Increased iNOS activity is essential for pulmonary epithelial tight junction dysfunction in endotoxemic mice

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Han, Xiaonan, Mitchell P. Fink, Takashi Uchiyama, Runkuan Yang, and Russell L. Delude. Increased iNOS activity is essential for pulmonary epithelial tight junction dysfunction in endotoxemic mice. Am J Physiol Lung Cell Mol Physiol 286: L259–L267, 2004. First published August 1, 2003; 10.1152/ajplung.00187.2003.—A murine endotoxia model and cultered Calu-3 monolayers were used to test the hypothesis that excessive nitric oxide (NO) production secondary to induction of inducible NO synthase (iNOS) is a key factor leading to altered tight junction (TJ) protein expression and function in the pulmonary epithelium. C57Bl/6J mice were injected with either Escherichia coli 0111:B4 lipopolysaccharide (LPS; 2 mg/kg) or vehicle. Twelve hours later, leakage of FITC-dextran (Mw 4 kDa; FD4) from blood into bronchoalveolar lavage fluid was significantly increased in endotoxemic but not control mice. This decrease in bronchoalveolar barrier function was associated with upregulation of iNOS protein expression and NF-κB activation in lung tissue. Expression of the TJ proteins, zonula occludens (ZO)-1, ZO-2, ZO-3, and occludin, as assessed by immunoblotting and/or immunofluorescence, decreased in lung after the injection of mice with LPS. Treatment of endotoxemic mice with an isoform-selective iNOS inhibitor [l-NAME-(l-iminoethyl)lysine; l-NIL] ameliorated LPS-induced changes in TJ protein expression and preserved bronchoalveolar epithelial barrier function. Incubating Calu-3 bronchiolar epithelial monolayers with cytomix (a mixture of 1,000 U/ml IFN-γ, 10 ng/ml TNF-α, and 1 ng/ml IL-1β) increased permeability to FD4, but adding l-NIL prevented this effect. These results suggest that decreased expression and mistargeting of TJ proteins in lung after systemic inflammation may be NO dependent. ZO-1, occludin, nitric oxide; permeability; multiple organ dysfunction syndrome; inducible nitric oxide synthase; lipopolysaccharide

A common consequence of sepsis and other conditions associated with systemic inflammation is multiple organ dysfunction syndrome (MODS) (11). Whereas the clinical manifestations of this syndrome are inconsistent, alterations in pulmonary function are almost always observed (3, 40, 42). Components of the pulmonary dysfunction associated with MODS include widening of the alveolar-arterial PO2 gradient, decreased lung compliance, and pulmonary edema.

The normal functioning of the lung depends on the establishment and maintenance of a milieu in the alveolar space that is distinct from the composition of the subjphelial compartment. This process depends on the formation and proper functioning of specialized structures, called tight junctions (TJ), between adjacent cells making up the epithelial sheet. The TJ is a complex of several integral membrane proteins and peripheral membrane proteins that interact strongly with the cytoskeleton (19). Integral membrane proteins involved in TJ formation include occludin and members of a large class of proteins called claudins (19). These proteins contain four transmembrane domains and are thought to be the points of cell-to-cell contact within the TJ (6). Cosedimentation assays of TJ proteins suggest that there is a strong interaction between occludin and another protein associated with TJ formation, zonula occludens (ZO)-1 (13, 36). ZO-1 has been shown to interact with the cytoplasmic tails of occludin and the claudins. In addition, ZO-1 interacts with two additional members of the membrane-associated guanylate kinase family of proteins, ZO-2 and ZO-3 (18). The TJ serves as a fence that differentiates the plasma membrane into apical and basolateral domains (51). TJs also serve as a regulated semipermeable barrier that limits the passive diffusion of solutes across the paracellular pathway between adjacent cells (2, 21). These properties of TJs, in combination with transepithelial transport processes, generate distinct internal environments in the opposing compartments.

Nitric oxide (NO), a simple diatomic free radical, acts as a mediator of immune system function and cell signal transduction (26). Relatively large amounts of NO can be synthesized by the enzyme inducible NO synthase (iNOS). Induction of iNOS expression and increased NO biosynthesis on this basis have been identified in a variety of cell types in response to exposure to inflammatory cytokines and/or lipopolysaccharide (LPS; endotoxin) (29). Within the lung, a variety of cell types are capable of synthesizing and releasing NO, including macrophages, neutrophils, endothelial cells, vascular smooth muscle cells, and epithelial cells (5, 22, 29).

