Inhibition of amiloride-sensitive sodium-channel activity in distal lung epithelial cells by nitric oxide

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Inhibition of amiloride-sensitive sodium-channel activity in distal lung epithelial cells; by nitric oxide. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L378–L387, 1998.—Distal lung epithelial cells (DLECs) play an active role in fluid clearance from the alveolus by virtue of their ability to actively transport Na+ from the alveolus to the interstitial space. The present study evaluated the ability of activated macrophages to modulate the bioelectric properties of DLECs. Low numbers of lipopolysaccharide (LPS)-treated macrophages were able to significantly reduce amiloride-sensitive short-circuit current (Isc) without affecting total Isc or monolayer resistance. This was associated with a rise in the flufenamic acid-sensitive component of the Isc. The effect was reversed by the addition of N-monomethyl-L-arginine to the medium, implying a role for nitric oxide. We hypothesized that macrophages exerted their effect by expressing inducible nitric oxide synthase (iNOS) in DLECs. The products of LPS-treated macrophages increased the levels of iNOS protein and mRNA transcripts in DLECs as well as causing a rise in iNOS activity. Immunofluorescence microscopy of LPS-stimulated macrophage-DLEC cocultures with anti-nitrotyrosine antibodies provided evidence for the generation of peroxynitrite in macrophages but not in DLECs. Data indicate that activated macrophages in the lung may contribute to impaired resolution of acute respiratory distress syndrome and suggest a novel mechanism whereby nitric oxide might alter cell function by altering its ion-transporting phenotype.

ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS) is characterized by the presence of hypoxemia, reduced lung compliance, and increased permeability pulmonary edema that results in diffuse alveolar infiltrates on chest radiographs. This pathological process develops as a result of the disruption of the alveolar-capillary membrane with leakage of protein-rich fluid exudate and migration of inflammatory cells (neutrophils and macrophages) into the air space (reviewed in Ref. 40). Under normal circumstances, the distal lung epithelial cells (DLECs) that line the terminal airways and alveoli provide a tight physical barrier to the movement of interstitial fluid into the alveolar space. Injury to this layer may result in alveolar edema that contributes to the clinical characteristics of ARDS.

Recent studies have demonstrated that the DLECs also play an active role in fluid clearance from the alveolus by virtue of their ability to actively transport Na+ from the alveolus to the interstitial space. Vectorial transport of Na+ (with Cl− and water following) across monolayers of these cells is mediated by the presence of Na+ channels on their apical surface that permit Na+ entry down their electrochemical gradient into the cell and Na+ − K+ -adenosinetriphosphatase (ATPase) on the basolateral surface of the cell that extrudes Na+ (3, 30). Both in vitro and in vivo experiments suggest a physiological role for this transport mechanism. Studies performed under conditions where the bioelectric properties may be measured show that monolayers grown on porous supports exhibit unidirectional apical-to-basolateral transport of Na+ and are able to establish a significant potential difference (apical negative) across the monolayer. These processes are inhibited by Na+-channel blockers amiloride and benzamil as well as by the Na+-K+-ATPase inhibitor ouabain and are stimulated by β-adrenergic agonists and membrane-permeant analogs of adenosine 3', 5' - cyclic monophosphate (3, 27–30). The existence of this process in vivo is supported by several lines of evidence. First, tracer studies in isolated perfused rat lung preparations using 22Na have clearly demonstrated active Na+ reabsorption from the alveolar spaces (9). Second, physiological studies in animals have shown a role for an amiloride-sensitive process in fluid clearance from the alveolus (1, 2, 20). Finally, there is evidence supporting the existence of a comparable system in humans. Sakuma et al. (38) have reported amiloride- and ouabain-inhibitable alveolar fluid clearance in resected human lung at rates comparable to those observed in animal experiments. Considered together with the in vitro data, these observations suggest that this fluid-resorptive mechanism may play an active role in the resolution phase of ARDS. In this regard, a study by Matthay and Wiener-Kronish (26) correlated the clinical resolution of ARDS with the ability of patients to concentrate alveolar fluid protein, an indirect measure of Na+ transport-mediated fluid clearance from the alveolar space.

