Regulatory Effects of iNOS on Acute Lung Inflammatory Responses in Mice

Cecilia L. Speyer,* Thomas A. Neff,*
Roscoe L. Warner,* Ren-Feng Guo,*
J. Vidya Sarma,* Niels C. Riedemann,*
Megan E. Murphy,[†] Hedwig S. Murphy,^{**} and
Peter A. Ward*

From the Department of Pathology,* University of Michigan
Medical School, Ann Arbor; Veterans Administration Ann Arbor
Health Service,[†] Ann Arbor; and Hope College,[‡] Holland,
Michigan

The role of endogenous NO in the regulation of acute lung injury is not well defined. We investigated the effects of inducible nitric oxide synthase (iNOS) and endothelial NOS (eNOS) on the acute inflammatory response in mouse lungs. Acute lung injury was induced by intratracheal instillation of bacterial lipopolysaccharide (LPS) into wild-type (WT) mice and mice deficient in iNOS ($iNOS^{-/-}$) or eNOS ($eNOS^{-/-}$). Endpoints of inflammatory injury were myeloperoxidase (MPO) content and leak of albumin into lung. Inflammatory injury was similar in WT and $eNOS^{-/-}$ mice but was substantially increased in $iNOS^{-/-}$ mice. Bronchoalveolar lavage (BAL) fluids of $iNOS^{-/-}$ and WT mice showed similar levels of CXC chemokines (MIP-2, KC) but enhanced levels of CC chemokines (MCP-1, MCP-3). Increased lung content of MPO in $iNOS^{-/-}$ mice was reduced by anti-MCP-1 to values found in WT mice. *In vitro* stimulation of microvascular endothelial cells with LPS and IFN γ revealed elevated production of CXC and CC chemokines in cells from $iNOS^{-/-}$ mice when compared to endothelial cells from $iNOS^{+/+}$ mice. Peritoneal macrophages from $iNOS^{-/-}$ donors also revealed increased production of CC chemokines after stimulation with LPS and interferon (IFN γ). These data indicate that absence of iNOS causes enhanced lung inflammatory responses in mice which may be related to enhanced production of MCP-1 by endothelial cells and macrophages. It appears that iNOS affects the lung inflammatory response by regulating chemokine production. (*Am J Pathol* 2003, 163:2319–2328)

There is abundant evidence that lung inflammatory injury, occurring after a variety of insults, is due to activation of lung phagocytic cells, which produce numerous chemokines (reviewed).¹ CXC chemokines are thought to attract neutrophils into the lung, while CC chemokines attract

lymphocytes and monocytes and also have the ability to activate macrophages.² It is well known that a variety of lung cells other than phagocytic cells have the ability to generate cytokines and chemokines.³ For inflammatory reactions that lead to injury, the role of NO is controversial, with evidence for pro-inflammatory as well as anti-inflammatory effects.⁴

In various lung injury models, neutrophil recruitment, cytokine production, and oxygen radical production were reduced in the presence of either inhaled NO or exogenous NO donors, suggesting a beneficial role for NO in acute lung injury.^{5–7} In contrast, a decrease in lung permeability and neutrophil recruitment was shown in various models of lung injury using NO synthase (NOS) inhibitors, suggesting a harmful effect of NO on lung injury.^{8–10} At high concentrations, NO is known to induce DNA strand breakage and base alterations.¹¹ NO also reacts with oxygen and superoxide anion to form nitrogen dioxide and peroxynitrite anion, which are known cytotoxic oxygen radicals that can also interfere with a variety of lung functional parameters. The discrepancy in the nature of NO effects may be due to the lack of isoform-specific NOS inhibitors, the amount of NO being released by the various NO donors, or the type of lung injury model used.

Under normal physiological conditions, endogenous NO is produced by the constitutive NOS isoforms, eNOS and nNOS (neuronal NOS). After exposure to various inflammatory stimuli such as lipopolysaccharide (LPS), tumor necrosis factor (TNF α), or interleukin (IL)-1 β , an inducible form of NOS (iNOS) is expressed by many cells within the lung parenchyma.¹² Studies involving the use of mice deficient in specific NOS isoforms has shed some light on the role of the various NOS synthases in inflammation. In mice lacking eNOS, basal leukocyte rolling and adhesion were elevated in mesenteric postcapillary venules, suggesting a possible role for eNOS in attenuating the inflammatory response.¹³ This finding is supported by numerous studies in which pharmacological inhibition of constitutive NO production produced significant increases in adherent leukocytes in the microcirculation of various organs including lung, heart, mesentery,

