



Published in final edited form as:

Am J Physiol Lung Cell Mol Physiol. 2002 April ; 282(4): L872–L880. doi:10.1152/ajplung.00217.2001.

Activation of type II alveolar epithelial cells during acute endotoxemia

VASANTHI R. SUNIL, AGNIESZKA J. CONNOR, YAN GUO, JEFFREY D. LASKIN, and DEBRA L. LASKIN

Department of Toxicology and Pharmacology, Rutgers University and Environmental and Community Medicine, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey 08854-8020

Abstract

Lung injury induced by acute endotoxemia is associated with increased generation of inflammatory mediators such as nitric oxide and eicosanoids, which have been implicated in the pathophysiological process. Although production of these mediators by alveolar macrophages (AM) has been characterized, the response of type II cells is unknown and was assessed in the present studies. Acute endotoxemia caused a rapid (within 1 h) and prolonged (up to 48 h) induction of nitric oxide synthase-2 (NOS-2) in type II cells but a delayed response in AM (12–24 h). In both cell types, this was associated with increased nitric oxide production. Although type II cells, and to a lesser extent AM, constitutively expressed cyclooxygenase-2, acute endotoxemia did not alter this activity. Endotoxin administration had no effect on mitogen-activated protein kinase or protein kinase B- α (PKB- α) expression. However, increases in phosphoinositide 3-kinase and phospho-PKB- α were observed in type II cells. The finding that this was delayed for 12–24 h suggests that these proteins do not play a significant role in the regulation of NOS-2 in this model. After endotoxin administration to rats, a rapid (within 1–2 h) activation of nuclear factor- κ B was observed. This response was transient in type II cells but was sustained in AM. Interferon regulatory factor-1 (IRF-1) was also activated rapidly in type II cells. In contrast, IRF-1 activation was delayed in AM. These data demonstrate that type II cells, like AM, are highly responsive during acute endotoxemia and may contribute to pulmonary inflammation.

Keywords

type II cells; alveolar macrophages; nitric oxide synthase-2; cyclooxygenase-2; nuclear factor- κ B; interferon regulatory factor-1

Exposure of humans and animals to excessive amounts of bacterially derived endotoxin is known to elicit an inflammatory reaction and can cause severe damage to various organs, including the lung and liver (14). Alveolar macrophages are known to play a central role in initiating and regulating endotoxin-induced inflammatory responses in the lung. This is

accomplished through the release of a variety of mediators, including cytokines, reactive oxygen intermediates, reactive nitrogen intermediates, and eicosanoids by these cells (28, 49). Type II alveolar epithelial cells are important in maintaining the functional and structural integrity of the alveolus. These cells not only synthesize and secrete surfactant but also act as progenitors for injured type I epithelial cells (1, 19). A number of studies have suggested that type II cells also have the capacity to participate in inflammatory processes. Thus, in response to cytokines, type II cells, like alveolar macrophages, release tumor necrosis factor- α , interleukin-8, monocyte chemotactic protein, macrophage inflammatory protein-2, nitric oxide, hydrogen peroxide, and prostaglandins (13, 23, 31, 32, 39, 40, 43, 56, 57). The functional and biochemical responses of type II cells during acute endotoxemia are largely unknown, and an analysis of their activity when compared with alveolar macrophages represents the focus of the present studies. We found that administration of endotoxin to rats resulted in induction of nitric oxide synthase-2 (NOS-2) protein and increased nitric oxide production in both cell types. Nuclear factor- κ B (NF- κ B) and interferon regulatory factor-1 (IRF-1), two key transcription factors known to activate genes that generate inflammatory mediators, were also induced in type II cells and alveolar macrophages by endotoxin; however, the kinetics of their responses were different. The results of our studies provide support for the concept that type II cells, like alveolar macrophages, participate in pulmonary inflammatory responses to irritants although their actions may be distinct.

MATERIALS AND METHODS

Animals and treatment

Female specific pathogen-free Sprague-Dawley rats (200–225 g, 6–8 wk) were purchased from Taconic (Germantown, NY). Animals were housed in microisolator cages and maintained on sterile food and pyrogen-free water ad libitum. Acute endotoxemia was induced by intravenous injection of rats with 5 mg/kg *Escherichia coli* lipopolysaccharide (LPS, serotype 0128:B12; Sigma Chemical, St. Louis, MO).

Cell isolation

Rats were killed by intraperitoneal injection of Nembutal (125 mg/kg). The lungs were perfused with 50 ml of warm (37°C) Ca²⁺/Mg²⁺-free Hanks' balanced salt solution (HBSS) (pH 7.4) containing 2.5 M HEPES, 0.5 M EGTA, and 4.4 M NaHCO₃ at a rate of 22 ml/min followed by perfusion with 50 ml HBSS without EGTA. Lungs were then lavaged five to six times with HBSS to collect alveolar macrophages. Cells were washed three to four times with HBSS containing 2% FCS. Cell viability was 98%, as determined by trypan blue dye exclusion, and cell purity was >97%, as determined morphologically after Giemsa staining.

Type II cells were isolated from lavaged lungs as previously described (17, 40). After being washed two times with 10 ml of buffer (in mM: 140 NaCl, 5 KCl, 2.5 Na₂HPO₄, 10 HEPES, 1.3 MgSO₄·7H₂O, pH 7.4, and 2 CaCl₂), 30 ml of elastase (4.2 U/ml; Worthington Biochemicals) were infused in the lungs using a 60-ml syringe. The tissue was then incubated at 37°C for 20 min, the trachea and major bronchi were removed, and the lungs were minced in the presence of 4 ml DNase (1 μ g/ml) and then digested for 5 min at 37°C

with 10 ml of elastase. The reaction was stopped by the addition of 5 ml FCS. The tissue was then sequentially filtered through 220, 60, 30, and 15 μm nylon mesh. Cells were collected, washed, and purified by negative selection (1 h, 37°C) on IgG-coated plates. Nonadherent cells were collected and washed with DMEM containing 10% FCS. Cell purity, assessed by modified Papinicolou staining, was 95%, and viability, determined by trypan blue dye exclusion, was >98%.

Measurement of nitric oxide production

Alveolar macrophages (2×10^5 cells/well) and type II cells (3×10^5 cells/well) were inoculated in 96-well dishes in phenol red-free DMEM containing 10% FCS and interferon- γ (IFN- γ ; 10 U/ml), LPS (10 ng/ml), IFN- γ plus LPS, or medium control with and without 0.5–5 μM pyrrolidine dithiocarbamate (PDTC). Nitric oxide was quantified 24 h later by nitrite accumulation in the culture medium using a procedure based on the Greiss reaction with sodium nitrite as the standard (22). For nitrate determinations, samples were treated with nitrate reductase and NADPH for 2 h before analysis using a nitrate/nitrite colorimetric assay kit (Cayman Chemical, Ann Arbor, MI). We found that, in medium from cells treated for 24 h with LPS and IFN- γ , the ratio of nitrate to nitrite was 2:1 for alveolar macrophages and 2.5:1 for type II cells, and this ratio did not change after endotoxin administration.