Our laboratory has previously investigated the ability of alveolar macrophages (Mφs) to modulate DLEC Na+-transport activity as a model whereby activated alveolar Mφs might contribute to the development and persistence of ARDS in the septic patient. These studies demonstrated that lipopolysaccharide (LPS)-treated alveolar Mφs were able to reduce total and amiloride-
sensitive short-circuit current ($I_{sc}$) in primary cultures of DLECs (7). The observation that this inhibitory effect was dependent on L-arginine metabolism suggested a role for nitric oxide (NO) as a key mediator of this effect. Because DLECs incubated with the supernatants of LPS-treated M$\delta$s exhibited alterations in $I_{sc}$ similar to those seen in the coculture system, we hypothesized that NO derived from DLECs might, at least in part, be responsible for the effect. The present studies demonstrate that the soluble products of LPS-treated M$\delta$s decrease the amiloride-sensitive Na$^+$ transport of the DLECs without affecting total $I_{sc}$. These changes correlated with an increase in the expression of inducible NO synthase (iNOS) at both the mRNA and protein levels in DLECs. However, treatment of DLECs with the NO donor S-nitroso-N-acetylpenicillamine (SNAP) alone did not reproduce the effect, suggesting that NO production is necessary but not sufficient to account for the observed alterations in amiloride-sensitive $I_{sc}$ in DLECs exposed to M$\delta$ products. These data therefore suggest a mechanism whereby NO might contribute to altered cell function by altering its ion-transporting phenotype.

**MATERIALS AND METHODS**

**Materials and Solutions**

Tissue culture media and additives, including RPMI 1640, Hank’s balanced salt solution (HBSS; with and without Ca$^{2+}$ and Mg$^{2+}$), Dulbecco’s phosphate-buffered saline (PBS), fetal bovine serum, Eagle’s minimum essential medium (MEM), MEM selectamine kits, trypsin, and penicillin-streptomycin, were all obtained from Gibco BRL (Life Technologies, Grand Island, NY). Collagenase and deoxyribonuclease for epithelial cell harvest were purchased from Worthington Biochemical (Freehold, NJ). Heparin (Hepalean; 1,000 U/ml) was from Organon Teknika (Toronto, Canada), and pentobarbital sodium (Somnotol) was from MTC Pharmaceuticals (Cambridge, Canada). Endotoxin (Escherichia coli O111:B4) was from Difco Laboratories (Detroit, MI). N$^G$-monomethyl-L-arginine (L-NMMA) was purchased from Calbiochem Behring (La Jolla, CA). Amlodipine, bumetanide, and flufenamic acid were from Sigma (St. Louis, MO).

**Alveolar M$\delta$ Isolation**

Adult male Wistar rats (300–350 g) were anesthetized with halothane-nitrous oxide and then exsanguinated after the administration of intravenous heparin (500 U). The trachea were then cannulated (14-gauge catheter; Becton Dickinson Vascular Access, Sandy, UT), and heart-lung blocks were extracted for ex vivo bronchoalveolar lavage. Bronchoalveolar lavage was performed by instilling 10-ml aliquots of PBS with 1 mM EDTA and recovering the fluid by gentle suction as previously described (6). This was repeated five times to yield a recovered volume of ~45 ml. Lavaged cells were pelleted by centrifugation (200 g for 10 min), pooled, and resuspended in RPMI medium containing 10% fetal bovine serum. Wright’s staining of cytospin-prepared cell populations demonstrated >92% alveolar M$\delta$s by morphology. Cell viability was routinely >95% as determined by trypan blue exclusion. Total cell numbers were counted with a hemocytometer (Improved Neubauer, American Optical, Buffalo, NY).

**Epithelial Cell Isolation and Culture**

DLECs were harvested from late-gestation fetal rats and grown in primary culture according to methods previously described (28). In brief, the lungs were excised from timed-gestation 20- or 21-day (term = 22 days) Wistar rat fetuses and minced into 1-mm$^3$ pieces. The lung fragments were incubated at 37°C with 0.125% trypsin and 0.002% deoxyribonuclease, and the dissociated cells were then passed through a Nitex 100 (B. and S. H. Thompson, Scarborough, Canada) mesh filter. The cells were then incubated with 0.1% collagenase and purified with differential adhesion techniques. These cells are >99% pure epithelial cells and consist of mature and precursor type II epithelial cells or distal airway cells, and hence we refer to these cells as DLECs (30). Previous experiments have demonstrated that epithelial cells cultured in this manner transport Na$^+$ via amiloride-sensitive and -insensitive mechanisms (28). These cells possess amiloride-sensitive whole cell Na$^+$ currents but no detectable Cl$^-$ currents (43) and have amiloride-sensitive nonselective cation (NSC) and Na$^+$-selective channels on their apical membranes (31, 42).

