Effects of Acute Hypoxia and Lipopolysaccharide on Nitric Oxide Synthase-2 Expression in Acute Lung Injury

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The potential role of nitric oxide synthase-2 (NOS2) in acute lung injury (ALI) has gained increasing attention. This study evaluates the effects of hypoxia, an important feature of ALI, on NOS2 expression in a rat model of ALI caused by exposure to hypoxia and LPS. Exposure to hypoxia alone had no effect on the expression of NOS2 in rat lungs. LPS treatment resulted in a significant increase in NOS2 in the lungs, which was further enhanced by concomitant exposure to hypoxia. Immunohistochemical analysis and *in situ* hybridization showed no changes in the expression of NOS2 in lung resident cells under any conditions. The increase in NOS2 levels is mainly due to the influx of NOS2-expressing inflammatory cells. By morphologic analysis, these inflammatory cells were identified as neutrophils, lymphocytes, and monocytes. *In vitro* experiments of lung epithelial and endothelial cell lines showed no detectable expression of NOS2 with any of the treatments. In a macrophage cell line, LPS-induced NOS2 expression was not affected by the concomitant exposure to hypoxia. In conclusion, LPS increases NOS2 expression in rat lungs through the recruitment of NOS2-producing leukocytes. Simultaneous exposure to LPS and hypoxia results in a greater influx of inflammatory cells that further enhances NOS2 expression.

Keywords: acute respiratory distress syndrome; endotoxin; leukocytes; sepsis

Nitric oxide (·NO) is a multifunctional, short-lived gas that may contribute to toxic free radical production and potentiate lung injury. ·NO is synthesized by a family of enzymes called nitric oxide synthases (NOS). There are three NOS isoforms, namely, NOS1 (neuronal NOS), NOS2 (inducible NOS), and NOS3 (endothelial NOS). These isoenzymes were first purified from rat brain (1), murine macrophages (2), and bovine aortic endothelial cells (3), respectively. NOS enzymes are classically classified as either constitutive (NOS1 and NOS3) or inducible (NOS2), although it is now known that the expression of NOS1 and NOS3 can be induced (4), and that NOS2 is constitutively expressed in numerous cell types (5). The so-called constitutive isoforms (NOS1 and NOS3) release low amounts of ·NO and their activity is dependent on calcium and calmodulin (6). On the other hand, NOS2 produces large amounts of ·NO after induction by stimuli such as endotoxin and inflammatory cytokines (7–9). All three isoenzymes have been found in the lung (10–12) and mediate a wide variety of biological events in the respiratory system,

such as bronchodilation (13, 14), pulmonary vasodilation (15, 16), and cytotoxicity (17).

Acute respiratory distress syndrome (ARDS), the most severe manifestation of acute lung injury (ALI), is clinically characterized by severe hypoxemia, diffuse pulmonary infiltrates, and increased pulmonary capillary permeability (18). Although significant advances have been achieved in the treatment of ARDS, the mortality associated with this syndrome still exceeds 40% (19). ·NO administered by inhalation has been shown to have beneficial physiologic effects in ARDS, such as decreased pulmonary pressures and improved oxygenation, although clinical trials have failed to show improvements in survival (20). Paradoxically, endogenous ·NO produced mainly by NOS2 is thought to have deleterious effects and has been implicated in the pathophysiologic mechanisms of ALI (21–25). It has been reported that hypoxia, the main clinical consequence of ALI, has important effects on the regulation of NOS2 via hypoxia response elements present in the NOS2 gene promoter (26). To better understand the potential role of ·NO in ALI, and the importance of hypoxia as an added cofactor, we used a rat model of LPS-induced ALI combined with exposure to normobaric hypoxia. These *in vivo* studies were followed by *in vitro* experiments using various rat lung cell lines treated with LPS and/or hypoxia. The expression of NOS2 in these models was determined by Northern blot, *in situ* hybridization, Western blot, and immunohistochemical analyses. Some of the results of these studies have been previously reported in the form of an abstract (27).

METHODS

See the online data supplement for additional detail on the methods used.

Animals and Treatments

Female Fischer Harlan rats were treated with an intraperitoneal injection of saline or LPS (0.1 mg/kg *Escherichia coli* O111:B4, phenol extract, L-2630; Sigma, St. Louis, MO) and immediately exposed to one of the following two conditions: (*1*) normoxia: room air (21.8°C and relative humidity of 40%); or (*2*) normobaric hypoxia: rats were placed in a Plexiglas chamber at barometric pressure, and gassed with a mixture of 9% O_2–91% N_2 at a constant flow throughout the experiment (22.1°C and relative humidity of 50.2%). Rats were killed by cervical dislocation after 3, 6, 12, or 24 hours of treatment.

Left lobes of lungs were fixed either in 10% formalin or 4% paraformaldehyde and embedded in paraffin or Epon, respectively. Right lobes were divided into two portions and frozen in liquid nitrogen for protein and RNA analyses. The study protocol conforms to National Institutes of Health guidelines.

Wet/Dry Weight Determinations

To confirm the presence of lung injury, six animals per group were exposed to the different treatment combinations for 6 hours, after which the lung wet-to-dry weight ratio was determined.

Cell Lines and Exposures

Normal rat lung epithelial cells (CCL-149; American Type Tissue Collection [ATCC], Manassas, VA), alveolar macrophages (CRL-2192; ATCC),

and rat pulmonary microvascular endothelial cells (RPMVECs; kindly donated by U. Ryan, Avant Immunotherapeutics, Needham, MA) were used.

Cell cultures were exposed to 20% O_2, 5% CO_2, 75% N_2 (normoxia), or were placed in an incubation chamber (Billups-Rothenberg, Del Mar, CA) and gassed with 1% O_2, 5% CO_2, 94% N_2 (hypoxia). LPS (100 ng/ml) was added to the cell culture medium just before initiating exposure to normoxia or hypoxia. Cells were exposed to each treatment for 0.5, 1, 3, 6, 12, or 24 hours. Total RNA or protein was extracted immediately after each treatment.

Northern Blot

Northern blot was performed as previously described (28) with slight modifications.

In Situ Hybridization

The protocol followed was similar to that applied by García and coworkers (29).

