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Journal of Toxicology and Environmental Health, Part A

ISSN: 1528-7394 (Print) 1087-2620 (Online) Journal homepage: http://www.tandfonline.com/loi/uteh20

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To cite this article: Hongwen Zhao , Joseph K. Ma , Mark W. Barger , Robert R. Mercer , Lyndell Millecchia , Diane Schwegler-Berry , Vince Castranova & Jane Y. Ma (2009) Reactive Oxygen Species- and Nitric Oxide-Mediated Lung Inflammation and Mitochondrial Dysfunction in Wild-Type and iNOS-Deficient Mice Exposed to Diesel Exhaust Particles, Journal of Toxicology and Environmental Health, Part A, 72:8, 560-570, DOI: 10.1080/15287390802706330

To link to this article: https://doi.org/10.1080/15287390802706330

Published online: 06 Mar 2009.

Journal of Toxicology and Environmental Health, Part A, 72: 560–570, 2009

ISSN: 1528-7394 print / 1087-2620 online
DOI: 10.1080/15287390802706330

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Reactive Oxygen Species- and Nitric Oxide-Mediated Lung Inflammation and Mitochondrial Dysfunction in Wild-Type and iNOS-Deficient Mice Exposed to Diesel Exhaust Particles

Hongwen Zhao[1,2], Joseph K. Ma[3], Mark W. Barger[1], Robert R. Mercer[1], Lyndell Millecchia[1], Diane Schwegler-Berry[1], Vince Castranova[1], and Jane Y. Ma[1]

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Pulmonary responses to diesel exhaust particles (DEP) exposure are mediated through enhanced production of reactive oxygen species (ROS) and nitric oxide (NO) by alveolar macrophages (AM). The current study examined the differential roles of ROS and NO in DEP-induced lung injury using C57B/6J wild-type (WT) and inducible NO synthase knockout (iNOS KO) mice. Mice exposed by pharyngeal aspiration to DEP or carbon black particles (CB) (35 mg/kg) showed an inflammatory profile that included neutrophil infiltration, increased lactate dehydrogenase (LDH) activity, and elevated albumin content in bronchoalveolar lavage fluid (BALF) at 1, 3, and 7 d postexposure. The organic extract of DEP (DEPE) did not induce an inflammatory response. Comparing WT to iNOS KO mice, the results show that NO enhanced DEP-induced neutrophils infiltration and plasma albumin content in BALF and upregulated the production of the pro-inflammatory cytokine interleukin 12 (IL-12) by AM. DEP-exposed AM from iNOS KO mice displayed diminished production of IL-12 and, in response to ex vivo lipopolysaccharide (LPS) challenge, decreased production of IL-12 but increased production of IL-10 when compared to cells from WT mice. DEP, CB, but not DEPE, induced DNA damage and mitochondria dysfunction in AM, however, that is independent of cellular production of NO. These results demonstrate that DEP-induced immune/inflammatory responses in mice are regulated by both ROS- and NO-mediated pathways. NO did not affect ROS-mediated mitochondrial dysfunction and DNA damage but upregulated IL-12 and provided a counterbalance to the ROS-mediated adaptive stress response that downregulates IL-12 and upregulates IL-10.

Diesel exhaust particles (DEP), a major component of airborne particulate, contain fine particulate matter (PM) and adsorbed organic compounds including polycyclic aromatic hydrocarbons (PAH). These particles deposit in the airways and alveoli of the lungs. The acute diesel engine exhaust exposures produce transient irritation and inflammation that exacerbate existing allergies and asthma symptoms, whereas chronic inhalation exposure may lead to lung cancer (Health Assessment Document for Diesel Engine Exhaust, 2002). In addition to environmental concerns, DEP exposure also presents an occupational health concern for workers exposed to high concentrations of DEP, especially for underground miners (Department of Labor, 1980).

Animal studies showed that DEP produced lung inflammation and suppressed the lung's defense against infection (Yang et al., 2001; Yin et al., 2003, 2004). Alveolar macrophages (AM) are the principal cell type in the lung that mediates the immune/inflammatory responses against inhaled particles, chemicals, or microorganisms through phagocytosis, reactive oxygen species (ROS), and nitric oxide (NO) generation, and liberation of chemokines and cytokines to protect the lung's susceptibility to infection. The *in vivo* action of DEP is marked by increased expression of inducible nitric oxide synthase (iNOS) in AM, which produces NO and peroxynitrite that play an important role in host defense against intracellular pathogens (Yang et al., 2001; Takano et al., 1999; Zhao et al., 2006). The intracellular ROS, superoxide anion, is generated mainly through mitochondria, and is accompanied by controlled antioxidant defenses. However, excessive ROS not only may damage mitochondria but can also overwhelm the antioxidant defense

Received 20 October 2008; accepted 5 December 2008.

The findings and conclusions in this article have not been formally disseminated by the National Institute for Occupational Safety and Health and should not be construed to represent any agency determination or policy.

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and lead to oxidative lung injury. Studies using macrophage cell lines showed that DEP organic extract (DEPE) induces ROS generation, which results in structural damage to the mitochondrial inner membrane (Hiura et al., 2000), induces heme oxygenase1 (HO-1), decreases superoxide dismutase (SOD) (Kumagai et al., 1995), and results in apoptosis (Hiura et al., 1999). One of the key consequences of the DEP-induced and ROS-mediated adaptive stress response is the weakened host immunity against respiratory pathogens. Previous studies found that DEP exposure induced a threat of ROS damage, which is countered by coordinated cellular responses that modulate the expression of sets of gene products including HO-1 leading to IL-10 production from AM (Yin et al., 2004). The study also demonstrated that DEP induced IL-10 significantly suppressed *Listeria monocytogenes* clearance in AM, suggesting that the inhibition of the innate immune responses of AM leading to weakened host defense against pathogens. DEP are known to (1) suppress mucociliary clearance of bacteria, (2) decrease interferon production in response to viral infection, and (3) increase susceptibility to bacterial infection through altered cytokine production by AM and T-lymphocyte differentiation in favor of the T helper (TH) 2 response (Castranova et al., 2001; Yin et al., 2003).