Preparation of cytoplasmic and nuclear extracts

Cytoplasmic extracts for Western blotting and nuclear extracts for the electrophoretic mobility shift assays (EMSA) were prepared as previously described (10). Briefly, either freshly isolated cells or cells cultured in six-well plates (2×10^6 cells/well) for 24 h in the presence or absence of LPS (10 ng/ml) plus IFN- γ (10 U/ml) with and without PDTC (1.0 or 2.5 μM) were lysed in buffer (in mM: 10 HEPES, pH 7.4, 10 KCl, 2 MgCl_2 , and 2 EDTA) on ice for 10 min with intermittent mixing. Nonidet P-40 was added to give a final concentration of 10%. After 5 min on ice, the cells were centrifuged (4°C, 16,000 g, 5 min), and supernatants containing cytoplasmic extracts were collected. To prepare nuclear extracts, pellets were resuspended in extraction buffer (50 mM HEPES, pH 7.4, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, and 10% glycerol) and incubated on ice for 20 min with periodic mixing. The cells were then centrifuged (4°C, 16,000 g, 5 min), and supernatants containing nuclear extracts were collected. Aliquots of cytoplasmic and nuclear extracts were frozen at -70°C until analysis. Protein determinations were performed using the BCA Protein Assay kit (Pierce, Rockford, IL) with BSA as the standard.

Western blot analysis

Cytoplasmic proteins were fractionated on SDS-PAGE (7.5–15%). The proteins were then transferred to nitrocellulose paper. Nonspecific binding was blocked by incubation of the blot for 30 min at room temperature with 5% nonfat dry milk in enhanced chemiluminescence (ECL) buffer (Amersham Life Sciences, Arlington Heights, IL). This was followed by incubation for 16–24 h at 4°C with primary antibody (Transduction Laboratories, San Diego, CA, or New England Biolabs, Beverly, MA). After extensive washing with ECL buffer, the blot was incubated with a 1:4,000 dilution of horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Proteins were

detected using an ECL detection system. The dilutions of primary antibodies and amounts of protein analyzed per lane were as follows: p38 mitogen-activated protein kinase (MAPK) and p44/42 MAPK, 1:500, 5 µg; phospho-protein kinase B (PKB)-α, 1:500, 8 µg; cyclooxygenase-2 (Cox-2) and phosphoinositide 3-kinase (PI 3-kinase, p85 subunit), 1:250, 10 µg; PKB-α, 1:200, 20 µg; phospho-p38 MAPK, 1:100, 10 µg; NOS-2, 1:100, 20 µg; and phospho-p44/42 MAPK, 1:50, 20µg.

EMSA

EMSA were performed as described previously (10) with some modifications. Binding reactions were carried out at room temperature for 30 min in a total volume of 15 µl containing 2–5 µg of nuclear extracts, 5 µl of 5× gel shift binding buffer (20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, and 50 mM Tris·HCl, pH 7.5), 2 µg poly(dI-dC) and 3 × 10⁴ counts·min⁻¹·µl⁻¹ [γ -³²P]ATP (3,000 Ci/mmol at 10 mCi/ml)-labeled NF-κB (5'-AGT TGA GGG GAC TTT CCC AGG C-3'; Promega Gel Shift Assay Systems, Madison, WI), or IRF-1 (5'-GGA AGC GAA AAT GAA ATT GAC T-3'; Santa Cruz Biotechnology, Santa Cruz, CA) consensus oligonucleotides. Protein-DNA complexes were separated on 5 or 7% nondenaturing polyacrylamide gels run at 250 V in 0.5× TBE (45 mM Tris-borate and 1 mM EDTA, pH 8.0), visualized after the gels were dried, and autoradiographed. For supershift reactions, 1 µg of antibodies to NF-κB subunits (p50 and p65) or IRF-1 (Santa Cruz Biotechnology) was added to the reaction mixtures and incubated on ice for 15 min before the addition of labeled oligonucleotide. For competitor reactions, 40-fold excess of the respective unlabeled oligonucleotide was added to the mixture before the addition of the labeled probe.

RESULTS

Effects of acute endotoxemia on NOS-2 expression and nitric oxide production

In initial studies, we analyzed the effects of endotoxin treatment of rats on expression of NOS-2, the enzyme mediating the generation of nitric oxide in type II cells and alveolar macrophages (31, 33, 38, 40). NOS-2 protein was not detectable in freshly isolated type II cells or alveolar macrophages from control animals (Fig. 1). Endotoxin administration resulted in a time-dependent induction of NOS-2 in both cell types. However, the kinetics of their responses were distinct. Thus, although in type II cells NOS-2 protein was expressed as early as 1 h after endotoxin administration and persisted for at least 48 h, in alveolar macrophages this response was delayed. Moreover, in these cells NOS-2 protein declined to control levels after 24 h (Fig. 1).

We also analyzed the effects of acute endotoxemia on nitric oxide production by cultured cells. In the absence of stimulation, type II cells and alveolar macrophages from control animals produced negligible quantities of nitric oxide. Although IFN-γ either alone or in combination with LPS caused a small increase in nitric oxide production by type II cells, LPS by itself had no major effect on this response (Fig. 2). In contrast, although alveolar macrophages from control animals were unresponsive to IFN-γ or LPS alone, the combination of these mediators was synergistic in inducing nitric oxide production (Fig. 2). Alveolar macrophages were found to produce two to three times more nitric oxide than type

II cells. Induction of acute endotoxemia resulted in a significant increase in nitric oxide production by both cell types. This was evident in unstimulated and IFN- γ - and/or LPS-treated cells and was maximum 24 h after endotoxin administration in type II cells and 12 h posttreatment in alveolar macrophages (Fig. 2). Cells from endotoxin-treated rats were significantly more sensitive to IFN- γ and/or LPS than were cells from control animals.

Production of nitric oxide by both type II cells and alveolar macrophages in response to IFN- γ and LPS was correlated with an increase in NOS-2 protein expression (Fig. 3). As observed with nitric oxide production, treatment of rats with endotoxin resulted in an increase in the sensitivity of the cells to IFN- γ and LPS in terms of NOS-2 induction. Type II cells from endotoxin-treated rats were more responsive to these mediators than alveolar macrophages (Fig. 3).

Effects of acute endotoxemia on Cox-2 expression

In our next series of studies, we determined if type II cells and alveolar macrophages were activated after endotoxin administration to express increased amounts of Cox-2, the enzyme mediating inducible prostaglandin biosynthesis (36, 47). Freshly isolated type II cells from control animals were found to constitutively express Cox-2 protein (Fig. 1). Two protein bands were detected in the blots, with the lower band most likely reflecting a Cox-2 breakdown product. Although similar results were observed in alveolar macrophages, significantly greater quantities of Cox-2 protein were identified in type II cells. In contrast to its effects on NOS-2, induction of acute endotoxemia had no major effects on Cox-2 expression in either cell type (Fig. 1).

Biochemical activation of type II cells and alveolar macrophages during acute endotoxemia

In further studies, we determined if acute endotoxemia was associated with biochemical activation of the cells. Initially, we analyzed expression of p38 and p44/42 MAPK, which are thought to be involved in the regulation of both NOS-2 and Cox-2 (8, 11, 20). Freshly isolated type II cells from control animals were found to constitutively express both total and phosphorylated p38 and p44/42 MAPK (Fig. 4). Endotoxin administration had no major effect on expression of these proteins. Similarly, freshly isolated alveolar macrophages from control animals were found to constitutively express total p38 and p44/42 MAPK, as well as phospho-p38 MAPK but not phospho-p44/42 MAPK. Moreover, endotoxin administration had no effect on expression of MAPK proteins in alveolar macrophages (Fig. 4).

PI 3-kinase and its downstream target PKB- α have been implicated in functional activation of epithelial cells and macrophages (15, 24, 52, 55), and expression of these proteins was analyzed next. Both type II cells and alveolar macrophages from control animals were found to constitutively express PI 3-kinase and PKB- α (Fig. 4). Induction of acute endotoxemia resulted in a time-related increase in expression of PI 3-kinase in both cell types, which was evident 12–48 h after treatment of the animals. In contrast, endotoxin administration to the animals did not significantly alter levels of PKB- α in the cells (Fig. 4). In general, type II cells expressed greater quantities of both PI 3-kinase and PKB- α compared with alveolar macrophages. Type II cells, but not alveolar macrophages from control animals, also

constitutively expressed activated phospho-PKB- α (Fig. 4). Although endotoxin administration to the rats resulted in increased phospho-PKB- α expression in type II cells, there was no effect in alveolar macrophages.