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Address reprint requests to Peter A. Ward, M.D., Department of Pathology, University of Michigan Medical School, 1301 Catherine Road, Ann Arbor, Michigan 48109-0602. E-mail: pward@umich.edu.

and skeletal muscle.^{14–16} With the use of mice deficient in iNOS, a role for iNOS in regulating neutrophil migration during inflammation is beginning to emerge.^{17,18} Neutrophil trafficking during inflammation is a complex process which involves leukocytic and endothelial adhesion molecules as well as several types of chemotactic factors which may include lipid mediators,¹⁹ complement components,^{20,21} and chemokines.^{1,22} NO has been shown to regulate expression of certain cytokines and chemokines, but the literature is very confusing.^{23–27}

The presence of various chemokines during lung injury has been demonstrated in many lung inflammatory models. However, the mechanisms by which these chemokines mediate neutrophil recruitment into the inflamed lung are not altogether clear. The ability of endogenous NO to inhibit neutrophil recruitment via reducing adhesive interactions to the endothelium and by modulating chemokine production are possible mechanisms for attenuation of lung injury. The goal of the present study was to examine a role for both iNOS and eNOS in the pathogenesis of lung injury, using iNOS^{−/−} and eNOS^{−/−} mice. The LPS-lung injury model was used because neutrophils are the predominant infiltrating leukocyte responsible for associated tissue damage.^{28,29} To characterize a role for iNOS or eNOS, mice deficient in these isoforms were subjected to LPS-induced lung injury and neutrophil migration (MPO assay), lung injury (assessed by albumin leakage), and CXC (MIP-2, KC) and CC (MCP-1, MCP-3) chemokine protein expressions were assessed. Companion *in vitro* studies using iNOS^{+/+} and iNOS^{−/−} endothelial cells and macrophages were also performed. The results of these studies suggest that products of iNOS suppress neutrophil recruitment and tissue damage in acute lung injury and may be the consequence of the ability of iNOS-derived NO to suppress chemokine generation *in vivo*.

Materials and Methods

Reagents

LPS isolated from *Escherichia coli* (sterile serotype 026:B6 and 0111:B4), myeloperoxidase (MPO) assay reagents, and gelatin were purchased from Sigma (St. Louis, MO). Fetal calf serum (FCS) was purchased from Hyclone (Logan, UT). Endothelial cell growth supplement (ECGS) and recombinant mouse (rm) IFN γ were purchased from BD Biosciences (Bedford, MA). Capture, blocking, and detection antibodies for KC, MIP-2, and MCP-3 (MARC) were from R&D Systems, Inc. (Minneapolis, MN) and antibodies for MCP-1 were from Pharmingen, Inc. (San Diego, CA). Capture and detection antibodies for mouse albumin were obtained from Bethyl Laboratories, Inc. (Montgomery, TX).

LPS-Induced Lung Injury Model

NOS2^{−/−} (iNOS^{−/−}), NOS3^{−/−} (eNOS^{−/−}), and wild-type (WT) male C57BL/6 mice (6 weeks old, 20 to 25 g) were purchased from Jackson Laboratories (Bar Harbor, ME).

Mice were anesthetized by intraperitoneal injection of 150 mg/kg ketamine HCl and 65 μ g/kg xylazine hydrochloride. LPS from *E. coli* (serotype 0111:B4; Sigma Aldrich) was instilled intratracheally (25 μ g in 50 μ l sterile saline) during inspiration. In some experiments MCP-1 was blocked by intratracheal instillation of anti-MCP-1 (10 μ g in 50 μ l; Pharmingen) at the time of LPS instillation. Control mice received 10 μ g isotype-matched IgG together with LPS (25 μ g in 50 μ l). Six hours after LPS instillation, mice were euthanized.

BAL Fluid Collection and Cell Counts

BAL fluid was collected by bronchoalveolar lavage, performed three times with 0.8 ml sterile saline. The recovered lavage was centrifuged at 4500 rpm for 10 minutes at 4°C. The cell-free supernatants from the first wash were stored at −20°C for further analysis of chemokine and mouse albumin content by ELISA. BAL fluid cell populations were found, in all experiments, to contain at least 95% neutrophils as demonstrated by cyto-spin and differential stain analysis.