NO and other related reactive nitrogen species, notably peroxynitrite, are thought to play an important role in pathogenesis of pulmonary dysfunction associated with sepsis and other conditions associated with systemic inflammation (4, 23, 38, 45, 53). When cultured monolayers of various epithelial cell lines, including A549 pneumocyte-like (31) and Caco-2 enterocyte-like cells (10, 47), are stimulated with proinflammatory cytokines in vitro, barrier function is impaired. Increased levels of NO, whether generated endogenously after iNOS upregulation or derived from the spontaneous decomposition of an exogenous donor, interfere with the proper expression and targeting of several TJ proteins in cultured epithelial cells (8, 24, 52). Promoted by these findings, we hypothesized that increased production of NO might lead to structural and functional alterations in pulmonary TJ function in vivo as a result of a systemic inflammatory response induced by inject-
ing mice with LPS. Herein, we show that endotoxemia is associated with decreased expression and function of several TJ proteins in the epithelium of lung.

**METHODS**

**Materials.** All chemicals were purchased from Sigma-Aldrich Chemical (St. Louis, MO) unless otherwise noted. L-N^6-(1-iminoethyl)lysine (L-NIL) was from A. G. Scientific (San Diego, CA). Anti-occludin and anti-ZO-1 polyclonal antibodies were from Zymed Laboratories (South San Francisco, CA). Anti-β-actin monoclonal antibody and rabbit polyclonal antibodies against ZO-2 and ZO-3 were from Santa Cruz Biotechnology, (Santa Cruz, CA). Anti-iNOS monoclonal antibody was from TransLabs (Lexington, KY). All secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

**Animals.** This research complied with regulations regarding animal care as published by the National Institutes of Health and was approved by the Institutional Animal Use and Care Committee of the University of Pittsburgh. Male C57Bl/6j mice weighing 20–25 g were from Jackson Laboratories (Bar Harbor, ME). All animals were maintained in the University of Pittsburgh Animal Research Facility on a 12-h light-dark cycle with free access to standard laboratory chow and water. Animals were not fasted before experiments. Animals were anesthetized with abnormal saline before surgery by intramuscular injection with pentobarbital sodium (60–90 mg/kg; Abbott Labs, Chicago, IL).

To induce a systemic inflammatory response, mice were injected intraperitoneally with *Escherichia coli* (strain O111:B4) LPS (50 μg/mouse; 2 mg/kg) dissolved in 1.0 ml of PBS. Control animals were injected with a similar volume of PBS without LPS. Some mice were treated with two 5-mg/kg doses of L-NIL administered by intraperitoneal injection 2 and 8 h after the injection of LPS or PBS. Some mice were treated with L-NIL according to the same schedule in the absence of a prior injection of LPS. Groups of mice were anesthetized 6, 12, or 18 h after injection of LPS or PBS for measuring lung epithelial barrier function or harvesting tissue specimens for various biochemical or histological assays.

**Effect of cytomex on the permeability of Calu-3 monolayers.** The Calu-3 cell line was obtained from American Type Culture Collection (no. HTB-55) and cultured on collagen I-coated Biocoat tissue culture vessels. Calu-3 cells are derived from a human lung adenocarcinoma and form epithelial monolayers when grown on permeable supports in culture (1). This cell line has been used to study lung epithelial permeability in vitro (1, 48). For permeability assays, 5 × 10^4 Calu-3 cells were seeded onto 24-well Transwell microporous inserts. Monolayers growing on Transwell inserts were used 14 days after seeding. The medium bathing the apical surface of the monolayers was replaced with 200 μl of DMEM complete medium (pH 6.8) containing FIC-dextran (average molecular mass 4,000 Da; FD4) at 25 mg/ml. The medium bathing the basolateral surface was replaced with 500 μl of DMEM complete medium alone or DMEM supplemented with cytomeg (1,000 U/ml IFN-γ, 10 ng/ml TNF-α, and 1 ng/ml IL-1β) in the absence or presence of 100 μM L-NIL. The cells were then incubated for 6 or 12 h. The concentrations of FD4 in the apical and basolateral media were determined spectrophotometrically with a Packard Fusion Reader (Packard Bioscience, Boston, MA) using an excitation filter of 492 nm and an emission wavelength of 515 nm. The permeability of the monolayers was expressed as a clearance (nL/cm^2·h·1) as described previously (24).

