown). The short-term exposure model described in this study differs therefore from chronic/high dose DEP inhalation models, where gross increases in airway inflammation are seen in OVA-sensitized mice (9–12). All considered, we have established a short-term exposure model in which OVA-specific IgG1 and IgE are helpful for demonstrating the adjuvant effects of DEP by an inhalation sensitization procedure. This mimics the adjuvant effects of DEP previously depicted in humans and animals (4–12).

Our next experiment tested the effect of thiol antioxidants in the inhalation-sensitization model. Compared with mice receiving saline only, animals exposed to aerosolized OVA alone showed no effect on OVA-specific IgE (Fig. 4A) and IgG1 (Fig. 4B). The DEP-only control was omitted from this experiment, because this treatment did not show an effect on OVA-specific Ab levels (Ta-

ble I). In contrast to the OVA-only group, animals exposed to DEP plus OVA showed 19- ($p < 0.005$) and 80-fold ($p < 0.001$) increases in OVA-specific IgE (Fig. 4A) and IgG1 (Fig. 4B) levels. Importantly, the adjuvant effect was suppressed in a statistically significant fashion ($p < 0.001$) in DEP- plus OVA-treated animals by daily peritoneal administration of NAC or BUC (Fig. 4). These antioxidants did not exert an effect in the saline- or OVA-only controls (Fig. 4). As in the previous experiment, we also performed lung histology and BAL to look for evidence of gross airway inflammation. No evidence was obtained for a significant increase in eosinophil, IL-5, or GM-CSF levels in the BAL fluid (data not shown). There were no morphological changes observed in the lungs of mice exposed to OVA, DEP, or DEP plus OVA during H&E staining (data not shown). We also did not observe an increase in mucin production in the airways using Alcian blue or periodic acid-Schiff staining, or an increase in MBP deposition as determined by immunohistochemistry (data not shown). This confirms that the increase in IgE and IgG1 isotype switching during the sensitization period is not accompanied by a significant efferent inflammatory response.

NAC and BUC suppressed generation of oxidative stress in the lung of mice exposed to DEP plus OVA

Because the data in Fig. 4 suggest that the adjuvant effects of DEP are dependent on ROS production, we performed several assays that reflect generation of oxidative stress in BAL fluid and lung tissue. First, we could not discern any DEP or drug effects on the total or fractional glutathione levels in BAL fluid or lung tissue (data not shown). However, we were able to show that DEP and thiol antioxidants affect the carbonyl protein and lipid peroxide content in the lungs of the animals used in Fig. 4. First, there was a 6-fold increase in carbonyl protein content in mice exposed to DEP and OVA compared with that in mice exposed only to OVA ($p < 0.001$; Fig. 5A). Both NAC and BUC were able to significantly ($p < 0.05$) suppress protein oxidation in these animals (Fig. 5A). The lipid peroxidation assay verified the protein data (Fig. 5B). Thus, there was a 2.9-fold increase ($p < 0.001$) in lipid peroxide levels in animals exposed to DEP plus OVA compared with those exposed to OVA alone (Fig. 5B). Moreover, both thiol antioxidants were able to significantly ($p < 0.01$) reduce lipid peroxide levels in the lung (Fig. 5B). All considered, this shows an excellent correlation between the *in vitro* and *in vivo* antioxidant effects of thiol antioxidants as well as their ability to interfere with DEP adjuvant effects in the lung.

Discussion

In this communication we demonstrate that it is possible, through the use of thiol antioxidants, to inhibit the adjuvant effects of DEP on OVA-induced IgG1 and IgE production. The thiol antioxidants also suppressed protein oxidation and lipid peroxidation in the lungs of mice receiving DEP and OVA, which agrees with their *in vitro* ability to inhibit oxidative stress by organic DEP chemicals. Although other classes of antioxidants could affect protein and lipid oxidation *in vitro*, these agents do not prevent the decrease in intracellular GSH:GSSG ratios or the induction of HO-1 expression (with the exception of luteolin). Taken together, these data indicate that the oxidative stress effect of DEP is directly linked to the ability of these particles to exert adjuvant effects in the lung. Thiol antioxidants are effective in reversing the adjuvant effects of DEP *in vivo*.