Determination of MPO Activity

After BAL, lungs were perfused via the right ventricle with 3 ml of sterile PBS, snap-frozen in liquid nitrogen and stored at −70°C. To measure MPO activity, whole lungs were homogenized and sonicated in 50 mmol/L potassium phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (HTAB) and 5 mmol/L ethylene diaminetetraacetic acid (EDTA). After centrifugation at 12000 $\times$ g for 10 minutes at 4°C, the supernatant fluids containing MPO were incubated in a 50 mmol/L potassium phosphate buffer containing the substrate, H₂O₂ (1.5 mol/L). In the presence of *o*-dianisidine dihydrochloride (167 μ g/ml; Sigma Aldrich), the enzymatic activity was determined spectrophotometrically by measuring the change in absorbance at 460 nm over 3 minutes using a 96-well plate reader (Molecular Devices, Sunnyvale, CA).

Determination of Albumin Content in BAL Fluid

Mouse albumin levels in BAL fluid were measured using a mouse albumin ELISA kit purchased from Bethyl Labs. The detection limit for this ELISA was 7 ng/ml.

Morphological Assessment of Lung Injury

To morphologically assess lung injury, 6 hours after intratracheal instillation of LPS, lungs were fixed by intratracheal instillation of 1 ml buffered (pH 7.2) formalin (10%). The lungs were further fixed in a 10% buffered formalin solution for histological examination by tissue sectioning and staining with hematoxylin and eosin.

Isolation and Culture of Microvascular Endothelial Cells and Peritoneal Macrophages

Because of the difficulty in obtaining a pure population of microendothelial cells from mouse lung, microvascular endothelial cells were isolated from mouse ear dermis as previously described.³⁰ Twenty-eight-day-old male C57BL/6 WT and iNOS^{-/-} mice were purchased from Jackson Laboratories. Ears were removed, split into two pieces and incubated in 5 mg/ml dispase II for 45 minutes to loosen the dermis. The dermis was removed from the epidermis using tweezers and the individual microendothelial cells were released into plating medium (RPMI containing 20% FCS, 50 U/ml penicillin/streptomycin, 0.25 μ g/ml fungizone, 1 mmol/L L-glutamine and 50 μ g/ml ECGS) using the blunt end of a scalpel. After 65 hours, tissue was removed from both cultures and the remaining endothelial cells were maintained in plating medium until confluent. Their identity was confirmed by their uptake of Dil-Ac-LDL³¹ as demonstrated by immunofluorescent microscopy and/or flow cytometry.

When confluent, cells were seeded at a density of 2.0×10^5 cells/ml into gelatin-coated 6-well plates at 1 ml/well, 24-well plates at 0.5 ml/well or into 60-mm tissue culture dishes at 3 ml/dish. Cells were stimulated with various concentrations of LPS $\pm$ IFN γ (25 U/ml) and in the presence or absence of NO donors. At desired time points, supernatants fluids were removed and stored at -80°C for chemokine analysis.

Macrophages were isolated from the peritoneal cavity of 4- to 6-week-old C57BL/6 WT and iNOS^{-/-} mice 4 days after peritoneal injection with 0.5 ml of 3% thioglycollate medium, yielding greater than 95% macrophages as demonstrated by cyto-spin and differential stain analysis. The cells were seeded at a density of 1×10^6 cells/ml and plated into 24-well plates at 1 ml/well. After 2 to 3 hours incubation, the cells were washed and stimulated with various concentrations of LPS in the presence and absence of IFN γ (25 U/ml). At desired time points, supernatants were removed and stored at -80°C for chemokine analysis.

Because LPS has been shown to affect cell growth in certain cell types³² and toxicity has been reported with certain NO donors, both microendothelial cells and macrophages were counted after each experiment with the aid of a hemocytometer and cell viability assessed by trypan blue exclusion.

Measurement of NO Production

NO production by both microendothelial cells and macrophages was measured using the NO fluorescent indicator, DAF-2. On reaction with an active intermediate (N_2O_3) formed in the oxidation of NO to nitrite, DAF-2 is converted to its fluorescent triazole form.³³ At various time points, supernatants from 24-well plates were removed and replaced with 500 μ l of a 10 μ mol/L DAF-2 solution diluted in Krebs buffer (120 mmol/L NaCl, 4.8 mmol/L KCl, 0.54 mmol/L CaCl_2 , 1.2 mmol/L MgSO_4 , 11 mmol/L glucose and 15.9 mmol/L NaH_2PO_4 at pH 7.2).

Fresh stimulants were added back at 100X and after 45 minutes incubation at 37°C , 200 μ l culture supernatant was removed from each well and transferred to a 96-well black plate with a clear bottom. Fluorescent intensity was measured using a fluorescent plate reader (Molecular Devices, Sunnyvale, CA) at excitation and emission wavelengths of 485 and 538 nm, respectively. The detection limit of NO by DAF-2 was 5 nmol/L.