While there is a strong correlation between DEP-induced oxidant generation and the pulmonary immune/inflammatory responses, the differential roles of these oxidants and their potential interplay to produce the overall effect remain unclear. This is of interest because the *in vivo* action of DEP may vary if there exist counterbalancing pathways for the DEP-induced immune/inflammatory responses, and thus may show dose and time sensitivity regulated by the relative production of reactive oxygen/nitrogen species. In a previous study, it was shown that exposure of rats to DEPE for 24 h produced severe cytotoxicity, ROS generation, and altered cytokine production by AM (Zhao et al., 2006). These DEPE-mediated responses were NO dependent, as they were significantly reduced in animals treated with aminoguanadine (AG), a selective iNOS inhibitor (Zhao et al., 2006), suggesting that ROS and NO may exhibit cooperative and/or opposite effects on given cellular responses.

Certain cooperative activities of ROS and NO have been established. iNOS, a hemoprotein (White & Marletta, 1992), possesses a flavin-containing reductase domain that is capable of producing superoxide by reacting with compounds such as quinones and nitroarenes that are present in DEPE (Kumagai et al., 1997, 1998). The iNOS-derived superoxide may interact with NO produced by the same enzyme to form peroxynitrite, a reactive intermediate that is known to produce protein damage through nitration of tyrosine, tryptophan, or cysteine residues (Rao, 2000; Sawa et al., 2000). Studies showed that the inactivation of CYP 2B1 is through peroxynitrite-mediated nitration of Tyr 190 in CYP 2B1 (Lin et al., 2003). Our previous studies demonstrated that exposure of rats to DEP significantly attenuated pulmonary CYP 2B1 (Rengasamy et al., 2003). In addition to intracellular mitochondria-dependent ROS generation, DEP also activate AM to generate superoxide anion through NADPH oxidase during the respiratory burst (Segal, 1989), and the organic component of DEP generates ROS through a redox cycle mediated by NADPH-cytochrome P-450 reductase (Dellinger, 2001). Together, these studies show that ROS and NO may separately or in combination modulate the pulmonary immune and inflammatory responses.

Although DEPE was found to mediate ROS- and NO-mediated cellular responses, its bioavailability in relation to DEP exposure is unknown. *In vivo* studies showed some significant deviation in pulmonary responses between DEP and DEPE exposure, suggesting that the physical–chemical characteristics of DEP play a crucial role in DEP-induced lung toxicity. The objectives of the present study were to (1) characterize and differentiate the roles of ROS and NO in mediating mitochondrial dysfunction in AM, alveolar damage, neutrophil recruitment, and AM production of pro- and anti-inflammatory cytokines during a 7-d postexposure time course using WT and iNOS KO mice, and (2) provide insight into the particulate- versus organic component-mediated cellular responses in relation to DEP exposure.

MATERIALS AND METHODS

Animal Treatment

DEP (National Institute of Standards and Technology, Gaithersburg, MD; Standard Reference Material 2975) were suspended in pyrogen-free sterile saline. The suspensions were sonicated for 5 min using an ultrasonic processor with a micro-tip (Branson Sonifier 450, Danbury, CT) prior to mouse pharyngeal aspiration (ACUC 05-JM-M-014). Specific-pathogen-free male wild-type (WT) (C57BL/6J, ~25 g) and breeding pairs of iNOS knockout mice (iNOS KO) (B6.129P2-NOS2^{TM1LAV}, ~25 g) were purchased form Jackson Laboratories (Bar Harbor, ME). Mice were kept in cages ventilated with HEPA-filtered air, housed in an AAALAC-approved facility under controlled environmental conditions and a 12-h light/dark cycle, and were provided food and water ad libitum. Breeding of iNOS KO mice was conducted in the NIOSH animal facility. After a 1-wk acclimation period, mice were anesthetized with combination of ketamine and xylazine (50 and 2 mg/kg, respectively, ip) and placed on an inclined restraint board. The suspensions of DEP or CB were given by pharyngeal aspiration at a dose of 35 mg/kg, an equivalent amount of DEPE contained in the DEP dose, or the appropriate volume of saline as the control for both WT and iNOS KO mice. Mice were sacrificed at 1, 3, or 7 d postexposure.

Isolation of Alveolar Macrophages (AM) and AM Cultures

Animals were anesthetized with sodium pentobarbital (0.2 g/kg) and exsanguinated by cutting the renal artery. AM were obtained by bronchoalveolar lavage (BAL) with a Ca^{2+},Mg^{2+}-free phosphate-buffered medium (145 m*M* NaCl, 5 m*M* KCl,

1.9 m*M* NaH_2PO_4, 9.35 m*M* Na_2HPO_4, and 5.5 m*M* glucose; pH 7.4) as described previously (Yang et al., 2001). The first lavage was saved separately from the subsequent lavages, and a total of 10 ml bronchoalveolar lavage (BAL) fluid per mouse was pooled and collected in sterile centrifuge tubes. Typically, BAL fluid from five mice of the same treatment was pooled to obtain a sufficient cell number for experiments. BAL fluid was centrifuged and cell pellets were combined, washed, and resuspended in a HEPES-buffered medium (145 m*M* NaCl, 5 m*M* KCl, 10 m*M* HEPES, 5.5 m*M* glucose, and 1 m*M* $CaCl_2$, pH 7.4). The acellular supernate of the first lavage from the same treatment group was combined and saved for further analysis. Cell counts and purity were measured using an electronic cell counter equipped with a cell sizing attachment (Coulter model Multisizer 3 with a 256C channelizer, Beckman Coulter, Fullerton, CA).

For cell culture lavage, cells were pellet by centrifugation and resuspended in Eagle's minimum essential medium (EMEM, BioWhittaker, Walkersville, MD), containing 1 m*M* glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 10% heat-inactivated bovine serum. AM-enriched cells were obtained by adherence to the tissue culture plate for 2 h as described previously (Yang et al., 1999). The adherence AM was washed 3 times to remove the nonadherent cells, and then cultured in fresh EMEM for an additional 24 h. AM-conditioned media were collected, centrifuged, and saved in aliquots at –80ºC for further analysis of cytokines. To ensure that the number of adherent cells was the same in various exposure samples, the adherent cells were treated with 0.5% Triton X100 at 37ºC for 30 min and the media were collected and centrifuged. The supernates were used for the protein determination using BCA protein assay (Thermo Scientific, Rockford, IL). The results did not show a significant difference among the samples form various treatment groups (data not shown).

Lactate Dehydrogenase (LDH) and Albumin Determination

The acellular LDH activity in BAL fluid was monitored using Roche Diagnostic reagents and procedures (Roche Diagnostic Systems, Indianapolis, IN) on an automated Cobas MIRA PLUS analyzer (Roche Diagnostic Systems). The albumin content in the acellular BAL fluid was measured based on albumin binding to bromcresol green with Sigma Diagnostic regents and procedures (Sigma-Aldrich, St. Louis, MO) following the manufacturer's protocol.