Antibodies

Polyclonal anti-NOS2 antiserum sc-650 (Santa Cruz Biotechnology, Santa Cruz, CA) was selected from previous studies in which other antibodies (i.e., N32020–050; Transduction Laboratories, Heidelberg, Germany) were tested. Adsorption controls were performed. Type II pneumocytes were detected with monoclonal antibody MNF116 recognizing cytokeratins 5, 6, 8, 17, and 19 (M 0821; Dako, High Wycombe, UK).

Western Blot

The Western blot procedure was performed as previously described (30) with minor modifications.

Immunohistochemistry

Paraffin sections of lung tissue were microwave-pretreated for antigen retrieval. Sections were revealed with the EnVision system (Dako). Adsorption controls for NOS2 were performed. The expression of NOS2 and MNF116 was quantified with the assistance of computer-aided image analysis (analySIS; Soft Imaging System, Munich, Germany).

After plastic removal, semithin sections were processed in the same manner as paraffin sections.

Statistical Analysis

The most representative times of exposure (3–24 hours in the *in vivo* model and 12 hours in the *in vitro* model) were chosen for statistical analysis (n ≥ 3). Densitometric values were normalized to S9 (Northern blot) and β-actin (Western blot). For statistical analyses, a Kruskall–Wallis test was applied; in those cases in which significant statistical variations were found, groups were compared using a Mann–Whitney U test with the Bonferroni adjustment (SPSS, Chicago, IL).

RESULTS

In Vivo Model

Assessment of lung permeability after various treatments. Rats were treated with LPS or saline and exposed to either normoxia or hypoxia for 6 hours. Histologic examination of slides from rats of different groups revealed differences only in the number of inflammatory cells migrating to the lung (data not shown). Whereas lungs treated with normoxia plus saline and with hypoxia plus saline showed only scattered resident macrophages and inflammatory cells, a considerable increase in the number of inflammatory cells was observed in lungs treated with normoxia plus LPS. The number of inflammatory cells was further augmented in LPS-treated rats concomitantly exposed to hypoxia. Because no other morphologic differences such as alveolar wall thickening or edema were histologically evident, lung injury was quantified by assessment of changes in vascular permeability.

The wet-to-dry lung weight ratios for each group were measured after 6 hours of treatment. Only the hypoxia plus LPS lung group (4.79 ± 0.3, n = 6) showed a significantly greater ratio than
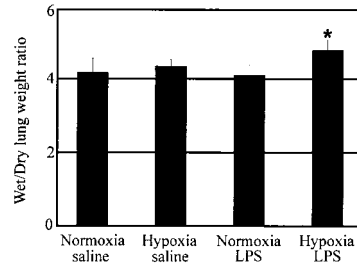

Figure 1. Changes in lung wet/dry weight ratios after 6 hours of treatment. Values are expressed as means (*columns*) ± SD (*error bars*) (n = 6 for each group). *p < 0.05 compared with normoxia plus saline group.

the normoxia plus saline group (4.18 ± 0.40, n = 6, p = 0.006). The wet-to-dry lung weight ratios of rats treated with hypoxia or LPS alone were similar to those of normoxic control lungs (Figure 1).

NOS2 mRNA levels in rat lungs after hypoxia and/or LPS exposure. Northern analysis of NOS2 mRNA expression in rats treated with saline and exposed to normoxia or hypoxia revealed a similarly weak but consistent 4.1-kb band (Figure 2), indicating that acute normobaric hypoxia alone did not induce NOS2 gene expression. In contrast, in lungs from rats treated with LPS and exposed to normoxia there was a significant increase in the expression of NOS2 mRNA. This expression peaked at 3 hours and began to decline after 6 hours of treatment, reaching control values after 24 hours of exposure. When LPS-treated rats were simultaneously exposed to hypoxia, NOS2 mRNA expression was even greater than in rats treated with LPS and exposed to normoxia (Figure 2). The survival rates of rats treated with hypoxia plus LPS for 12 and 24 hours were 80 and 0%, respectively.
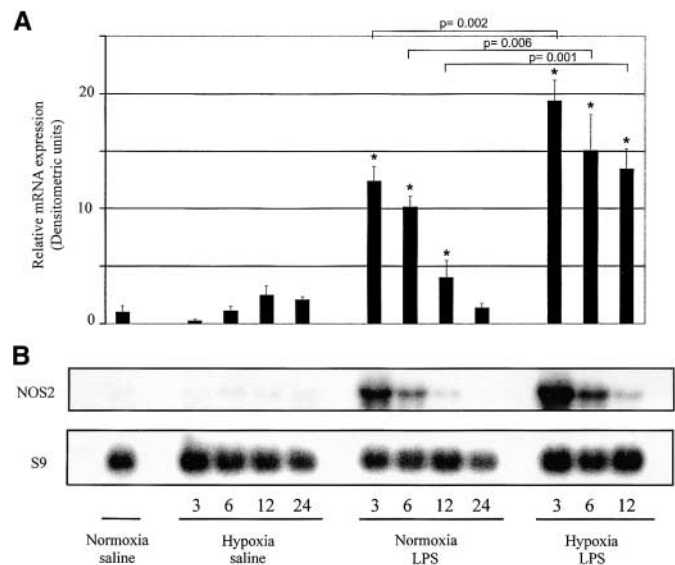

Figure 2. Northern blot analysis of nitric oxide synthase (NOS-2) messenger RNA (mRNA) in rat lungs. (*A*) Quantitation of NOS2 mRNA levels normalized to the signal for S9 mRNA. To standardize between experiments, an arbitrary density of one was assigned to the band obtained from lungs of rats exposed to normoxia plus saline. Values are expressed as means (*columns*) ± SD (*error bars*) (n = 4 for each group at each time point). *p < 0.05 compared with normoxia plus saline group. (*B*) Representative Northern blot analysis of NOS2 and S9 mRNA from lung extracts of rats exposed to normoxia (20% O_2) or hypoxia (9% O_2) and injected with saline or LPS (0.1 mg/kg) for 3, 6, 12, and 24 hours. Combined treatment of hypoxia and LPS was lethal at 24 hours of exposure. Fifteen micrograms of total RNA were loaded per lane.

