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Role of inducible nitric oxide synthase-derived nitric oxide in lipopolysaccharide plus interferon-γ-induced pulmonary inflammation

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Abstract

Exposure of mice to lipopolysaccharide (LPS) plus interferon-γ (IFN-γ) increases nitric oxide (NO) production, which is proposed to play a role in the resulting pulmonary damage and inflammation. To determine the role of inducible nitric oxide synthase (iNOS)-induced NO in this lung reaction, the responses of inducible nitric oxide synthase knockout (iNOS KO) versus C57BL/6J wild-type (WT) mice to aspirated LPS + IFN-γ were compared. Male mice (8–10 weeks) were exposed to LPS (1.2 mg/kg) + IFN-γ (5000 U/mouse) or saline. At 24 or 72 h postexposure, lungs were lavaged with saline and the acellular fluid from the first bronchoalveolar lavage (BAL) was analyzed for total antioxidant capacity (TAC), lactate dehydrogenase (LDH) activity, albumin, tumor necrosis factor-α (TNF-α), and macrophage inflammatory protein-2 (MIP-2). The cellular fraction of the total BAL was used to determine alveolar macrophage (AM) and polymorphonuclear leukocyte (PMN) counts, and AM zymosan-stimulated chemiluminescence (AM-CL). Pulmonary responses 24 h postexposure to LPS + IFN-γ were characterized by significantly decreased TAC, increased BAL AMs and PMNs, LDH, albumin, TNF-α, and MIP-2, and enhanced AM-CL to the same extent in both WT and iNOS KO mice. Responses 72 h postexposure were similar; however, significant differences were found between WT and iNOS KO mice. iNOS KO mice demonstrated a greater decline in total antioxidant capacity, greater BAL PMNs, LDH, albumin, TNF-α, and MIP-2, and an enhanced AM-CL compared to the WT. These data suggest that the role of iNOS-derived NO in the pulmonary response to LPS + IFN-γ is anti-inflammatory, and this becomes evident over time.
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Keywords: Inducible nitric oxide synthase; Lipopolysaccharide; Interferon-gamma; Lung inflammation

Introduction

Lipopolysaccharide (LPS), or endotoxin, is a structural component of the outer leaflet of the outer membrane of Gram-negative bacteria (Rietschel and Westphal, 1999). Bacterial invasion into the body leads to bacteriolysis which releases these structural components that, in turn, cause a complex immune response (Beutler and Rietschel, 2003). Particles generated from compost, grain, hay, silage, and cotton, for example, are often contaminated with bacteria and bacterial products (Castranova et al., 1996; Rylander, 1997). Acute inhalation of these organic particles may lead to a respiratory disease known as organic dust toxic syndrome (ODTS). Chronic exposure to organic particles, typically in cotton and flax processing plants, may result in the obstructive lung disease byssinosis (Merchant et al., 1975; Rylander and Morey, 1982).

Animal and cellular studies show LPS activates alveolar macrophages (AMs), which are the primary phagocytic cells in the lung. AM activation by LPS involves release of tumor necrosis factor-α (TNF-α), production of chemokines which cause polymorphonuclear leukocyte (PMN) infiltration into the alveolus, activation of nuclear factor-kB (NF-kB) and mitogen-activated protein kinase (MAPK) pathways, and generation of reactive species (Beutler et al., 1985; Han et al., 1994; Ryan and Karol, 1991; Shakhov et al., 1990). Guinea pigs exposed to LPS-contaminated cotton dust had increased TNF-α and PMNs in the bronchoalveolar lavage

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(BAL) fluid (Ryan and Karol, 1991). In addition, LPS administration in rats has been shown to increase inducible nitric oxide synthase (iNOS or NOS2) expression and subsequent production of nitric oxide (NO), nitrotyrosine, and chlorotyrosine in the lung (Hataishi et al., 2002; Huffman et al., 1997).

As mentioned, NO derived from iNOS is enhanced in response to LPS. Numerous studies suggest iNOS-derived NO plays a role in inflammation, but the pulmonary actions of NO, in response to LPS and other stimuli, remain incompletely understood. NO has been shown to increase proliferation of human lung fibroblasts in vitro, which suggests that NO plays a role in the development of pulmonary fibrosis (Romanska et al., 2002). Highly reactive intermediates, such as peroxynitrite ($OONO^-$), formed from NO and superoxide (O_2^-), have been implicated in the pathogenesis of pneumoconiosis (Castranova et al., 1998; Iguchi et al., 1996; Tanaka et al., 1998). In contrast, iNOS-derived NO possesses antimicrobial properties as demonstrated in vivo where protection against anthrax and *Bordetella pertussis* infection was observed in wild-type (WT) compared to iNOS knockout (KO) mice (Canthaboo et al., 2002; Kalns et al., 2002). In addition, NO from exogenous sources has been reported to attenuate LPS-induced acute lung injury in rabbits (Kang et al., 2002; Uchizumi et al., 1993).

Interferon-γ (IFN-γ) is a potent inflammatory cytokine produced mainly by T lymphocytes and natural killer cells. IFN-γ activates macrophages to release NO and other inflammatory mediators (Beutler et al., 1985; Boehm et al., 1997; Farrar and Schreiber, 1993). Primary mouse AMs, harvested from C57BL/6J mice, show an increase in NO production, as measured by nitrate and nitrite (NOx) release into the cellular supernatant, after exposure to IFN-γ. LPS stimulation further increases this NOx production (Zeidler et al., 2003). IFN-γ ± LPS-induced NOx does not occur in AMs harvested from iNOS KO mice (Zeidler et al., 2003). LPS administered intraperitoneally also increases iNOS mRNA in WT mice but not in iNOS KO mice (Zeidler et al., 2003).

