Alteration of Intracellular Cysteine and Glutathione Levels in Alveolar Macrophages and Lymphocytes by Diesel Exhaust Particle Exposure

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The purpose of this study was to characterize the effects of diesel exhaust particles (DEP) on thiol regulation in alveolar macrophages (AM) and lymphocytes. We obtained AM and lymph node (thymic and tracheal) cells (LNC) (at different time points) from rats exposed intratracheally to DEP (5 mg/kg) or saline, and measured inflammatory markers, thiol levels, and glutathione reductase (GSH-R) activity. DEP exposure produced significant increases in neutrophils, lactate dehydrogenase, total protein, and albumin content in the lavage fluid. AM from DEP-exposed rats showed a time-dependent increase in intracellular cysteine (CYSH) and GSH. In LNC the intracellular GSH reached peak level by 24 hr, declining toward control levels by 72 hr after exposure. LNC-CYSH and AM-CYSH and GSH were increased at both 24 and 72 hr. Both Sprague-Dawley and Brown Norway rats showed similar trends of responses to DEP exposure as per measurement of the inflammatory markers and thiol changes. AM and, to a lesser degree, LNC were both active in cystine uptake. The DEP exposure stimulated GSH-R activity and increased the conversion of cystine to CYSH in both cell types. The intracellular level of GSH in DEP-exposed AM was moderately increased compared with the saline control, and was further augmented when cells were incubated with cystine. In contrast, the intracellular level of GSH in DEP-exposed LNC was significantly reduced despite the increased CYSH level and GSH-R activity when these cells were cultured for 16 hr. DEP absorbed 23–31% of CYSH, cystine, and GSH, and only 8% of glutathione disulfide when incubated in cell free media. These results indicate that DEP exposure caused lung inflammation and affected thiol levels in both AM and LNC. *Key words:* cysteine, diesel exhaust particles, glutathione, glutathione reductase, inflammation, intratracheal instillation. *Environ Health Perspect* 110:349–353 (2002). [Online 1 March 2002] *http://ehpnet1.niehs.nih.gov/docs/2002/110p349-353al-humadi/abstract.html*

Diesel engine exhaust is an occupational and environmental health concern. The particulate phase of diesel engine exhaust is thought to contribute to the increased prevalence of acute and chronic airway diseases. Exposure to diesel exhaust particles (DEP) of > 2 mg/m^3 has been documented in underground mines, railroad, construction, and in auto repair industries (*1*). Such exposures have been associated with pulmonary inflammation, fibrosis, lung cancer, increased rate of respiratory infections, and enhanced allergic sensitization (*2–6*). DEP are a complex mixture of polycyclic aromatic hydrocarbons (PAHs), ash, transition metals, and a carbon core. Major PAH components of DEP include phenanthrenes, fluorenes, naphthalenes, fluoranthrenes, and pyrenes (*7*). Previously we demonstrated that both the organic and the particulate components play a major role in DEP-induced pulmonary toxicity. Although the particulate induces acute inflammatory responses, the organic component suppresses both secretion of proinflammatory cytokines by alveolar macrophages (AM) and cell-mediated immunity in response to bacterial infection (*4–6*). In addition, DEP exposure has been linked to increased IgE production (*3*) and isotype switching in B cells to produce IgE in humans (*8*) and in animals (*9*). These studies suggest that DEP may suppress cellular but enhance allergic immune responses.

Glutathione (GSH), long known for its protective function against oxidative cell damage (*10*), is thought to play a regulatory role in various lymphocyte functions. Depletion of intracellular GSH by buthionine sulfoximine decreases the proportion of CD8$^+$ cells, inhibits the generation of large blastlike CD8$^+$ cells, and decreases cytotoxic T-lymphocyte activity (*11*). The biosynthesis of GSH in lymphocytes requires intracellular cysteine (CYSH), an acid-soluble thiol produced and released by AM. Gmunder et al. (*12*) reported that exogenously added CYSH increases the intracellular GSH level and the activity of DNA synthesis in mitogen-stimulated lymphocytes, suggesting that CYSH also plays a regulatory role in mediating immunologically relevant functions of lymphocytes. Gmunder and Droge (*11*) demonstrated that GSH mediates cytokine-dependent DNA synthesis in T lymphocytes and that the depletion of intracellular GSH inhibits relevant DNA synthesis and alters T-lymphocyte activity.