In situ hybridization for NOS2 mRNA in lung sections from animals treated with saline and normoxia or hypoxia showed barely any labeling, whereas several cell types presented positive staining in the lungs of rats treated with LPS and exposed to either normoxia or hypoxia (Figure 3; only control and hypoxia plus LPS lungs are shown). These cells appeared to be consistent with inflammatory cells adhering to vascular endothelial cells and with cells that have migrated into the lung parenchyma. Labeling was absent when the hybridization was performed with the sense riboprobe (Figures 3E and 3F).

NOS2 protein expression. Western blot analysis of NOS2 protein expression was performed on the same lungs used for Northern blot analysis. NOS2 protein was not detectable in lungs of animals treated with saline and exposed to normoxia or hypoxia (Figure 4). Treatment with LPS caused a significant increase in the amount of NOS2 protein in the lungs of animals exposed to normoxia or hypoxia, although the expression was greater in the hypoxia plus LPS group. In the latter two groups, the induction of NOS2 protein levels followed the same pattern observed for NOS2 mRNA, with an approximate 3-hour delay (Figures 2 and 4). Parallel blots incubated with the anti-NOS2 antibody preadsorbed with the sc-650 peptide showed no immunoreactive band (data not shown).

In an attempt to determine the cell types in which NOS2 expression was increased, immunohistochemical localization of NOS2 was performed in rats subjected to the aforementioned treatments (Figure 5). Weak immunoreactivity for NOS2 in the normoxia plus saline group was present in bronchiolar and vascular smooth muscle, bronchiolar epithelium, and Clara cells. No immunostaining for NOS2 was detected in alveolar walls, endothelial cells, or inflammatory cells related to blood vessels (Figures 5A–5C). Acute hypoxia alone had no detectable effect on the pattern of expression of NOS2 as compared with control
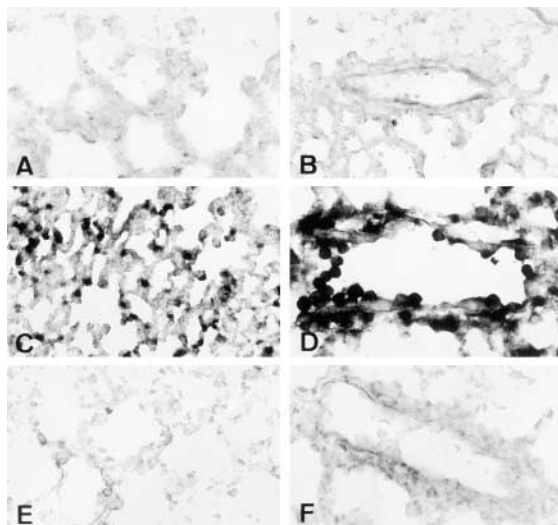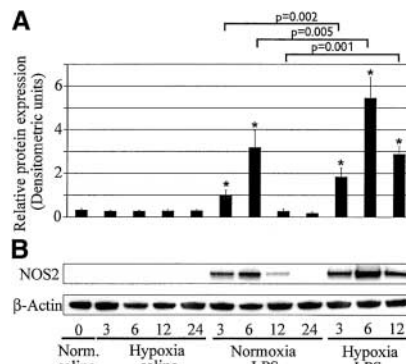

Figure 4. Western immunoblot of lung NOS2 expression. (*A*) Densitometric analysis of NOS2 protein expression with various treatments and times of exposure is shown as the ratio between NOS2 and β-actin optical densities at each point. Because no detectable bands were found in the control (normoxia plus saline) group, an arbitrary density of one was assigned to the band corresponding to the 3-hour normoxia plus LPS group. Data shown represent means (*columns*) ± SD (*error bars*) (n = 4 for each group at each time point). *p < 0.05 compared with normoxia plus saline group. (*B*) Representative Western immunoblot of lung NOS2 and β-actin protein content of normoxic and hypoxic rats injected with saline or LPS (0.1 mg/kg) for 3, 6, 12, and 24 hours. Hypoxia plus LPS treatment for 24 hours was lethal. NOS2 antibody detected a single immunoreactive band with an estimated molecular mass of 130 kD.

Figure 3. *In situ* hybridization for NOS2 mRNA in rat lungs after 3 hours of treatment. (*A* and *B*) *In situ* hybridization performed with the antisense riboprobe in normoxia plus saline-treated rat lungs. No detectable NOS2 mRNA is found in the lung parenchyma (*A*) or in blood vessels (*B*). In hypoxia plus LPS-treated lungs, *in situ* hybridization with the antisense riboprobe shows abundant positive cells within the parenchyma (*C*), as well as numerous positive cells within blood vessels, most of which adhered to the endothelium (*D*). Note the absence of labeling in the lung parenchyma (*E*) and in cells related to blood vessels (*F*) when using the sense riboprobe on hypoxia plus LPS-treated lungs. Original magnification: (*A, D, E,* and *F*) ×360; (*B* and *C*) ×180.

animals (Figures 5D–5F). NOS2 immunostaining was greatly increased in lungs of animals treated with LPS and exposed to either normoxia (Figures 5G–5I) or hypoxia (Figures 5J–5L), being more prominent and statistically significant (p < 0.001) in the latter. Immunohistochemical quantification of NOS2 expression by image analysis (Figure 6), corroborates the results from Western blot analyses. Maximal NOS2 protein expression occurred after 6 hours of treatment. The increased NOS2 immunostaining was found in many inflammatory cells adhered to vascular endothelial cells and in cells that appeared to have migrated into the lung parenchyma.