Therefore, the objective of this study was to determine the role of iNOS-derived NO in a model of acute and longer-term lung inflammation. The iNOS KO and the background strain (C57BL/6J) were exposed to a combination of LPS + IFN-γ via aspiration. Aspiration is a method of pulmonary exposure which results in uniform deposition of the exposure agent (Rao et al., 2003). Pulmonary response was evaluated at 24 and 72 h after aspiration of LPS + IFN-γ, and lung damage and inflammation were compared in iNOS KO and WT mice.

Methods

Animals. Breeder pairs of iNOS KO mice (B6.129P2–*Nos2*^{tm1Lau}) along with wild-type strain (C57BL/6J) were purchased from Jackson Laboratories (Bar Harbor, Maine). The iNOS KO status was verified in a previous study using in vivo and in vitro methods (Zeidler et al., 2003). Animals were housed in an AAALAC-accredited, specific pathogen-free, environmentally controlled facility and allowed to acclimate at least 5 days before use. The mice were free of endogenous viral pathogens, parasites, mycoplasms, Helicobacter, and CAR Bacillus. Mice were kept in ventilated cages, which were provided HEPA-filtered air, with Alpha-Dri virgin cellulose chips and hardwood Beta-chips for bedding. Food and tap water were given ad libitum. Male mice, 8–10 weeks old with an average body weight of 25 g, were used in this study.

Mouse pharyngeal aspiration. Mouse pharyngeal aspiration was performed as described by Rao et al. (2003). Briefly, after anesthetization with a mixture of ketamine and xylazine (50 and 2 mg/kg subcutaneous in the abdominal area, respectively), the mouse was placed on a board in a near vertical position and the animal's tongue extended with lined forceps. A suspension (approximately 30 μl) of LPS from *E. coli* serotype 026:B6 (Sigma, St. Louis, MO) at a dose of 1.2 mg/kg and a bolus dose of 5000 U/mouse recombinant murine IFN-γ (BioSource International, Inc. Camarillo, CA) was placed posterior in the throat and the tongue held until the suspension was aspirated into the lungs. Control mice were administered sterile Ca^{+2} + Mg^{+2}-free phosphate-buffered saline (PBS) vehicle. The mice revived unassisted after approximately 30–40 min. All mice in WT and iNOS KO groups survived this exposure procedure. Mice were sacrificed either 24 or 72 h following aspiration. The dose of LPS used has been shown previously in our lab to produce a consistent and significant yet nonmaximal increase in markers of lung injury and inflammation (unpublished results). Mice were exposed to LPS plus IFN-γ, because LPS alone failed to consistently induce NO production by AM from exposed WT mice. iNOS induction in response to LPS plus IFN-γ was confirmed by immunohistochemistry as described previously (Porter et al., 2002). Data indicate that LPS plus IFN-γ caused induction of iNOS at both 24 and 72 h post-exposure in AM from WT mice but not iNOS KO mice (data not shown).

Bronchoalveolar lavage. Mice were weighed and sacrificed with intraperitoneal injection of sodium pentobarbital (>100 mg/kg). The trachea was cannulated with a blunted 22 gauge needle and BAL was performed using cold sterile Ca^{2+} + Mg^{2+}-free PBS at a volume of 0.6 ml for first lavage (kept separate) and 1.0 ml for subsequent lavages. Approximately 10 ml of BAL fluid per mouse was pooled and collected in sterile centrifuge tubes. Typically, BAL fluid from two to three mice was pooled to obtain a sufficient cell number for experiments. Pooled BAL cells were washed in Ca^{+2} + Mg^{+2}-free PBS by alternate centrifugation (600 × *g* for 10 min at 4 °C) and resuspension. Acellular first fraction

BAL aliquots were frozen or kept on ice for later analysis. The final cell pellet was suspended in 10 mM HEPES buffer (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM $CaCl_2$, 5.5 mM glucose) at pH 7.4.

Cell counts and differentials. Cell counts were performed using an electronic cell counter equipped with a cell sizing attachment (Coulter model Multisizer II with a 256C channelizer, Coulter Electronics, Hialeah, FL). Alveolar macrophages (AM) or polymorphonuclear leukocytes (PMN) were identified by their characteristic cell diameter (Castranova et al., 1990). AM and PMN inflammation was confirmed by histopathology as described previously (Porter et al., 2002a,b).

First bronchoalveolar lavage fluid tumor necrosis factor-α and macrophage inflammatory protein-2 assays. After thawing at room temperature, the first BAL supernatants were assayed directly using a mouse TNF-α (BioSource International) or a mouse macrophage inflammatory protein-2 (MIP-2) ELISA kit (R&D Systems, Minneapolis, MN) according to manufacturers' instructions. Concentrations of TNF-α and MIP-2 were determined as pg/ml based on the appropriate TNF-α or MIP-2 standard curve. ELISA plates were read at 450 nm using a Spectramax 250 microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA).