Although epidemiological studies have clearly established a positive relationship between exposure to ambient PM and adverse health effects in susceptible human subpopulations (1–3), there is still a fundamental lack of understanding of the most toxic particle

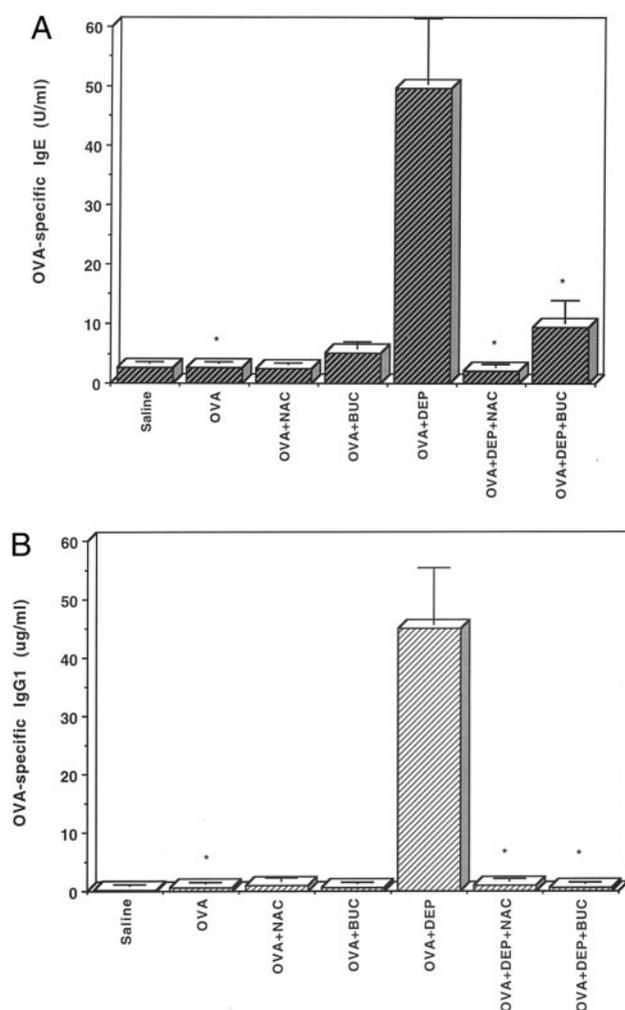


FIGURE 4. Thiol antioxidants interfere in the adjuvant effect of inhaled DEP in an OVA sensitization model. BALB/c mice (six animals per group) were exposed to saline, OVA, or OVA plus DEP by aerosolized inhalation daily for 10 days as described in *Materials and Methods*. NAC or BUC was given by i.p. injection 0.5 h before inhalation on every exposure day. Serum obtained from the animals 24 h after the last exposure was used to measure OVA-specific IgE (A) and IgG1 (B) values by ELISA. Values are the mean \pm SEM. This experiment was reproduced once using the same animal groups, except for the groups receiving BUC. In this experiment NAC also interfered in statistical significant fashion in the adjuvant effect of DEP plus OVA. *, $p < 0.001$ (statistically different when compared with OVA plus DEP).

