Preventing Endotoxin-Stimulated Alveolar Macrophages from Decreasing Epithelium Na⁺ Channel (ENaC) mRNA Levels and Activity

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ABSTRACT

The acute respiratory distress syndrome is characterized by impairment of the alveolar-capillary barrier. Our laboratory has shown that distal lung epithelial cell (DLEC) amiloride-sensitive Na⁺ transport is impaired by *in vitro* coculture with endotoxin (lipopolysaccharide)-stimulated alveolar macrophages (AM) through an L-arginine-dependent mechanism. To investigate the effect of this model on mRNA levels of the rat epithelial Na⁺ channel, mature fetal rat DLEC monolayers were incubated for 16 h with rat AM (1×10^7) and lipopolysaccharide (10 $\mu\text{g/mL}$), or the cell-free supernatant of lipopolysaccharide-stimulated rat AM. Such exposure resulted in a profound decrease in mRNA expression for all subunits (α , β , and γ) of the rat epithelial Na⁺ channel, without affecting 18S RNA levels. This effect was prevented by the antioxidant N-acetylcysteine. In separate experiments, confluent DLEC monolayers were exposed to lipopolysaccharide-stimulated AM supernatant for 16 h with or without N-acetylcysteine and studied in Ussing chambers. As previously demonstrated in our laboratory, AM supernatant resulted in a significant ($p < 0.05$) impairment of DLEC Na⁺ transport, as reflected by a decrease in the amiloride-sensitive component of short-circuit current (control, $3.96 \pm 0.18 \mu\text{A/cm}^2$ versus supernatant, $2.34 \pm 0.56 \mu\text{A/cm}^2$; $p < 0.05$). This effect was significantly reversed by N-acetylcysteine (3.55 ± 0.48

$\mu\text{A/cm}^2$), but not by DTT ($1.87 \pm 0.21 \mu\text{A/cm}^2$). N-acetylcysteine, but not DTT, increased DLEC thiol levels. These studies elucidate mechanisms by which activated AM impair alveolar epithelial barrier function in an *in vitro* model of acute lung injury. (*Pediatr Res* 48: 304–310, 2000)

Abbreviations

AM, alveolar macrophages
ARDS, adult respiratory distress syndrome
ALI, acute lung injury
DLEC, distal lung epithelial cells
ENaC, epithelial sodium channel
FBS, fetal bovine serum
GSH, glutathione
I_{sc}, short-circuit current
LPS, lipopolysaccharide
MEM, minimum essential medium
NAC, N-acetyl cysteine
NAME, N^ω-nitro-L-arginine methyl ester
NO, nitric oxide
PD, transepithelial potential difference
rENaC, rat epithelial sodium channel
ROS, reactive oxygen species

ARDS, or ALI, represents an important cause of mortality in critically ill patients (1). Both direct lung injury and distant insults (1), most commonly nonpulmonary sepsis, can initiate a process resulting in a fundamental derangement of the intrinsic barrier property of the lung, manifested clinically as high-permeability pulmonary edema, which is one characteristic of

ARDS. It is widely held that these devastating changes most commonly arise because of widespread and uncontrolled activation of inflammatory cells, including neutrophils and macrophages, with the release of a wide range of potentially injurious mediators, such as cytokines, proteolytic enzymes, biologically active lipids, and ROS (2). Activation of inflammatory cells could, in turn, have deleterious effects on the ability of alveolar epithelia to clear edema fluid.

The failure of the alveolar-capillary membrane, the structure responsible for preserving a fluid-free alveolar space vital for adequate gas exchange, has been the focus of increasing attention in continuing efforts to understand the basic mechanisms

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of ARDS. Evidence from numerous *in vitro* and *in vivo* studies has implicated alveolar epithelial active Na^+ transport, with resulting water movement out of the alveolus, as an integral component of alveolar pulmonary edema fluid clearance (3, 4). The favored model for this Na^+ transport involves apically located amiloride-sensitive Na^+ channels, which transport Na^+ down a favorable electrochemical gradient maintained by basolaterally located ouabain-sensitive $\text{Na}^+\text{-K}^+\text{-ATPases}$.