Studies show that lymphocytes exhibit strong membrane transport activity for CYSH, but only a weak transport activity for cystine (*13*). In contrast, AM have a strong capacity for cystine uptake and the conversion of cystine into CYSH (*14*). In fact, Gmunder et al. (*12*) have shown that macrophages, when stimulated, function as a CYSH pump that takes up cystine and releases CYSH, increasing the intracellular GSH level of activated lymphocytes in the vicinity. These studies suggest that AM may regulate T-lymphocyte responses through thiol regulation. To provide more insight into the effects of DEP exposure on pulmonary immune responses, AM and pulmonary lymph node cells (LNC) from saline- and DEP-exposed rats were isolated and studied for thiol regulations. In this study we report the effects of DEP exposure on cellular uptake and use of cystine, glutathione reductase (GSH-R) activity, and production of CYSH and GSH in AM and LNC.

Materials and Methods

Particulate samples preparation. We purchased DEP standardized sample (standard reference material 1650) from the National Institute of Standards and Technology (Gaithersburg, MD, USA). The particles were representative of heavy-duty diesel engine emissions with a mass median aerodynamic diameter of approximately 0.5 µm. We used endotoxin-free sterile saline (Baxter Healthcare Corporation, Deerfield, IL, USA) to suspend the particles. The DEP suspension was sonicated for 1 min using an ultrasonic processor with micro tip (Heat System-Ultrasonics, Plainview, NY, USA) before intratracheal (IT) instillation.

Animal exposure. Male Sprague-Dawley (SD) rats (200–250 g; Hilltop, Scottsdale, PA, USA) were used. Brown Norway (BN) rats (200–250 g; Charles River, Stoneridge, NY, USA) were used for strain comparison purposes. Animals were housed in an American Association for Accreditation of Laboratory Animal Care-approved facility and maintained at 23 ± 1°C with 50% relative humidity and a 12-hr light/dark cycle.

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Food and water were given *ad libitum*. Methohexital sodium, 35 mg/kg body weight, intraperitoneal (IP; Eli Lilly Co., Indianapolis, IN, USA), was used to lightly anesthetize the animals before their placement in a vertical position for IT instillation. We used a curved, ball-tipped cannula (18 gauge) to briskly inject the DEP suspension (5 mg/kg body weight) into the trachea. Control animals received the sterile saline vehicle. The instillation volume was 2 mL/kg body weight of each rat. AM and LNC were collected 4, 24, and 72 hr after exposure and used for CYSH and GSH determination. In separate experiments, cell samples were collected 72 hr after exposure either for GSH-R activity measurements or for culture in HEPES for 16 hr with or without cystine (24 µg/mL) for CYSH and GSH assessment.

Bronchoalveolar lavage and biochemical assay of bronchoalveolar lavage fluid. Rats were anesthetized with sodium pentobarbital, 50 mg/kg, IP (Butler, Columbus, OH, USA), and euthanized by exsanguination of the abdominal aorta. The trachea was cannulated and the lungs were lavaged with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS; 145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 9.35 mM Na_2HPO_4, and 5.5 mM glucose; pH 7.4) at a volume of 6 mL for the first lavage and 8 mL for the subsequent lavages. The process continued until a total of 80 mL was recovered. The bronchoalveolar fluid (BALF) was centrifuged at 500 × *g* for 10 min at 4°C. Supernatant fluid from the first lavage (4 mL/rat) was saved separately. All cell pellets from an individual rat were combined and suspended in 1 mL PBS to determine the total cell, macrophage, and granulocyte cell counts using an electronic cell counter (Coulter Electronics, Hialeah, FL, USA) equipped with a cell-sizing unit (*15*). AM and granulocytes were distinguished by their cell volumes. The AM were used for primary cell culture to determine the thiol levels. We used the acellular BALF from the first lavage to determine the lactate dehydrogenase (LDH) activity, total protein, and albumin content using an automated Cobas Fara II analyzer (Roche Diagnostic Systems, Montclair, NJ, USA) following standard diagnostic reagent and manufacturer's procedures.