Effects of acute endotoxemia on NF- κ B and IRF-1 nuclear binding activity

The transcription factors NF- κ B and IRF-1 are known to regulate the expression of numerous inflammatory genes, including NOS-2 and Cox-2 (6, 9, 20). We next determined if acute endotoxemia was associated with altered nuclear binding activity of these transcription factors. NF- κ B nuclear binding activity was not detectable in freshly isolated type II cells from control rats (Fig. 5, *left*). Treatment of the animals with endotoxin resulted in a rapid (within 1 h) and transient increase in NF- κ B binding activity. Two NF- κ B complexes (I and II) were detected in the cells. Antibodies to p50 and p65 were found to alter the migration of these complexes (Fig. 5, *middle*). Binding was eliminated by an excess of unlabeled NF- κ B, demonstrating the specificity of the probe. As observed with NF- κ B, IRF-1 binding activity was not detected in freshly isolated type II cells from control animals (Fig. 6, *left*). Treatment of animals with endotoxin resulted in a rapid (within 2 h) and prolonged induction of IRF-1 nuclear binding activity in these cells. Binding was decreased in the presence of anti-IRF-1 antibody and excess unlabeled IRF-1 (Fig. 6, *middle*).

The effects of acute endotoxemia on NF- κ B and IRF-1 nuclear binding activity in alveolar macrophages were also analyzed. Freshly isolated alveolar macrophages from control animals exhibited low levels of NF- κ B and IRF-1 nuclear binding activity (Figs. 5, *right*, and 6, *right*). Endotoxin administration to the animals resulted in a rapid (within 1 h) and prolonged activation of NF- κ B. In contrast, IRF-1 activation in alveolar macrophages was not observed until 24 h posttreatment. As observed in type II cells, antibodies to both p50 and p65 were found to alter the migration of both NF- κ B complexes (Fig. 5, *middle*). Moreover, binding was decreased in the presence of excess unlabeled competitor NF- κ B probe. Similarly, binding of IRF-1 was decreased in the presence of anti-IRF-1 antibody and excess unlabeled IRF-1 (Fig. 6, *middle*).

Effects of inhibiting NF- κ B on NOS-2 expression and nitric oxide production

To analyze the potential role of NF- κ B in NOS-2 expression and nitric oxide production, we used PDTC, which has been reported to block NF- κ B nuclear binding activity (7, 51). PDTC was found to cause a dose-related inhibition of NOS-2 protein expression in the cells, with maximum effects at 2.5 μ M (Fig. 3). Type II cells and alveolar macrophages from endotoxin-treated rats were less sensitive to the inhibitory effects of PDTC on NOS-2 protein expression than were cells from control animals. We also found that PDTC treatment caused a dose-dependent inhibition of nitric oxide production by both type II cells and alveolar macrophages, reaching a maximum with 2.5 μ M (Fig. 7). At this concentration, PDTC had no effect on cell viability (data not shown). Alveolar macrophages from endotoxin-treated rats were less sensitive to the inhibitory effects of PDTC on nitric oxide production than were cells from control animals. To exclude the possibility that PDTC was acting as a nitrite scavenger (44), we quantified nitrite levels in a standard solution containing increasing concentrations of sodium nitrite (5–35 μ M) in the presence and absence of 5 μ M PDTC. We found that PDTC had no effect on nitrite levels in the solution

(data not shown). Moreover, EMSA showed that PDTC was effective in blocking NF- κ B nuclear binding activity in type II cells and alveolar macrophages (data not shown).

DISCUSSION

The role of type II cells in inflammatory responses of the lung has received relatively limited attention. The fact that these cells release cytokines and reactive oxidants suggests that their participation in these responses may be significant. In this regard, recent studies have demonstrated that type II cells are activated to produce increased quantities of nitric oxide, hydrogen peroxide, and superoxide anion after exposure of animals to pulmonary irritants such as ozone or particulate matter (29, 40, 46). The present studies demonstrate that a number of functional and biochemical activities are upregulated or induced in type II cells after endotoxin administration to rats. In fact, these cells appear to be more responsive to endotoxin than alveolar macrophages. These findings are similar to our studies on interstitial macrophages (53, 54) and suggest that close association with the endothelium increases the sensitivity of cells to intravenously administered endotoxin.

Nitric oxide is a highly reactive molecule produced in type II cells and alveolar macrophages by the NADPH-dependent enzyme NOS-2 (31, 33, 38, 40). We found that acute endotoxemia caused a rapid- and time-dependent induction of NOS-2 in type II cells, suggesting that these cells are highly responsive to in vivo endotoxin. This is supported by our findings that type II cells from endotoxemic animals are sensitized to produce more nitric oxide and express greater amounts of NOS-2 after treatment with IFN- γ plus LPS, compared with cells from control animals. Our data are consistent with previous findings of increased NOS-2 in type II cells after ozone inhalation (40) and support the idea that these cells are an important part of the inflammatory response to pulmonary irritants. The relatively delayed production of nitric oxide in vitro by type II cells after endotoxin administration, compared with alveolar macrophages, suggests that they may need to be activated or primed for mediator production. As described previously (53), alveolar macrophages were also found to produce more nitric oxide in response to inflammatory mediators after endotoxin administration to rats. However, the response of these cells was more rapid compared with type II cells (12 vs. 24 h). This is consistent with a primary role of alveolar macrophages in the pulmonary inflammatory response (21, 26, 58).

Prostaglandins are highly reactive lipid mediators released during inflammatory responses from arachidonic acid via the enzyme Cox-2 (36, 47). We found that Cox-2 was constitutively expressed in type II cells and alveolar macrophages from control rats. This is in accord with previous studies examining rat and mouse lung macrophages (18, 50). These findings suggest that Cox-2 may be important in maintaining homeostatic pulmonary activity. In this regard, Cox-2-dependent pathways have been implicated in the regulation of lung vascular tone, alveolar epithelial permeability, surfactant homeostasis, and lung development (4, 16, 25, 30, 41, 48). Endotoxin administration to the animals had no significant effect on expression of Cox-2 in type II cells or alveolar macrophages. This indicates that Cox-2 expression in these cells does not limit prostaglandin production in the lung during endotoxin-induced inflammation.

MAPK signaling pathways are known to play a central role in the regulation of a number of inflammatory responses, including NOS-2 and Cox-2 expression (8, 11, 20). Our studies revealed that both p38 and p44/42 MAPK were constitutively expressed in type II cells from control animals, which may explain constitutive expression of Cox-2 in these cells. Endotoxin administration had no effect on expression of either total MAPK or phospho-MAPK. These findings are consistent with the lack of effects of endotoxin on Cox-2. It should be noted, however, that it is not known if the MAPK are functionally active in type II cells. Additional studies are required to determine the precise role of MAPK in these cells. Constitutive p38 MAPK and phospho-p38 MAPK were also detected in alveolar macrophages, and, as observed in type II cells, these proteins were unaffected by endotoxin administration. These results, together with the observation that alveolar macrophages did not express phospho-p44/42 MAPK in response to endotoxin, suggest that activation of these proteins may not be required for NOS-2 expression in these lung cells. This idea is supported by recent studies in mouse macrophage and epithelial cell lines demonstrating that activation of p44/42 MAPK is not essential for LPS-induced NOS-2 expression (27).