Immunohistochemical staining for NOS2 on semithin sections and serial consecutive sections, observed by electron microscopy (Figure 7), identified NOS2-immunoreactive cells in the microvasculature of the lung predominantly as lymphocytes and neutrophils. Neutrophils were recognized by the multilobulated nucleus and the presence of larger primary granules and specific granules (Figures 7C–7E). Mononucleated cells also showed immunoreactivity for NOS2 (Figures 7F and 7G). Because of the presence of an indented nucleus and relatively condensed chromatin, few primary granules, and a large number of free ribosomes in the cytoplasm, these cells probably correspond to lymphocytes. The size of these cells is that of medium-sized lymphocytes (about 7 μm in diameter). Furthermore, histologic examination of reverse-face hematoxylin- and eosin-stained sections serial to NOS2-immunostained sections (Figures 8A and 8B), showed that NOS2 immunoreactivity in large blood vessels of the lung corresponds to cells with histologic features of monocytes. In the lung parenchyma, a few cells other than leukocytes were also positive for NOS2. By studying serial reverse-face pair slides, one immunostained for NOS2 and the other for specific cytokeratins, these cells were identified as Type II pneumocytes (Figures 8C and 8D). After quantitative evaluation by image analysis, the proportion of Type II cells with NOS2 immunoreactivity was 1.5 ± 0.003 and 1.46 ± 0.008% in normoxia plus LPS and hypoxia plus LPS groups, respectively. The contribution of Type II pneumocytes to total NOS2 staining was also low: 1.6 ± 0.007% in the normoxia plus LPS group, and 0.18 ± 0.001% in the hypoxia plus LPS group.
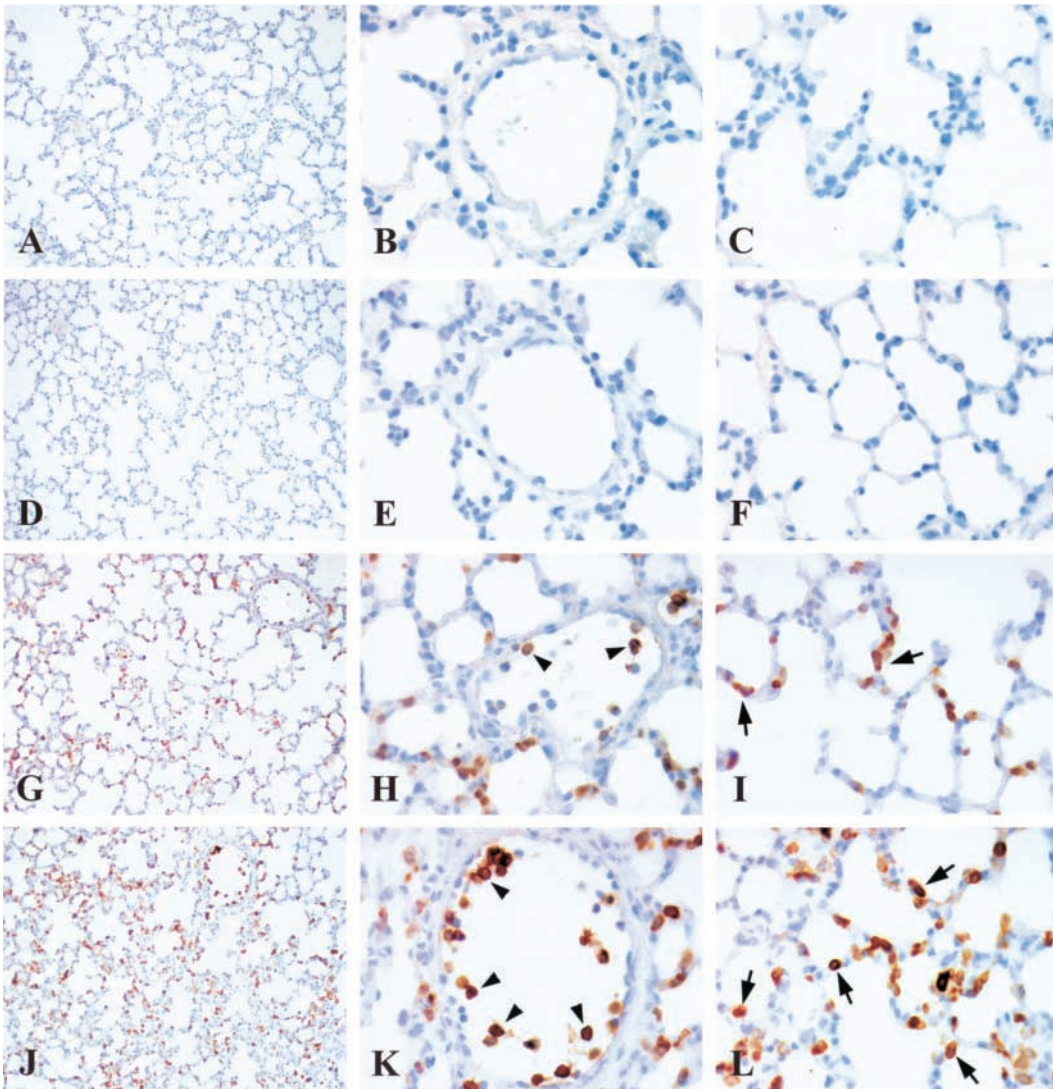
Figure 5. Immunolocalization of NOS2 in lungs of rats injected with saline or LPS (0.1 mg/kg) and exposed to normoxia (20% O_2) or normobaric hypoxia (9% O_2) for 6 hours. In normoxic lungs (*A–C*), no detectable immunostaining for NOS2 is found either in the blood vessels (*B*) or in the parenchyma (*C*). The same results are observed in rat lungs exposed to hypoxia (*D–F*). In LPS-treated lungs, NOS2 immunoreactivity is observed in inflammatory cells mainly adhered to blood vessels (*H, arrowheads*), and in some cells of the lung parenchyma (*I, arrows*). Hypoxia plus LPS treatment (*J–L*) dramatically increases the number of NOS2-immunoreactive cells (as can be observed by comparing *G* and *J*). In the latter group, immunoreactivity is localized to inflammatory cells in blood vessels (*K, arrowheads*), and the lung parenchyma (*L, arrows*). Original magnification: (*A, D, G,* and *J*) ×90; (*B, C, E, F, H, I, K,* and *L*) ×300.

In conclusion, the greater expression of NOS2 protein in lungs of rats treated with LPS and exposed to hypoxia, as compared with LPS-treated rats exposed to normoxia, is due to a greater number of NOS2-immunoreactive inflammatory cells present in the former (Figures 5, 7, and 8). The expression of NOS2 protein in bronchiolar epithelial cells was not affected by any of the treatments.

In Vitro Model

NOS2 mRNA levels in cell lines. Rat lung epithelial cells (CCL-149), RPMVECs, and alveolar macrophages (CRL-2192) were exposed to normoxia or hypoxia for 0.5, 1, 3, 6, 12, or 24 hours and treated concomitantly with or without LPS. Northern blot analysis of NOS2 mRNA levels in these cell lines revealed different responses according to cell type. NOS2 mRNA was not detectable in epithelial or endothelial cells after any of the treatment combinations (data not shown). In the rat alveolar macrophage cell line (Figure 9), NOS2 mRNA was barely observed in cells exposed to normoxia or hypoxia. However, treatment with LPS and exposure to normoxia resulted in the induction of NOS2 gene expression, which was already detected 30 minutes after the onset of treatment and gradually increased up to 12 hours. In comparison with normoxia plus LPS treatment, exposing

LPS-treated cells to hypoxia resulted in significant lower NOS2 mRNA levels (p = 0.004) (Figure 9).