Preparation of lymph node cells. Lymph nodes (parathymic and tracheal) were collected in 1 mL of 10 mM (pH 7.4) HEPES buffer. The lymph node capsules were broken with a glass bar using a tissue culture screen. LNC were washed with HEPES, transferred into a 15-mL tube, and centrifuged at 2,000 rpm for 10 min. Pellets were reconstituted with 1 mL HEPES buffer and lymphocytes were isolated by Histopaque (density, 1.083; Sigma Chemical Co., St. Louis, MO, USA). The samples were centrifuged for 30 min at 2,500 rpm, and lymphocytes were collected, washed twice, and resuspended with 1 mL HEPES buffer. Aliquots of 2×10^6 cells were either used immediately to determine GSH-R activity or further incubated in microcentrifuge tubes with or without cystine (24 µg/mL) for 16 hr in a humidified incubator (37°C). After incubation, cultures were centrifuged at 500 × *g* for 5 min. The pellets were resuspended in 1 mL HEPES buffer and used for cell count, differential count, and analysis of CYSH and GSH. LNC preparations were > 98% lymphocytes. For the determination of CYSH and GSH levels, 1 µL of 80 mM monobromobimane (mbb) solution was added to 100 µL cell suspension. Derivatization was allowed to proceed at 4°C, overnight; cells were then lysed by sonication for 30 sec using a sonic dismembrator with micro tip (Fisher Scientific, Pittsburgh, PA, USA). Cellular membranes and debris were removed by centrifugation at 10,000 rpm for 10 min and filtration through 0.2-µm filters. CYSH and GSH were measured using a reverse-phase high-performance liquid chromatography (HPLC).

Alveolar macrophage cell culture. AM from the BALF of each rat were suspended in Eagle Minimum Essential Medium culture medium (Sigma) containing 1 mM glutamine, 100 µg/mL streptomycin, 100 units/mL penicillin, 10% heat-inactivated fetal bovine serum, and 10 mM HEPES. We added aliquots of 1 mL cell suspensions from each rat, adjusted to 1×10^6 AM, to each well of a 12-well tissue culture plate. AM were allowed to adhere to the plastic plate for 2 hr in a humidified incubator (37°C and 5% CO_2) (*16*). The nonadherent cells were then removed by rinsing the monolayers three times with HEPES buffer. These AM-enriched cells were incubated (37°C and 5% CO_2) in fresh HEPES buffer with or without cystine (24 µg/mL) for 16 hr. After centrifugation at 500 × *g* for 5 min, both the pellets and supernatants were derivatized immediately with mbb (as described above) for CYSH and GSH determination.

High-performance liquid chromatography analysis. A Shimadzu HPLC system equipped with a RF-551 fluorometric detector (Shimadzu Scientific Instruments, Inc., Columbia, MD) and a C18 reverse-phase column (Luna, 5 µm; Phenomenex, Torrance, CA) was used for the analysis of CYSH and GSH. We separated the mbb-derivatized thiols using a mobile phase of 7% acetonitrile (in 0.1 M sodium acetate, pH 5.0) for the first 6 min, and a step-gradient system to 15, 25, and 40% after 10, 15, and 18 min, respectively, with a flow rate of 1 mL/min. We detected CYSH and GSH at 8.0 and 10.2 min, respectively, using an excitation of 380 nm and an emission of 480 nm.