Western Blot Analysis

At various time points after stimulation of cells with LPS and/or IFN γ , cells were collected by trypsinization, washed, and resuspended in lysis buffer containing 10 mmol/L Tris-HCl (pH 7.6), 50 mmol/L NaCl, 1% Triton X-100, and 1X complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Samples were extracted on ice for 30 minutes, sonicated twice for 5 seconds and centrifuged at $14000 \times g$ for 10 minutes. Protein concentration of cell lysate was determined using Bio-Rad D_c Protein Reagents and either 50 μ g (for iNOS) or 100 μ g (for eNOS) of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5%) and electrophoretically transferred to polyvinylidene fluoride membranes. Immunodetection was performed using a primary rabbit antibody to mouse iNOS or bovine eNOS (Calbiochem, San Diego, CA) with appropriate secondary antibodies and detected by enhanced chemiluminescence.

Quantification of Chemokine Production by ELISA

Chemokine levels in BAL fluid and cell supernatants were measured by sandwich ELISA. Briefly, Immulon ELISA plates were coated overnight with 5 μ g/ml capture antibody to either MIP-2, KC, MCP-1, or MCP-3. The plates were washed and blocked for 1 hour with PBS containing 3% bovine serum albumin. Various dilutions of samples with appropriate standards were added to the wells and incubated for 2 hours, followed by washing and incubation in appropriate biotinylated secondary antibody (2 μ g/ml) for 1 hour. Wells were washed and streptavidin-peroxidase was added for 30 minutes followed by washing and incubation in OPD substrate (Sigma Aldrich) for 10 minutes. The reaction was stopped by addition of 0.5 mol/L sulfuric acid. Absorbance was measured at 490 nm using a Molecular Devices plate reader (Sunnyvale, CA). The detection limit for all chemokines ranged between 30 and 120 pg/ml.

Statistical Analysis

All numerical results are expressed as mean $\pm$ SEM. For these assays, statistical analysis was performed using repeated measures analysis of variance followed by a multiple comparison procedure using the Student-Newman Keuls method. A value of $P < 0.05$ was considered significant.

Results

Albumin Permeability and MPO Buildup in Lungs after LPS Challenge

Increases in lung permeability in response to LPS intrapulmonary instillation was determined by measuring leakage of mouse albumin into BAL fluids. Six hours after LPS challenge, albumin levels in BAL fluids from WT, iNOS^{-/-}, and eNOS^{-/-} mice were significantly increased over uninjured lungs from control mice (Figure 1A). There was no significant difference in the level of albumin leakage between WT and eNOS^{-/-} mice (experiment 2, Figure 1A). However, in iNOS^{-/-} mice, there was almost a two-fold increase in albumin leakage over that found in WT mice (experiment 1, Figure 1A).

Increases in lung permeability correlated with increases in neutrophil accumulation in lungs as determined by MPO content (Figure 1B). As with albumin leakage, MPO content in iNOS^{-/-} mice was much higher (nearly threefold) when compared to values in WT mice. No significant differences in MPO were found between WT and eNOS^{-/-} mice (experiment 2, Figure 1B). Increased MPO activity in lungs of iNOS^{-/-} mice correlated with morphological changes in lungs from similarly treated mice (Figure 2). As expected, no evidence of neutrophils was detected in normal WT lungs (Figure 2, A and B). After LPS instillation into WT lungs, intraalveolar and interstitial accumulation of neutrophils were evident (Figure 2, C and D). In iNOS^{-/-} lungs, the LPS inflammatory response was considerably enhanced as shown by more intense accumulations of neutrophils (Figure 2, E and F). Taken together, these data suggest the inflammatory response to LPS-induced lung injury develops excessively in the absence of iNOS.

Chemokine Levels in BAL Fluids of Mice after LPS Challenge

CXC (MIP-2, KC) and CC (MCP-1, MCP-3) chemokines were measured in the BAL fluids from all three groups of mice (Figure 3). Under non-injury conditions, both CXC and CC chemokines were undetectable in BAL fluids (<100 pg/ml). However, after intratracheal instillation of LPS, both CXC (MIP-2, KC) and CC (MCP-1, MCP-3) chemokines appeared in detectable quantities. There was no significant difference in CXC chemokine content between injured WT, iNOS^{-/-} and eNOS^{-/-} mice. However, there was a significant elevation in CC chemokines in BAL fluids from LPS-instilled iNOS^{-/-} mice when compared to the values in WT and eNOS^{-/-} mice.