The harvested epithelial cells were immediately seeded (1 × 10$^5$ cells/cm$^2$) onto Transwell tissue culture-treated porous polycarbonate filters (total surface area 4.7 cm$^2$; Costar, Cambridge, MA). All cells were grown in MEM with 10% fetal bovine serum and penicillin-streptomycin at 37°C in a humidified 95% air-5% CO$_2$ environment. Nonadherent epithelial cells were removed 24 h after they were seeded. Epithelial monolayers were subsequently studied 3 or 4 days after they were seeded. Cells used in patch-clamp experiments were seeded (5 × 10$^5$ cells/cm$^2$) onto translucent porous Nunc filter inserts (Whatman Scientific) and were confluent when studied 4 days later.

**Epithelial Cell Monolayer Bioelectric Properties**

The bioelectric properties of epithelial cell monolayers were studied by placing the filters in Ussing chambers (MRA International, Clearwater, FL) that contained warmed HBSS and 22.4 mM NaHCO$_3$ that was circulated with an air lift of a 95% air-5% CO$_2$ gas mixture (7, 30). Measurements of the bioelectric properties of the monolayer were made with KCl agar-bridged half-cells and silver-silver chloride electrode-saline agar bridges that were connected to a high-impedance millivoltmeter that could function as a voltage-current clamp with automatic fluid-resistance compensation (VCC 600 Physiologic Instruments, San Diego, CA). The transepithelial potential difference (PD) was recorded continuously under open-circuit conditions with a linear-chart recorder (Linear Recorder 585, Baxter, Toronto, Canada). Every 10 s, a 0.5-s duration 1-µA pulse of current was delivered across the monolayer so that the measured change in PD enabled the calculation of resistance (R) using Ohm’s law. Transepithelial R is a sensitive measurement of epithelial monolayer permeability to ions and in large part reflects the barrier function of epithelial tight junctions. Every 15 min, the transepithelial PD was temporarily clamped to 0 mV so that $I_{sc}$ could be recorded. $I_{sc}$ represents the net movement of positive charge from the apical to the basolateral side of the epithelial membrane, with the basolateral side of the monolayer having a positive PD relative to the apical side of the monolayer.

Four groups of epithelial cell monolayers were studied to determine the effect of alveolar M$\delta$s and/or LPS on monolayer permeability (R) and ion transport ($I_{sc}$). The apical side of confluent epithelial monolayers was exposed to control medium, LPS alone (10 µg/ml), M$\delta$s alone (1–6 × 10$^5$ cells/cm$^2$),...
or both LPS (10 µg/ml) and Mφs (1–6 × 10^5 cells/cm²) for varying times. In some studies, the NO donor SNAP (0.1 mM) was added to the monolayers every 4 h for four doses before evaluation of the bioelectric properties. Monolayers were then rinsed with MEM and placed in Ussing chambers containing freshly prepared HBSS. After baseline bioelectric properties were determined, the Na⁺-transport blocker amiloride (0.1 mM apically), the Na⁺-K⁺-2Cl⁻ cotransport inhibitor bumetanide (0.1 mM basally), and the NOS inhibitor flufenamic acid (0.45 mM apically) were added sequentially to the monolayer, thus enabling the calculation of amiloride-sensitive current and nonselective current as a percentage of the total current in all experimental groups. The amiloride-sensitive I₁₉₅ likely reflects blockade of Na⁺ channels because our laboratory has previously shown that amiloride blocks 12- and 25-pS Na⁺-permeant channels (31, 42) and whole cell cation conductances (43) in the apical membrane of these epithelial cells. This amiloride dose was chosen based on previous dose-response studies performed on similar cells (28). Furthermore, dimethylamiloride, an amiloride analog with high potency for the Na⁺/H⁺ antiport, does not affect in vivo lung water clearance (28), DLEC Iₛₑ (30) or whole cell Na⁺ currents (43).

In some studies, alveolar Mφs were incubated in coculture but were physically separated from the DLEC monolayer. To accomplish this, alveolar Mφs (2 × 10⁶) were added to the bottom of the Ussing chambers and allowed to adhere for 2 h before insertion of the filters coated with the epithelial cell monolayers into the Ussing apparatus. This coculture setup was incubated for a further 16–24 h in the presence or absence of LPS, at which time the bioelectric properties were investigated.