Glutathione reductase activity. The GSH-R activity was determined by measuring the reduction of glutathione disulfide (GSSG) to GSH. In brief, a 200-µL sample was added to a cuvette containing 400 µL of

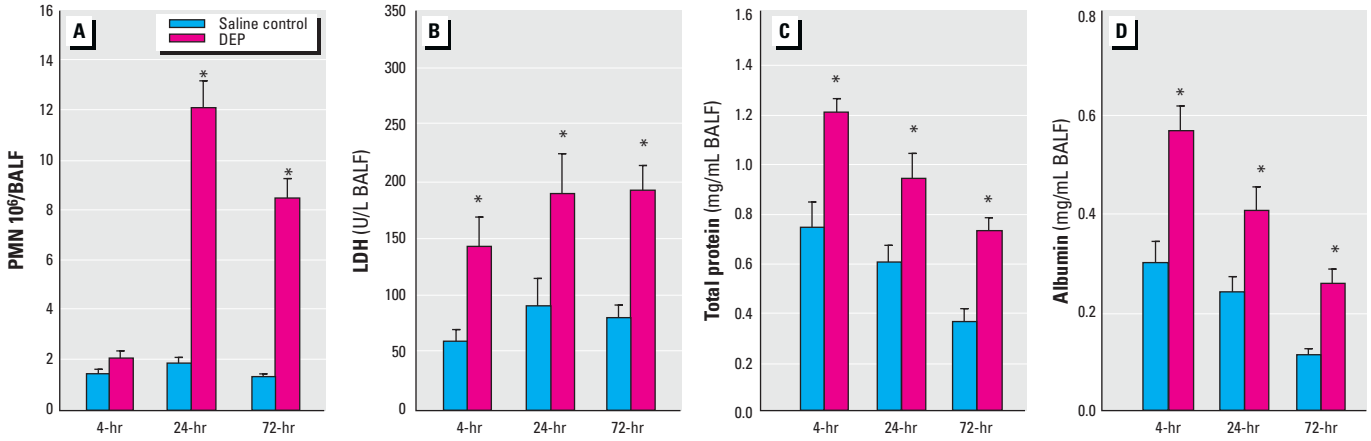

Figure 1. The effect of IT DEP on (*A*) neutrophil count, (*B*) LDH, (*C*) total protein, and (*D*) albumin in SD rats. PMN, polymorphonuclear leukocytes.
*Significantly different from saline control at $p < 0.05$ (*t*-test).

2.4 mM GSSG reagent (Sigma) and 200 µL of 3 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB, Sigma). At time zero, we added 400 µL nicotinamide adenine dinucleotide phosphate and recorded the rate of changes in ultraviolet absorbance at 412 nm over 6 min. The enzyme activity was determined and expressed as micromoles GSH per minute per million cells.

Data analysis. All data were expressed as mean ± SE of 2–4 separate experiments. Each analysis was run in duplicate or triplicate per experiment. We performed statistical analysis of data using Sigma Stat (version 2.0; Jandel Scientific Software, San Rafael, CA, USA) statistical software for Windows 95, NT, and 3.1. ANOVA and *t*-test were conducted, and values of $p < 0.05$ were considered statistically significant.

Results

Figure 1 shows the pulmonary inflammatory responses to DEP exposure as indicated by a time-dependent increase in neutrophil infiltration, LDH level, total protein, and albumin content in the lavage fluid. The neutrophil count peaked (6-fold over control) by 24 hr and was still elevated 72 hr after exposure. We observed significant lung damage, indicated by BALF LDH activity, protein, and albumin content, 4, 24, and 72 hr after exposure. The corresponding changes in intracellular CYSH and GSH in AM and LNC in relation to the saline control are shown in Figure 2. In response to DEP exposure, AM showed an acute elevation in CYSH (10-fold) and GSH (3-fold) levels (24 hr after exposure) that was still elevated 72 hr after exposure. In comparison, CYSH in LNC was increased starting at 4 hr and remained elevated at 24 and 72 hr after DEP exposure. There was a delay in the increase of GSH levels in LNC, with a peak concentration (1.5-fold over control) at 24 hr. The elevation of GSH levels in DEP-exposed lymphocytes declined toward control values by 72 hr postexposure.