**Measurement of bronchoalveolar epithelial permeability.** Mice were injected via the tail vein with either FD4 solution in PBS (25 mg/ml; 10 mg/kg) or FITC-labeled bovine serum albumin (FITC-albumin; 1.25 mg/ml; 5 mg/kg). Ten minutes later for mice injected with FD4 or 2 h later for mice injected with FITC-albumin, the animals were anesthetized. The trachea was exposed, and the lungs were lavaged three times with 1 ml of sterile saline per wash. The aliquots of bronchoalveolar lavage fluid (BALF) were pooled, and fluorescence was determined as described above. Blood was obtained by cardiac puncture, and the serum was collected. Ten microliters of serum were mixed with 200 μl of PBS, and fluorescence was determined as described above. The BALF:serum fluorescence ratio was calculated and used as a measure of pulmonary epithelial permeability.

**Measurement of NO production.** Aliquots of BALF and serum were cleared by centrifugation. The end products of NO metabolism, NO_2_/NO_3_, were quantitated using the Bioxytech Nitric Oxide Assay kit exactly as directed by the manufacturer (OXIS International, Portland, OR). Values were expressed as μg NO in 1 ml BALF.

**Nonidet P-40-insoluble and total protein extracts.** Lung tissue (150–200 mg) was homogenized on ice with a Polytron tissue homogenizer in 1 ml of Nonidet P-40 (NP-40) lysis buffer [25 mM HEPES, pH 7.4, 150 mM NaCl, 4 mM EDTA, 25 mM NaF, 1% NP-40, 1 mM Na_2VO_3, 1 mM 4-aminophenylmethanesulfonyl flu- oride (APMSF), 10 μg/ml leupeptin, and 10 μg/ml aprotinin]. The samples were gently rocked at 4°C for 30 min. The sample was centrifuged at 12,000 g for 30 min at 4°C. The pellets were resuspended in SDS-dissolving buffer (25 mM HEPES, pH 7.5, 4 mM EDTA, 25 mM NaF, 1% SDS, and 1 mM Na_2VO_3) using five strokes with a Dounce homogenizer (pestle B) followed by sonication with a M1.0 Fisher Scientific Sonic Dismembrator fitted with a microtip on settings 3–5 for 1 min. Sonication continued until the precipitate was completely dissolved. These were designated the NP-40-insoluble fractions.

For total cellular protein extracts, tissue specimens were homogenized in cold RIPA buffer [PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml APMSF, 1.0 mM sodium orthovanadate, and 1× mammalian protease inhibitor cocktail (Sigma-Aldrich cat. no. P 8340)] and sonicated until the sample was completely dissolved. These samples are referred to as total protein extracts.

**Immunoprecipitation.** One hundred micrograms of NP-40-insoluble fraction were immunoprecipitated with either anti-occludin or anti-ZO-1 polyclonal antibodies. The lysate was preclarified by adding 0.25 μg of normal mouse IgG together with 20 μl of suspended protein A/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA). After being incubated at 4°C for 30 min, the beads were collected by centrifugation at 2,500 rpm for 5 min at 4°C. The supernatant was transferred to a fresh tube, 3 μg of anti-occludin or anti-ZO-1 antibody were added, and the tube was incubated on a rocker platform for 2 h at 4°C. Twenty microliters of resuspended agarose A/G were added to the tube, and the incubation was continued overnight at 4°C with gentle shaking. The agarose beads were washed five times with 1 ml of NP-40 lysis buffer. Proteins were eluted by boiling in 1× Laemmli buffer (10% glycerol, 5% 2-mercaptoethanol, 2.5% SDS, 0.1 M Tris, pH 6.8, and 0.2% bromphenol blue) for 10 min. Equal volumes of sample were separated by gel electrophoresis followed by immunoblotting.