Primary mouse alveolar macrophage zymosan-stimulated chemiluminescence. Alveolar macrophage chemiluminescence was determined as described by Porter et al. (2002) using an automated luminometer (Berthold Autolumat LB 953, EG&G, Gaithersburg, MD) at 390–620 nm for 15 min with a total assay volume of 0.25 ml. Briefly, 1.0×10^6 alveolar macrophages/ml from control or exposed mice were incubated in HEPES buffer and resting chemiluminescence was determined by adding 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) at a final concentration of 0.08 μg/ml. Zymosan-stimulated alveolar macrophage chemiluminescence was determined by adding unopsonized zymosan A (Sigma) at 2 mg/ml immediately before measurement. Unopsonized zymosan has been shown to stimulate chemiluminescence from alveolar macrophages but not polymorphonuclear leukocytes, which only respond to opsonized stimulants (Allen, 1977; Castranova et al., 1987; Hill et al., 1977). Zymosan-stimulated alveolar macrophage chemiluminescence was calculated as counts per minute (cpm) in the zymosan-stimulated assay minus cpm in the resting assay.

First bronchoalveolar lavage fluid albumin. Albumin concentration (mg/ml) was determined colorimetrically at 628 nm based on albumin binding to bromcresol green (Albumin BCG diagnostic kit, Sigma) using a Cobas Mira Plus Transfer Analyzer (Roche Diagnostic Systems, Montclair, NJ).

First bronchoalveolar lavage fluid lactate dehydrogenase activity. Lactate dehydrogenase (LDH) activity (U/L) was determined by monitoring the LDH catalyzed oxidation of pyruvate coupled with the reduction of NAD at 340 nm using a commercial kit and a Cobas Mira Plus Transfer Analyzer (Roche Diagnostics Systems).

First bronchoalveolar lavage fluid total antioxidant capacity. Total antioxidant capacity was assessed using the Bioxytech AOP-490 assay (Oxis Research, Portland, OR), which is based upon the reduction of Cu^{2+} to Cu^{+} by all antioxidants in the sample. Using a SpectraMax 250 microplate spectrophotometer (Molecular Devices Corporation) set to 490 nm, data were obtained from a standard curve of known uric acid concentrations and expressed as "mM Uric Acid Equivalents."

Statistical analysis. Statistical significance was obtained using a Student's t test with $P \leq 0.05$. To achieve an adequate cell number for the experiments, BAL cells were pooled from two to three mice in most cases. Therefore, the

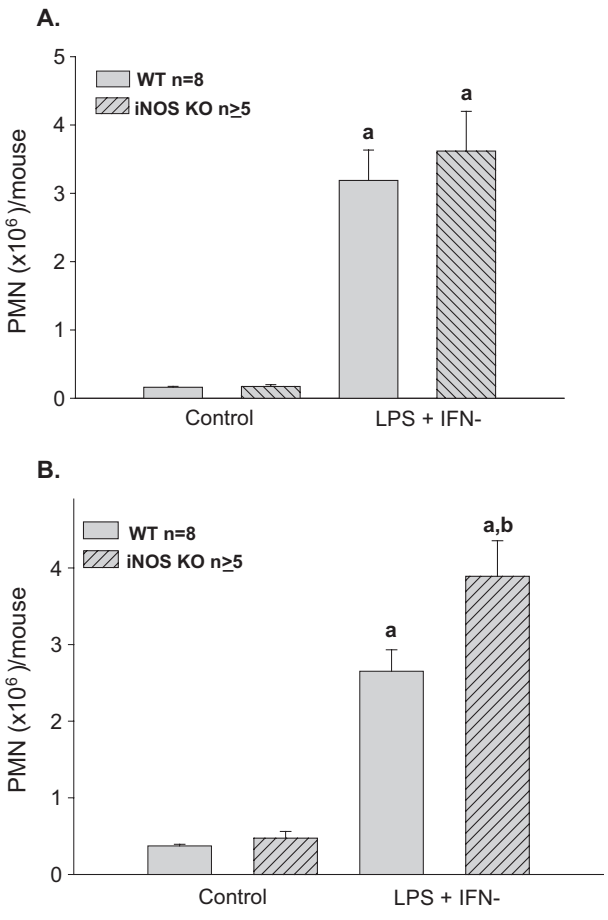

Fig. 1. Polymorphonuclear leukocyte yield per mouse measured 24 (panel A) and 72 (panel B) h after exposure to LPS + IFN-γ or saline vehicle. A significant increase from the corresponding control polymorphonuclear leukocyte yield is indicated by a; a significant difference between the iNOS KO and WT treated groups is indicated by b ($P \leq 0.05$).