Figure 3 shows the effects of DEP exposure on the capacity of AM to generate CYSH and GSH. Cells from rats exposed to IT-DEP or saline for 72 hr were incubated *ex vivo* with 24 µg/mL cystine for 16 hr. In saline-exposed cells the CYSH level was increased 6-fold over the control by the presence of cystine, indicating that AM exhibit a strong capacity to take up cystine and reduce it to CYSH. DEP exposure enhanced AM capacity to convert cystine to CYSH (Figure 3A). The addition of cystine (in DEP-exposed AM) increased the cellular level of CYSH 10-fold. The level of GSH in AM from control rats was not affected by *ex vivo* incubation with cystine for 16 hr despite the increase in CYSH, but was significantly increased by DEP exposure, particularly in the presence of cystine (Figure 3B). These results show that although CYSH is required for the synthesis of GSH, increased levels of intracellular CYSH did not necessarily produce greater GSH levels in cells from saline-exposed control animals at this 16-hr time point. The cells from DEP-exposed rats, on the other hand, exhibited increased uptake and conversion of cystine to CYSH as well as significantly elevated GSH level, suggesting an enhanced GSH synthesis.

Figure 4 shows the effects of DEP exposure on the thiol regulation in LNC. The *ex vivo* incubation with cystine increased CYSH production 2-fold, suggesting that these cells have the capacity to take up and convert cystine to CYSH. In comparison, the DEP exposure resulted in a 6-fold enhancement of CYSH production in LNC, with or without the addition of cystine (Figure 4A). Figure 4B shows that the GSH levels in LNC were not affected by *ex vivo* cystine, but were significantly reduced (60%) by the DEP exposure when cultured for 16 hr in thiol-free media.

We investigated the possibility that DEP may affect solution concentrations of various thiols through particle absorption.

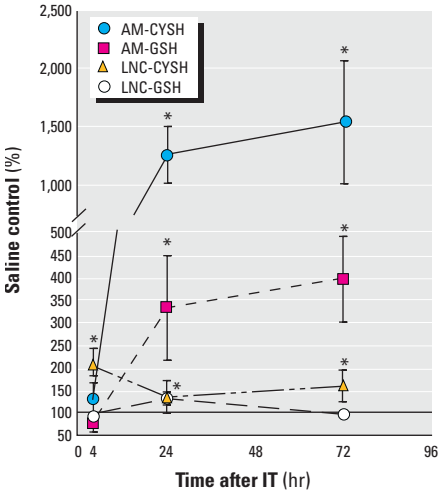

Figure 2. The effect of IT instillation of DEP on the levels of AM-cysteine and GSH and LNC-cysteine and GSH at 4, 24, and 72 hr after exposure in SD rats. Average percent control SE for three time points = 7.16, 10.4, and 14.5 for AM-GSH, AM-CYSH, and LNC-GSH, respectively.
*Significantly different from saline control at $p < 0.05$ (ANOVA).

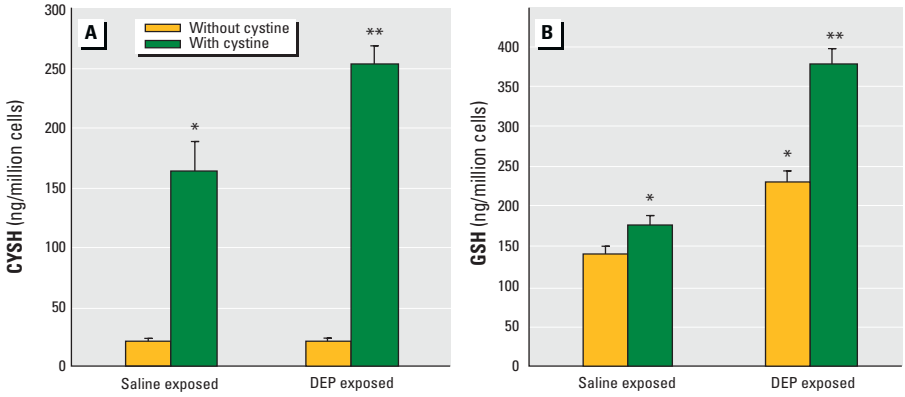

Figure 3. CYSH (*A*) and GSH (*B*) levels in AM of SD rats after *in vivo* IT-DEP and 16 hr *ex vivo* with or without cystine treatment.
*Significantly different from saline control at $p < 0.05$ (ANOVA). **Significantly different from DEP at $p < 0.05$ (ANOVA).