**Immunoblotting.** Equal amounts of total protein extract were mixed in 1× Laemmli buffer, boiled for 3 min, and centrifuged for 10 s. The supernatants were electrophoresed on 7.5% or 12% precast SDS-polyacrylamide gels (Bio-Rad, Hercules, CA). The proteins were electroblotted onto Hybond-P polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Leicester, UK) and blocked with Blotto (1× Tris-buffered saline, 5% milk, 0.05% Tween 20, and 0.2% NaN_3) or 1:10 normal donkey serum (ZO-2 and ZO-3) only for 60 min. The filters were incubated at room temperature for 1 h with anti-ZO-1 or anti-β-actin antibody at 1:4,000 dilution or anti-ZO-2, anti-ZO-3, or anti-iNOS antibody diluted 1:2,000 in PBST (PBS and 0.02% Tween 20). After being washed three times in PBST, immunoblots were exposed for 1 h to a 1:20,000 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody. After three washes in PBST and two washes in PBS, the membrane was impregnated with an enhanced chemiluminescence substrate (ECL, Amer- sham Pharmacia Biotech) and used to expose X-ray film. Autoradiographs were captured using a Hewlett Packard (Palo Alto, CA)
Significant differences test. 

Statistical analyses. Results are presented as means ± SE. Data were analyzed using analysis of variance followed by Fisher’s least significant differences test. P values <0.05 were considered significant.

RESULTS

Endotoxemia decreases pulmonary epithelial barrier function. Prompted by the assumption that the effective small pore radius in the alveolar epithelial sheet (0.55–1.5 nm) is smaller than in the endothelial layer (~4.4 nm) (28, 34), we reasoned that bronchoalveolar epithelial barrier function would be better measured using FD4 as a tracer (solute radius ~1.3 nm) (34) rather than a larger molecule, such as albumin (solute radius 3.6 nm) (9). To obtain some preliminary data in support of this assumption, we employed the human Calu-3 cell line, derived from a bronchiolar carcinoma and possessing many characteristics of normal secretory pulmonary epithelium (15, 44). Calu-3 cells were cultured on permeable Transwell supports for 12 days to allow the formation of the epithelial barrier. Monolayer permeability was determined by measuring the apical-to-basolateral flux of FD4 essentially as described previously by our group and others (1, 24, 41). The permeability of the monolayers to FD4 increased slightly after 6 h and increased still further after 12 h of exposure to cytokinx (Fig. 1). In contrast, when monolayers were incubated with cytokinx in the presence of the isoform-selective iNOS inhibitor 1-NIL (37), permeability to FD4 was not statistically different from the control value. These results support the view that FD4 is a reasonable probe to detect changes in pulmonary epithelial permeability in vivo. These data are consistent with the idea that pulmonary epithelial barrier function decreases after exposure to proinflammatory cytokines via an iNOS-dependent mechanism.

We extended these observations by examining the effect of E. coli LPS (2 mg/kg) on the leakage of FD4 or FITC-albumin from the plasma compartment into the alveolar space in C57Bl/6j mice. At various time points after injection of LPS or PBS, we injected mice with FD4 in saline. Within 6 h after the induction of endotoxemia, the BALF:serum FD4 ratio increased significantly (Fig. 2A). At 12 h, the BALF:serum FD4 ratio increased still further. By 18 h after the injection of LPS, the BALF:serum FD4 ratio had returned toward normal. Delayed treatment with 1-NIL significantly ameliorated the increase in lung permeability caused by LPS. When we used FITC-albumin instead of FD4 as permeability probe, we observed similar changes, although the magnitude of the effect was much smaller, and the results were less reproducible. Twelve hours after the injection of LPS, the BALF:serum FITC-albumin ratio increased but the change was not statistically significant. Treatment of mice with 1-NIL ameliorated the slight increase in lung-to-BALF leakage of FITC-albumin induced by injection of LPS (Fig. 2B). We interpret these findings as indicating that the BALF:serum FD4 ratio is more sensitive than the BALF:serum FITC-albumin ratio for detecting changes in bronchoalveolar epithelial permeability, presumably because leakage of the larger probe is probably also influenced by the integrity of the alveolar endothelial barrier. We also interpret these findings as showing that injecting mice with LPS transiently impairs bronchoalveolar epithelial barrier function. Finally, these data support the view that LPS-induced bronchoalveolar barrier dysfunction is mediated, at least in part, by iNOS-dependent NO synthesis.