Protein levels. To determine the effects of the four different treatments on the expression of NOS2 protein in the lung cell lines studied, Western blot techniques were applied to protein homogenates. NOS2 protein was not detected in epithelial or endothelial cells after any of the treatments (data not shown). As seen in Figure 10, NOS2 protein levels were higher in rat
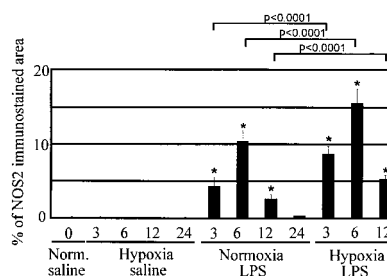

Figure 6. Quantitative evaluation of NOS2 immunohistochemical staining. Staining is depicted as the percentage of stained area versus total tissue area. Data shown represent means (*columns*) ± SD (*error bars*) (n = 4, 5 slides per animal, and 10 fields [×200] per slide were analyzed for each group at each time point).

alveolar macrophages exposed to hypoxia as compared with those exposed to normoxia. In addition, exposing cells to LPS plus normoxia resulted in a strong induction of NOS2 protein expression, which increased gradually, reaching maximal levels at 24 hours of treatment. As compared with LPS alone, the concomitant exposure to LPS plus hypoxia resulted in a somewhat lower induction of NOS2 protein, although no significant differences were found after 12 hours of treatment. Maximal NOS2 protein induction in the alveolar macrophage cell line was delayed in relation to the increase in mRNA expression, as was also previously observed in the *in vivo* model.

DISCUSSION

There are two main findings in this study. First, in an *in vivo* rat model, the injection of LPS caused an increase in the production of NOS2 in the lung, mainly due to an influx of NOS2-producing leukocytes and not because of the upregulation of NOS2 expression in lung resident cells. Second, when treatment

Figure 7. (*A* and *B*) Semithin section of rat lung exposed to hypoxia plus LPS for 6 hours, immunostained for NOS2, and (*C–G*) serial consecutive ultrathin section of the boxed area in (*A*). (*A*) Panoramic semithin section in which numerous NOS2-positive cells are observed. (*B*) Detail of the boxed area in (*A*). Identity of NOS2-immunostained and ultrastructurally observed cells is shown by the *arrow* (*B* and *C*) and the *arrowhead* (*B* and *F*). Note that both cells are localized within blood vessels (*C* and *F*) (*e* = endothelial cell). (*D* and *E*) Higher magnification of the cell shown by the *arrow* in (*B*) and (*C*). This cell presents characteristic features of a neutrophil, such as the multilobulated nucleus (*D*) and the presence of larger primary granules (*arrow* in *E*) and specific (secondary) granules (*arrowhead* in *E*). (*G*) Ultrastructural detail of the cell shown by the *arrowhead* in (*B*) and (*F*), probably corresponding to a lymphocyte. The cytoplasm is occupied by a multitude of free polyribosomes and a few profiles of granular endoplasmic reticulum. A small amount of primary granules is also observed (*triangles*). Original magnification: (*A*) ×190; (*B*) ×380; (*C* and *F*) ×2400; (*D*) ×11300; (*E*) ×22600; (*G*) ×9000.

with LPS was combined with exposure to acute normobaric hypoxia, there was an even greater migration of NOS2-expressing leukocytes, which further increased the expression of NOS2 mRNA and protein levels in whole lung tissue; furthermore, no induction or upregulation of NOS2 expression in any lung resident cell type occurred.

Acute lung injury is defined as acute and persistent lung inflammation, and is characterized clinically by hypoxemia, pulmonary edema secondary to nonhydrostatic capillary permeability, and diffuse pulmonary infiltrates on chest radiographs (31). Acute respiratory distress syndrome (ARDS) refers to the most severe end of the spectrum of ALI. The pathogenic mechanisms of this disease are not well understood, but the role of reactive oxygen species and ·NO has gained increasing attention. Previous studies show that NOS2 expression and ·NO end products are increased in lungs of patients with ARDS (25, 32). Whether ·NO has a beneficial role or participates in the mechanisms of injury is still debated (24). Several investigations suggest that NOS2 plays a harmful role in ALI, because released ·NO causes tissue damage through reactions with reactive oxygen species, such as superoxide, resulting in the formation of peroxynitrite (33, 34). Kristof and colleagues showed that NOS2 knockout mice were more resistant to LPS-induced lung injury than were wild-type mice (23). Moreover, treatment with NOS2 inhibitors seems to prevent ALI in sheep after burn and smoke inhalation injury (35, 36). On the other hand, there are reports suggesting a beneficial role of ·NO in ALI based on the following: (*1*) it downregulates the expression of adhesion molecules, resulting in a reduction in the migration and accumulation of neutrophils (37); (*2*) it inhibits cytokine production (38); and (*3*) in a clinical trial, as had been previously shown for some animal models (39), the use of NOS inhibitors in patients with sepsis resulted in an increase in mortality (40). As suggested by Yang and colleagues (41), the detrimental or beneficial role of ·NO in inflammatory diseases might depend on the oxidant–antioxidant balance and the severity of oxidative stress. Most investigations do agree that in ALI there is an increased expression of NOS2 in the lungs. However, most studies have used techniques such as Northern blot and Western blot analyses of whole lung tissue, which are not capable of determining the cell types responsible for the NOS2 overexpression. The novelty of the work presented herein is the use of parallel molecular and *in situ* techniques (*in situ* hybridization and immunohistochemistry), which have led to the identification of systemic inflammatory cells as the source of the increased NOS2 levels in the lungs of normoxia plus LPS-treated rats and hypoxia plus LPS-treated rats. Besides, immunohistochemical and electron microscopy techniques show that the increased NOS2 expression in the lungs is due to the presence of monocytes in large blood vessels and neutrophils and lymphocytes in the microvasculature. In addition, no upregulation of NOS2 expression in pulmonary resident cells occurs. Our experiments with *in vitro* cell models confirm that in response to LPS or hypoxia plus LPS, pulmonary epithelial and microvascular endothelial cells do not express detectable NOS2, whereas in a macrophage cell line the induction of the enzyme is evident under both experimental conditions.