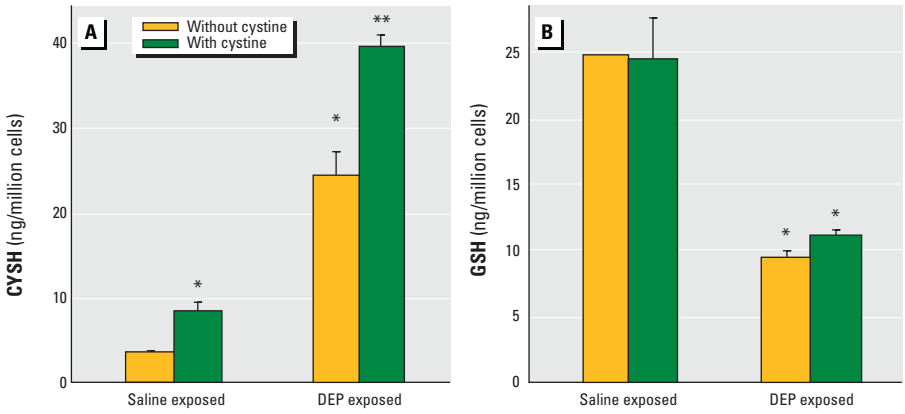

Figure 4. CYSH (*A*) and GSH (*B*) level in parathymic and tracheal LNC of SD rats after *in vivo* IT-DEP and 16 hr *ex vivo* with or without cystine treatment.
*Significantly different from saline control at $p < 0.05$ (ANOVA). **Significantly different from DEP at $p < 0.05$ (ANOVA).

Figure 5 shows that DEP exhibited relatively strong affinities toward cystine, moderate absorptive capacity toward CYSH and GSH, but no effect on GSSG. DEP did not show any absorptive effect on GSH-R (data not shown).

Figure 6 shows the effect of DEP on GSH-R activity in AM (Figure 6A) and lymphocytes (Figure 6B). Cells from rats exposed to saline or DEP for 72 hr were used. The GSH-R activities in DEP-exposed AM and lymphocytes increased 1.8- and 4.0-fold over the saline controls, respectively.

Because the BN rats have been used extensively as a model for allergic sensitization, we conducted similar studies to evaluate thiol changes in the BN rat model. Table 1 compares DEP-induced inflammatory responses in the SD and BN rats. These results show that although they differed in magnitude, both rat models showed similar trends of inflammatory responses to DEP exposure. Thiol regulation also showed similar trends in both strains. It is interesting to note that DEP thiol alteration in the AM of SD rats was notably much greater than that seen in AM from the BN rats.

Discussion

DEP exposure induces pulmonary inflammation, including elevated levels of neutrophils, LDH, and total protein in the airways (*5*). These increases have been associated with increased production in oxygen free radicals in the alveolar space (*17*). AM from DEP-exposed rats show decreased response to lipopolysaccharide and bacterial stimulation in the production of interleukin-1 and tumor necrosis factor-α (*5,6*), suggesting that DEP alters innate immune responses, leading to increased susceptibility to bacterial infection. In addition, several recent studies have linked DEP exposure to allergic inflammation (*18–21*) and increased IgE production (*3*). Other studies have shown that DEP exposure promotes isotype switching to produce IgE in humans (*8*) and in animals (*9*). Together, these studies indicate that DEP may suppress cellular but enhance humoral pulmonary immune responses.