Constitutive expression of PI 3-kinase, PKB- α , and phospho-PKB- α was also observed in type II cells from control animals. It has been suggested that PI 3-kinase is involved in the regulation of MAPK and Cox-2 (34). Our findings that expression of PI 3-kinase and phospho-PKB- α increased after endotoxin administration to the animals provides additional support for the concept that type II cells are activated during acute endotoxemia. The observation that PI 3-kinase and phospho-PKB- α expression is induced in type II cells during acute endotoxemia is novel. At the present time, the functions of these proteins in type II cell activation are unknown. Although they may be involved in constitutive Cox-2 expression, PI 3-kinase and PKB- α may also contribute to type II cell survival, proliferation, protein synthesis, and/or glucose transport, and this remains to be determined (3, 37, 45). Alveolar macrophages from control animals also constitutively expressed PI 3-kinase and PKB- α , but not phospho-PKB- α . Although endotoxin treatment of the animals induced PI 3-kinase, it had no effect on either PKB- α or phospho-PKB- α expression. This suggests distinct functions for PKB- α in type II cells and alveolar macrophages during endotoxin-induced lung injury.

The promoter regions of both the NOS-2 and Cox-2 genes contain consensus sequences for the transcription factors NF- κ B and IRF-1, which presumably regulate the activity of these genes (6, 9, 20). We found that the time course of induction of NOS-2 in type II cells after endotoxin administration correlated with activation of NF- κ B and IRF-1 nuclear binding activity. Of interest is our finding that NF- κ B activation in type II cells after endotoxin administration was transient, whereas IRF-1 activity was prolonged. This suggests that the combined actions of these transcription factors may be required for prolonged expression of NOS-2 in type II cells during acute endotoxemia. In this regard, recent studies have demonstrated that multiple transcription factors are involved in regulating NOS-2 gene activity (35). The fact that constitutive NF- κ B and IRF-1 were not evident in type II cells indicates that other pathways control Cox-2 and MAPK expression. Our observation that NF- κ B activity is increased after endotoxin administration is in accord with previous studies demonstrating activation of this transcription factor in type II cells and alveolar macrophages after exposure of animals to ozone, particulate matter, or asbestos (29, 42) and

suggests that upregulation of transcription factors may be a general response to pulmonary injury (12).

Alveolar macrophages from control animals exhibited low levels of constitutive NF- κ B binding activity. Endotoxin administration caused a rapid and prolonged activation of NF- κ B in these cells, which is also consistent with increased NOS-2 expression and nitric oxide production. Our findings that blocking NF- κ B with PDTC inhibited nitric oxide production and NOS-2 expression in both type II cells and alveolar macrophages provide additional support for an involvement of this transcription factor in the regulation of NOS-2 in these cells. Our results are consistent with previous reports showing activation of NF- κ B in alveolar macrophages after endotoxin treatment of animals and during sepsis (2, 5). The observation that IRF-1 activation was delayed in alveolar macrophages after endotoxin administration suggests that this transcription factor does not play a major role in induction of NOS-2 in these cells. In alveolar macrophages, constitutive IRF-1 activity was correlated with expression of MAPK, PKB- α , and PI 3-kinase. One can speculate that this transcription factor may regulate signaling pathways mediated by these proteins under homeostatic conditions (6, 20).

The present studies demonstrate that endotoxin administration to rats activates type II cells and alveolar macrophages for increased nitric oxide production. However, distinct signaling molecules appear to be involved in the regulation of NOS-2 in these two cell types during acute endotoxemia, and this may be attributed to differences in their origin and function. Thus, although type II cells are epithelial in origin, alveolar macrophages are derived from myeloid precursors. Type II cells also have a more limited life span compared with alveolar macrophages, which are relatively long-lived cells and can persist within inflammatory lesions. Further studies are needed to determine the precise signaling pathways activated in each cell type during acute endotoxemia and their relative contributions to pulmonary inflammation.

Acknowledgments

This work was supported by National Institute of Environmental Health Sciences Grants ES-04738, ES-06897, and ES-05022 and by a Career Development Award from the Burroughs Wellcome Fund.

References

1. Adamson IY, Bowden DH. The type 2 cell as progenitor of alveolar epithelial regeneration. A cytodynamic study in mice after exposure to oxygen. *Lab Invest.* 1974; 30:35–42. [PubMed: 4812806]
2. Amory-Rivier CF, Mohler J, Bedos JP, Azoulay-Dupuis E, Henin D, Muffat-Joly M, Carbon C, Moine P. Nuclear factor-kappaB activation in mouse lung lavage cells in response to *Streptococcus pneumoniae* pulmonary infection. *Crit Care Med.* 2000; 28:3249–3256. [PubMed: 11008989]
3. Ardeshtna KM, Pizzey AR, Devereux S, Khwaja A. The PI3 kinase, p38 SAP kinase, and NF-kappaB signal transduction pathways are involved in the survival and maturation of lipopolysaccharide-stimulated human monocyte-derived dendritic cells. *Blood.* 2000; 96:1039–1046. [PubMed: 10910920]
4. Arias-Diaz J, Vara E, Garcia C, Balibrea JL. Tumor necrosis factor-alpha-induced inhibition of phosphatidylcholine synthesis by human type II pneumocytes is partially mediated by prostaglandins. *J Clin Invest.* 1994; 94:244–250. [PubMed: 8040266]

5. Blackwell TS, Lancaster LH, Blackwell TR, Venkatakrisnan A, Christman JW. Differential NF- κ B activation after intratracheal endotoxin. *Am J Physiol Lung Cell Mol Physiol*. 1999; 277:L823–L830.
6. Blanco JC, Contursi C, Salkowski CA, DeWitt DL, Ozato K, Vogel SN. Interferon regulatory factor (IRF)-1 and IRF-2 regulate interferon gamma-dependent cyclooxygenase 2 expression. *J Exp Med*. 2000; 191:2131–2144. [PubMed: 10859338]
7. Brennan P, O’Neill LA. 2-Mercaptoethanol restores the ability of nuclear factor kappa B (NF kappa B) to bind DNA in nuclear extracts from interleukin 1-treated cells incubated with pyrrolidine dithiocarbamate (PDTC). Evidence for oxidation of glutathione in the mechanism of inhibition of NF kappaB by PDTC. *Biochem J*. 1996; 320:975–981. [PubMed: 9003388]
8. Caivano M, Cohen P. Role of mitogen-activated protein kinase cascades in mediating lipopolysaccharide-stimulated induction of cyclooxygenase-2 and IL-1 beta in RAW264 macrophages. *J Immunol*. 2000; 164:3018–3025. [PubMed: 10706690]
9. Callejas NA, Casado M, Bosca L, Martin-Sanz P. Requirement of nuclear factor kappaB for the constitutive expression of nitric oxide synthase-2 and cyclooxygenase-2 in rat trophoblasts. *J Cell Sci*. 1999; 18:3147–3155. [PubMed: 10462530]
10. Carter AB, Monick MM, Hunninghake GW. Lipopolysaccharide-induced NF-kappaB activation and cytokine release in human alveolar macrophages is PKC-independent and TK- and PC-PLC-dependent. *Am J Respir Cell Mol Biol*. 1998; 18:384–391. [PubMed: 9490656]
11. Chen C, Chen YH, Lin WW. Involvement of p38 mitogen-activated protein kinase in lipopolysaccharide-induced iNOS and COX-2 expression in J774 macrophages. *Immunology*. 1999; 97:124–129. [PubMed: 10447723]
12. Christman JW, Sadikot RT, Blackwell TS. The role of nuclear factor-kappa B in pulmonary diseases. *Chest*. 2000; 117:1482–1487. [PubMed: 10807839]
13. Cott GR, Westcott JY, Voelkel NF. Prostaglandin and leukotriene production by alveolar type II cells in vitro. *Am J Physiol Lung Cell Mol Physiol*. 1990; 258:L179–L187.
14. Deitch EA. Multiple organ failure. Pathophysiology and potential future therapy. *Ann Surg*. 1992; 216:117–134. [PubMed: 1503516]
15. Dekker LV, Segal AM. Perspectives: signal transduction signals to move cells. *Science*. 2000; 287:982–985. [PubMed: 10691572]
16. Devalia JL, Sapsford RJ, Cundell DR, Rusznak C, Camp-bell AM, Davies RJ. Human bronchial epithelial cell dysfunction following in vitro exposure to nitrogen dioxide. *Eur Respir J*. 1993; 6:1308–1316. [PubMed: 8287947]
17. Dobbs LG, Gonzalez R, Williams MC. An improved method for isolating type II cells in high yield and purity. *Am Rev Respir Dis*. 1986; 134:141–145. [PubMed: 3637065]
18. Ermert L, Ermert M, Goppelt-Struebe M, Walmrath D, Grimminger F, Steudel W, Ghofrani HA, Homberger C, Duncker H, Seeger W. Cyclooxygenase isoenzyme localization and mRNA expression in rat lungs. *Am J Respir Cell Mol Biol*. 1998; 18:479–488. [PubMed: 9533935]
19. Evans MJ, Cabral LJ, Stephens RJ, Freeman G. Transformation of alveolar type 2 cells to type 1 cells following exposure to NO₂. *Exp Mol Pathol*. 1975; 22:142–150. [PubMed: 163758]
20. Faure V, Hecquet C, Courtois Y, Goureau O. Role of interferon regulatory factor-1 and mitogen-activated protein kinase pathways in the induction of nitric oxide synthase-2 in retinal pigmented epithelial cells. *J Biol Chem*. 1999; 274:4794–4800. [PubMed: 9988718]
21. Gauldie J, Jordana M, Cox G. Cytokines and pulmonary fibrosis. *Thorax*. 1993; 48:931–935. [PubMed: 8236078]
22. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. *Anal Biochem*. 1982; 126:131–138. [PubMed: 7181105]
23. Grimminger F, von Kurten I, Walmrath D, Seeger W. Type II alveolar epithelial eicosanoid metabolism: predominance of cyclooxygenase pathways and transcellular lipoxigenase metabolism in co-culture with neutrophils. *Am J Respir Cell Mol Biol*. 1992; 6:9–16. [PubMed: 1728301]