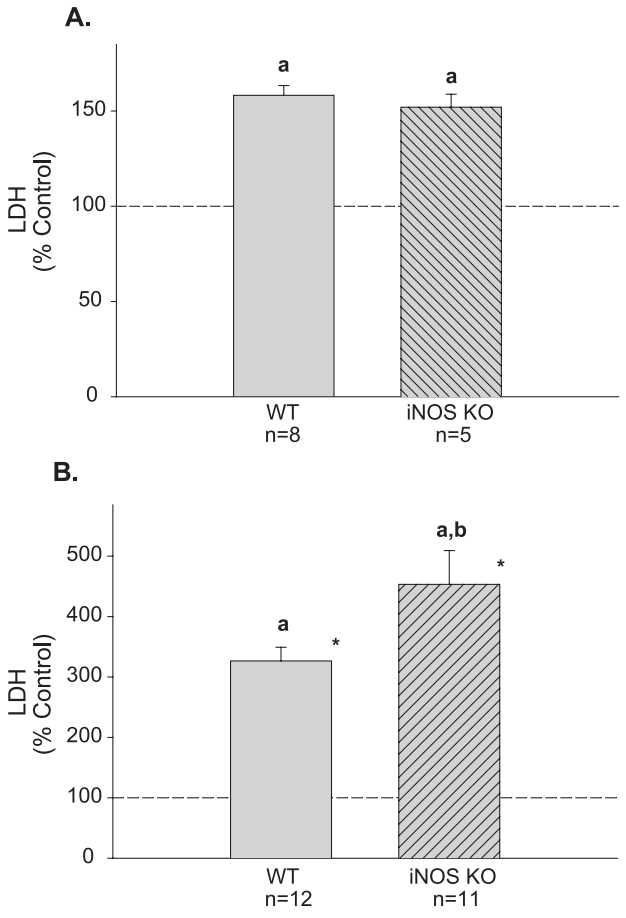

Fig. 2. First bronchoalveolar lavage lactate dehydrogenase activity measured 24 (panel A) and 72 (panel B) h after exposure to LPS + IFN-γ or saline vehicle. Data are presented as percent of control (dashed line indicating 100%). A significant increase from the corresponding control lactate dehydrogenase activity is indicated by a; a significant difference between the iNOS KO- and WT-treated groups is indicated by b; * indicates a significant difference between the 24 and 72 h corresponding treated groups ($P \leq 0.05$).

experimental *n* is indicative of the number of independent experiments and not the total number of mice used (i.e., 2–3 mice pooled equaled an $n = 1$).

Results

Bronchoalveolar lavage alveolar macrophage yield

The yield of AM harvested by bronchoalveolar lavage of WT and iNOS KO mice treated with LPS + IFN-γ was determined at 24 and 72 h postexposure. There was a significant increase (1.7-fold), from the respective control, in AMs at 24 h which further increased (2.5-fold) at 72 h postexposure. However, there was no significant difference in AM yield between the WT and iNOS KO under control or LPS + IFN-γ-treated conditions at 24 or 72 h (data not shown). Histopathology confirmed the dramatic increase in AM in both WT and iNOS KO mice at 24 h postexposure (data not shown).

Bronchoalveolar lavage polymorphonuclear leukocyte yield

Fig. 1 shows the PMN yield from WT and iNOS KO mice exposed to LPS + IFN-γ at 24 (panel A) and 72 (panel B) h postexposure. No significant difference was found between the WT and iNOS KO control PMN counts at 24 or 72 h. There was a significant increase, from the respective control, in PMNs at the 24 h time point for both mice. Further increases were seen for the iNOS KO at 72 h postexposure. The iNOS KO had a significantly higher PMN yield than the WT mice at this time point. Histopathology confirmed the dramatic PMN infiltration in both WT and iNOS KO mice at 24 h postexposure (data not shown).

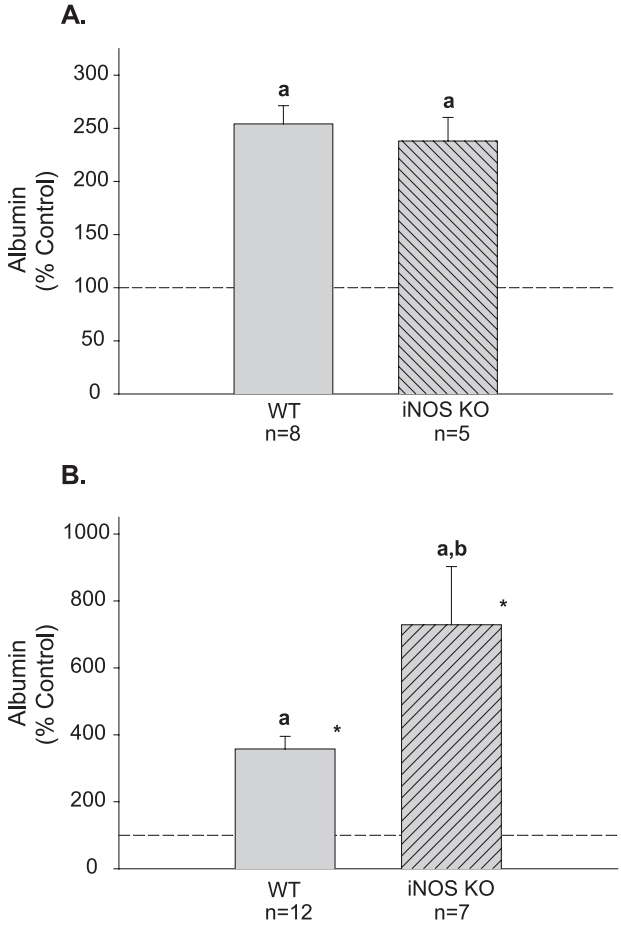

Fig. 3. First bronchoalveolar lavage fluid albumin measured 24 (panel A) and 72 (panel B) h after exposure to LPS + IFN-γ or saline vehicle. Data are presented as percent of control (dashed line indicating 100%). A significant increase from the corresponding control albumin levels is indicated by a; a significant difference between the iNOS KO and WT treated groups is indicated by b; * indicates a significant difference between the 24 and 72 h corresponding treated groups ($P \leq 0.05$).