Injecting mice with LPS significantly increased the concentration of the NO breakdown products NO2 and NO3 in BALF (Fig. 3A) and serum (Fig. 3B). Treatment with 1-NIL only partially inhibited the accumulation of NO2 and NO3 in blood, whereas treatment with the iNOS inhibitor almost completely blocked accumulation of these NO metabolites in BALF.

Endotoxemia is associated with NF-κB activation in lung tissue and increased pulmonary iNOS expression. Induction of iNOS expression in macrophages (27) and pulmonary epithelial cells (38) is at least partially controlled by the transcription factor NF-κB. As expected, we documented LPS-induced NF-κB activation and upregulation of iNOS expression using EMSA (Fig. 4A) and Western blotting (Fig. 4B) in lung tissue extracts, respectively.
Endotoxemia is associated with alterations in the expression of TJ proteins in the lung. NP-40-insoluble occludin and ZO-1 levels decreased within 6 h after the injection of LPS and were maximally decreased at 12 h after the induction of endotoxemia (Fig. 5A). By 18 h, NP-40-insoluble occludin and ZO-1 levels had begun to return toward normal. Total extractable ZO-2 and ZO-3 levels also decreased (Fig. 5B), and the observed changes in the expression of these proteins were similar, both with regard to kinetics and magnitude, to the observed changes in the expression of ZO-1 and occludin. As assessed by direct immunofluorescence, ZO-1 was localized in lung tissue from control mice as a continuous line along the boundaries between neighboring bronchial and alveolar epithelial cells (Fig. 6). The intensity of this staining was dramatically reduced in lung tissue harvested from mice that were challenged with LPS 12 h earlier (Fig. 6).

Pharmacological inhibition of iNOS activity ameliorates deranged TJ protein expression and targeting. Treating endotoxemic mice with l-NIL modulated the decrease in ZO-1 and occludin levels in NP-40-insoluble complexes (Fig. 7). Treatment with l-NIL failed to completely normalize levels of these proteins. Similarly, l-NIL treatment tended to preserve the normal ZO-1 staining pattern in lung tissue from mice exposed to LPS, but the staining pattern remained different from that observed in control specimens (Fig. 6).

**DISCUSSION**

Although MODS is the most common cause of death for patients requiring intensive care, the molecular and cellular mechanisms underlying this phenomenon remain elusive. On the basis of the results of autopsies performed shortly after death, we know that lethal sepsis in humans is associated with extensive apoptosis of lymphoid tissues, but the pathology of many other organs, including the ileum, lung, liver, and kidney, is remarkably bland (25). In particular, the lung shows evidence of inflammation, edema formation, and accumulation of proteinaceous fluid in alveoli, but the epithelial architecture is well preserved. Thus overwhelming cell death is almost certainly not the cause of parenchymal organ system failure in sepsis; rather, more subtle derangements in cellular structure and function are probably critical in the pathogenesis of MODS. Prompted by our laboratory’s findings using a reductionist model of intestinal epithelial dysfunction induced by proinflammatory cytokines (24), we have carried out a series of studies to determine whether endotoxemia causes alterations in the structure and function of TJs in the epithelia of multiple organs. In one study, we showed that the structure and function of TJs in the liver were altered in mice injected 12–18 h earlier.
with LPS (24a). These changes were ameliorated when mice were treated with L-NIL to inhibit iNOS-dependent NO synthesis. Equivalent results were obtained in studies using ileal or colonic mucosa from endotoxemic mice (24b). In the study reported here, we obtained similar results and showed that the structure and function of TJs in the lung were altered in mice injected 12 h earlier with LPS. Moreover, these changes were ameliorated by treatment with L-NIL. As a result of these efforts, we now present a unifying hypothesis for the pathogenesis of MODS. We propose that sepsis- or systemic inflammation-induced alterations in the function of multiple organs, including the gut, liver, and lungs, are due, at least in part, to massive changes in the expression and localization of key TJ proteins. Furthermore, we propose that induction of iNOS leading to excessive production of NO is an important component of this process in each of these organs.