iNOS Expression in Alveolar Mφs and DLECs

To evaluate separately iNOS expression in alveolar Mφs and DLECs, a coculture system in which these cells were physically separated was established. A petri dish (60 mm; Falcon) was secured with silicone in the center of a larger petri dish (150 mm). Epithelial cells were seeded and grown to confluence over a 3-day period in the outer well. At this time, alveolar Mφs (1.0 × 10⁶) were added to the central well and allowed to adhere for 2 h at 37°C before being washed to remove nonadherent cells. The level of the culture medium was then raised so that the inner and outer wells were bathing within the same medium. The double-well system was slowly agitated on a plate shaker, gently enough to prevent Mφs from detaching and reaching the outer well. The DLEC monolayer, thus enabling the calculation of amiloride-sensitive current and nonselective current as a percentage of the total current in all experimental groups. The amiloride-sensitive I₁₉₅ likely reflects blockade of Na⁺ channels because our laboratory has previously shown that amiloride blocks 12- and 25-pS Na⁺-permeant channels (31, 42) and whole cell cation conductances (43) in the apical membrane of these epithelial cells. This amiloride dose was chosen based on previous dose-response studies performed on similar cells (28). Furthermore, dimethylamiloride, an amiloride analog with high potency for the Na⁺/H⁺ antiport, does not affect in vivo lung water clearance (28), DLEC Iₛₑ (30) or whole cell Na⁺ currents (43).

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Northern blot analysis. Total RNA was extracted with the method of Chomczynski and Sacchi (4). Briefly, cells were washed with cold Ca²⁺- and Mg²⁺-free HBSS and lysed with guanidine thiocyanate. The cell lysate was then recovered from the dish with a sterile cell scraper and transferred to Eppendorf tubes. After RNA extraction and spectrophotometric quantitation, 10 µg of total RNA were electrophoresed in a 1.2% agarose gel containing formaldehyde, blotted transferred to a nylon membrane, and then ultraviolet cross-linked. Membranes were hybridized with the ³²P random-labeled cDNA probe for the murine iNOS (kindly provided by Dr. Dennis Stuehr, Cleveland Clinic, OH; see Ref. 10), washed at room temperature in 1× sodium chloride-sodium phosphate-EDTA and 0.1% sodium dodecyl sulfate for 30 min, and exposed overnight to Kodak film at −70°C. The nitrite content of the cell-free supernatants was measured by reacting samples (100 µl) with the Griess reagent (100 µl) for 10 min at 37°C according to established methods (12). Absorbance at 540 nm was then measured, and nitrite concentrations were calculated from a linear standard curve generated between 0 and 128 mM sodium nitrite.

Detection of Nitrotyrosine Residues

Cells were cultured on glass coverslips in six-well tissue culture dishes. Coverslips were then washed three times with ice-cold PBS and fixed in 100% methanol (−20°C for 10 min). Coverslips were blocked in 5% donkey serum-PBS for 2 h at room temperature, then incubated with primary antibody overnight at 4°C (titer of 1:100 diluted in 1% bovine serum albumin-PBS). To demonstrate the specificity of immunofluorescence, the antibodies were coincubated with the antigenic fusion protein (1 mg fusion protein/mg antibody) in a volume of 100 µl of antibody buffer (1% bovine serum albumin-PBS) overnight at 4°C on a rotating shaker. Coverslips were then washed three times in ice-cold PBS, and secondary antibodies were applied (6 µg/ml of fluorescence-labeled anti-rabbit immunoglobulin G) for 2 h at room temperature. The cells were washed and mounted for confocal-microscopic visualization with Slow Fade (Molecular Probes, Eugene, OR).

Statistics

The results are means ± SE of n experiments unless otherwise indicated. The statistical significance of the differences between the means of multiple groups or the means of an individual group at multiple time points was determined by one-way analysis of variance followed by Newman-Keuls multiple intergroup comparisons. Student’s unpaired two-tailed t-test was used to assess significance between two groups. P < 0.05 was considered statistically significant.

RESULTS

Effect of Mφs on the Bioelectric Properties of the Epithelium

In a previous study, Compeau et al. (7) demonstrated that treatment of a Mφ-DLEC coculture system with LPS significantly reduced both Iₛₑ and R of the DLEC monolayers. To study Iₛₑ independent of alterations in monolayer R, we performed initial studies in which we...
varied the number of Mϕs in the coculture. As reported, high numbers of Mϕs (3 × 10^6/filter) significantly reduced I_sc compared with control monolayers or monolayers exposed to LPS or Mϕs in the absence of LPS (Fig. 1A). By contrast, lesser numbers of Mϕs (0.5–2.0 × 10^6/filter) stimulated with LPS had no effect on total I_sc. Similarly, LPS-stimulated Mϕs at concentrations ≤ 2 × 10^6/filter did not alter baseline resistance of the DLEC monolayers (Fig. 1B). These studies thus established conditions in which we were able to study the effect of stimulated Mϕs on DLEC monolayer I_sc independent of an alteration in monolayer R.