LPS administration was chosen for the present study because of the widely used animal model of LPS-induced ALI (42, 43) and the well known mechanisms by which LPS induces NOS2 and causes inflammation (44–47). In comparison with other LPS-induced ARDS models, lower doses of LPS were chosen in our study (0.1 mg/kg) to allow the combination of LPS and hypoxia. Injection of LPS in experimental animals elicits a potent infiltration of neutrophils and lymphocytes in the lung, beginning in the induction phase (up to 4 hours after LPS injection) and maintained in the acute phase of the process (from 6 to 12 hours)
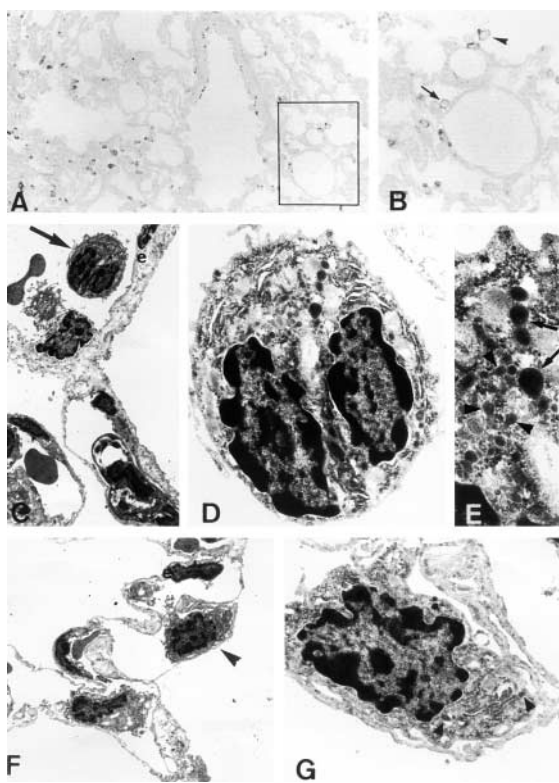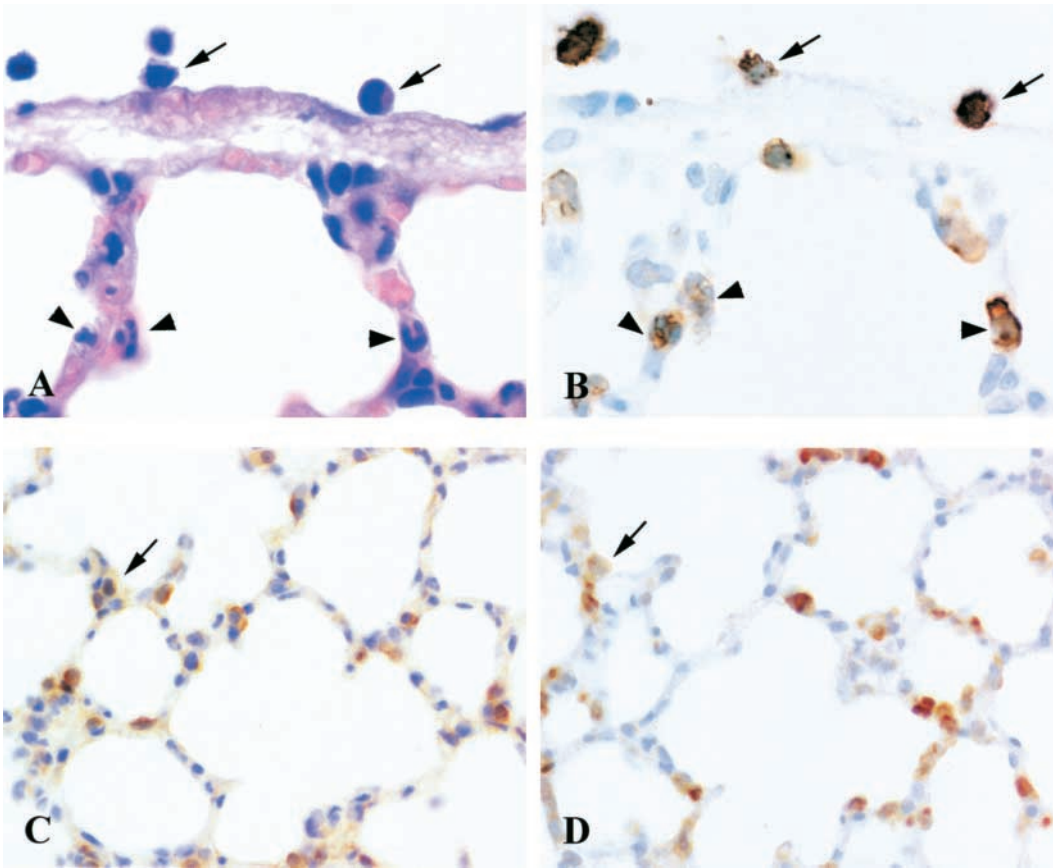
Figure 8. Serial reverse-face sections of rat lungs treated with hypoxia plus LPS for 6 hours, and stained with hematoxylin and eosin and for NOS2 (*A* and *B*), and for cytokeratins (MNF116 antibody) and NOS2 (*C* and *D*). Note that NOS2-positive cells shown by *arrows* in (*B*) present histologic features of monocytes in (*A*). NOS2-immunoreactive cells, shown by *arrowheads* in (*B*), present histologic characteristics of neutrophils in (*A*). (*C*) Panoramic view of immunostaining with MNF116 (used as a marker of Type II pneumocytes in the lung parenchyma). (*D*) Serial reverse section to (*C*) immunostained for NOS2. Note that few Type II pneumocytes are immunoreactive for NOS2 (*arrow* in *C* and *D*). Original magnification: (*A* and *B*) ×1200; (*C* and *D*) ×500.