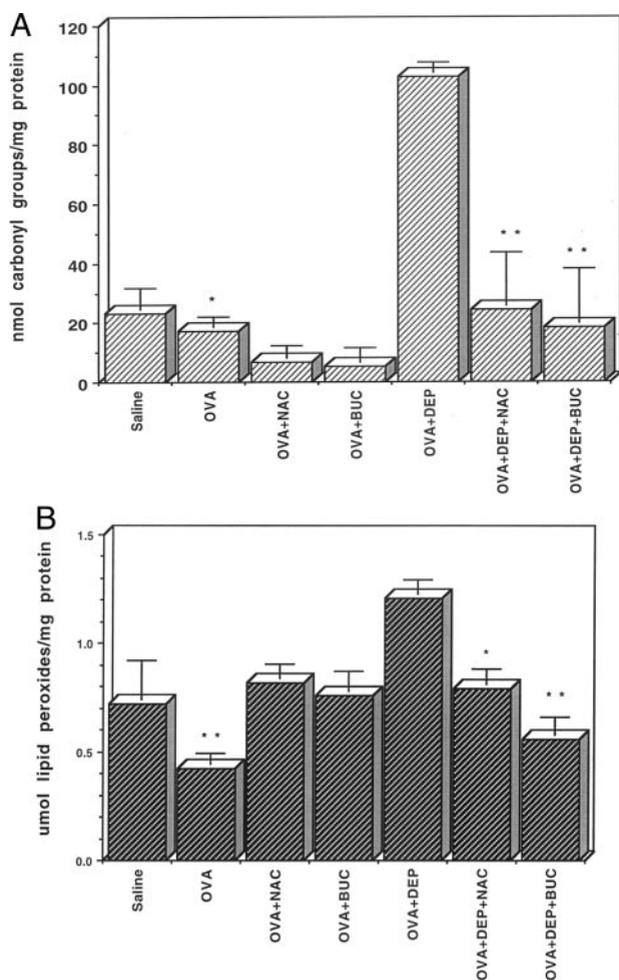


FIGURE 5. Assays for carbonyl protein content and lipid hydroperoxides in the lung homogenates of the animals described in Fig. 4. Approximately 200 mg lung tissue from each animal was homogenized as described in *Materials and Methods*. These homogenates were used to determine carbonyl protein content (A) and lipid hydroperoxides (B) as previously described (41, 42). Values are the mean \pm SEM. *, $p < 0.05$; **, $p < 0.001$ (statistically different when compared with OVA plus DEP).

constituents and the toxicological mechanisms through which they act (50). Research into these issues is of key importance for understanding the disease mechanism (50) as well as developing rational treatments that will reverse the pulmonological effects of these particles. Our studies and the work of others have highlighted the role of ROS, catalyzed by organic chemical compounds, in the proinflammatory effects of DEP and PM in the respiratory tract (4–6, 8–15, 21, 51, 52). While we still need to learn a great deal about the drug transformation pathways by which DEP chemicals generate ROS in the human lung, studies of lung microsomes have shown that redox cycling quinones are involved in O_2^- generation by DEP extracts (17). Moreover, our own studies have confirmed that aromatic and polar chemical groups fractionated from DEP induce HO-1 expression in tissue culture macrophages (26). These chemical groups are enriched for PAH and oxy-PAHs, respectively (26, 53). HO-1 expression is a sensitive oxidative stress response (48) that is dependent on the transcriptional activation of the HO-1 gene via antioxidant response elements in its promoter (49). This response is particularly relevant to the lung, in which HO-1 has been shown to exert potent antioxidant effects (47). In addition, it has been demonstrated that CO in exhaled air is a marker for oxidative stress in asthma and the most sensitive biological response

marker for human subjects exposed to DEP in vivo (54, 55). CO is a catalytic product that is generated when heme is converted to biliverdin by HO-1 (48). In addition to the role of redox cycling DEP quinones, organic DEP extracts also generate O_2^- production by a mitochondrial pathway that follows perturbation of the mitochondrial permeability transition (PT) pore and disruption of one electron transfers in the mitochondrial inner membrane (24, 25). This pathway is involved in the induction of apoptosis by oxidizing DEP chemicals (25).