Cytokine Production

The cytokines interleukin (IL)-12p40 and IL-10, in AM-conditioned media with or without ex vivo lipopolysaccride (LPS, 0.1 μg/ml) stimulation, were determined using enzyme-linked immunosorbent assays (ELISA) (Biosource International, Inc., Camarillo, CA) according to the manufacturer's protocol.

Histological Examination

Lung tissues were fixed immediately after sacrifice as described previously (Ma et al., 1999). Briefly, tissues were routinely processed, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin for light microscopic examinations.

Confocal Microscopy

AM were plated on circular glass cover slips in 24-well plates that were pretreated for 1 h at 37°C with EMEM + 10% fetal bovine serum (FBS) to allow cell attachment, then washed with phosphate-buffered saline (PBS) once. Dihydroethidium (DHE, 5 μ*M*) (Invitrogen, Carlsbad, CA) was added to AM and incubated for 15 min; then cells were washed with PBS, fixed with 10% buffered formalin phosphate for 10 min, and mounted on glass slides using Prolong Antifade (Invitrogen). Slides were imaged using red fluorescence (excitation 518; emission 605 nm) with a Zeiss LSM 510 confocal microscope (Carl Zeiss, Inc., Thornwood, NY) at a 512 × 512 pixel size with a 40 × water immersion objective.

Mito Tracker Red CMXRos (200 n*M*) was used to detect the change of mitochondrial membrane potential in AM, monitored using confocal microscopy (excitation 579; emission 599 nm). Slides were imaged at 1024 × 1024 pixel size with a 100 × oil immersion objective.

Transmission Electron Microscopy (TEM)

For electron microscopic analysis of AM, cells isolated from different exposure groups were fixed in Karnovsky's fixative (2.5% glutaraldehyde–3% paraformaldehyde in 0.1 *M* sodium cacodylate, pH 7.4) and postfixed with osmium tetroxide. AM were dehydrated in graded alcohol solutions and propylene oxide and embedded in LX-112 (Ladd, Williston, VT). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (TEM) (JEOL 1220, Tokyo).

ATP Content

The levels of ATP in AM were determined using ATPlite 1step provided by Perkin Elmer (Waltham, MA) according to the manufacturer's instructions. This is a luminescence assay for the quantification of viable cells based on firefly (*Photinus pyralis*) luciferase. The luminescence generated was monitored using an FLx800 fluorescence microplate reader from Bio-Tek Instruments (Winooski, VT).

Comet Assay

The Comet assay was performed according to the manufacturer's instructions using a CometAssay kit (Trevigen, Gaithersburg, MD) under alkaline conditions. The slides were stained with SYBR green dye. Comets were visualized

and photographed at 200 × magnification using a fluorescence microscope (Olympus AX70) with an image capture system (SamplePCI, Compix Inc., Cranberry Township, PA). Tail length was defined as the distance between the leading edge of the nucleus and the end of the tail; 50–100 determinations were made for each sample using an image analysis system (Optimas 6.51, Media Cybernetics Inc., Silver Spring, MD). Data represent a mean of three separate experiments.

Statistical Analysis

Data are presented as means ± standard errors. Comparisons were made using analysis of variance (ANOVA) with means testing by Dunnett's test ($p < .05$ being considered as significant). For the comet assay, comparison of DNA migration between exposed and control groups were performed with one-way ANOVA, followed by Student's *t*-test.

RESULTS

Role of NO on DEP-Induced Lung Inflammation and Injury

Particle-induced inflammatory responses, including PMN infiltration, the presence of plasma albumin, and increased LDH activity in the lavage fluid, are markers for inflammation, air/blood barrier leakage and cytoxicity, respectively. Figure 1 shows that exposure of WT or iNOS KO mice to DEP resulted in significant PMN infiltration (Figure 1A), elevated LDH activity (Figure 1B), and marked increase in albumin content (Figure 1C) in lung lavage fluid at 1, 3, or 7 d postexposure. The cytotoxic effect of DEP exposure, determined as LDH activity, remained at high levels throughout the study period, whereas the albumin content peaked at 3 d post exposure. Strong recruitment of PMN occurred at d 1 and gradually decreased at 7 d postexposure, but remained significantly above the control level. The results show that iNOS KO mice exhibited a significant reduction in PMN infiltration at 1 d postexposure and reduced albumin content at 1 and 3 d postexposure. These inflammatory responses were also investigated after exposure to CB, representing the carbonaceous core, or DEPE, representing the organic component of DEP, to evaluate their role in DEP-induced lung inflammation. Figure 1, A–C, shows that exposure of mice to CB, but not DEPE, resulted in significant PMN infiltration, cytotoxicity, and epithelial damage at similar levels with both WT and iNOS KO mice at all time points. These findings suggest that the particle core, but not the organic adsorbed on the particle, plays a major role in DEP-induced lung inflammation and injury.

Pulmonary Histopathology

Pulmonary inflammatory changes in both WT and iNOS KO mice exposed to DEP were further examined in histopathologic lung sections. Figure 1D shows that the control lungs of iNOS KO mice (panel b) are normal with similar morphology to WT vehicle control (panel a). DEP exposure shows (Figure 1, panels c and d) that neutrophil influx occurred in both WT and KO mice; however, fewer areas with inflammation were seen in KO mice, which is consistent with the less PMN infiltration as shown in Figure 1A.

ROS Generation and Mitochondrial Damage

Figure 2A shows a progressive increase with exposure time in intracellular superoxide anion in DEP-exposed AM from both WT and iNOS KO mice. Our results demonstrate that NO does not mediate induction of ROS, although at 1 and 3 d postexposure AM from iNOS KO mice appears to exhibit greater levels of oxidant than those of WT mice, which may be attributed to a partial reduction of superoxide by NO through the formation of peroxynitrite in AM from WT but not iNOS KO mice. In contrast to DEP-mediated effects, neither CB nor DEPE induced superoxide anion generation in AM compared to saline control, suggesting that particles with bound organic components such as DEP must be internalized by AM to mediate induction of intracellular superoxide. Confocal microscopy study (Figure 2B) shows that DEP and CB, but not DEPE, significantly reduced mitochondrial membrane potential in AM from both WT and iNOS KO mice to a similar level. These results demonstrate that the particle component of DEP produced mitochondria damage that is independent of cellular NO production.