Effect of MCP-1 Blockade on Lung Neutrophil Migration

Recent studies suggest a role for MCP-1 and/or its ligand, CCR2, in mediating neutrophil influx in various inflammatory models.^{34–36} To examine a possible role for MCP-1 in mediating neutrophil migration in this study, WT and iNOS^{-/-} mice were treated intratracheally with either

Figure 1. Lung vascular permeability and neutrophil accumulation (MPO content) in iNOS^{-/-} (EXP 1) and eNOS^{-/-} (EXP 2) mice compared to wild-type (WT) mice after LPS-induced lung injury. Mouse albumin levels were measured in BAL fluids (A) as an index for vascular leakage and MPO activity in whole lungs (B) as an index for neutrophil influx. Results are means $\pm$ SEM from at least five mice, where # is $P < 0.05$ compared to uninjured control and * is $P < 0.05$ compared to WT treated mice.

anti-MCP-1 blocking antibody or an isotype-matched IgG, which were given intratracheally together with the LPS. In WT mice, anti-MCP-1 had no effect on MPO activity (Figure 4). However, in iNOS^{-/-} mice, treatment with anti-MCP-1 reduced MPO levels back to those found in WT mice. Thus, in the absence of iNOS, MCP-1 appears to enhance recruitment of neutrophils into LPS-injured lungs.

NO Production and iNOS Protein Expression in Stimulated Dermal Microendothelial Cells

Since endothelial cells are known to express iNOS in response to LPS with IFN γ , a possible role for iNOS in

Figure 2. Hematoxylin and eosin staining of lung tissues obtained from $iNOS^{+/+}$ mice treated with saline (**A**) or LPS (**C**), and $iNOS^{-/-}$ mice treated with LPS (**E**). Interstitial neutrophil infiltration is present in lungs from $iNOS^{+/+}$ treated mice (**C** and **D**), and substantially increased in the $iNOS^{-/-}$ -treated mice (**E** and **F**). Magnification: **A**, **C**, and **E**, $\times 20$; **B**, **D**, and **F**, $\times 40$.

regulating chemokine production by endothelial cells was examined. Treatment of dermal microendothelial cells from WT mice for 24 hours with LPS (10 μ g/ml) produced little increase in NO production compared to unstimulated control cells (Figure 5A). The same was true for IFN γ (25 U/ml). However, the combination of IFN γ (25 U/ml) and LPS (10 μ g/ml) resulted in very robust NO production that was 20-fold above that found in non-

stimulated endothelial cells. NO production was associated with an increase in iNOS protein as demonstrated by Western blot analysis, whereas no consistent change in eNOS protein was found (Figure 5B). As recently pointed out in a study by Jourdain³⁷ both peroxynitrite and horseradish peroxidase can oxidize DAF-2, making it quantitatively difficult to measure exact amounts of NO. Therefore, caution must be taken when estimating

Figure 3. CXC (MIP-2, KC) and CC (MCP-1, MCP-3) chemokine levels in BAL fluid of iNOS^{-/-} and eNOS^{-/-} mice compared to WT mice after LPS-induced lung injury. Results are the mean $\pm$ SEM from at least five mice where # is $P < 0.05$ compared to uninjured and * is $P < 0.05$ compared to WT treated.

amounts of NO generated in cells undergoing both oxidative and/or nitrosative stress.

The time course for iNOS-induced NO production was determined in mouse microvascular endothelial cells. After exposure to LPS and IFN γ (10 μ g/ml and 25 U/ml, respectively), NO production in endothelial cells from WT mice was significantly increased at 6 hours (Figure 6A). As expected, no increase in NO production was detected in iNOS^{-/-} mice. The increase in NO production in WT mice correlated with an increase in iNOS protein levels as a function of time after exposure to LPS and IFN γ , as determined by Western blot analysis (Figure 6B). No

Figure 4. Ability of anti-MCP-1 to reduce lung neutrophil influx in iNOS^{-/-} mice after LPS-induced lung injury determined by MPO activity in whole lungs. Results are the mean $\pm$ SEM from at least five mice where # is $P < 0.05$ compared to uninjured and * is $P < 0.05$ compared to WT treated.

A

B

Figure 5. NO production (A) and NOS protein expression (B) in dermal microvascular endothelial cells after 24-hour exposure to LPS (10 μ g/ml) and/or IFN γ (25 U/ml). Protein expression was determined by Western blot analysis. Values in (A) are means $\pm$ SEM based on three separate experiments performed using triplicate samples. The data in (B) are representative of results from two separate and independent experiments.

significant change in eNOS protein was detectable over the 36 hours period in stimulated WT endothelial cells (data not shown). Western blot analysis confirmed the ability of LPS and IFN γ to induce iNOS protein in endothelial cells from WT but not from iNOS^{-/-} mice (Figure 6C). Peritoneal macrophages (mac) from WT mice stimulated with LPS and IFN γ (10 μ g/ml and 25 U/ml, respectively) for 24 hours also demonstrated appearance of iNOS protein (Figure 6, B and C). As expected, stimulated macrophages from iNOS^{-/-} mice expressed no measurable iNOS protein (Figure 6C).