24. Hirsch E, Katanaev VL, Garlanda C, Azzolino O, Pirola L, Silengo L, Sozzani S, Mantovani A, Altruda F, Wymann MP. Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science*. 2000; 287:1049–1053. [PubMed: 10669418]
25. Holtzman MJ. Arachidonic acid metabolism. Implications of biological chemistry for lung function and disease. *Am Rev Respir Dis*. 1991; 143:188–203. [PubMed: 1898844]
26. Kooguchi K, Hashimoto S, Kobayashi A, Kitamura Y, Kudoh I, Wiener-Kronish J, Sawa T. Role of alveolar macrophages in initiation and regulation of inflammation in *Pseudomonas aeruginosa* pneumonia. *Infect Immun*. 1998; 66:3164–3169. [PubMed: 9632581]
27. Lahti A, Lahde M, Kankaanranta H, Moilanen E. Inhibition of extracellular signal-regulated kinase suppresses endotoxin-induced nitric oxide synthesis in mouse macrophages and in human colon epithelial cells. *J Pharmacol Exp Ther*. 2000; 294:1188–1194. [PubMed: 10945876]
28. Laskin DL, Pendino KJ. Macrophages and inflammatory mediators in tissue injury. *Annu Rev Pharmacol Toxicol*. 1995; 35:655–677. [PubMed: 7598511]
29. Laskin DL, Sunil V, Guo Y, Heck DE, Laskin JD. Increased nitric oxide synthase in the lung after ozone inhalation is associated with activation of NF-kappa B. *Environ Health Perspect*. 1998; 5:1175–1178. [PubMed: 9788894]
30. Lassus P, Wolff H, Andersson S. Cyclooxygenase-2 in human perinatal lung. *Pediatr Res*. 2000; 47:602–605. [PubMed: 10813584]
31. Marletta MA, Yoon PS, Iyengar R, Leaf CD, Wishnok JS. Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochemistry*. 1988; 27:8706–8711. [PubMed: 3242600]
32. McRitchie DI, Isowa N, Edelson JD, Xavier AM, Cai L, Man HY, Wang YT, Keshavjee SH, Slutsky AS, Liu M. Production of tumor necrosis factor alpha by primary cultured rat alveolar epithelial cells. *Cytokine*. 2000; 12:644–654. [PubMed: 10843740]
33. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev*. 1991; 43:109–142. [PubMed: 1852778]
34. Munir I, Fukunaga K, Miyazaki K, Okamura H, Miyamoto E. Mitogen-activated protein kinase activation and regulation of cyclooxygenase 2 expression by platelet-activating factor and hCG in human endometrial adenocarcinoma cell line HEC-1B. *J Reprod Fertil*. 1999; 117:49–59. [PubMed: 10645245]
35. Murphy, WJ. *Cellular and Molecular Biology of Nitric Oxide*. New York: Dekker; 1999. Transcriptional regulation of the genes encoding nitric oxide synthase; p. 1-56.
36. O'Banion MK. Cyclooxygenase-2: molecular biology, pharmacology, and neurobiology. *Crit Rev Neurobiol*. 1999; 13:45–82. [PubMed: 10223523]
37. Pece S, Chiariello M, Murga C, Gutkind JS. Activation of the protein kinase Akt/PKB by the formation of E-cadherin-mediated cell-cell junctions. Evidence for the association of phosphatidylinositol 3-kinase with the E-cadherin adhesion complex. *J Biol Chem*. 1999; 274:19347–19351. [PubMed: 10383446]
38. Pendino KJ, Laskin JD, Shuler RL, Punjabi CJ, Laskin DL. Enhanced production of nitric oxide by rat alveolar macrophages after inhalation of a pulmonary irritant is associated with increased expression of nitric oxide synthase. *J Immunol*. 1993; 151:7196–7205. [PubMed: 7505023]
39. Peters-Golden M, Feyssa A. Transcellular eicosanoid synthesis in cocultures of alveolar epithelial cells and macrophages. *Am J Physiol Lung Cell Mol Physiol*. 1993; 264:L438–L447.
40. Punjabi CJ, Laskin JD, Pendino KJ, Goller NL, Durham SK, Laskin DL. Production of nitric oxide by rat type II pneumocytes: increased expression of inducible nitric oxide synthase following inhalation of a pulmonary irritant. *Am J Respir Cell Mol Biol*. 1994; 11:165–172. [PubMed: 7519435]
41. Seeger W, Walter H, Suttorp N, Muhly M, Bhakdi S. Thromboxane-mediated hypertension and vascular leakage evoked by low doses of *Escherichia coli* hemolysin in rabbit lungs. *J Clin Invest*. 1989; 84:220–227. [PubMed: 2500455]
42. Shukla A, Timblin C, BeruBe K, Gordon T, McKinney W, Driscoll K, Vacek P, Mossman BT. Inhaled particulate matter causes expression of nuclear factor (NF)-kappaB-related genes and oxidant-dependent NF-kappaB activation in vitro. *Am J Respir Cell Mol Biol*. 2000; 23:182–187. [PubMed: 10919984]