The significant reduction of mitochondrial membrane potential may produce cumulative damage to the mitochondrial membrane and uncouple oxidative phosphorylation, leading to interference of ATP synthesis (Green & Reed, 1998). An assessment of cellular ATP levels showed the ATP content in AM from various exposure groups was not markedly different from the controls. In addition, it was also not affected by NO deficiency, as shown in Table 1.

TEM

Confocal analysis showed DEP, but not CB, significantly modified mitochondrial potential in AM. However, the correlation of particle deposition in relation to mitochondrial damage was not demonstrated; thus, the correlation of particle-bound organic component of DEP with DEP-mediated mitochondrial dysfunction in AM from WT mice was further investigated by TEM. Figure 3 shows the typical EM profile of AM from mice receiving polystyrene microspheres (used as a particle control, Figure 3A), CB (particle core control for DEP, Figure 3B), or DEP (Figure 3C), respectively, at 7 d postexposure. Both CB- and DEP-exposed AM show particle-filled vacuoles (solid arrows) that indicate phagocytosis, whereas the polystyrene bead-exposed AM demonstrate clear vacuoles due to the dissolution of polystyrene during embedding. The internalization of CB or DEP through phagocytosis was found to rise with increased exposure time (data not shown). Our data also showed that in the presence of DEP, mitochondria

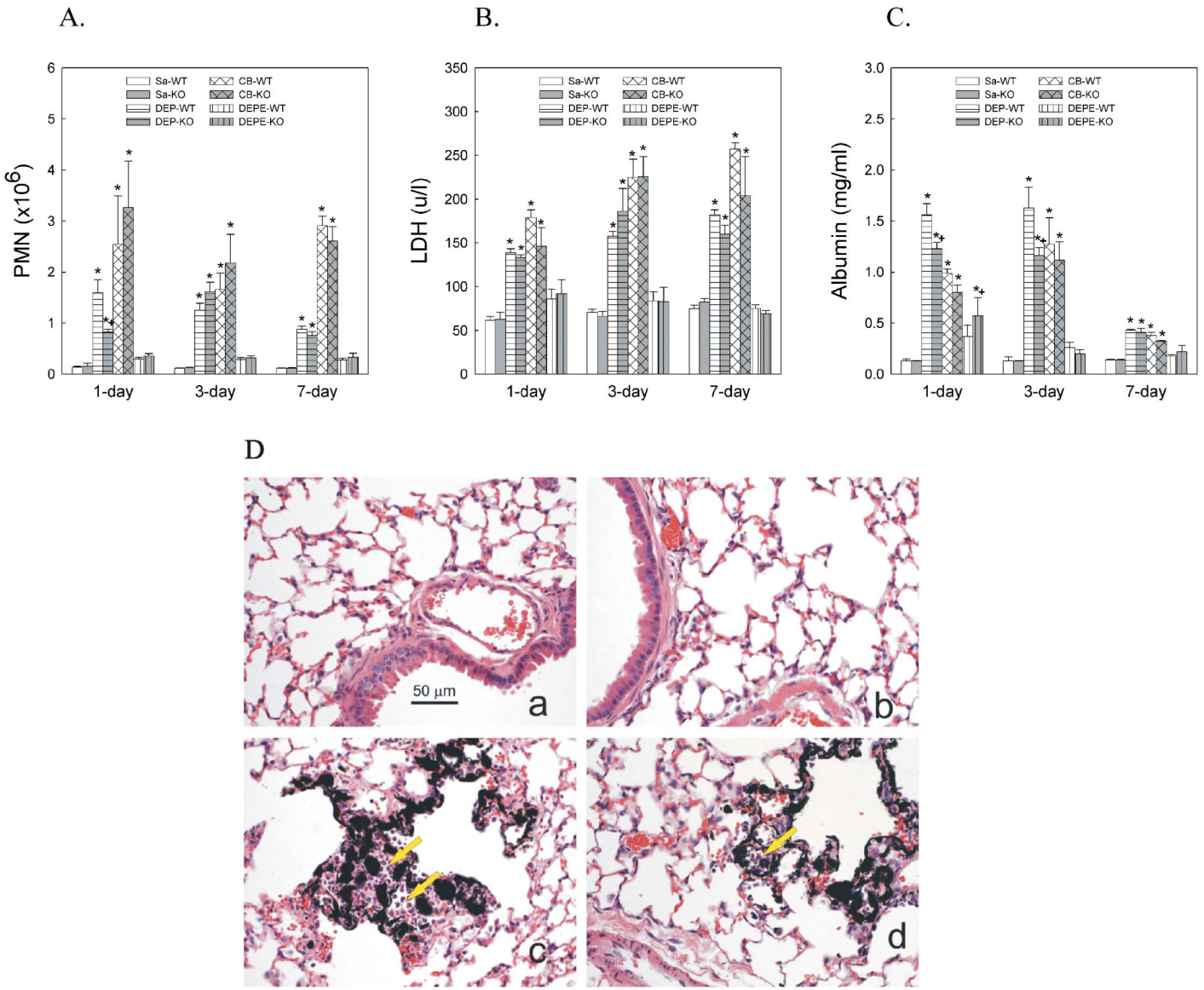

FIG. 1. DEP-, CB- and DEPE-induced pulmonary inflammatory profile in WT and NOS2 KO mice at 1, 3, and 7 d postexposure compared to the saline (Sa) control. PMN infiltration in BAL fluid (A) was determined to indicate lung inflammation; the first acellular BAL fluid was analyzed for LDH activity (B) to indicate the cytotoxicity, and plasma albumin content (C) to indicate air/blood barrier damage. Values are expressed as mean ± SE (n = 6–8). Asterisk indicates significantly different from control group, $p < .05$; = indicates significantly different from the NOS2 KO group, $p < .05$. (D) Photomicrographs of hematoxylin- and eosin-stained lung sections from mice exposed to saline or DEP. WT mice are panels (a) and (c), and iNOS KO mice are panels (b) and (d). (a) and (b) are from mice exposed to saline, (c) and (d) to DEP. All were taken with a 40 × objective lens; the bar is 50 µm. The arrows indicate regions of neutrophil influx.