CXC and CC Chemokine Generation in LPS/IFN γ -Stimulated Microendothelial Cells

As shown in Figure 7, after exposure (at the times indicated) of dermal microendothelial cells to LPS and IFN γ (10 μ g/ml and 25 U/ml, respectively), both CXC (MIP-2, KC) and CC (MCP-1, MCP-3) chemokines were significantly increased in endothelial cells derived from

A

B

C

Figure 6. Time course for NO production and iNOS protein expression in dermal microendothelial cells stimulated with LPS and IFN γ (10 μ g/ml and 25 U/ml, respectively). **A:** NO production in endothelial cells derived from iNOS $^{+/+}$ and iNOS $^{-/-}$ mice as a function of duration of stimulation. **B:** Western blot analysis of iNOS protein in iNOS $^{+/+}$ dermal microendothelial cells and peritoneal macrophages. **C:** Lack of iNOS protein expression in iNOS $^{-/-}$ endothelial cells compared to iNOS $^{+/+}$ endothelial cells after 24-hour stimulation. iNOS protein is also shown in peritoneal macrophages from iNOS $^{+/+}$ mice. Values in **A** are means $\pm$ SEM based on three experiments performed in triplicate. Data in **B** and **C** are representative of two separate and independent experiments, where * is $P < 0.05$.

iNOS $^{-/-}$ mice, proteins for CXC as well as CC chemokines were significantly increased over those found in endothelial cells from WT mice, suggesting a regulatory role for iNOS in synthesis of both CXC and CC chemokines. In iNOS $^{-/-}$ cells, MIP-2 and KC levels were significantly increased at 12 hours (with fourfold and threefold increases, respectively) over those found in stimulated WT cells. On the other hand, increases in MCP-1 and MCP-3 in iNOS $^{-/-}$ cells, when compared to results with WT cells, were somewhat delayed, the latter not being significantly elevated above WT cells until 36 and 24 hours, respectively. These data suggest that the absence of iNOS results in enhanced expression of both CXC and CC chemokines.

Figure 7. Time-course analysis of LPS- and IFN γ -induced (10 μ g/ml and 25 U/ml, respectively) KC and CXC protein expression in dermal microendothelial cells derived from iNOS $^{+/+}$ and iNOS $^{-/-}$ mice. Results are the mean $\pm$ SEM based on three separate and independent experiments with triplicate samples for each data point, where * is $P < 0.05$, compared to stimulated iNOS $^{+/+}$ cells.

To assess effects of the exogenous NO donors, DETA (1 mmol/L) and GSNO (1 mmol/L), on chemokine production in endothelial cells, these donors were added together with LPS and IFN γ and the supernatant fluids collected 36 hours later. In the presence of either NO donor, chemokine production was substantially reduced (Table 1). MIP-2 and MCP-3 production in iNOS $^{-/-}$ cells was reduced to levels found in stimulated WT cells while KC and MCP-1 production was decreased but not as much as that found for MIP-2 and MCP-3 in iNOS $^{-/-}$ cells. While these data confirm that NO has regulatory effects on chemokine production from stimulated endothelial cells, caution must be observed since the concentrations of NO donors were high compared to intrinsic levels of NO.

Table 1. Effect of NO Donors on Chemokine Production in Microvascular Endothelial Cells after 36-Hour Stimulation with LPS/IFN γ (10 μ g/ml:25 U/ml)

Chemokine measured	NO donor added (1 mM)	Concentration (ng/ml)	
		iNOS $^{+/+}$	iNOS $^{-/-}$
KC	None	31.3 $\pm$ 0.4	107.3 $\pm$ 5.0*
	DETA	19.2 $\pm$ 0.9	64.4 $\pm$ 3.1*
	GSNO	19.6 $\pm$ 1.1	62.3 $\pm$ 0.5*
MIP-2	None	36.9 $\pm$ 1.1	78.3 $\pm$ 2.9*
	DETA	23.4 $\pm$ 0.4	27.0 $\pm$ 0.6
	GSNO	22.5 $\pm$ 0.8	27.5 $\pm$ 0.6
MCP-1	None	37.9 $\pm$ 0.8	77.4 $\pm$ 5.4*
	DETA	16.9 $\pm$ 0.1	52.7 $\pm$ 2.7*
	GSNO	18.4 $\pm$ 2.0	49.3 $\pm$ 2.0*
MCP-3	None	47.0 $\pm$ 1.2	71.7 $\pm$ 3.0*
	DETA	42.8 $\pm$ 0.9	49.2 $\pm$ 5.0
	GSNO	26.4 $\pm$ 1.7	21.2 $\pm$ 2.6

* $P < 0.05$ compared to iNOS $^{+/+}$ values.