The results of our study are consistent with the concept that CYSH and GSH may play a dual role in DEP-induced pulmonary immune/inflammatory responses. The increase in CYSH and GSH levels in AM (Figure 2) appears to be an acute response to DEP exposure. As antioxidants, these molecules are produced and secreted by AM to protect the lung from particle-induced oxidative stress and injury. Interestingly, our results showed that DEP are capable of absorbing cystine and CYSH and possibly altering the extracellular thiol balance through a noninflammatory mechanism. During the inflammatory process, oxidized thiols such as cystine may be internalized by AM and converted to CYSH. The fact that the DEP induces GSH-R activity in AM (Figure 6) is also consistent with an increased production of CYSH. The DEP exposure also significantly increased AM production of GSH at 24 hr postexposure and persistently increased GSH levels at longer postexposure times (72 hr). The effect of DEP on GSH in AM (*ex vivo*) may be attributed at least in part to increased GSH-R activity.

As shown in Figure 2, the GSH level in LNC increased at 24 hr and declined at 72 hr after DEP exposure. When cultured *ex vivo,* LNC from DEP exposed animals had a significant decrease in GSH. Compared with AM, there was a delayed increase in GSH level in LNC and no increase in GSH from control LNC cultured with cystine. In all cases lymphocytes were the predominant (> 95%) LNC. These data suggest that lymphocytes depend on extracellular GSH released from cells such as macrophages.

To provide more insight into DEP effects on cellular regulation of thiols, AM and lymphocytes from saline and DEP-exposed animals (72 hr after exposure) were isolated and tested for their production of CYSH and GSH (Figures 3 and 4). These results show a clear difference between AM and lymphocytes in their responses to DEP exposure. AM exhibited a strong capacity in the uptake and conversion of cystine to cysteine (CYSH) (Figure 3). This is in line with the results reported by Watanabe and Bannai (*14*), who showed that macrophages exhibit strong membrane transport activity for cystine uptake and are capable of releasing reduced thiol into the extracellular space. DEP exposure increased AM production of CYSH and GSH 2-fold. AM from rats exposed to DEP showed a significant increase in GSH-R activity (Figure 6). The DEP exposure did not affect the intracellular level of CYSH in the

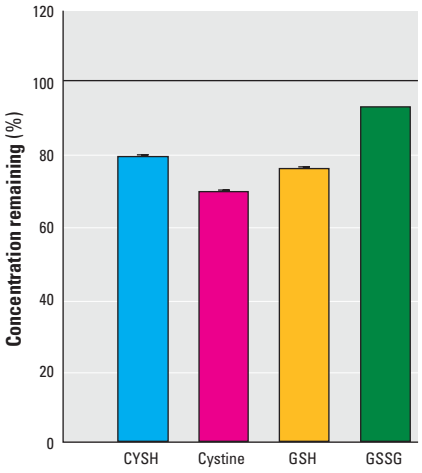

Figure 5. Percent absorption of CYSH, cystine, GSH, and GSSG in a cellular culture media (no cells added) after 4-hr incubation with (50 µg/mL) DEP.

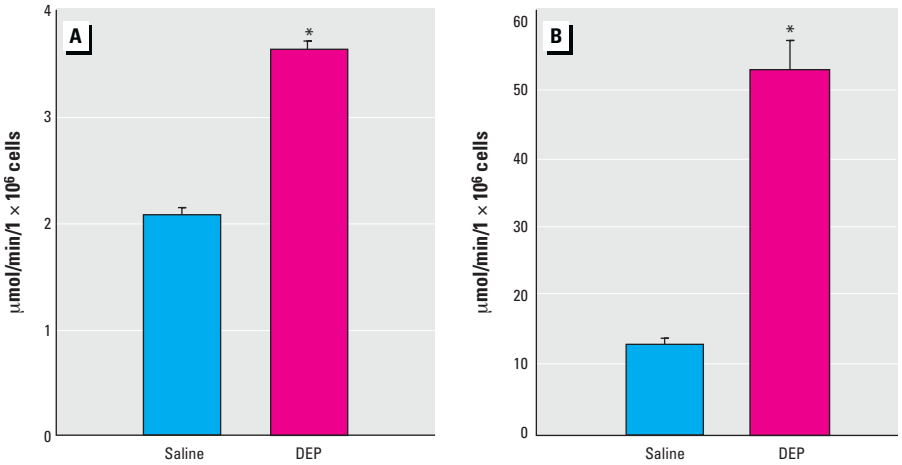

Figure 6. The effect of DEP (3 days after IT instillation) on (*A*) GSH-R activity in AM and (*B*) parathymic and tracheal LNC cells of SD rats.
*Significantly different from saline control at $p < 0.05$ (ANOVA).