(9, 48, 49). In the recovery phase (12 to 48 hours), the number of neutrophils decreases, being substituted by monocytes and macrophages. This may explain why in this experimental model NOS2-immunoreactive monocytes are found in large blood vessels, whereas neutrophils and lymphocytes are found in the microvasculature. A similar pattern of inflammatory cell response was found in response to LPS instilled intratracheally (50). Previous studies have also reported an increase in the number of NOS2-expressing inflammatory cells in the lung parenchyma (8, 51). Using immunohistochemical techniques, Buttery and colleagues suggest that the induction of NOS2 after the injection of LPS in rodents occurs predominantly in macrophages present in many organs, including the lungs (8). By reciprocal bone marrow transplantation between $NOS2^{+/+}$ and $NOS2^{-/-}$ mice, and determining NOS2 activity in the lung, Wang and colleagues suggest that both parenchymal and inflammatory cells contribute
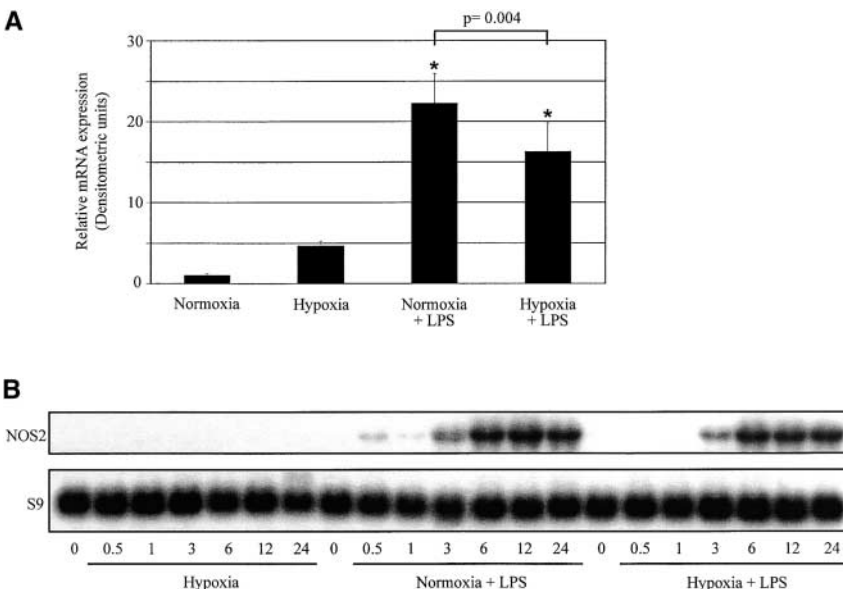

Figure 9. Northern blot analysis of NOS2 mRNA expression in the CRL-2192 macrophage cell line. (*A*) Optical density of NOS2 mRNA after 12 hours of treatment, expressed as the ratio of NOS2 and S9 mRNA. To standardize between experiments, an arbitrary density of one was assigned to the band obtained from cells exposed to normoxia (control group; zero time point). Values are expressed as means (*columns*) $\pm$ SD (*error bars*) (n = 3 for each group). *$p < 0.05$ compared with control group. (*B*) Northern blot analysis of a representative experiment using the rat alveolar macrophage cell line CRL-2192 cultured under normoxic (20% O_2) or hypoxic (1% O_2) conditions and treated with or without LPS (100 ng/ml) for the indicated times (in hours). Fifteen micrograms of total RNA were loaded per lane.
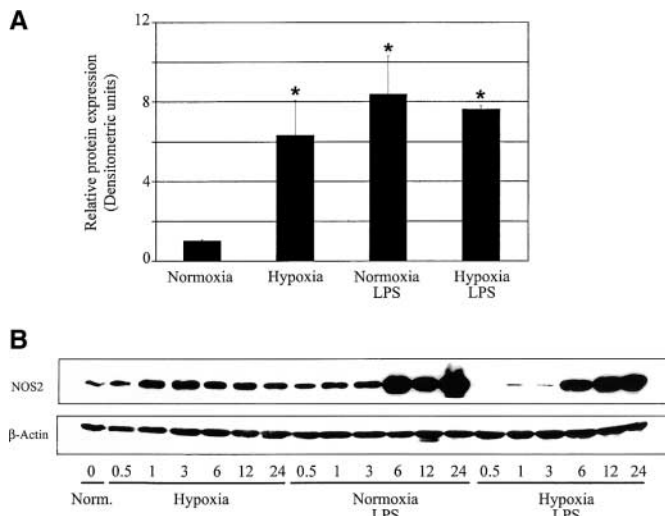
Figure 10. Western blot analysis of NOS2 in rat alveolar macrophage cell line CRL-2192. (*A*) Relative NOS2 protein levels after 12 hours of treatment, determined by normalization to the correspondent β-actin optical density, are shown as means (*columns*) ± SD (*error bars*) (n = 3 for each group). *p < 0.05 compared with control group. There is no significant difference between expression within hypoxia, LPS, and hypoxia plus LPS treatments. To standardize between experiments, an arbitrary density of one was assigned to the band obtained from cells exposed to normoxia. (*B*) Representative Western blot analysis of NOS2 and β-actin protein content of rat alveolar macrophage cell line CRL-2192, exposed either to normoxia or hypoxia and treated with or without LPS (100 ng/ml) for the indicated times.

to the increased NOS2 production in lungs after the injection of LPS (52). In a subsequent publication, the same group further defines that the injury leading to microvascular leakage of proteins is caused by NOS2 produced by bone marrow–derived inflammatory cells (51). In the study presented herein, immunohistochemical and electron microscopy techniques show that the cells responsible for the increased production of NOS2 after LPS insult in lung tissue are monocytes, neutrophils, and lymphocytes. As previously reported (53–55), a few Type II pneumocytes were also found to be immunoreactive for NOS2 after LPS insult, although no clear upregulation of the enzyme was observed in this cell type. Our *in vitro* experiments also failed to show induction of NOS2 in alveolar epithelial cells and RPMVECs in response to LPS. Other investigations have shown induction of NOS2 in RPMVECs, although in response to tumor necrosis factor-α and interleukin-1β (56). This may suggest that stimuli other than LPS are needed for NOS2 induction in this cell line. As also reported for other macrophage cell lines (57), the CRL-2192 cell line showed a high upregulation of NOS2 mRNA and protein after LPS administration. This induction was higher than that obtained with the hypoxia or hypoxia plus LPS treatments.