In addition to the role of PM, H_2O_2 is derived from activated eosinophils, neutrophils, and APC recruited to the airways of asthmatics (31–36). These inflammatory cells generate NO, which may combine with O_2^- to form the peroxynitrite anion ($ONOO^-$). Peroxynitrite is important in the lung because it induces lipid peroxidation and epithelial injury (56, 57). ROS production also contributes to airway inflammation through the induction of cytokines, chemokines, and adhesion molecules (18, 21, 58, 59). In this regard it is known that DEP and oxidative stress lead to activation of the NF- κ B and mitogen-activated protein kinase cascades in macrophages and epithelial cells (18, 60–62). These signaling cascades regulate transcriptional activation of cytokine, chemokine, and adhesion molecule genes (27, 60). Apoptosis of epithelial cells, through the effects of DEP chemicals on the mitochondrial PT pore (25) and $ONOO^-$ generation (56, 57), may contribute to airway hyperreactivity. In this regard it has been shown that oxidative injury of the epithelial layer contributes to bronchial hyperresponsiveness in humans (31). Moreover, improvement in airway hyperreactivity during corticosteroid treatment is accompanied by reduced chemiluminescence in BAL cells obtained from asthmatics (34). In the setting of PM exposure, thiol agents may interfere in oxidative stress that contributes to the generation of airway inflammation as well as the oxidative stress that sustains airway inflammation once established. The possible pathways by which oxidative stress may contribute to Ag-specific IgE synthesis include effects on cytokine/chemokine production as well as enhanced Ag presentation. More specifically, this may include a role for oxidative stress in the expression of CD80, RANTES, TNF- α , IL-8, and adhesion molecules (18, 60, 63, 64).

The relative selectivity of the thiols in reversing the oxidative stress effects of DEP chemicals is of considerable interest. Although the thiols, similar to the flavanoids and vitamin C, act as radical scavengers that prevent protein and lipid oxidation (Fig. 2), this class of antioxidants also stimulates glutathione synthesis. The effect of the thiols on glutathione synthesis may explain the preservation of the GSH:GSSG ratio in cells exposed to DEP in vitro (Fig. 1). A third mechanism of action includes covalent binding of thiols to oxy-PAHs and quinones by a 1,4-Michael addition reaction (65). This may lead to active removal of the quinones from the cell as well as protect the mitochondrial PT pore against oxidizing DEP chemicals (66). PT pore opening is regulated by vicinal thiol groups, which, upon cross-linking, induce PT (66). Protection of these vicinal sulfhydryl groups by thiol antioxidants may avert PT pore opening and protect the cell against apoptosis and further O_2^- generation (66). It is interesting that NAC, a Food and Drug Administration-approved drug for treatment of acetaminophen toxicity, prevents toxic damage to the liver by a benzoquinone derivative of that drug (67). In addition, NAC has also been used to reverse mutagenic effects of particulate air pollutants in rat lungs (68). All considered, the unique biochemical effects of thiol antioxidants might explain their effectiveness in suppressing the adverse effects of DEP in this study.

An interesting feature of our study is that short-term DEP plus OVA exposure exerts a profound effect on Ab production, yet induces a relatively small increase in BAL neutrophils without

evidence of gross airway inflammation or eosinophilia. This stands in contrast to chronic DEP exposure, where high particle doses delivered daily for 6 wk or longer can induce eosinophilia and gross airway inflammation (9–12). While the exact explanation for these differences is unknown, it is possible that the 10-day exposure period is too short to lead to a significant efferent response after the initial sensitization. Another possibility is that limited tissue inflammation is not picked up by random tissue sectioning. In this regard, it is known that high particle deposition rates at airway bifurcation points create hot spots where biological effects can occur (69). This includes APC activation by DEP chemicals, which allows these cells to ingest OVA and transfer the Ag to regional lymph nodes for presentation to Th2 cells. Because the total surface area of these high impact sites may be limited in size (a few square millimeters), widespread pulmonary inflammation may fail to develop. While it has not been estimated with any degree of certainty what the minimal mucosal surface area is that leads to allergen sensitization, it is conceivable that a summation of the deposition hot spots may suffice to generate a systemic OVA-IgE or IgG1 response. The number of exposures and the level of sensitization that are required for subsequent induction of an efferent response, including airway hyperreactivity, are currently being studied.

In conclusion, we have shown that thiol antioxidants are highly effective in reversing the adjuvant effects of DEP in the murine lung. While it still needs to be demonstrated that this will lead to a reduction of airway hyperreactivity, these results are of key importance in designing rational asthma therapy, particularly interfering in the adjuvant effects of particulate air pollutants on allergic inflammation.

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