Table 1. Comparison of BN and SD rats in their responses to DEP exposure times.

	4 hr		24 hr		72 hr	
Analyte	BN[a]	SD[a]	BN[a]	SD[a]	BN[a]	SD[a]
GSH-LNC	109.1	96.3	260	130.2	159.0	95.4
CYSH-AM	221.7	125.0	143.3	1276.0	175.9	1551.7
GSH-AM	147.9	79.5	121.3	330.1	115.1	392.8
PMN	206.4	148.5	526.4	671.9	477.3	696.3
LDH			199.1	256.2	336.7	265.8
Total protein			164.7	163.1	231.3	220.0

[a]Percent of saline control.

absence of (added) cystine, but a moderate increase in GSH levels was observed. The mechanism by which DEP induces an increase in intracellular GSH levels is not yet clear, but the enhancement of GSH-R activity appears to play a major role in AM responses to DEP exposure. In the presence of cystine, the intracellular levels of CYSH and GSH in DEP-exposed cells were markedly higher than the controls, which can be attributed primarily to increased production of CYSH through increased GSH-R activity.

In lymphocytes, the DEP effect on thiol regulation was quite different from that observed in macrophages. These cells exhibited a moderate capacity in the uptake and conversion of cystine to CYSH. Studies by Ishii et al. (*13*) have shown that lymphocytes exhibit strong membrane transport activity for CYSH, but only a weak transport activity for cystine. Unlike its effect on AM, the DEP exposure strongly increased the level of CYSH in LNC with or without added cystine. This may be in part because DEP also induces GSH-R activity in these cells (Figure 6). It is possible that DEP-exposed lymphocytes contain oxidized thiols that were produced during the exposure. These compounds were subsequently reduced to CYSH through increased GSH-R activity. However, despite the increase in CYSH level and GSH-R activity, the intracellular concentration of GSH in lymphocytes was significantly decreased by DEP exposure when cultured *ex vivo*. The mechanism by which DEP depletes GSH in lymphocytes is not yet clear. DEP may have increased catabolism of GSH by the lymphocyte, which was not able to replenish its intracellular stores when cultured in GSH-free media. These possibilities are currently under investigation.

GSH levels affect the activation of nuclear factor kappa B (*22,23*), and DEP exposure could alter the function of this important intracellular messenger. DEP exposure has been linked to increased IgE production (*3*) and isotype switching in B cells to produce IgE in humans (*8*) and in animals (*9*). The cellular activity of GSH in regulating B-cell functions has not been studied in detail. It is likely, however, that GSH plays an important role in the alteration of both cellular and humoral immune responses by DEP exposure. The BN rat strain, which is noted for its Th2 (IgE) phenotypic responses, was compared with SD rats. The pattern and magnitude of the inflammatory response to DEP was similar. The thiol response pattern was also similar, but the magnitude of the AM-thiol increases after DEP exposure was drastically greater in the SD rats. It is not known whether the strain difference in the magnitude of the AM-thiol response is related to differences in antibody isotype production.

In summary, this study showed that AM and LNC responded to DEP exposure with alteration in their thiol metabolism. Exposure to DEP increased the GSH-R activity of AM and LNC and thus increased the level of CYSH. The intracellular level of GSH in DEP-exposed AM was moderately increased when compared with the saline control and was further augmented when cells were incubated with cystine. Lymphocytes from DEP-exposed rats also showed a significant increase in GSH, but in contrast to AM, DEP-exposed lymphocytes could not maintain or replenish their GSH when cultured in GSH-free media, indicating that DEP exposure may decrease either lymphocyte production or uptake of GSH. Other possibilities, such as increasing GSH catabolism or shunting CYSH to other synthetic pathways such as antibody synthesis, should also be considered.

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