43. Stringer B, Kobzik L. Environmental particulate-mediated cytokine production in lung epithelial cells (A549): role of preexisting inflammation and oxidant stress. *J Toxicol Environ Health*. 1998; 55:31–44.
44. Togashi H, Sasaki M, Frohman E, Taira E, Ratan RR, Dawson TM, Dawson VL. Neuronal (type I) nitric oxide synthase regulates nuclear factor- κ B activity and immunologic (type II) nitric oxide synthase expression. *Proc Natl Acad Sci USA*. 1997; 94:2676–2680. [PubMed: 9122255]
45. Ueki K, Yamamoto-Honda R, Kaburagi Y, Yamauchi T, Tobe K, Burgering BM, Coffey PJ, Komuro I, Akanuma Y, Yazaki Y, Kadowaki T. Potential role of protein kinase B in insulin-induced glucose transport, glycogen synthesis, and protein synthesis. *J Biol Chem*. 1998; 273:5315–5322. [PubMed: 9478990]
46. Van der Vliet A, Cross CE. Oxidants, nitrosants, and the lung. *Am J Med*. 2000; 109:398–421. [PubMed: 11020397]
47. Vane JR, Bakhle YS, Botting RM. Cyclooxygenases 1 and 2. *Annu Rev Pharmacol Toxicol*. 1998; 38:97–120. [PubMed: 9597150]
48. Walmrath D, Ghofrani HA, Rosseau S, Schutte H, Cramer A, Kaddus W, Grimminger F, Bhakdi S, Seeger W. Endotoxin “priming” potentiates lung vascular abnormalities in response to *Escherichia coli* hemolysin: an example of synergism between endo- and exotoxin. *J Exp Med*. 1994; 180:1437–1443. [PubMed: 7931076]
49. Ward PA. Phagocytes and the lung. *Ann NY Acad Sci*. 1997; 832:304–310. [PubMed: 9704058]
50. Wardlaw SA, March TH, Belinsky SA. Cyclooxygenase-2 expression is abundant in alveolar type II cells in lung cancer-sensitive mouse strains and in premalignant lesions. *Carcinogenesis*. 2000; 21:1371–1377. [PubMed: 10874016]
51. Watanabe K, Kazakova I, Furniss M, Miller SC. Dual activity of pyrrolidine dithiocarbamate on kappa B-dependent gene expression in U937 cells. I. Regulation by the phorbol ester TPA. *Cell Signal*. 1999; 11:479–489. [PubMed: 10405758]
52. Weinstein SL, Finn AJ, Dave SH, Meng F, Lowell CA, Sanghera JS, DeFranco AL. Phosphatidylinositol 3-kinase and mTOR mediate lipopolysaccharide-stimulated nitric oxide production in macrophages via interferon-beta. *J Leukoc Biol*. 2000; 67:405–414. [PubMed: 10733102]
53. Wizemann TM, Gardner CR, Laskin JD, Quinones S, Durham SK, Goller NL, Ohnishi ST, Laskin DL. Production of nitric oxide and peroxynitrite in the lung during acute endotoxemia. *J Leukoc Biol*. 1994; 56:759–768. [PubMed: 7527832]
54. Wizemann TM, Laskin DL. Enhanced phagocytosis, chemotaxis, and production of reactive oxygen intermediates by interstitial lung macrophages following acute endotoxemia. *Am J Respir Cell Mol Biol*. 1994; 11:358–365. [PubMed: 8086172]
55. Wymann MP, Sozzani S, Altruda F, Mantovani A, Hirsch E. Lipids on the move: phosphoinositide 3-kinases in leukocyte function. *Immunol Today*. 2000; 21:260–264. [PubMed: 10939787]
56. Xavier AM, Isowa N, Cai L, Dziak E, Opas M, McRitchie DI, Slutsky AS, Keshavjee SH, Liu M. Tumor necrosis factor-alpha mediates lipopolysaccharide-induced macrophage inflammatory protein-2 release from alveolar epithelial cells. Autoregulation in host defense. *Am J Respir Cell Mol Biol*. 1999; 21:510–520. [PubMed: 10502561]
57. Zhao MQ, Stoler MH, Liu AN, Wei B, Soguro C, Hahn YS, Enelow RI. Alveolar epithelial cell chemokine expression triggered by antigen-specific cytolytic CD8(+) T cell recognition. *J Clin Invest*. 2000; 106:R49–R58. [PubMed: 10995793]
58. Zsengeller Z, Otake K, Hossain SA, Berclaz PY, Trapnell BC. Internalization of adenovirus by alveolar macrophages initiates early proinflammatory signaling during acute respiratory tract infection. *J Virol*. 2000; 74:9655–9667. [PubMed: 11000238]

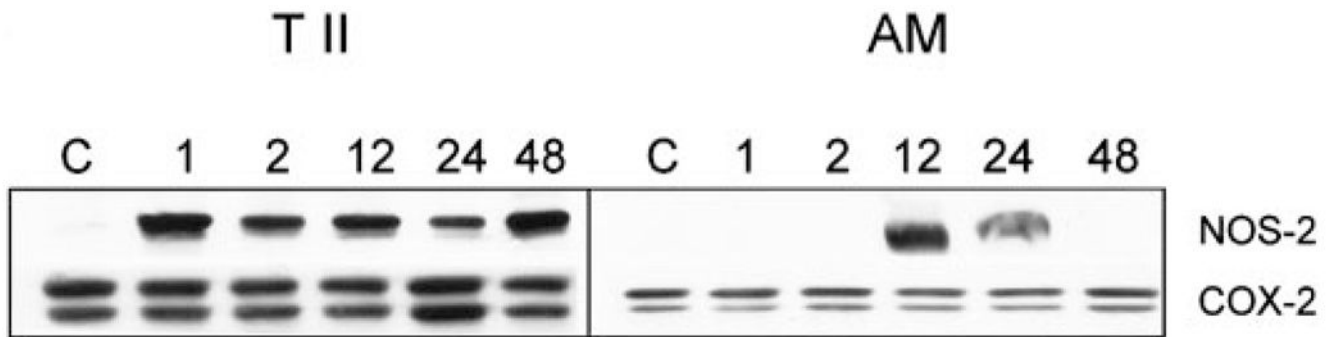


Fig. 1.

Effects of acute endotoxemia on nitric oxide synthase (NOS)-2 and cyclooxygenase (Cox)-2 protein expression. Type II cells (T II) and alveolar macrophages (AM) were isolated from control animals (C) or 1–48 h after endotoxin administration. Cytoplasmic extracts were prepared from the cells immediately after isolation, and equal amounts were analyzed by Western blotting. One representative gel from three separate experiments is shown. The bands on the NOS-2 gel were scanned using Scanalytics (CSPI, Fairfax, VA). The relative densities for type II cells were as follows: C, 0.06; 1 h endotoxin (ETX), 6.47; 2 h ETX, 3.60; 12 h ETX, 4.44; 24 h ETX, 2.63; 48 h ETX, 6.53. The densities for AM were as follows: C, 0; 1 h ETX, 0; 2 h ETX, 0; 12 h ETX, 6.98; 24 h ETX, 3.59; 48 h ETX, 0.