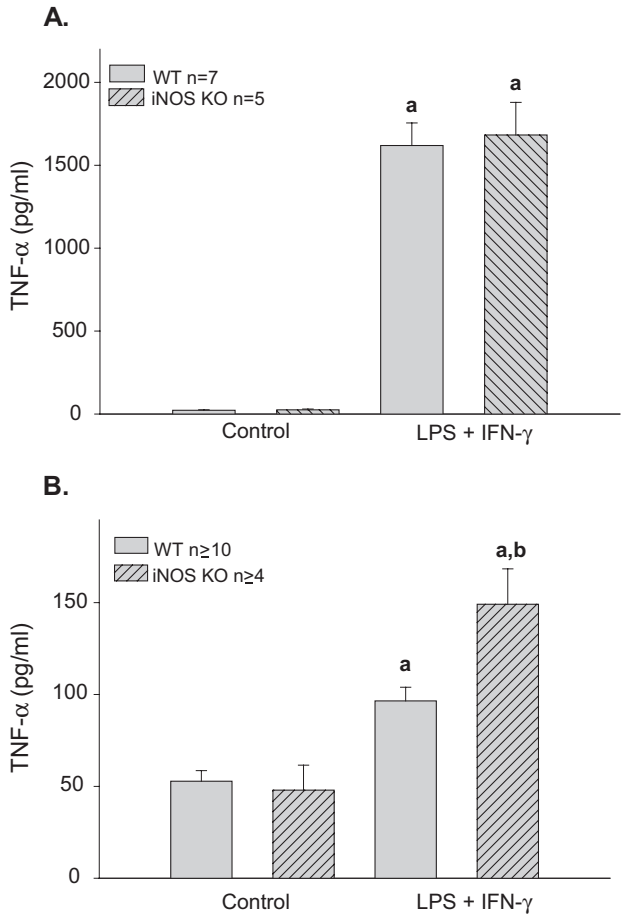

Fig. 4. First bronchoalveolar lavage fluid TNF-α measured 24 (panel A) and 72 (panel B) h after exposure to LPS + IFN-γ or saline vehicle. A significant increase from the corresponding control TNF-α levels is indicated by a; a significant difference between the iNOS KO- and WT-treated groups is indicated by b ($P \leq 0.05$).

First bronchoalveolar lavage fluid lactate dehydrogenase activity

First BAL fluid LDH activity, a measure of cytotoxicity, is shown in Fig. 2. Panels A and B represent 24 and 72 h postexposure, respectively. LDH was significantly increased (approximately 50%) from the corresponding WT or iNOS KO control at 24 h postexposure. Further increases were observed at 72 h (approximately 230% and 350% above the WT or iNOS KO control, respectively) with the iNOS KO having a significantly higher cytotoxic response compared to the WT at this time point.

First bronchoalveolar lavage fluid albumin

An indicator of damage to the air–blood barrier (Beck et al., 1982) is first BAL fluid albumin concentration shown in Fig. 3. Albumin was significantly elevated in both WT and iNOS KO mice at 24 h (panel A) with an even greater increase at 72 h postexposure (panel B). The 72 h time point revealed a significantly higher albumin concentration in iNOS KO compared to WT. Note: no differences were found among the control levels of the WT and iNOS KO mice at either time point.

First bronchoalveolar lavage fluid tumor necrosis factor-α

Concentrations of the inflammatory cytokine TNF-α reached approximately 1690 pg/ml for both the WT and iNOS KO mice 24 h after LPS + IFN-γ treatment (Fig. 4, panel A). At 72 h postexposure (Fig. 4, panel B), TNF-α levels remained increased versus the corresponding control, but the LPS + IFN-γ-treated iNOS KO had significantly higher levels than the WT mice (approximately 150 and 96 pg/ml, respectively). Note: control levels of TNF-α did not differ between the WT and iNOS KO mice at either time point. Statistical comparisons, for the corresponding groups as percent of control, between the 24 and 72 h time points