Previous work in our laboratory has resulted in the development of an *in vitro* model to study the relationship between sepsis and the ability of the alveolar epithelium to transport Na^+ and water (5). Primary monolayer cultures of DLEC, which have similar bioelectric properties to adult type II epithelial cells (6, 7), were exposed to LPS-stimulated AM, and their bioelectric properties were subsequently studied in Ussing chambers. Coculture of DLEC with LPS-activated AM resulted in a significant decrease in amiloride-sensitive epithelial I_{sc} , indicating impaired Na^+ transport. Studies in both our laboratory and those of others have suggested a role for NO in this impaired Na^+ transport (5, 8–10).

The amiloride-sensitive component of I_{sc} is a surrogate measurement for the function of amiloride-sensitive Na^+ channels in the apical membranes of DLEC. The functional cloning of the three subunits, α , β , and γ , of the rENaC (11–13) enabled us to investigate potential mechanisms by which activated AM decrease DLEC I_{sc} . This channel complex has been identified in a number of Na^+ -transporting epithelia (12), including lung epithelium (14), with αENaC being essential for channel function (12). Amiloride-sensitive Na^+ transport (15), mediated by ENaC (16), is critical for normal fluid clearance from the alveolar space at birth.

The goal of the present studies was to determine the effect of direct or indirect contact with LPS-activated AM on rENaC mRNA levels. Because our studies demonstrated that LPS-activated AM decreased rENaC mRNA levels, as well as amiloride-sensitive I_{sc} , additional experiments were performed to determine whether antioxidants could modulate the effect of the LPS-activated AM on Na^+ transport by DLEC.

METHODS

Materials and solutions. Tissue culture media and additives including Hanks' balanced solution (HBSS with and without Ca^{2+} and Mg^{2+}), FBS, Dulbecco's PBS, Eagle's minimum essential media (MEM), and MEM selectamine kits were all obtained from GIBCO Laboratories (Grand Island, NY, U.S.A.). Collagenase and deoxyribonuclease for epithelial cell harvest were purchased from Worthington Biochemicals (Freehold, NJ, U.S.A.). Trypsin and penicillin-streptomycin were obtained from GIBCO Laboratories. Endotoxin (*Escherichia coli* 0111:B4) was from Difco Laboratories (Detroit, MI, U.S.A.). NAC was obtained from Bristol Laboratories of Canada (Montreal, Quebec), DTT was purchased from Sigma Chemical Co.-Aldrich Canada (Mississauga, Ontario), and NAME was purchased from Calbiochem (La Jolla, CA, U.S.A.). [^{14}C]adenine used in epithelial cytotoxicity assays ([8- ^{14}C]adenine hydrochloride, 58.5 mCi/mL) was obtained from Du Pont (Boston, MA, U.S.A.). Amiloride, bumetanide,

flufenamic acid, and Triton X-100 were all obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Epithelial cell isolation and culture. DLEC were harvested from late-gestation fetal rats (20- or 21-d gestation; term, 22 d) and grown in primary culture according to methods previously described (6). These cells are 99% epithelial as assessed by cytokeratin immunohistochemistry. The harvested epithelial cells were immediately seeded onto 100- or 150-mm-diameter tissue culture dishes (Falcon, Mississauga, Ontario), or tissue culture-treated Snapwell porous polycarbonate filters (Costar, Cambridge, MA, U.S.A.). Seeding density was 3×10^5 cells/cm² for tissue culture dishes, and 1×10^6 cells/cm² for Snapwell filters. All cells were grown in MEM with 10% FBS and penicillin-streptomycin at 37°C in a humidified 95% air/5% CO_2 environment. Nonadherent epithelial cells were removed 24 h after seeding. Epithelial monolayers used in Ussing chamber experiments were studied after 3 or 4 d in culture, whereas those used for total RNA extraction were grown to confluence for a minimum of 2 d before study.