Herein, we showed that incubating Calu-3 pulmonary epithelial cell monolayers with a cocktail of proinflammatory cytokines (i.e., cytomix) markedly increased permeability to FD4. We also showed that injecting mice with LPS to induce systemic inflammation increased the leakage of FD4 from the blood compartment into the alveolar space. An increase in the BALF:serum FD4 ratio could be indicative of derangements in pulmonary endothelial or pulmonary epithelial barrier function or some combination of the two. However, our in vitro studies using Calu-3 bronchiolar-like epithelial cells support the view that an inflammatory milieu (produced in vitro by adding cytomix or produced in vivo by injecting LPS) increases the

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Fig. 4. Assessment of NF-κB nuclear translocation (A) and iNOS expression (B) in pulmonary tissue extracts. Mice were injected with LPS or PBS (Con), and tissue extracts were assayed at various time points thereafter. Results are representative of 4–6 assays (EMSA or Western blot) using samples from different mice. Bar graph presents mean area density ± SE.

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Fig. 5. A and B: effect of in vivo LPS administration on the expression of tight junction proteins in pulmonary tissue extracts. Mice were injected with LPS or PBS (Con), and tissue extracts were assayed at various time points thereafter. Results are representative of 4–6 blots using samples from different mice. Bar graphs present mean area density ± SE. NP-40, Nonidet P-40; ZO-1, zonula occludens-1; OCC, occludin.
permeability of the pulmonary epithelial barrier. It is noteworthy that we were unable to clearly document an increase in the BALF:serum FITC-albumin ratio after the injection of mice with LPS. Because the pulmonary epithelium represents the major barrier to the movement of proteins like albumin (20) and horseradish peroxidase (43) from the blood compartment into the pulmonary air spaces (or vice versa), these results suggest that the magnitude of the inflammatory response in our murine model of acute endotoxemia was not sufficient to completely abolish the barrier provided by the epithelium. Further studies will be required to determine whether other components of TJs (e.g., claudins) remain properly assembled.

The magnitude of the functional alteration in pulmonary epithelial barrier function peaked at 12 h after the injection of LPS.
LPS. By 18 h, the BALF:serum FD4 ratio was almost back to normal. This temporal pattern of changes in the barrier function of the bronchoalveolar epithelium was paralleled by changes in the expression of key proteins involved in the formation of TJs. For example, at 12 h after LPS injection, ZO-1 expression, as quantitated by immunoprecipitation and Western blotting, was virtually undetectable. However, at 18 h, a clear ZO-1 band was again apparent. The findings with respect to occludin and ZO-2 were similar.

Consistent with findings previously reported by Franek and colleagues (16), iNOS protein expression increased in total lung homogenates throughout the 18-h period of observation after injection of LPS. Nevertheless, the concentration of NO metabolites in BALF peaked at 12 h after the induction of endotoxemia and decreased at the 18-h time point. The discordance between iNOS protein levels in lung tissue and NO2 and NO− concentrations in BALF could be due to decreased enzymatic activity of iNOS or increased clearance of NO metabolites into the blood. In any event, however, we observed temporal associations between the concentration of NO metabolites in BALF on one hand and the functional changes in BALF and the expression of TJ proteins in the lungs on the other hand. This temporal relationship suggests that the observed LPS-induced derangements in bronchoalveolar epithelial TJ structure and function were mediated in some way by increased local production of NO. This idea is further supported by the data we obtained using L-NIL to selectively inhibit iNOS enzymatic activity and thereby decrease NO biosynthesis. Treatment of mice with L-NIL ameliorated both the functional changes in pulmonary epithelial permeability induced by LPS and the changes in expression and localization of TJ proteins associated with endotoxemia.

The source(s) of NO in this animal model still needs to be determined. Others have demonstrated that rat alveolar and bronchial epithelial cells express iNOS after exposure to LPS (12). This finding suggests that the pulmonary epithelium, like the intestinal and hepatic epithelia, is a major source of NO in endotoxemia. However, Fujii et al. (17) showed that pretreating mice with gadolinium chloride, which depletes macrophages, decreases NO production and iNOS protein levels in endotoxemic mice. On the basis of these findings, these authors argued that infiltrating pulmonary macrophages were the major pulmonary source of iNOS and increased pulmonary NO biosynthesis in endotoxemic mice. An alternative explanation for the findings made by Fujii et al. can be articulated. Decreased production of iNOS in the lung in gadolinium chloride-treated animals could reflect decreased induction of iNOS in pulmonary epithelial cells as a result of a decreased production of proinflammatory cytokines by infiltrating or resident macrophages in the lung.