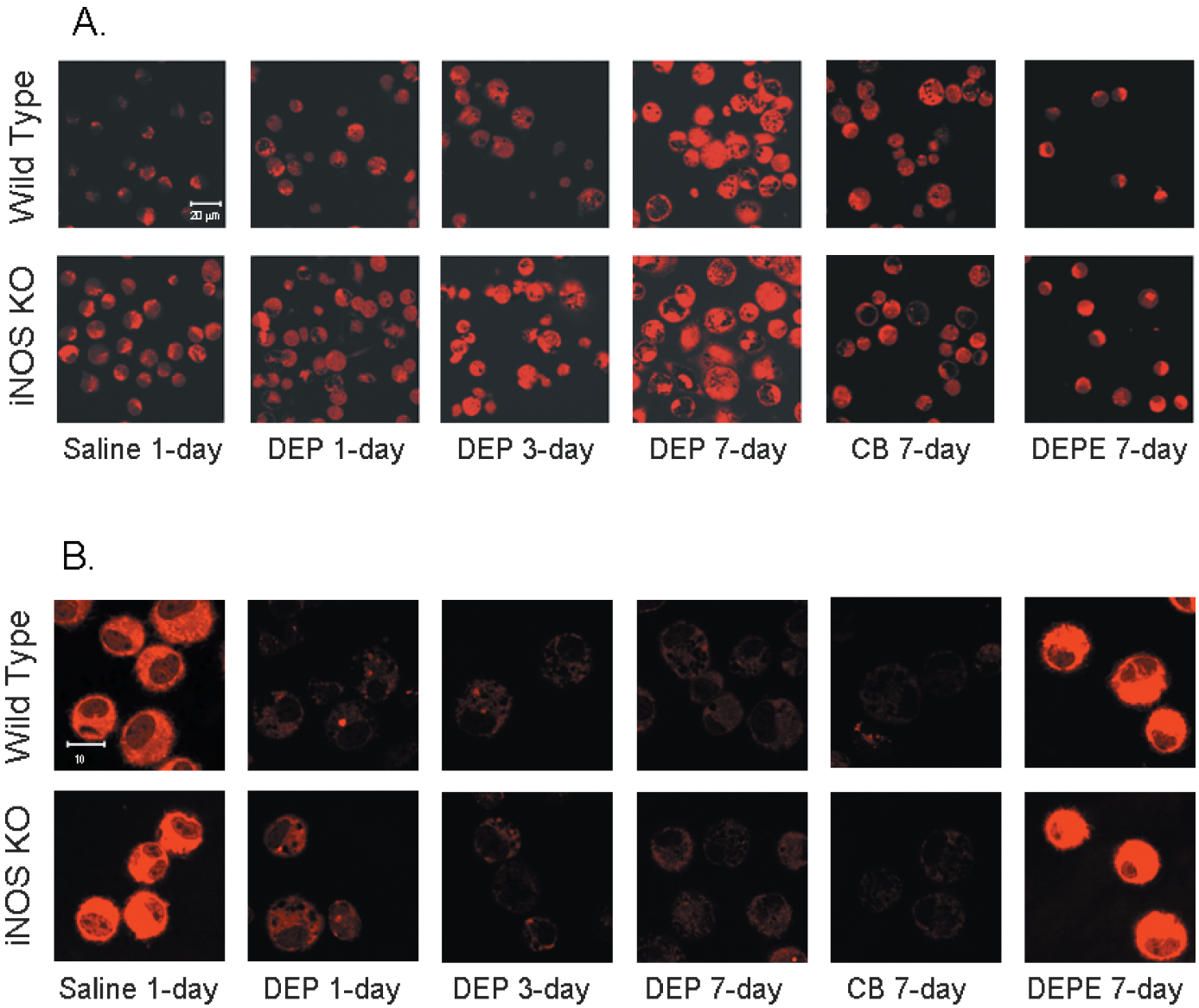

FIG. 2. Effects of different exposures of WT or iNOS KO mice on intracellular superoxide anion generation (A) and mitochondria membrane potential change (B) monitored from harvested AM using confocal microscopy. The images in (A) were taken with 40 × water immersion objective (bar is 20 μm), while the images in (B) were taken with a 100 × oil immersion objective lens (bar is 10 μm).

or mitochondrial fragments were found within vacuoles as illustrated in the higher magnification view of Figure 3D. This effect was not observed in CB-exposed AM. A significant portion of the DEP-mediated increase in mitochondria may be functionally impaired, as some of the mitochondria were in direct contact with autolytic vacuole or vacuoles (unfilled arrow), while others appeared totally enclosed inside the vacuoles (Figure 3D).

DNA Damage

DEP exposure induced time-dependent ROS generation and mitochondrial dysfunction in AM, which may lead to DNA damage. Monitoring DNA damage using the comet assay (Figure 4) shows that DEP induced comet tails in AM at 1 d postexposure, which remained at the same level through 7 d postexposure. This DEP-induced DNA damage was also found in CB-exposed AM at a similar level, but not in DEPE-exposed AM. These results suggest that the particle core of DEP is responsible for the induction of DNA damage.

NO-Mediated Cellular Production of Pro- and Anti-inflammatory Cytokines

Figure 5A shows that the production of IL-12p40 by AM in response to early DEP exposure (1 and 3 d postexposure)

TABLE 1
ATP Content in AM from Different Treatment Groups

	Wild-type			iNOS KO		
Treatment	1 d	3 d	7 d	1 d	3 d	7 d
Saline	0.297 ± 0.016	0.317 ± 0.024	0.322 ± 0.019	0.326 ± 0.017	0.337 ± 0.036	0.316 ± 0.320
DEP	0.407 ± 0.056	0.350 ± 0.070	0.342 ± 0.025	0.528 ± 0.091	0.453 ± 0.037	0.290 ± 0.027
CB	0.313 ± 0.040	0.358 ± 0.104	0.342 ± 0.025	0.345 ± 0.036	0.345 ± 0.036	0.305 ± 0.014
DEPE	0.364 ± 0.026	0.352 ± 0.029	0.318 ± 0.025	0.391 ± 0.022	0.352 ± .025	0.318 ± .018

Note. Mice were exposed by pharyngeal aspiration to DEP, CB, or DEPE, and AM were harvested at various days postexposure. The results are presented as means ± SE of ATP ($\mu M/2.5 \times 10^4$ AM); no significant exposure-dependent changes were noted.