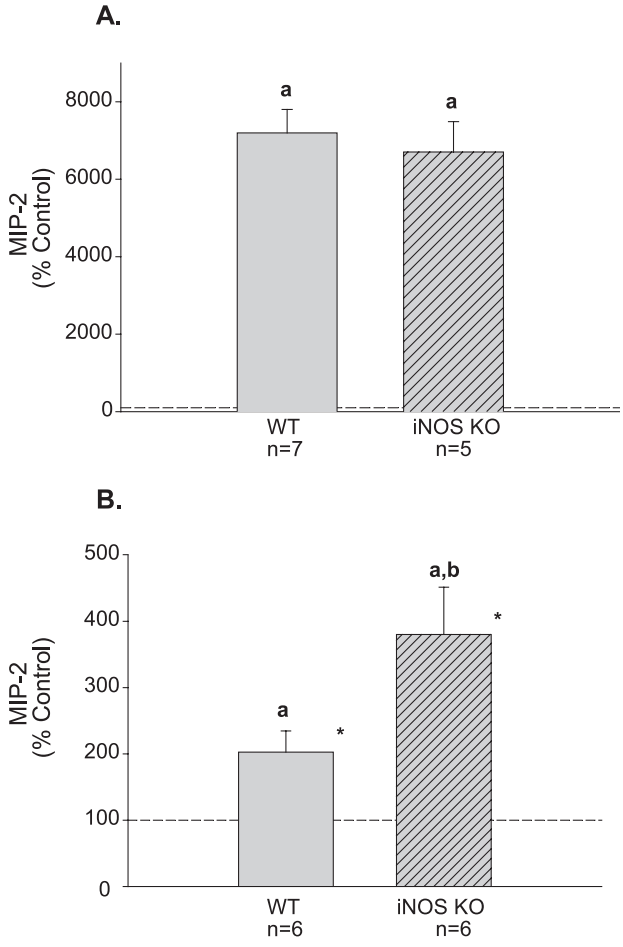

Fig. 5. First bronchoalveolar lavage fluid MIP-2 measured 24 (panel A) and 72 (panel B) h after exposure to LPS + IFN-γ or saline vehicle. Data are presented as percent of control (dashed line indicating 100%). A significant increase from the corresponding control MIP-2 levels is indicated by a; a significant difference between the iNOS KO and WT treated groups is indicated by b; * indicates a significant difference between the 24 and 72 h corresponding treated groups ($P \leq 0.05$).

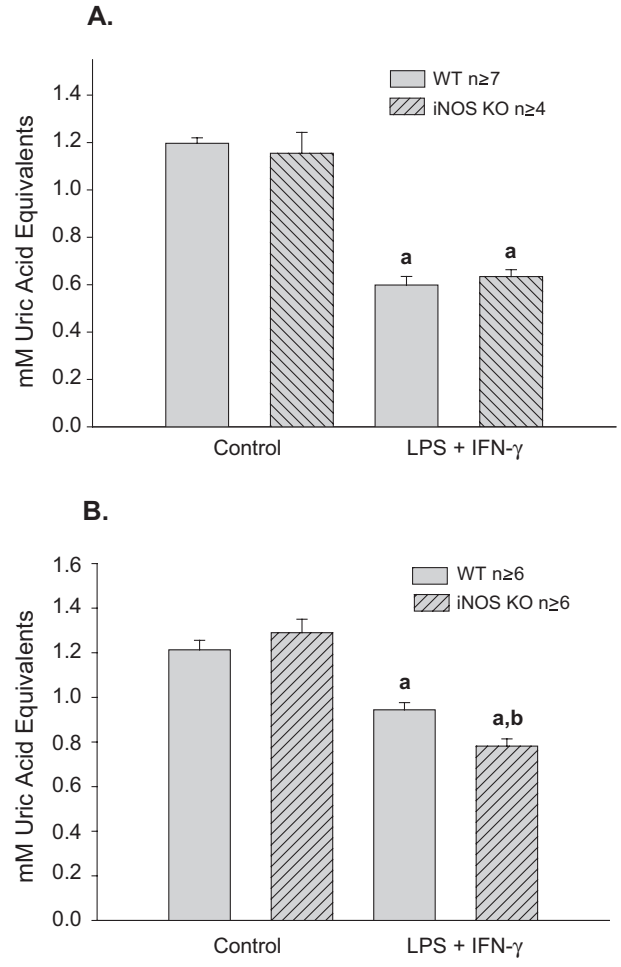

Fig. 6. First bronchoalveolar lavage fluid total antioxidant capacity measured 24 (panel A) and 72 (panel B) h after exposure to LPS + IFN-γ or saline vehicle. A significant decrease from the corresponding control total antioxidant capacity is indicated by a; a significant difference between the iNOS KO and WT treated groups is indicated by b ($P \leq 0.05$).

were also performed and TNF-α levels significantly decreased in both the WT and iNOS KO mice by 72 h (percent of control data not shown).

First bronchoalveolar lavage fluid macrophage inflammatory protein-2

At 24 h postexposure, MIP-2 levels were approximately 190 pg/ml for the WT and iNOS KO mice compared to control values of 2–5 pg/ml (Fig. 5, panel A). Again, a significant increase was found from the corresponding WT or iNOS KO control, but no difference was noted between the WT and iNOS KO mice. At 72 h postexposure, levels of MIP-2 in the first BAL had decreased toward control levels; however, the iNOS KO had significantly higher MIP-2 levels compared to the WT at this time point (Fig. 5, panel B). Note: control levels of MIP-2 did not differ between the WT and iNOS KO at either time point.

First bronchoalveolar lavage fluid total antioxidant capacity

The total antioxidant capacity of BAL fluid is represented in Fig. 6 for the 24 (panel A) and 72 (panel B) h postexposure to LPS + IFN-γ or saline vehicle. At 24 h postexposure, levels of antioxidants were significantly decreased to the same extent in the WT and iNOS KO mice. At 72 h postexposure, levels of antioxidants had increased toward control, but less of an increase was found in the iNOS KO, resulting in a significant difference between the WT and iNOS KO mice. Note: control antioxidant capacity at 24 or 72 h did not differ between the WT and iNOS KO mice.