Because our previous studies had demonstrated that LPS-stimulated Mϕs induced a reduction in amiloride-sensitive I_sc several hours before having an effect on total I_sc, we postulated that the lower numbers of Mϕs might exert a selective effect on amiloride-sensitive I_sc.

To test this possibility, amiloride was added after the measurements had stabilized to determine the amiloride-sensitive component of I_sc. O’Brodovich and colleagues (28, 30) and others (3, 34) have previously demonstrated that amiloride decreases Na^+ transport without affecting total transepithelial R. Although total I_sc did not differ among groups, DLEC monolayers incubated with LPS-treated Mϕs (2 × 10^6/filter) demonstrated a marked reduction in the amiloride-sensitive component of the I_sc compared with all other groups (Fig. 2A, Table 1). A time course of this effect demonstrated that some degree of inhibition occurred by 4 h and then was progressive over the next several hours (Fig. 2B). By 16 h, DLEC exposed to LPS-treated Mϕs exhibited essentially no measureable amiloride-sensitive I_sc. This effect was present whether Mϕs were in direct contact with the DLECs (contact) or were physi-
Table 1. Effect of Mφs on short-circuit current in DLECs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Total, μA/cm²</th>
<th>%Amiloride sensitive</th>
<th>%Flufenamic acid sensitive</th>
<th>%Residual</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control DLECs</td>
<td>43</td>
<td>4.26 ± 0.21</td>
<td>67 ± 3</td>
<td>19 ± 3</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>LPS + DLECs</td>
<td>22</td>
<td>4.84 ± 0.34</td>
<td>58 ± 6</td>
<td>17 ± 5</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>Mφs + DLECs</td>
<td>12</td>
<td>3.23 ± 0.20</td>
<td>66 ± 5</td>
<td>10 ± 2</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>LPS + Mφs + DLECs</td>
<td>15</td>
<td>4.89 ± 0.34</td>
<td>7 ± 2†</td>
<td>44 ± 6*</td>
<td>49 ± 8</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>4.93 ± 0.34†</td>
<td>10 ± 3†</td>
<td>52 ± 7†</td>
<td>38 ± 6†</td>
</tr>
</tbody>
</table>

Data are means ± SE; n, no. of experiments. Mφs, macrophage; DLEC, distal lung epithelial cell; LPS, lipopolysaccharide. %Residual, percentage of short-circuit current remaining after addition of amiloride (10⁻⁴ M), flufenamic acid (5 × 10⁻⁴ M), and bumetanide (10⁻⁴ M). In all groups, bumetanide had little (<1%) effect on short-circuit current. Significantly different from control DLECs: *P < 0.01; †P < 0.001 (by analysis of variance).

Induction of DLEC iNOS Expression by Stimulated Mφs

Figure 3 demonstrates the ability of L-NMMA to prevent the loss of amiloride-sensitive I_sc caused by LPS-treated Mφs in coculture as previously reported (7). L-NMMA also reversed the flufenamic acid-inhibitable component back toward control levels [18.0 ± 2.1% (SE); n = 8]. The ability of Mφs to alter amiloride-sensitive I_sc in DLEC monolayers combined with our previous studies indicating a role for NO in this process suggested the possibility that soluble Mφ factors might upregulate iNOS expression in DLECs. To test this hypothesis, Mφs and DLECs were cultured physically separated but bathing within the same medium in the presence or absence of LPS (see MATERIALS AND METHODS). At various time points, Mφs and DLECs were separately recovered and analyzed for iNOS mRNA and protein expression. Figure 4A demonstrates the level of iNOS mRNA at 16 h in DLEC monolayers cultured in the presence or absence of Mφs with or without LPS treatment. iNOS mRNA was detected in DLEC monolayers exposed to both Mφs and LPS but not in any of the other groups. The time course of this induction is shown in Fig. 4B. iNOS expression in DLECs cultured in the presence of Mφs and LPS was evident by 2 h, reached a maximum at 6 h, and began to fall by 12 h. LPS-treated Mφs recovered from the same study showed clear evidence of iNOS mRNA expression at 2 h, and this increased steadily over the 12-h time course studied (Fig. 4C).