Hypoxemia and local tissue hypoxia are important pathophysiologic consequences of ALI. A number of investigations have reported that hypoxia transcriptionally upregulates NOS2 expression through hypoxia-inducible factor-1, which binds a hypoxia response element in the promoter of the gene (58). However, data from studies of NOS2 expression in animal models of hypoxia are controversial. There are reports of increased (11, 58–60) and maintained (61–63) NOS2 lung expression in response to low oxygen concentrations. In our rat model, NOS2 expression in the lung was not altered by exposure to acute hypoxia alone (Figures 2, 4, and 5), which is consistent with the

observation by Gess and colleagues (61). A number of groups have studied *in vitro* models to assess the effect of hypoxia on NOS2 production, also finding conflicting results in different cell types. Whereas in bovine pulmonary artery endothelial cells hypoxia induces NOS2 promoter activity (58), results presented herein and by others (56) show that hypoxia has no effect on RPMVECs. These differences could be explained by the phenotypic heterogeneity of endothelial cells obtained from different pulmonary blood vessels or from different species (64–66). In macrophages, prior studies have shown that hypoxia alone does not induce NOS2 expression, and that reoxygenation (67, 68), or the combination of hypoxia with other stimuli, such as LPS or IFN-γ (69), is required for its induction. Our results show that acute hypoxia alone is not capable of inducing NOS2 mRNA expression in a cultured rat lung macrophage cell line (Figure 9); however, at the protein level, an increase is observed after 12 hours of treatment (Figure 10). Yu and colleagues reported that hypoxia-inducible factor-1α protein is upregulated in several cell types of the lung (pulmonary arterial endothelial cells, smooth muscle and bronchial epithelial cells, alveolar macrophages, and RPMVECs) and in ferret lungs exposed to acute hypoxia, and that this upregulation is dependent on the concentration of oxygen and the duration of the exposure (70). These investigators could not detect hypoxia-inducible factor-1α protein in lungs ventilated with 7% O_2. This could explain the lack of NOS2 induction in our lung model, in which rats were exposed to 9% O_2.

In our opinion, the main achievement of this work is the analysis of the concomitant effect of two of the main factors involved in ALI (i.e., LPS and hypoxia), both *in vivo* and *in vitro*. Although LPS treatment itself is thought to induce hypoxemia (via lung injury), the administration of LPS alone does not completely mimic the pulmonary effects of endotoxin-associated ALI, as noted by a National Heart, Lung, and Blood Institute working group on acute lung injury (71). In fact, this working group suggests that two-hit models might better reflect actual conditions present in patients with ALI (71). In this sense, the concomitant exposure to hypoxia and LPS has allowed us to more effectively compare the relative importance of both factors and their synergistic effect in NOS2 induction. To expose animals to hypoxia and LPS simultaneously without causing their death, lower doses of LPS as compared to other LPS-based ALI models were used. As seen in Figure 1, the combination of hypoxia and LPS in our experimental conditions elicited a modest but statistically significant increase in lung wet-to-dry ratio after 6 hours of treatment, which suggests that our model may represent the early stages of ALI. With this combination, hypoxia did cause an increase in the LPS-dependent expression of NOS2 mRNA and protein in whole lungs as observed by Northern and Western blot analyses (Figures 2 and 4). Again, by the parallel use of molecular and *in situ* techniques (NOS2 mRNA hybridization and immunohistochemistry), this increase in NOS2 expression was observed to be due to an even greater influx of leukocytes into the lung parenchyma (Figures 3 and 5). Finally, by immunocytochemical and morphologic analyses in serial sections, these NOS2-expressing inflammatory cells were identified as monocytes, neutrophils, and lymphocytes (Figures 7 and 8). Studies report that neutrophils adhering to the lung vasculature after LPS injection are activated cells in which apoptosis is decreased (72). In endothelial cells, the combination of hypoxic stress and LPS or cytokines increases the expression of specific adhesion molecules such as intercellular adhesion molecule-1 (73, 74) and E-selectin (75). In addition, several studies have demonstrated that hypoxia increases leukocyte adhesion to endothelial cells through the induction of integrins on the surface of leukocytes (76, 77). Moreover, high bronchoalveolar lavage levels of interleukin-6 have been found in patients with ARDS (78), and interleukin-6 treatment in rabbits

results in the release of less deformable polymorphonuclear leukocytes, which are preferentially sequestered in lung microvessels (79). Therefore, because of the interaction of adhesion molecules in leukocytes and endothelial cells, we suggest that the velocity of leukocyte rolling might be diminished under hypoxic conditions, favoring the permanence of the inflammatory cells in the lung. It is possible that by concomitant treatment with LPS and hypoxia, not only is the recruitment of LPS-induced NOS2-producing inflammatory cells enhanced, but the time spent by these cells in the pulmonary vasculature is prolonged, thus resulting in enhanced expression of NOS2 in whole lung tissue as compared with LPS administration alone. In fact, the synergistic effect of hypoxia and LPS on the influx of NOS2-producing inflammatory cells to the lung might have been underestimated in the pathogenesis of ARDS. Furthermore, the severity of this syndrome might be mediated by the inflammatory products released by leukocytes, rather than by the intrinsic toxicity of ·NO, as has already been suggested in various studies (25, 34).

As shown in the *in vitro* experiments, in no individual cell lines used in this study did the concomitant exposure to hypoxia plus LPS result in an increase in NOS2 production as compared with LPS treatment alone. If anything, in the macrophage cell line (CRL-2192) hypoxia attenuated the LPS-dependent increase in NOS2 mRNA (Figure 9). Others have also shown in RPMVECs that hypoxia attenuates LPS-dependent NOS2 upregulation (56). This further suggests that the increased expression of NOS2 in whole lung tissue after treatment with LPS and hypoxia is due to the recruitment of a greater number of cells that express NOS2 (monocytes, neutrophils, and lymphocytes), and not because of upregulated expression in pulmonary resident cells.

In conclusion, in a model of ALI that combines treatment with LPS and exposure to hypoxia, NOS2 expression is increased in the lung parenchyma due to an increase in the recruitment of leukocytes expressing NOS2. Exposure to hypoxia enhances the LPS-dependent influx of NOS2-expressing inflammatory cells. Neither LPS nor hypoxia, alone or in combination, seems to upregulate NOS2 expression in lung resident cells. *In vitro* studies using epithelial, endothelial, and macrophage rat pulmonary cell lines further corroborate these results.

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