Primary mouse alveolar macrophage zymosan-stimulated chemiluminescence

Although significantly elevated from the respective controls, the reactive species production between the WT and iNOS KO AMs did not differ upon ex vivo stimulation with zymosan 24 h after exposure to LPS + IFN-γ in vivo (Fig. 7,

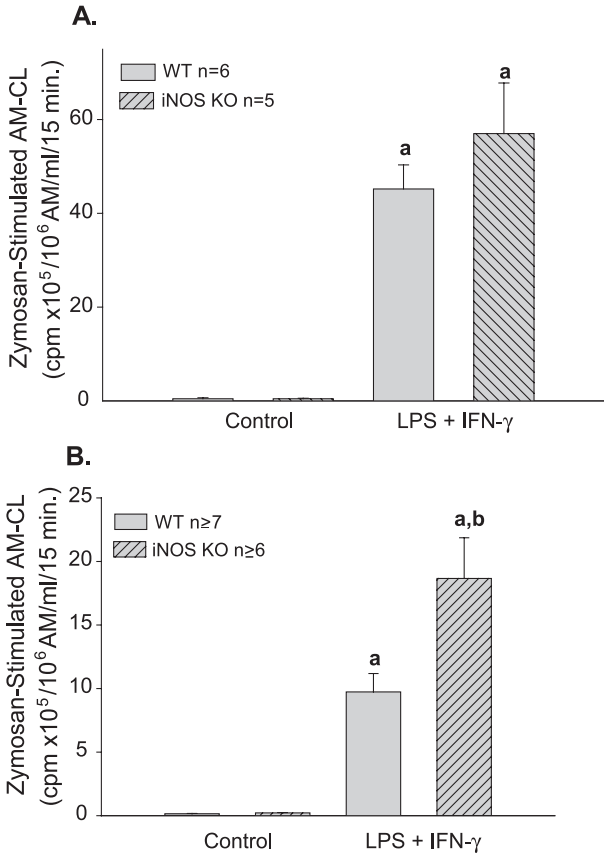

Fig. 7. Zymosan-stimulated alveolar macrophage chemiluminescence measured 24 (panel A) and 72 (panel B) h after exposure to LPS + IFN-γ or saline vehicle. A significant increase from the corresponding control zymosan-stimulated chemiluminescence is indicated by a; a significant difference between the iNOS KO and WT treated groups is indicated by b ($P \leq 0.05$).

panel A). Approximately, a 45- and 55-fold increase more than the control AM-CL was observed for the WT and iNOS KO LPS + IFN-γ treated AMs, respectively. At 72 h postexposure, levels of AM-CL had decreased in both types of mice, but were still significantly elevated from control (panel B). At 72 h postexposure, the iNOS KO AMs demonstrated a significantly enhanced reactive species production compared to the WT AMs. Note: control chemiluminescence did not differ at either time point between the WT and iNOS KO mice. The 24 and 72 h responses between the corresponding groups were also compared as percent of control (data not shown). The WT exhibited a significant decline in AM-Cl at 72 h postexposure, while the decline was not found to be statistically significant in the iNOS KO mice.

Discussion

The present study demonstrates a strong inflammatory response is association with aspiration of LPS + IFN-γ in WT and iNOS KO mice. It was observed that the response to LPS plus IFN-γ did not differ between the WT and iNOS KO mice at 24 h postexposure. However, at 72 h postexposure, significant changes between the WT and iNOS KO response were observed for virtually all parameters in this study. After 72 h, significantly greater inflammation and damage were evident in the iNOS KO compared to the WT mice, suggesting an anti-inflammatory role of iNOS-derived NO in this experimental model.

Exposure of WT and iNOS KO mice to LPS + IFN-γ significantly increased LDH activity and albumin levels in the first BAL fluid, indicating cytotoxicity and damage to the alveolar air–blood barrier, respectively. The cellular injury and damage caused by aspirated LPS + IFN-γ was found to be greater at 72 h postexposure compared to 24 h. Furthermore, at 72 h postexposure, the response in the iNOS KO mice was more severe than for the WT.

The findings for AM-CL and total antioxidant capacity also suggest that NO may play a protective role in this experimental model. LPS + IFN-γ-induced generation of reactive species and decrease in antioxidant capacity in the iNOS KO mice were significantly greater than the WT at 72 h postexposure. This implicates NO as a possible antioxidant engaged in attenuating lung damage induced by LPS + IFN-γ. It is interesting that the basal production of superoxide and hydrogen peroxide from AM in vitro was found to be higher in iNOS KO mice than WT mice (Zeidler et al., 2003). This supports the suggested antioxidant role of NO. However, LPS plus IFN-γ-induced reactive oxygen level did not differ in this in vitro model between WT and iNOS KO AM. It is likely that these differences between the in vitro and in vivo responses reflect mediator interactions present in the whole animal model.

NO is produced, via iNOS, by AMs, alveolar type II epithelial cells, and PMNs in response to inflammatory stimuli such as LPS and cytokines (Gutierrez et al., 1995; Palmer et al., 1988; Salvemini et al., 1989; Wright et al., 1989). Currently, the majority of data suggest that NO derived from iNOS is a potent inflammatory mediator that causes significant lung injury and damage. For example, NO and its reactive intermediates are involved in the pulmonary damage associated with cotton dust, silica, and asbestos exposure (Chao et al., 1996; Iguchi et al., 1996; Mongan et al., 2000; Porter et al., 2002; Thomas et al., 1994). Lung injury and damage during inflammation have been associated with excess production by lung cells, including AMs and PMNs, of reactive species, such as NO and O_2^- which may form $OONO^-$, OH, H_2O_2, as well as other inflammatory mediators. Thus, iNOS KO mice would be expected to exhibit less injury and damage due to the lack of production of certain reactive species. The present study indicates that an alternative, perhaps protective, action of NO may exist during pulmonary exposure to the Gram-negative bacteria derivative LPS and the inflammatory cytokine IFN-γ. These data contrast with those reported by Stanley et al. (2002), where LPS was found to be less inflammatory in iNOS KO mice. Several differences in methodology may explain this difference, that is, a 6 h postexposure evaluation vs. 24 and 72 h measurements and LPS vs. LPS plus IFN-γ exposure in the Stanley et al. study vs. the present study, respectively. In addition, Stanley et al. (2002) reported that induction of IL-10 was absent in iNOS KO mice in response to LPS. One would expect the absence of IL-10 to result in a more prolonged inflammatory response, which was noted in the present study at 72 h postexposure.