All values were corrected for levels found in supernatant fluid of nonstimulated endothelial cells (in which case values were <2 ng/ml).

Figure 8. Analysis of LPS- and IFN γ -induced (10 ng/ml and 25 U/ml, respectively) CXC and CC protein expression in peritoneal macrophages derived from iNOS^{+/+} and iNOS^{-/-} mice. Results are the mean $\pm$ SEM based on three separate and independent experiments with triplicate samples for each data point where # is $P < 0.05$ compared to uninjured and * is $P < 0.05$ compared to iNOS^{+/+} treated.

CXC and CC Chemokine Generation by Peritoneal Macrophages

A regulatory role for iNOS on chemokine production in macrophages was found to more closely resemble the *in vivo* response observed in this study. Similar to the endothelial cell response, stimulation of macrophages with various concentrations of LPS up to 10 μ g/ml produced little increase in NO production (data not shown). However, in the presence of IFN γ , NO production and iNOS protein appearance were significantly up-regulated in WT, but not in iNOS^{-/-} macrophages, after stimulation with LPS (10 ng/mL) and IFN γ (25 U/ml) (data not shown). The increase in NO production after stimulation of macrophages for 18 hours with 10 ng/ml LPS and 25 U/ml IFN γ correlated with increased protein for both CXC and CC chemokines (Figure 8). In iNOS^{-/-} macrophages, stimulation with LPS/IFN γ led to substantially greater production of MCP-1 and MCP-3 when compared to production in WT cells. MCP-1 generation was fivefold above that in stimulated WT cells and almost threefold greater in the case of MCP-3. Somewhat surprisingly, macrophage production of KC and MIP-2 was the same in iNOS^{+/+} and iNOS^{-/-} cells. These data suggest that products from iNOS in some manner regulate CC chemokine production in macrophages.

Discussion

The results of this study demonstrate a suppressive role for iNOS (but not for eNOS) in mediating the lung inflammatory response induced by airway administration of LPS. Numerous studies involving both NOS inhibitors and NO donors have suggested a role for eNOS in suppress-

ing leukocyte recruitment in various microvascular compartments under normal physiological conditions.^{15,38–40} Similar observations have also been made in a recent study using eNOS-deficient mice¹³ where neutrophil rolling under basal conditions and in response to thrombin was significantly elevated in mesenteric venules. In our experiments, the inability of eNOS deficiency to affect either albumin leak or MPO accumulation in LPS-injured lung suggests that products of iNOS, but not of eNOS, significantly affect the inflammatory response by causing its attenuation. Interestingly, in a recent study by Sanz et al,⁴¹ increased levels of nNOS were found in both brain and skeletal muscle of eNOS^{-/-} mice and appeared to compensate for the lack of eNOS in regulating leukocyte-endothelial cell interactions under normal physiological conditions in the cremasteric microcirculation. Therefore, it is possible in the present study that nNOS might be compensating for eNOS loss in regulating leukocyte-endothelial cell interactions in the eNOS^{-/-} mice after LPS-induced lung injury. However, in the study just mentioned, nNOS was not able to produce sufficient amounts of NO to mimic eNOS effects after oxidative stress, raising the question as to whether nNOS, under conditions of inflammation, would be able to compensate for the loss of eNOS. The ability of iNOS to reduce lung injury and attenuate neutrophil recruitment in our study is in agreement with other reports in which NO donors or enhancers of endogenous NO production were shown to attenuate LPS-induced lung injury and lung neutrophil migration.^{6,42} The role of iNOS as an endogenous suppressor of lung neutrophil migration has also been demonstrated in iNOS deficient mice using intravital microscopy.¹⁸ In a study by Mulligan et al,⁸ protection against acute lung injury was demonstrated using intratracheal instillation of iNOS antagonists although BAL chemokines were not measured. Mulligan's studies involved IgG-immune complex injury in rat lung while our current studies involved LPS in mouse lung injury. Until data are available using iNOS inhibitors in LPS induced lung injury in mice, it will be premature to reconcile these observations. Previous studies⁴³ have also implicated a proinflammatory role for iNOS where iNOS^{-/-} mice were found to be more resistant to LPS-induced lung injury than their WT counterparts. Because that study also used ventilated rats, it is difficult to compare the outcomes involving different experimental conditions.