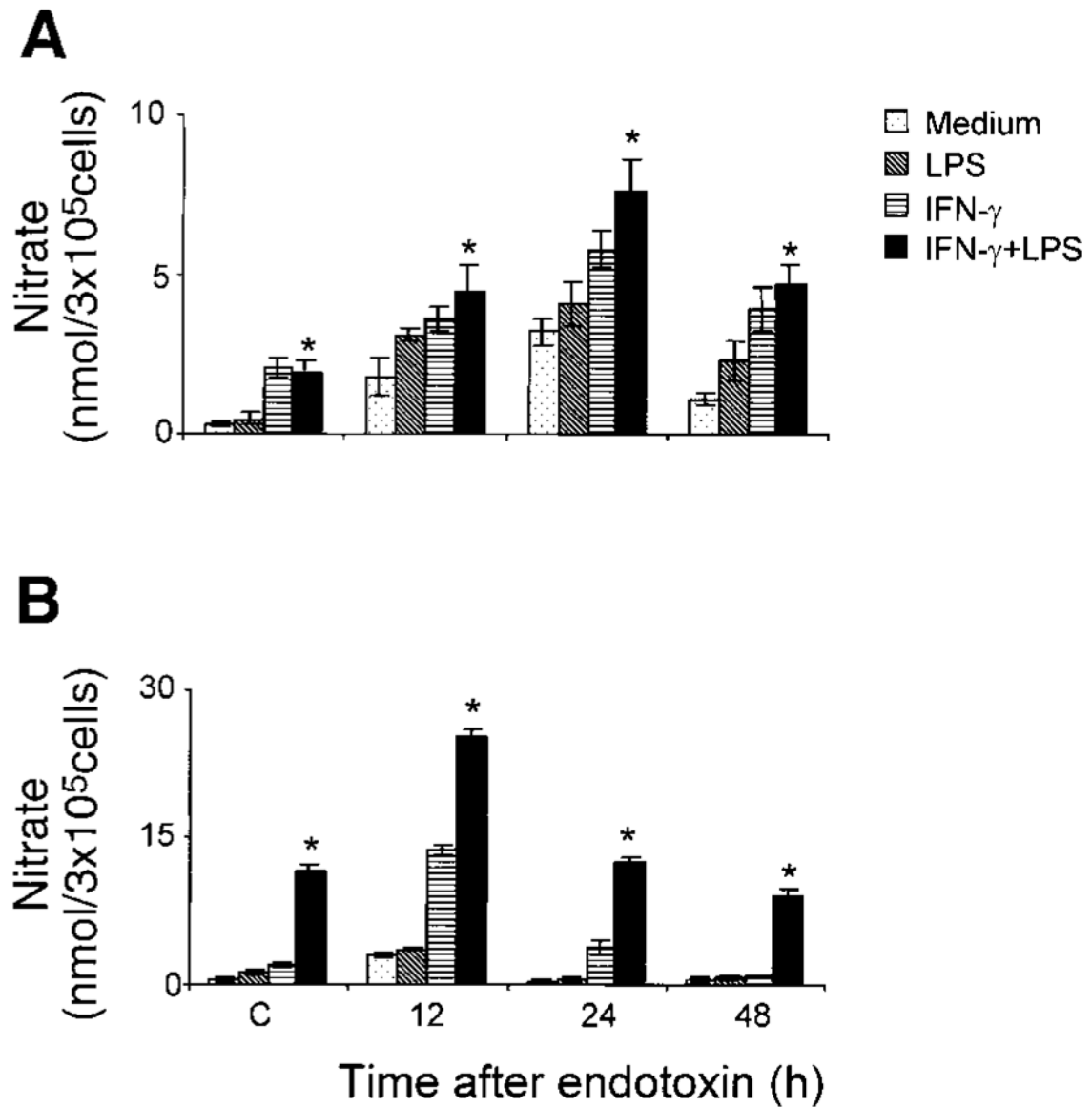
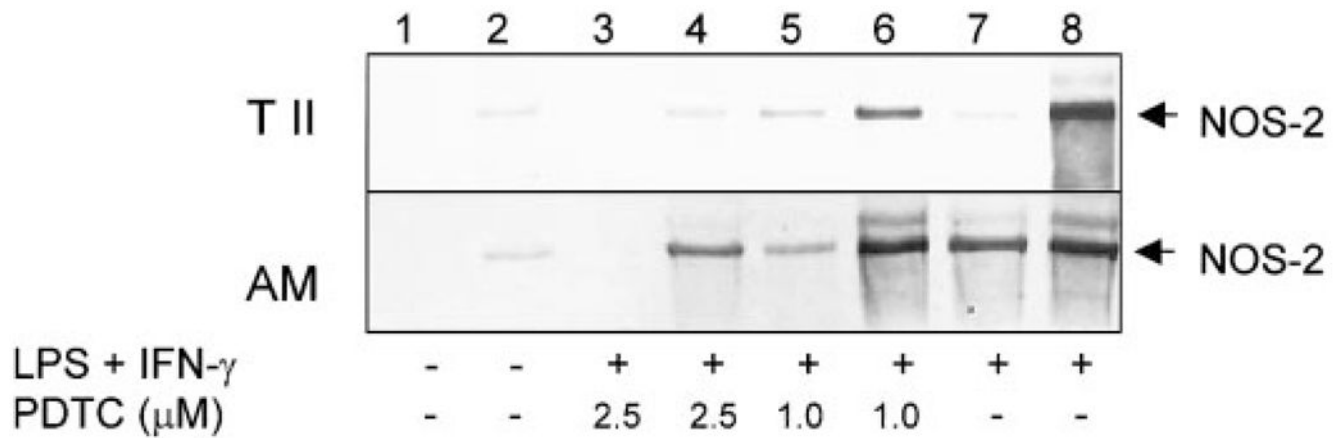


Fig. 2.

Effects of acute endotoxemia on nitric oxide production by type II cells (A) and alveolar macrophages (B). Cells isolated from control animals or 12, 24, or 48 h after endotoxin administration were incubated with medium, 100 ng/ml lipopolysaccharide (LPS), 10 U/ml interferon (IFN)- γ , or 10 U/ml IFN- γ + 100 ng/ml LPS. Nitrite was quantified in culture supernatants 24 h later. Each point represents the mean \pm SE of 3 experiments.

*Significantly different ($P < 0.01$) from cells treated with medium, LPS, or IFN- γ .

**Fig. 3.**

NOS-2 protein expression in type II cells and alveolar macrophages. Cells isolated from control animals (*lanes 1, 3, 5, and 7*) or 24 h after endotoxin administration (*lanes 2, 4, 6, and 8*) were treated with medium (*lanes 1 and 2*) or LPS + IFN- γ (*lanes 3–8*) in the absence (*lanes 1, 2, 7, and 8*) or presence of 1 μ M (*lanes 5 and 6*) or 2.5 μ M (*lanes 3 and 4*) pyrrolidine dithiocarbamate (PDTC). Cytoplasmic extracts were prepared 24 h later, and equal amounts of protein were analyzed by Western blotting. One representative gel from 3 separate experiments is shown. The bands on the gel were scanned using Scanalytics. The relative densities for type II cells were as follows: *lane 1*, 0.34; *lane 2*, 1.02; *lane 3*, 0.47; *lane 4*, 1.23; *lane 5*, 1.48; *lane 6*, 3.68; *lane 7*, 1.27; *lane 8*, 11.80. The densities for AM were as follows: *lane 1*, 0.13; *lane 2*, 0.56; *lane 3*, 0.05; *lane 4*, 1.39; *lane 5*, 0.88; *lane 6*, 3.22; *lane 7*, 2.41; *lane 8*, 3.92.

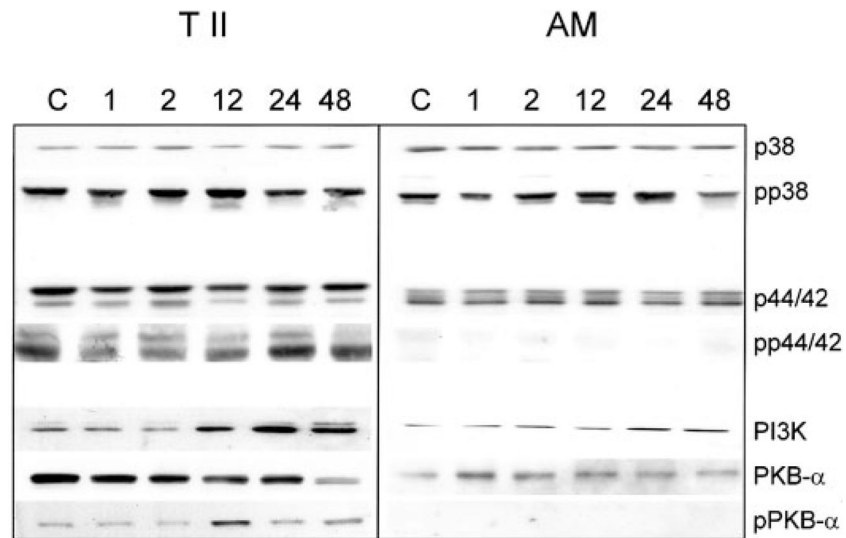


Fig. 4.