The time course of iNOS protein expression is illustrated in Fig. 5. No detectable iNOS protein was seen in control DLECs over the 10-h study period. By contrast, epithelial cells cultured in the presence of Mφs and LPS demonstrated a small amount of iNOS protein by 4 h,

![Image](http://ajplung.physiology.org/)
which increased to maximum values between 6 and 10 h. At 6 h, Mφs recovered from the same study expressed a large amount of iNOS protein. The induction of iNOS protein activity was reflected in increased nitrite release by the Griess reaction. To detect nitrite produced by DLECs, two approaches were taken. First, we generated Mφ supernatants by treating cells with LPS or vehicle overnight and then added this cocktail to DLECs. The cocktail was washed away after 6 h, the DLECs were thoroughly washed, and then the DLECs were further incubated in fresh medium for 16 h. Nitrite levels in the supernatant were then measured. The nitrite level increased from undetectable in studies in which the cocktails were derived from unstimulated Mφs to 14.6 ± 3.9 µM (n = 6 experiments) in studies in which the cocktails were from LPS-treated Mφs. Second, Mφs were cocultured physically separated from DLECs but bathing in the same medium in the presence or absence of LPS for 6 h. The cells were then washed, and the production of nitrite was detected in DLECs cultured separately in fresh medium for 16 h. Exposure of DLECs to LPS-stimulated Mφs increased nitrite release to 71.6 ± 4.2 µM (n = 6 experiments). By contrast, LPS-treated Mφs (3 × 10⁶) released 204.6 ± 3.0 µM (n = 3 experiments). LPS-treated Mφs in the absence of DLECs repeatedly released small concentrations of nitrites (<5 µM). These data thus support the iNOS gene and protein expression studies, demonstrating that iNOS activity is less in DLECs than in Mφs.

Role of NO as a Mediator of Altered Bioelectric Properties in DLECs

After iNOS expression in LPS-stimulated DLECs was demonstrated, studies were performed to discern the role of NO as the effector molecule of the observed changes in amiloride-sensitive Iₘ. A previous study (7) showed that L-NMMA was able to prevent Mφ-mediated inhibition of DLEC Iₘ, suggesting that NO was involved in the inhibitory effect. To determine whether NO generation was sufficient to account for the reduction in amiloride-sensitive Iₘ, DLEC monolayers cultured on porous substrata were treated with the NO donor SNAP and evaluated for alterations in bioelectric properties. SNAP (0.1 mM) had no effect on amiloride-sensitive Iₘ, whereas higher concentrations of SNAP (1 mM) reduced baseline R to <50 Ω·cm². At the higher

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Fig. 4. Inducible nitric oxide synthase (iNOS) gene expression in DLECs. A: level of iNOS mRNA expression in DLECs cultured in presence (+) or absence (−) of Mφs with or without LPS treatment. In DLE-Mφ cocultures, cells were physically separated to permit recovery of DLECs alone. At 16 h, DLECs were recovered and subjected to Northern analysis for iNOS as described in MATERIALS AND METHODS. Blots were stripped and reprobed with cDNA probe for α-tubulin. Data are representative of 3 separate studies. B: time course of induction of iNOS mRNA in control DLECs or DLECs incubated in presence of LPS and Mφ. In DLEC-Mφ cocultures, cells were physically separated to permit recovery of DLECs alone. Data are representative of 3 separate studies. C: time course of induction of iNOS mRNA in Mφs exposed to LPS. Mφs were treated with LPS for the indicated times and recovered for Northern blot analysis as described in MATERIALS AND METHODS. Data are representative of 3 separate studies.
concentration of SNAP, nitrite release by DLECs approximated that generated by DLECs exposed to LPS-treated Mφs. Combined with the findings from using L-NMMA (Fig. 3A), these data suggest that NO is necessary but not sufficient to account for the changes in amiloride-sensitive $I_{sc}$ seen in DLECs after exposure to LPS-treated Mφs.

A recent study (17) has implicated peroxynitrite as playing an important role in NO-mediated effects on target cells including DLECs. To discern the generation of peroxynitrite in DLECs when cultured with LPS-stimulated Mφs, cocultures were blotted with anti-nitrotyrosine antibodies and studied by confocal microscopy. As shown in Fig. 6, nitrotyrosine residues were detected in Mφs in LPS-treated Mφ-DLEC cocultures but not in DLECs. Specificity was confirmed by the lack of fluorescence when the primary antibody was omitted (Fig. 6C) or competed with peptide (Fig. 6D).