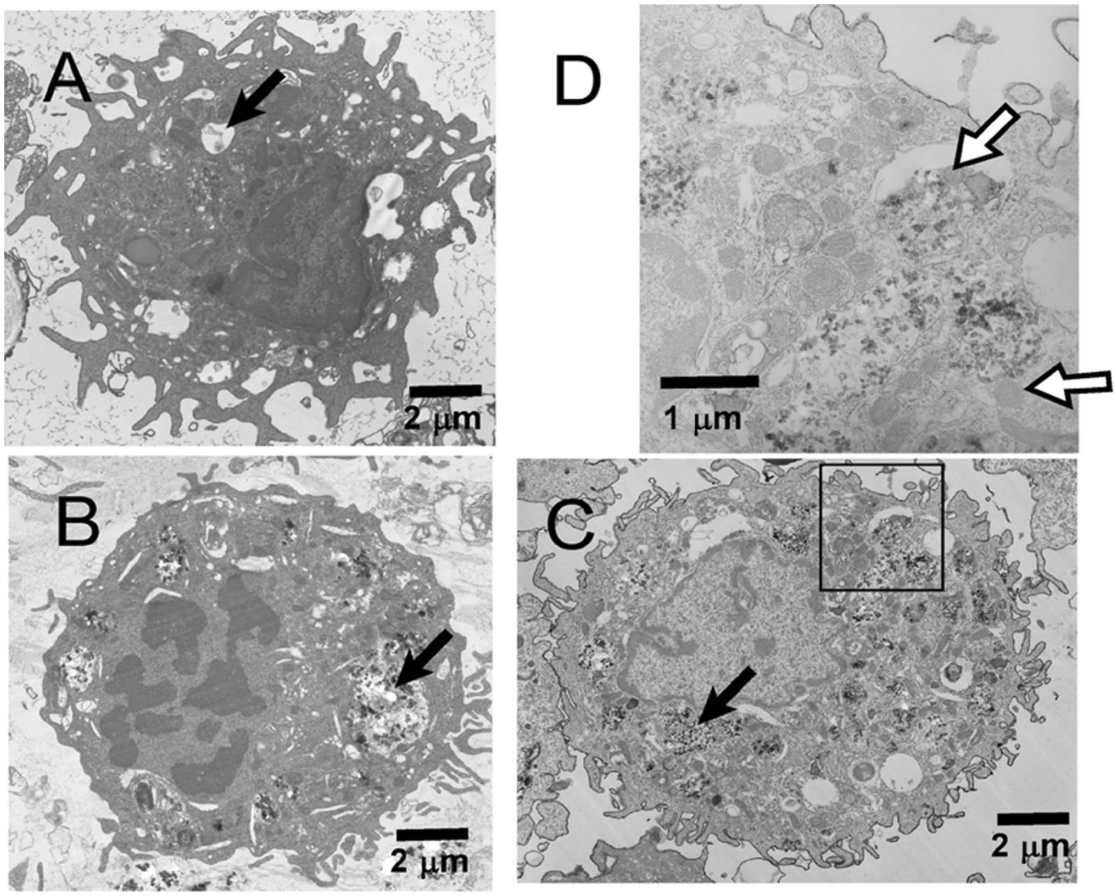

FIG. 3. Electron micrographs of AM harvested from WT mice at 7 d postexposure to DEP, CB, or polystyrene. The typical profiles of AM from WT mice receiving polystyrene microspheres (A, particle control), CB (B, particle core control for DEP) or DEP (C). Animals exposed to CB and DEP were heavily laden with particle filled vacuoles (solid arrows). Polystyrene-filled vacules appear clear due to the dissolution of polystyrene during embedding (A). (D) A higher magnification of the boxed area of (C). Vacuoles containing DEP were frequently found to contain mitochondria or be in direct membrane-to-membrane contact with mitochondria (unfilled arrows, D).

was independent of cellular production of NO. At 7 d postexposure, however, secretion of IL-12p40 by AM from iNOS KO mice was significantly attenuated when compared to WT mice (panel A), due to NO deficiency. IL-12 is known to play a key role in the initiation of T-cell-mediated immunity, the suppressed IL-12 in iNOS KO mice may significantly modify host defense against infection. DEP-exposed AM at 3 and 7 d postexposure showed marked increase in IL-10 production (panel B), an anti-inflammatory cytokine that was found to be a product of ROS-mediated adaptive

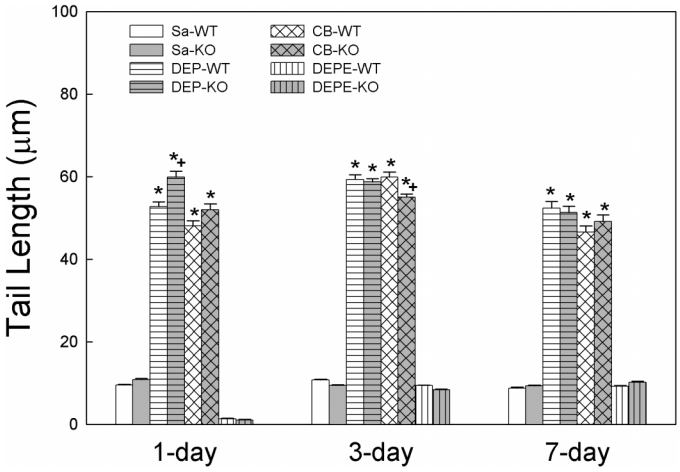

FIG. 4. Effects of NO on DNA damage in AM from saline control or DEP-, CB-, or DEPE- exposed WT and iNOS KO mice, with AM isolated from the different treatment groups at 1, 3, or 7 d postexposure. DNA damage was monitored using a comet assay.

stress response (Yin et al., 2004). Upon ex vivo challenge with LPS, a potent activator of the immune system, DEP-exposed AM from iNOS KO mice showed consistently decreased production of IL-12 (panel C), but increased production of IL-10 at day 3 (panel D), compared to the WT control. The elevated production of IL-10 by AM from iNOS KO mice peaked at day 3, whereas the downregulation of IL-12 occurred at d 3 and 7 post DEP exposure. These results suggest that NO plays a significant role in modulating the pro- and anti-inflammatory cytokines production by AM in response to DEP and LPS challenge.

DISCUSSION

Both *in vitro* (Baulig et al., 2003; Hiura et al., 2000; Pan et al., 2004) and *in vivo* (Risom et al., 2003; Tsurudome et al., 1999) studies showed that DEP-induced lung injury and cellular responses stem from increased production of intracellular oxidants; however, the mechanisms through which DEP induce ROS and NO and the roles of these reactive species to elicit cellular responses remain unclear. Our previous studies reported that the organic extract of DEP (DEPE) induced oxidative stress in rats 24 h after exposure, mainly through induction of iNOS and increased NO production, whereas DEP induced oxidant generation through phagocytosis by AM, which occurs even in rats treated with aminoguanidine (Zhao et al., 2006). The current study is designed to demonstrate and differentiate the roles of ROS and NO and possible interplay between these species in DEP-induced inflammatory responses and cell injury in time-dependent studies using WT and iNOS KO mice.