The mechanism(s) through which NO decreases the expression and interferes with the targeting of TJ proteins in pulmonary epithelial cells remains unknown. However, the results of some prior in vitro studies using Caco-2 human enterocyte-like epithelial cells suggest that the deleterious moiety is not NO per se, but rather ONOO−, a potent oxidizing and nitrating agent that is formed when NO reacts with the superoxide radical (O2−) (39). The permeability of Caco-2 monolayers increases when the cells are incubated with various NO donors, such as S-nitroso-N-acetylpenicillamine (SNAP) or sodium nitroprusside (35, 41). SNAP-induced hyperpermeability, however, is further augmented by the addition of diethylthiocarbamate, a superoxide dismutase inhibitor, or pyrogallol, an O2− generator (35). Furthermore, SNAP-induced hyperpermeability is blocked by Tiron, an agent that scavenges O2− (35). SNAP-induced hyperpermeability is also prevented by various ONOO− scavengers, such as urate and deferoxamine (35).

When Caco-2 monolayers are incubated with IFN-γ or a combination of IFN-γ, TNF-α, and IL-1β, NO2− expression is induced and permeability is increased (10, 47). The increase in permeability can be blocked by inhibiting NO production or scavenging ONOO− (10, 47). Collectively, these findings support the view that cytokine-induced intestinal epithelial hyperpermeability is mediated, at least in part, by the formation ONOO−.

Additional evidence can be mustered to support a role for ONOO− as a mediator of inflammation-induced lung epithelial hyperpermeability. The permeability of A549 human pulmonary epithelial monolayers increases after exposure to authentic ONOO− (46). Furthermore, in both experimental animals (23, 33) and humans (23, 30), acute pulmonary injury is associated with nitrotyrosine formation in the lung. Nitrotyrosine is a marker for the interaction of ONOO− (or other related reactive nitrogen species) with cellular proteins (39). Despite these data, further studies are required to determine whether ONOO− or some other reactive nitrogen intermediate is the actual moiety responsible for the deleterious effects of NO on TJ protein expression that we have observed in organs, including the lungs, of endotoxemic animals. Moreover, we can only speculate about the ways that ONOO− might induce alterations in the expression and localization of TJ proteins. It is reasonable, however, to hypothesize that highly reactive nitrogen species covalently modify amino acid residues in key proteins involved in regulating the synthesis and assembly of TJs (32). Conceivably, ONOO−-mediated posttranslational events interfere with key signal transduction pathways or otherwise compromise the normal trafficking and interaction of proteins that are involved in the formation of TJs. Our laboratory is actively pursuing this line of investigation.

Acute lung injury and acute respiratory distress syndrome (ARDS) are common complications in critically ill patients. Considerable research has focused on endothelial dysfunction in the pathogenesis of pulmonary dysfunction. However, a relatively less studied component of the blood-air barrier is the alveolar epithelium, which plays an essential role in the clearance of fluid from the alveolar space (7). Alveolar fluid clearance is the result of ion gradients established, at least in part, by pumping Na+ across the basolateral membrane by Na+− K+− ATPase. The resulting ionic gradient results in passive movement of water through water channels in alveolar epithelial cells (14). Ware and Matthay (49) reported that alveolar fluid clearance is impaired in patients with acute lung injury and ARDS, and decreased alveolar fluid clearance is associated with extended mechanical ventilation and increased mortality. Our laboratory has obtained preliminary data indicating that Na+− K+− ATPase expression and activity are decreased in endotoxemic mice. Thus alveolar epithelial dysfunction is probably a significant component of the pathophysiology of pulmonary dysfunction in the critically ill patient. Decreased expression and function of TJ proteins in the alveolar epithelium would result in dissipation of ionic gradients and loss of motive force for the movement of water out of alveolar air
spaces. Furthermore, the loss of the fence function of the TJ could also lead to a loss of the normal polarization of the alveolar epithelium into apical and basolateral domains and, therefore, derangements in transport function on this basis as well.

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REFERENCES


25. Han X, Fink MP, Yang R, and Delude RL. Increased iNOS activity is essential for intestinal epithelial tight junction dysfunction in endotoxemic mice. Shock. In press.


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