**DISCUSSION**

Resolution of pulmonary edema after acute lung injury is dependent on the successful treatment of the primary process responsible for fluid leakage into the air space coupled with efficient fluid reabsorption from the alveolar space. Fluid transport out of the alveolar space is largely mediated by epithelial cell vectorial transport of Na$^+$ from the apical to the basolateral surface (reviewed in Ref. 27). The clinical importance of this epithelial Na$^+$ transport mechanism is illustrated by data showing that survival from either high pressure (congestive heart failure) or high permeability (ARDS) correlated with the ability of the lungs to concentrate its air space fluid (26), presumably via the active absorption of Na$^+$ and Cl$^-$, with water following. Such a speculation was supported by a recent report (38) that demonstrated that the distal lung regions of...
the human lung also absorb fluid by active Na\(^+\) transport. In the present study, we have utilized an in vitro model to investigate cellular interactions that might contribute to reduced Na\(^+\) transport by DLECs, leading to impaired recovery from pulmonary edema. We originally chose to study M\(\phi\)s because of data showing their potential role in the pathogenesis of lung injury during the early stages before neutrophil influx. In addition, their ability to release a diverse range of inflammatory mediator molecules coupled with their close apposition to DLECs in vivo suggested the possibility that they might be capable of modulating DLEC function. Using a M\(\phi\)-DLEC co-culture system, we demonstrated that M\(\phi\) products released in response to LPS are able to induce iNOS gene and protein expression in DLECs and stimulate iNOS activity in these cells. The NO generated in this system is necessary but not sufficient to cause the observed reduction in the amiloride-sensitive \(I_{sc}\) and the increase in the flumenamic acid-sensitive component of total \(I_{sc}\) in DLECs. This occurred without affecting total \(I_{sc}\) or the R of the monolayer. These observations suggest a mechanism whereby NO produced locally in the lung might interact with other proinflammatory molecules to exert effects that impair Na\(^+\) transport during acute lung inflammation.

Increased iNOS expression with enhanced local production of NO in the lung has been demonstrated after systemic LPS administration in rats (21, 25, 41, 46). Several possible cellular sources for NO generation in this setting have been suggested, including both interstitial and alveolar M\(\phi\)s, DLECs, bronchial epithelial cells, fibroblasts, and pulmonary vascular smooth muscle cells. The present study confirms the ability of M\(\phi\)s and DLECs to express iNOS in response to appropriate stimuli. Importantly, they provide a physiological model whereby cells might interact to augment NO production. Specifically, although LPS alone was unable to induce iNOS in DLECs, co-culture with M\(\phi\)s in the presence of LPS resulted in iNOS expression. This occurred when M\(\phi\)s and DLECs were physically separated to allow evaluation of the cell source of iNOS. These data suggest that M\(\phi\)s products released in response to LPS treatment induced iNOS expression in the DLECs. This is supported by the temporal delay in iNOS expression by the DLECs, presumably the time required for LPS to stimulate the synthesis and release of M\(\phi\) products into the medium. The nature of these products was not evaluated in the present study. However, other investigators have defined various cytokines that either alone or in combination induce DLEC iNOS expression. These include interleukin-1\(\beta\), tumor necrosis factor-\(\alpha\), and interferon-\(\gamma\) (13, 33, 35, 36). A recent study by Gutierrez et al. (13) reported that LPS alone is capable of stimulating a modest amount of NO release from DLECs. However, this effect was observed at a later time point (>24 h) than that evaluated in the present study. In relative terms, M\(\phi\)s appear to be much more potent generators of NO than DLECs in the in vitro setting. In the context of the in vivo setting, the close apposition of M\(\phi\)s to DLECs in vivo would readily permit M\(\phi\)-derived NO as well as endogenous production by DLECs to contribute to NO-mediated events occurring in the DLECs.

Although NO appears to be necessary for the reduction in amiloride-sensitive \(I_{sc}\) in DLEC monolayers, the data suggest that it must act in concert with other cellular products to exert its effects. One possible candidate is peroxynitrite. Peroxynitrite is generated through a chemical reaction of NO with superoxide anion (18). In separate studies, this molecular species was shown to be secreted by activated M\(\phi\)s into the epithelial cell lining fluid (19) and also to inhibit amiloride-sensitive \(^{22}\text{Na}\) uptake in alveolar type II cells (17). The half-life of this molecule is <1 s, making it unlikely that M\(\phi\)-derived peroxynitrite is the active species when the cells are physically separated in the in vitro separate-culture model. However, this does not preclude an effect in vivo where the cells are closely apposed. In this regard, the presence of nitrotyrosine residues has been reported in histological sections of lung tissue with ARDS due to sepsis (14). Localization along the blood-gas barrier is consistent with an effect of peroxynitrite on DLECs (14). In the present study, we were unable to detect nitrotyrosine residues in DLECs as evidence of their exposure to peroxynitrite. Although this suggests that peroxynitrite was not involved, we cannot totally rule out the possibility of an inadequate sensitivity of the immunohistochemistry in the DLECs. Alternatively, S-nitrosothiols produced by the interaction of NO with thiols may mediate the effect of NO on DLECs. S-nitrosothiolase, the predominant form found in alveolar lining fluid, has a prolonged half-life (<3 h) and bioactivity that includes bronchodilation, inhibition of receptor-ligand interaction, and inhibition of enzyme function (11). Importantly, S-nitrosothiolase levels were found to be markedly increased in the lavage fluid of patients with pneumonia, suggesting a possible role for this product in vivo (11).