AM isolation and culture. AM were isolated from adult female Wistar rats (190–210 g) as previously described (5). Cell viability, as determined by trypan blue exclusion, was consistently >95%, and >92% of cells were AM, as demonstrated with Wright's staining. AM were then immediately cultured, either in the presence of DLEC (see below), or alone, for the purposes of supernatant acquisition. Those cultured alone were either plated on 100-mm-diameter tissue culture dishes (1×10^7 cells/dish) in 10 mL of MEM with 10% FBS and penicillin-streptomycin, or in six-well plates (Costar, 2.25×10^5 cells/well) in 2.25 mL of the same medium, and exposed to 10 $\mu\text{g/mL}$ LPS for 18–24 h. Cell-free supernatants, after centrifugation at $8000 \times g$ for 30 s, were then added to DLEC monolayers previously cultured in 100-mm tissue culture dishes or porous filters (see below). All animal research protocols were approved by the Animal Care Committee of the Toronto Hospital (Toronto General Division) and The Hospital for Sick Children Animal Research Ethics Committee, and animals were cared for according to the "Guide to the Care and Use of Experimental Animals" formulated by the Canadian Council of Animal Care.

Northern analyses. DLEC total RNA was extracted (17), and 15 μg of total RNA underwent electrophoresis in a 1% agarose gel containing formaldehyde, then was blot-transferred to a Hybond-N⁺ nylon membrane (Amersham, Buckinghamshire, UK), baked at 80°C for 10 min, and then UV cross-linked. Membranes were then prehybridized and hybridized with solutions containing 50% deionized formamide, $5\times$ Denhardt's solution, 1% SDS, $5\times$ sodium chloride sodium citrate buffer (SSC), and 0.2 mg/mL herring sperm DNA. Hybridization was performed overnight with ^{32}P random-labeled (Multiprime DNA kit, Amersham) full-length or subunit-specific short cDNA probes for one or more of α -, β -, and γENaC (11, 12). Blots were then washed with the high-stringency solution consisting of $0.1\times$ SSC and 0.1% SDS at 50°C for 30 min. Transcripts were visualized using standard autoradiography or phosphorimaging. After stripping the blots in a solution containing 1 mM Tris-HCl, 1 mM EDTA, and 20% SDS, RNA integrity and comparable RNA loading between lanes was

assessed by hybridization of membranes with a cDNA probe for mouse 18S RNA (American Type Culture Collection, Rockville, MD, U.S.A.). Final wash used $0.1 \times$ SSC and 0.1% SDS at 65°C for 15 min.

Bioelectric properties of the DLEC monolayer. As previously described (5), DLEC monolayers cultured on Snapwell filters were studied by placing them in Ussing chambers (MRA, Clearwater, FL, U.S.A.) that contained warmed HBSS and 22.4 mM NaHCO_3 , which was circulated using an air lift of 95% air/5% CO_2 gas mixture. Measurements of the bioelectric properties of the monolayer were made with a high-impedance millivoltmeter that could function as a voltage-current clamp with automatic fluid resistance compensation (VCC 600 Physiologic Instruments, San Diego, CA, U.S.A.). The transepithelial PD was recorded continuously under open circuit conditions, and 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 calculation of transepithelial resistance using Ohm's law. After stabilization of PD, both at baseline and after the addition of various ion transport inhibitors (see below), transepithelial PD was temporarily clamped to 0 mV so that I_{sc} could be recorded. At the end of each experiment, EDTA (10 mM) was added to both surfaces of the monolayer, and previous measurements were corrected for the observed residual PD.