The results of this study demonstrate a regulatory effect of NO generated by iNOS on chemokine expression both *in vitro* and *in vivo*. The differing pattern of chemokine expression *in vivo* and *in vitro* with respect to the endothelial cells may be due to tissue-specific responses between lung and dermal microendothelial cells which has been shown to have differing susceptibility to neutrophil-mediated damage.³⁰ The ability of iNOS to regulate MCP-1 expression is consistent with a recent study by Hogaboam et al²³ in which L-NAME inhibition of LPS-induced NO generation in mouse alveolar and peritoneal macrophages was associated with two-fold and five-fold increases in MCP-1 production, respectively. In the present study, increased levels of MCP-1 protein in microvascular endothelial cells of iNOS^{-/-} mice correlated

with increased mRNA levels detected by real-time reverse transcription-polymerase chain reaction (data not shown). Since the CC chemokines possess NF κ B binding sites in their promotor region, it is likely that the effect of iNOS-derived NO on MCP-1 is being transcriptionally regulated via its well known effect on NF κ B.

The appearance of CXC chemokines, MIP-2 and KC, in BAL fluids in the current study is consistent with previous studies showing a role for both MIP-2 and KC in the pathogenesis of direct LPS-induced lung injury.^{43–45} Increased neutrophil migration into pleural cavity after instillation of MIP-2 and KC has been described⁴⁶ and may account for mediation of neutrophil recruitment in LPS models.

CXC (MIP-2, KC) and CC (MCP-1 or MCP-3) chemokine production in an LPS-induced lung injury model occurs in the absence of iNOS or eNOS protein. Increased production of the CC chemokines in iNOS^{-/-} mice and the ability of anti-MCP-1 to abolish increased MPO buildup in lungs of mice suggests a novel pathway by which iNOS attenuates neutrophil recruitment during LPS-induced lung injury. What is of special interest in the BAL fluid data are that in iNOS^{-/-} mice production of MCP-1 and MCP-3 is enhanced while levels of KC and MIP-2 in the BAL fluid are no different from those levels found in iNOS^{+/+} mice. Furthermore, administration of anti-MCP-1 resulted in "normalization" of the lung MPO levels to those found in iNOS^{+/+} mice, indicating some type of link between MCP-1 and neutrophil accumulation in lung after instillation of LPS. A role for CC chemokines in mediating PMN accumulation *in vivo* has been suggested. In the IgG immune complex model of acute lung injury, blockade of MIP-1 β was shown to attenuate vascular permeability and lung neutrophil influx.⁴⁷ Recent findings suggest that neutrophils can respond directly to the CC chemokines. For instance, in a model of adjuvant-induced vasculitis,³⁶ increased responsiveness to MCP-1 correlated with increased CCR1 and CCR2 receptor expression on neutrophils. *In vitro* studies of stimulated neutrophils were also shown to change their receptor expression pattern and become responsive to certain CC chemokines on stimulation with GM-CSF or IFN γ .^{48,49} Based on these findings, increased expression of CC chemokines in the absence of iNOS could serve as an additional chemotactic factor to help direct neutrophils into the alveolar space.

An indirect role of CC chemokines in mediating neutrophil migration has also been suggested. In a recent study by Matsukawa et al,³⁵ MCP-1 was shown to mediate peritoneal neutrophils via LTB₄ production in a model of septic peritonitis. This phenomenon is consistent with another recent report demonstrating a possible role for CCR2 in mediating lung neutrophil recruitment.³⁴ However, in the latter study the mechanism responsible for neutrophil recruitment was not elucidated. In addition to macrophages, endothelial cells and smooth muscle cells have recently been shown to possess CCR2 receptors.^{50,51} Based on these findings, it is conceivable that increased MCP-1 production in iNOS^{-/-} mice mediates neutrophil recruitment indirectly by its effect on other neighboring cells. Further studies into the mechanism by

which MCP-1 production in iNOS^{-/-} mice mediates neutrophil recruitment are needed.

The results of our study demonstrate a regulatory role for iNOS (but not eNOS) in neutrophil recruitment into LPS-injured lungs. In addition, a role for MCP-1 in mediating the enhanced inflammatory response in the iNOS^{-/-} mice has been demonstrated. Further studies examining the mechanism(s) by which iNOS or the CC chemokines regulate LPS-induced lung injury and neutrophil migration would further enhance our understanding of the lung inflammatory process.

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