The mechanism whereby NO and its cofactor might exert their effects on DLECs requires further study. Many of the effects of NO and its by-products are known to be mediated via a guanosine 3',5'-cyclic monophosphatase (cGMP)-dependent pathway. There are well-characterized NSC channels in epithelial cells of the rod retina (8) and inner medulla collecting duct of the kidney (24) that are respectively upregulated and downregulated by cGMP. Studies from our group demonstrated that neither atrial natriuretic peptide (the second messenger of which is cGMP) nor 8-bromo-cGMP (a membrane-permeant analog of cGMP) altered the bioelectric properties of DLECs within 30 min of exposure (29). However, our present and previous work has demonstrated that the LPS-treated M\(\phi\) effect requires several hours of incubation. This longer time-dependent effect may indicate additional mechanisms, possibly related to cGMP generation. Another possible explanation relates to the recent report by Rotin et al. (37) indicating that cytoskeleton-Na\(^+\)-channel interactions determine the apical localization of the \(\alpha\)-subunit of the epithelial cell Na\(^+\) channel in DLECs.
with a previous study by Compeau et al. (7) demonstrating that LPS-treated Mφs induced cytoskeletal changes in DLECs, these data suggest an effect related to altered traffic to or retention of Na+ channels at the apical membrane. In this regard, S-nitrosoglutathione has been shown to stimulate ADP ribosylation of F-actin in neutrophils (5). Finally, NO or its metabolites may modulate both transcriptional and posttranscriptional events in the cell (16, 44), suggesting a possible effect on expression of the Na+ channel itself or of its regulatory proteins.

The Ussing chamber studies demonstrated that the ion-transport characteristics of the DLECs were markedly changed as a result of exposure to LPS-stimulated Mφs; there was a virtual disappearance of amiloride-sensitive Isc with a concomitant increase in its clalenameric acid sensitivity. Normally, ~70% of the Isc of the DLEC monolayer is amiloride and benzamil sensitive (28–30). It has been previously demonstrated that the amount of amiloride-sensitive Isc is increased when DLECs are cultured in serum-free medium (29), whereas it is decreased when DLECs are cultured from more immature fetal rat lungs (34) or when DLECs are grown on an immature fetal lung cell-derived matrix (32). Exposure of rats to sublethal hyperoxia has also been shown to upregulate non-amiloride-inhibitable Isc (15, 47). The ionic nature of the amiloride-insensitive Isc is not completely understood. The weight of evidence suggests that the amiloride-insensitive Isc is Na+ transport. Previous studies have demonstrated that the Isc of the DLECs is entirely dependent on the presence of Na+ in the bathing medium (28), that amiloride-insensitive $^{22}$Na+ transport is present in tracheal epithelium (23), and that flufenamic acid, which inhibits NSC channels in colonic epithelium (39), markedly decreased the Isc in LPS-Mφ-exposed DLEC monolayers. In addition, as previously described (28, 34), the classic inhibitor of Cl− secretion, bumetanide, had no influence on DLEC Isc. However, Pitkanen et al. (32) have noted that Cl− depletion has a modest effect on the DLEC Isc and flufenamic acid has also been reported to block Ca2+–activated Cl− channels in Xenopus laevis oocytes (45). Therefore, one cannot rule out the possibility that some Cl− secretion is present in the DLECs but that the basolateral entry pathway for Cl− is via an Na+–dependent bumetanide-insensitive transporter.

In summary, the present study demonstrates that cell-cell interactions in the lung during inflammation may impair fluid resorption and thus resolution of pulmonary edema. The central role for NO generation in this process suggests alternative approaches to the prevention and treatment of lung injury.

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REFERENCES


17. Hu, P., H. Ischiropoulos, J. S. Beckman, and S. Matalon. Peroxynitrite inhibition of oxygen consumption and sodium...