DLEC cytotoxicity. Confluent epithelial monolayers in primary culture, as described above, were preincubated with 0.2 mCi/mL [^{14}C]adenine for 2 h. DLEC cytotoxicity resulting from specific interventions (see below) was assessed by measuring [^{14}C]adenine levels in cell-free supernatants. At the end of each experiment, epithelial cell monolayers were lysed with Triton X-100 (0.1%) and collected. The percentage of [^{14}C]adenine release during culture was calculated after determination of total radioactivity in both the supernatant and the cell lysate by liquid scintillation counting (1219 Rack Beta Liquid Scintillation Counter, LKB Wallac, Sweden).

Measurement of cellular nonprotein thiols. Cellular non-protein and protein reduced sulfhydryls were quantitated (18) by reaction with acid-soluble and acid-precipitable fractions, respectively, with dithio-bis-nitrobenzoic acid. After washing twice in ice-cold PBS, cells were harvested by scraping into 50 mg/mL ice-cold sulfosalicylic acid. After centrifugation, the supernatant was taken for assay of acid-soluble (nonprotein) reduced sulfhydryl content. To assay, 200 μL of sample was added to 1000 μL of 0.2 M phosphate, 2 mM EDTA, 1 mg/mL SDS, pH 9, and 20 μL of 10 mM dithio-bis-nitrobenzoic acid, mixed, and then allowed to stand for 10 min at room temperature. Sulfhydryl content was determined by measurement at absorption at 412 nm, with results being expressed as nanomoles of reduced sulfhydryl groups as determined from a standard curve constructed using known amounts of the non-protein thiol GSH, one mole of reduced sulfhydryl per mole of reduced GSH.

Effect of products of LPS-stimulated AM on DLEC rENaC mRNA levels. To determine whether a soluble factor(s) released from endotoxin-stimulated AM could modulate DLEC

α -, β -, or γ rENaC mRNA levels, DLEC monolayers cultured for a minimum of 2 d in 100-mm tissue culture dishes were exposed to either AM alone (1×10^7), LPS (10 $\mu\text{g}/\text{mL}$) alone, or AM and LPS together. Control groups consisted of DLEC exposed to culture medium alone. After 2–16 h, total RNA was extracted, and Northern analysis was performed.

Similar experiments were performed involving the physically separated coculture of DLEC and AM. A 60-mm-diameter tissue culture dish was secured with silicone in the center of a larger, 150-mm-diameter tissue culture dish. DLEC were seeded and grown to confluence for at least a 2-d period in the outer well. AM (1.0×10^7) were then added to the central well and allowed to adhere for 30 min at 37°C. Additional culture medium was then added so that common medium bathed both the inner and outer wells. LPS (10 $\mu\text{g}/\text{mL}$) was added to the system, and gentle agitation of these dishes allowed circulation of medium without the displacement of adherent AM into the outer well. Control groups consisted of DLEC exposed to culture medium alone. Again, after 2–16 h, total RNA was extracted, and Northern analysis was performed.

Finally, studies were undertaken to examine the effect of the cell-free supernatant of LPS-stimulated AM alone on DLEC rENaC mRNA levels. DLEC monolayers were cultured for a minimum of 2 d in 100-mm tissue culture dishes. Bathing media was then removed and replaced with the cell-free supernatant of AM (1×10^7) that had been cultured for 18–24 h in the presence of LPS (10 $\mu\text{g}/\text{mL}$), as described above. Control groups consisted of DLEC exposed to culture medium alone. After 16 h, total RNA was extracted, and Northern analysis was performed.

Studies performed previously in our laboratory have demonstrated no evidence of DLEC cytotoxicity resulting from 16 h of direct coculture with LPS-stimulated AM (5). To ensure that such exposure had not in fact initiated a process that would ultimately lead to cell death, similar studies were performed in the physically separated coculture system at later time points, after removal of LPS-stimulated AM-conditioned medium. Confluent epithelial monolayers grown in the outer well of tissue culture dishes, as described above, were exposed to soluble factors released from LPS-stimulated AM grown in the inner well (1×10^7 AM). Coculture in common bathing medium was continued for 16 h. Cell-free supernatants were collected, and sufficient medium was added to bathe only the epithelial cells in the outer well, thereby effectively ending coculture. Culture of DLEC alone was continued for a further 24 h. Cell-free supernatants were again collected, and [^{14}C]adenine release at both times was then determined as an index of DLEC injury.