The sources for intracellular oxidant generation include mitochondria, cytosolic iNOS and the cytochrome P-450 isozymes that are capable of producing superoxide and hydrogen peroxide (Bondy & Naderi, 1994). Mitochondria are the major cellular organelles for oxidant generation and key players in regulating programmed cell death. Our data show that DEP exposure resulted in a time-dependent increase in the production of superoxide anion and a concomitant reduction of mitochondrial potential in AM, which was correlated with elevated DEP mass that peaked at 7 d postexposure, observed from scanning electron microscopy. NO did not appear to play a significant role in DEP-mediated ROS generation or mitochondrial dysfunction, since AM from iNOS KO mice produced similar increases in intracellular ROS and decreasing mitochondrial potential when compared to WT mice. However, AM from DEP-exposed WT mice showed a quantitatively reduced level of superoxide compared to cells from iNOS KO mice at 1 and 3 d postexposure, but rose to a similar level at 7 d postexposure in both mouse types. This early quantitative reduction of superoxide might be due to the partial removal of superoxide by iNOS-derived NO via the formation of peroxynitrite. Similar findings were found in studies of silica- or LPS-stimulated AM of the removal of superoxide by iNOS-derived NO through peroxynitrite formation (Zeidler et al., 2003).

TEM analysis showed phagocytosis following DEP and CB, but not DEPE, by AM in a time-dependent manner with the DEP mass, which peaked in AM at 7 d postexposure. EM analysis demonstrated a time-dependent increase of mitochondria or mitochondrial fragments in the cytoplasm AM from DEP- but not CB-exposed lungs, suggesting that the organic component of DEP does play a role in mediating mitochondrial activity. In addition, these increased mitochondria in DEP-exposed AM are often in direct contact with vacuoles or enclosed within the vacuoles, suggesting that the number of mitochondria may be increased but they are functionally impaired due to restricted access to the cytoplasm. In comparison, in AM from mice exposed to polysterene beads (as control particles) there was no apparent interaction between phagocytic vacuoles and mitochondria. The results also reported that AM from DEPE-exposed mice showed ultrastructure similar to controls (data not shown), suggesting that the organic compounds of DEP in extracted form do not directly affect mitochondrial function. These findings demonstrated that the *in vivo* action of DEP is derived from particles containing the adsorbed organic component as a whole rather than from each component, thus providing evidence that naked particles or extract alone may induce some biological responses, but not the full spectrum of responses induced by DEP.

It should be mentioned that a number of *in vitro* studies using primary AM or macrophage cell lines showed that DEPE was the main contributor for DEP-induced ROS generation (Baulig et al., 2003; Hiura et al., 1999), and mitochondrial perturbation followed by cytochrome *c* release resulted in membrane asymmetry and apoptosis (Hiura et al., 2000). These significant discrepancies for the role of particle core or organic component in DEP-induced cellular responses are mainly due to the *in vitro* versus *in vivo* studies, suggesting extreme caution