Western blot analysis of type II cells and alveolar macrophages. Cells were isolated from control animals or 1–48 h after endotoxin administration. Cytoplasmic extracts were prepared immediately after isolation, and equal amounts were analyzed by Western blotting. One representative gel from 3 separate experiments is shown. PI 3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B.

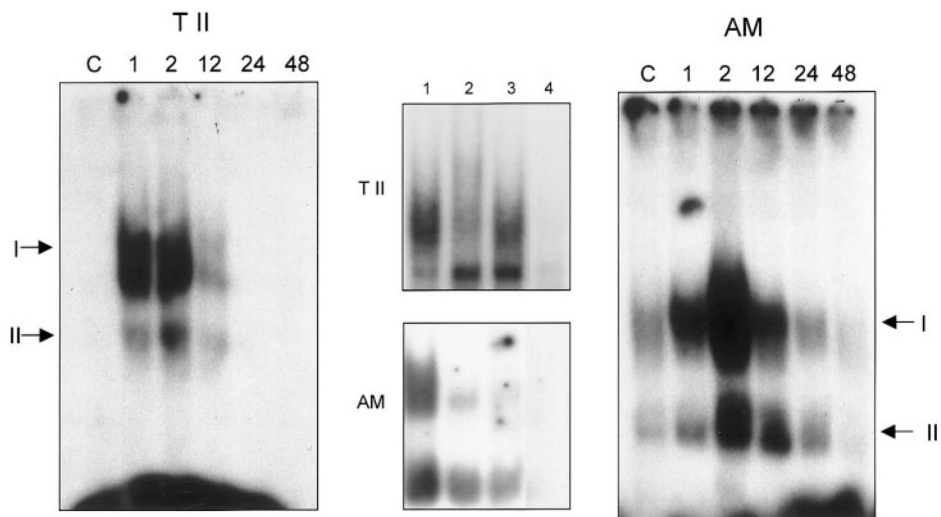


Fig. 5. Effects of acute endotoxemia on nuclear factor (NF)- κ B nuclear binding activity. Type II cells and alveolar macrophages were isolated from control animals or 1–48 h after endotoxin administration. Nuclear extracts were prepared immediately after isolation, and equal amounts were analyzed for NF- κ B binding activity by electrophoretic mobility shift assay (EMSA). *Center:* extracts prepared from cells collected 2 h after endotoxin administration (*lane 1*) were preincubated with antibodies to p50 (*lane 2*), p65 (*lane 3*), or 40-fold excess unlabeled NF- κ B competitor probe (*lane 4*). One representative gel from 3 separate experiments is shown.

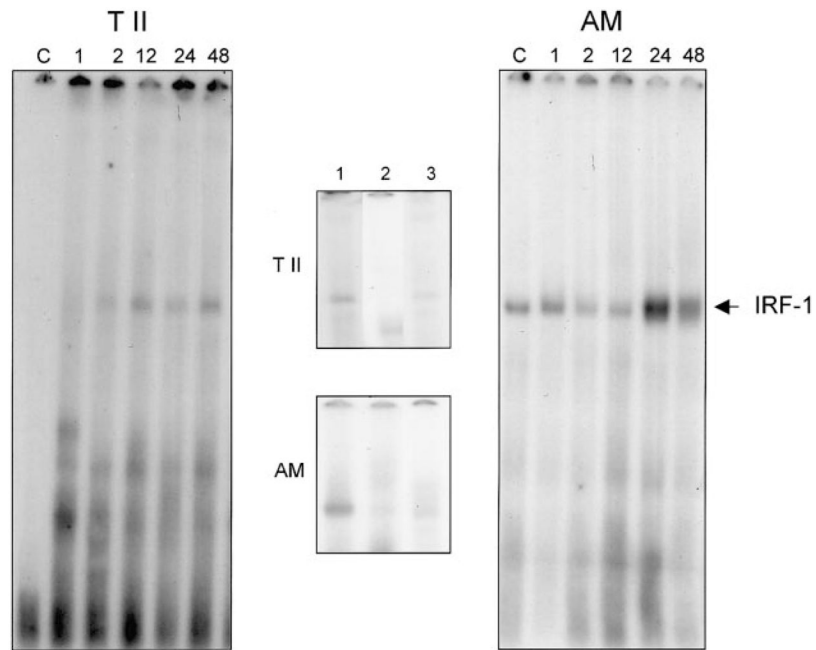


Fig. 6. Effects of acute endotoxemia on interferon regulatory factor (IRF)-1 nuclear binding activity. Type II cells and alveolar macrophages were isolated from control animals or 1–48 h after endotoxin administration. Nuclear extracts were prepared immediately after isolation, and equal amounts were analyzed for IRF-1 binding activity by EMSA. *Center:* extracts prepared from cells collected 24 h after endotoxin administration (*lane 1*) were preincubated with antibody to IRF-1 (*lane 2*) or 40-fold excess unlabeled IRF-1 oligonucleotide (*lane 3*). One representative gel from 3 separate experiments is shown.

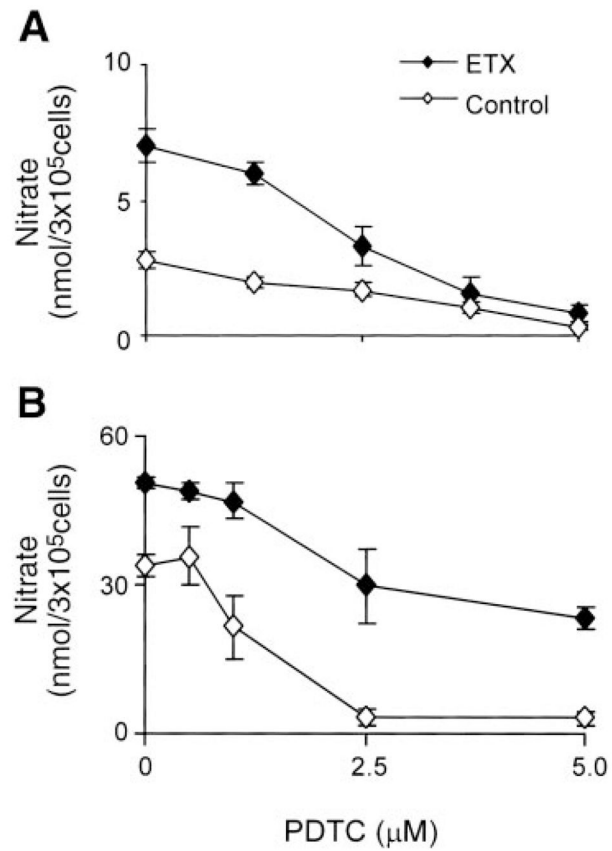


Fig. 7. Dose-dependent inhibition of nitric oxide production by PDTC. Type II cells (A) and alveolar macrophages (B) isolated from control animals or 24 h after endotoxin (ETX) administration were cultured with LPS + IFN- γ with and without PDTC. Nitrite release was quantified in culture supernatants 24 h later. Each point represents the mean \pm SE of 3 experiments.