Studies show NO may act to counteract oxidative lung injury by decreasing the reactivity of lipid radicals and the extent of lipid peroxidation (Rubbo et al., 1994). It has also been shown that exogenous and endogenous NO attenuates leukocyte adhesion and sequestration into the lung, both in vivo after i.v. endotoxin administration and in vitro, decreasing the overall lung inflammatory mileu (Gaston et al., 1994; Hickey and Kubes, 1997; Lin et al., 2001; Sato et al., 1999). Evidence for the protective actions of NO in the lung during certain inflammatory states have previously been reported. For example, acute lung injury induced by ozone or hyperoxia resulted in significantly greater lung injury and damage, as measured by total protein, LDH, albumin, and PMNs in the BAL fluid, in iNOS KO compared to WT mice (Kenyon et al., 2002; Kobayashi et al., 2001). The present study also reported a similar protective action of NO in response to LPS + IFN-γ-induced inflammation, as mentioned above.

Both TNF-α and MIP-2 levels in the first BALF were significantly greater in the iNOS KO compared to the WT mice at 72 h postexposure to LPS + IFN-γ, although levels had decreased in both groups from 24 h postexposure. TNF-α and MIP-2 are central to the prompt initiation and progression of the lung inflammatory response. TNF-α release causes PMN influx, vascular endothelial adhesion molecule expression, and release of inflammatory mediators

from multiple pulmonary cell types (Albelda et al., 1994; Springer, 1994; Standiford et al., 1990; Strieter et al., 1989). MIP-2 is a potent chemoattractant factor for PMNs and the heightened levels of MIP-2 in the iNOS KO mice versus WT coincide with the greater PMN yield observed at 72 h postexposure. This observation was also reported by Kenyon et al. (2002) in a model of ozone-induced acute lung injury in iNOS KO mice.

NF-κB activation is associated with the production of TNF-α and MIP-2, and when inhibited, LPS induction of these inflammatory mediators decreases (Raychaudhuri et al., 1999). An action recently proposed for NO is the inhibition of this transcription factor. Data show that NO increases the levels of the inhibitory subunit IκBα and decreases NF-κB binding to gene promoter regions in the DNA by nitrosylating cysteine residues on the p50 subunit of this transcription factor (Matthews et al., 1996; Peng et al., 1995). In vivo, exogenous NO (10 ppm) has been reported to attenuate pulmonary damage and inflammation in rabbits exposed to intravenous LPS through suppression of NF-κB (Kang et al., 2002).

Surprisingly, this study demonstrated lack of a vital role for iNOS-derived NO in the immediate inflammatory response (i.e., 24 h) to LPS + IFN-γ. However, after 72 h, the differences in the WT and iNOS KO mice became apparent, suggesting the NO plays a more pronounced role in lung inflammation and damage after the initial phase. An explanation for this may be that the initial and extended responses to aspirated LPS + IFN-γ differ. All parameters significantly increased from the respective controls in response to LPS + IFN-γ, except total antioxidant capacity which showed a significant decline, in the 24-h phase of the experiment. Typically, after initial injury, there is resolution of the inflammation and damage, but this was not observed in the present study for all parameters at 72 h postexposure. For example, AMs, PMNs (only in the iNOS KO), LDH, and albumin continued to increase 72 h postexposure to LPS + IFN-γ. In contrast, initial elevations in TNF-α, MIP-2, and AM-CL declined, while the initial decline in total antioxidants in the first BAL returned toward control levels, from 24 to 72 h. As mentioned, iNOS KO mice showed significantly greater changes in these parameters at 72 h postexposure. Therefore, the extended response in terms of cellular influx, cytotoxicity, and blood air–barrier damage (most likely due to increased inflammatory cell influx) worsened but cellular activity (measured as cytokines and reactive species production) and antioxidant status were recovering.

It is possible that the protective action of NO at 72 but not 24 h postexposure reflects the time course of NO production following LPS + IFN-γ treatment. The source of NO pertinent in this study results from transcription and translation of inducible NOS, which takes adequate time to complete possibly allowing differences only to be seen after a sufficient induction time. Reports show that iNOS mRNA is detectable in rats approximately 4 h after stimulation with LPS and significant NO production is observed after about 8 h (Castranova et al., 1998). In the present study, immunohistochemistry indicates that iNOS was induced at 24 and 72 h postexposure to LPS plus IFN-γ in AM from WT but not iNOS mice (data not shown). During hyperoxia, iNOS mRNA in C57BL/6J mice was also reported to be expressed between 24 and 72 h postexposure (Kobayashi et al., 2001).

In summary, NO may not always cause lung damage and inflammation. The present study revealed protective (i.e., anti-inflammatory) effects of NO derived from iNOS, i.e., the absence of NO in iNOS KO mice enhanced pulmonary inflammation and damage in response to LPS + IFN-γ exposure. These effects were observed at 72 h postexposure but not after 24 h, which suggests the effects of iNOS are more prominent in the later phases of disease progression. The reported findings are consistent with a select group of models of acute lung injury that also suggest lung inflammatory responses may be mitigated by iNOS-derived NO.

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