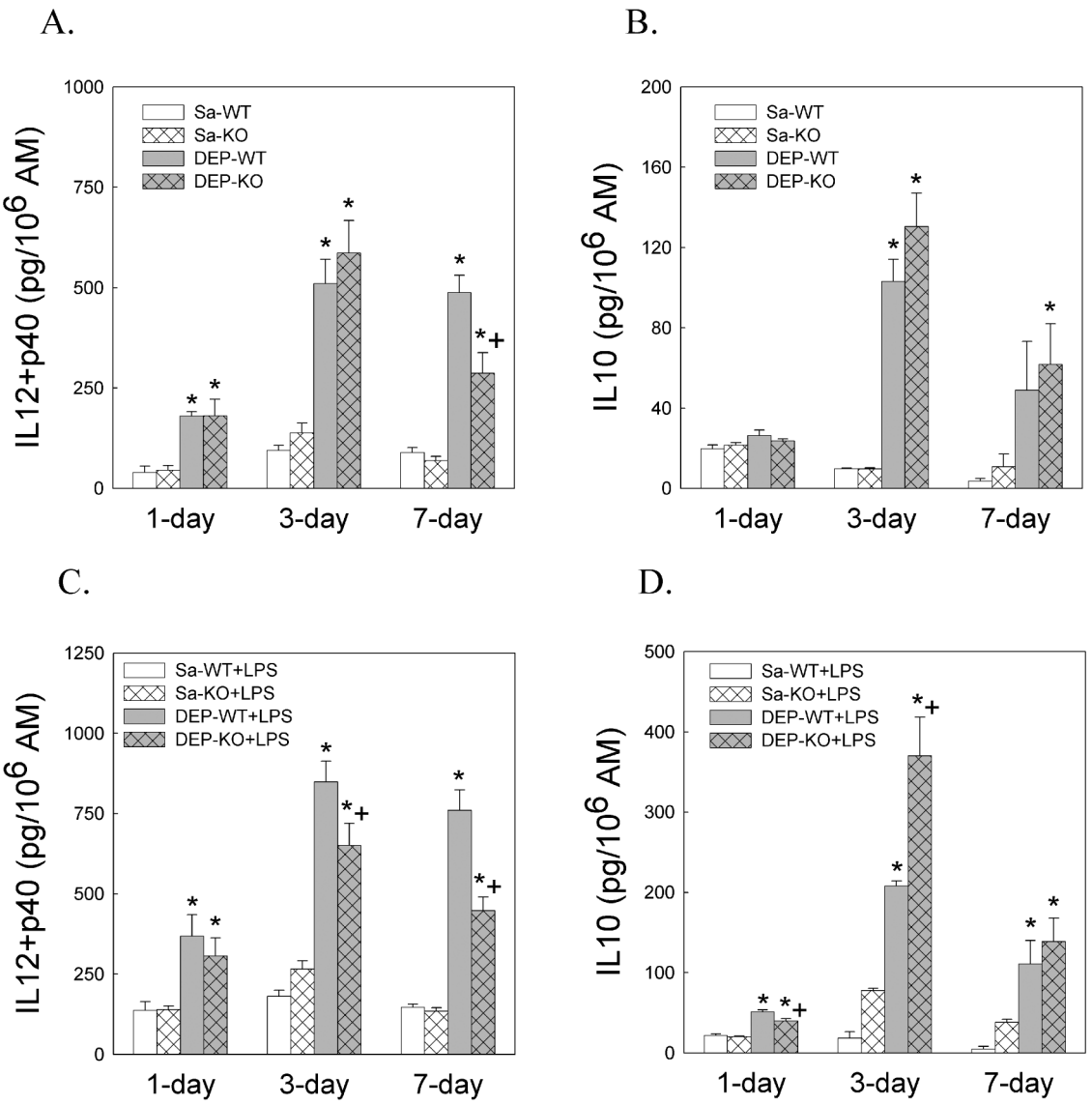

FIG. 5. Role of NO in DEP-mediated pro- and anti-inflammatory cytokine production by AM with or without LPS challenge. AM were isolated from different exposure groups in both WT and iNOS KO mice. (A) IL-12 and (B) IL-10 production in the supernatant of the AM culture medium at 37ºC for 24 h was assayed using ELISA kits. The other groups of AM were isolated at the time points as indicated in the graph with an additional 3 h of LPS stimulation, and the secretion of (C) IL-12 and (D) IL-10 in the AM cultured medium was determined. Asterisk indicates significantly different from control group, $p < .05$; + indicates significantly different from the WT group, $p < .05$.

is required when extrapolating *in vitro* findings to *in vivo* responses. It is interesting to point out that although DEP produced severe mitochondrial dysfunction, they did not significantly affect cellular ATP content from either WT or iNOS KO mice when compared to controls. This constant ATP level suggests that DEP-exposed AM may exhibit enhanced glycolysis in the presence of mitochondrial damage, an energy generation process that is preferred by AM and many cancer cell types over the mitochondrial electron transport in their handling of oxidative stress (Fisher, 1980; Brandon et al., 2006). In addition, a recent study showed that macrophages were protected from oxidative stress through enhanced glycolysis as a mechanism for cellular survival (Kondoh et al., 2007). Together, our studies show that phagocytosis of the organic laden DEP is a key event in DEP-induced intracellular production of superoxide and mitochondrial dysfunction, whereas NO may result in partial removal of superoxide through formation of peroxynitrite but does not play a role in mitochondrial dysfunction.

Respirable particles are known to induce a pulmonary inflammatory profile manifested by neutrophil infiltration, increased albumin content, and elevated LDH activity in BAL fluid. Both DEP and CB, which represents the carbonaceous core of DEP, induced similar inflammatory profile, but not

DEPE, suggesting that phagocytosis of particles is central to the inflammatory process. Our studies further showed that NO plays a significant role in the early inflammatory responses to DEP, as both the onset of infiltration of neutrophils (at 1 d postexposure) and pulmonary capillary leakage (at 1 and 3 d postexposure) induced by DEP in iNOS KO mice were significantly lower than observed in WT mice. These results suggest that DEP-induced production of NO may initiate a signaling pathway for the recruitment of PMN into the lung, and its excess contributes to greater air/blood barrier damage. NO does not appear to significantly affect DEP-induced cytotoxicity as indicated by the measured LDH activities in BAL fluid. In comparison, CB induced a similar inflammatory profile in WT and iNOS KO mice, indicating that NO does not play a role in CB-mediated inflammatory responses, whereas DEPE does not induce an inflammatory response. These findings further suggest that phagocytosis is key to the cellular responses, and the particle-bound organic component, but not DEPE, defines the *in vivo* DEP toxicity. Since DEP induce both ROS and NO production by AM, it is likely that the NO-dependent neutrophil infiltration and epithelial/capillary barrier damage may result from a cooperative action of ROS- and NO-mediated cell activities.

Macrophages also play an important role in host defense against foreign invaders including bacterial infections that involve pro- and anti-inflammatory cytokine production in addition to oxidant generation. Studies have demonstrated that after prolonged (7 d) exposure to DEP NO mediates IL-12 production by AM, since NO deficiency markedly reduced IL-12 secretion; however, it did not significantly affect IL-10 production. This effect is more pronounced when DEP-exposed AM are challenged ex vivo with LPS, a bacterial product. Under these conditions, DEP-exposed AM from iNOS KO mice show lowered IL-12 but increased IL-10 production than cells from WT mice in response to LPS stimulation. IL-12, which may be induced by bacterial products, is a key cytokine that bridges the innate and adaptive immune responses (Trinchieri & Gerosa, 1996). IL-10, on the other hand, is a potent inhibitor of monocyte/macrophage function, which is known to suppress the production of many pro-inflammatory cytokines including IL-12 (Chung, 2001; Raychaudhuri et al., 2000). Our study shows that NO is crucial for AM to maintain their pro-inflammatory function. The potential interactive effect of NO- and ROS-mediated inmmune responses may provide insightful information on *in vivo* DEP toxicity. It was previously shown that DEP exposure, at relatively low doses, suppressed pulmonary clearance of *Listeria monocytogenes* in rats through a mechanism that involves ROS-mediated expression of adaptive stress response protein HO-1 by AM (Yin et al., 2004) and results in increased secretion of IL-10 and downregulated IL-12. The current study demonstrates that NO promotes IL-12 production and has a tendency to reduce IL-10 in response to LPS, thus favoring inflammatory responses (Li et al., 2003; Otterbein et al., 1995; Yin et al., 2004). Together, these studies indicate a definitive role for DEP-induced iNOS on NO production that modulate ROS-mediated cellular responses, including the balance of the pulmonary immune/inflammatory responses.

In summary, this study showed that AM respond to *in vivo* DEP exposure through both ROS- and NO-mediated pathways. DEP, when present with particulate and organic components, resulted in a time-dependent increase in intracellular ROS generation and mitochondrial dysfunction that correlated with a time-dependent rise in DEP mass in these phagocytes. Cell deficiency in NO production did not prevent DEP-induced ROS generation and mitochondrial damage. Nitric oxide, however, was shown to play a significant pro-inflammatory role in DEP-exposed lung that includes enhanced initial neutrophil recruitment, increased air/blood barrier leakage, and AM production of IL-12. The induction of NO by DEP provides a counterbalance to DEP-induced ROS generation in AM, which is known to elicit an adaptive stress response that downregulates IL-12 and upregulates IL-10, an anti-inflammatory cytokine that is induced and employed by many intracellular pathogens to lessen their removal from the host system.

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