Modulation of the decrease in DLEC rENaC mRNA levels by antioxidants. Because our experiments demonstrated a decrease in α -, β -, and γ rENaC DLEC mRNA expression as a result of exposure to a soluble factor(s) released from LPS-stimulated AM and coculture with activated AM, experiments were undertaken to investigate how this reduction in steady-state mRNA levels could be prevented. To determine whether treatment with the thiol antioxidants NAC or DTT could modulate this effect, experiments were undertaken involving exposure of DLEC to either LPS-stimulated AM supernatant or

direct coculture with activated AM, as described above, with the addition of NAC (20 mM) or DTT (100 μ M). The supernatant model was used so that the experiment would not be confounded by any effect of the antioxidants on AM activation. Control groups consisted of monolayers exposed to medium alone. After 16 h, DLEC total RNA was extracted, and Northern analysis was performed.

Effect of antioxidants in modulating the LPS-stimulated AM-induced impairment of DLEC Na^+ transport. Given that NAC prevented the decrease in rENaC mRNA levels resulting from DLEC exposure to LPS-stimulated AM supernatant, experiments were conducted to examine the effects of this agent on the L-arginine-dependent impairment of DLEC amiloride-sensitive Na^+ transport induced by soluble AM products after LPS stimulation, previously demonstrated in our laboratory (5). Monolayers, in culture for 3–4 d on porous filters, were exposed to either medium alone or the cell-free supernatant of LPS (10 μ g/mL)-stimulated AM (2.25×10^5 cells/well), with or without NAC (20 mM) or DTT (100 μ M). After 16 h, filters were mounted in Ussing chambers. After determining baseline bioelectric properties, the Na^+ transport blocker amiloride (0.1 mM apically) was added to the monolayer.

Effect of NAC and DTT on DLEC thiol levels. To determine whether NAC or DTT treatment could alter DLEC thiol levels, monolayers of cultured DLEC were exposed to 1 mM NAC or 100 μ M DTT for 24 h. DLEC were then harvested, and reduced nonprotein and protein sulfhydryl levels were measured.

Effect of NO synthase inhibitor on activated macrophage-induced decrease in DLEC mRNA levels. Because our laboratory has shown that AM modulate DLEC bioelectric properties by an L-arginine-dependent mechanism (5), we incubated DLEC in the presence of AM with and without 0.2 mM NAME and measured steady-state mRNA levels of α -, β -, and γ rENaC using standard Northern blotting methods.

Statistical analysis. Results are presented as mean $\pm$ 1 SD of n experiments, unless otherwise indicated. The statistical significance of differences between the means of multiple groups or the means of an individual group at multiple time points was determined by one-way ANOVA followed by Newman-Keuls multiple intergroup comparisons. Probability <0.05 was considered statistically significant.

RESULTS

Effect of products of LPS-stimulated AM on DLEC rENaC mRNA expression. DLEC exposed to control medium only, as well as AM alone, expressed significant levels of α -, β -, and γ rENaC (Fig. 1; α rENaC, 0.73 ± 0.19 for AM control versus 0.38 ± 0.16 activated AM; β rENaC, 0.82 ± 0.33 versus 0.44 ± 0.21 ; γ rENaC, 0.64 ± 0.28 versus 0.25 ± 0.16 ; $n = 8$; $p < 0.05$; all values were normalized first to the 18S RNA and then to the media control). Although LPS alone decreased the level of mRNA for all three subunits of the rENaC, the effect of LPS was not statistically significant from that of cells incubated with media alone. (1.00 ± 0.00 versus 0.55 ± 0.21 for α rENaC; 1.00 ± 0.00 versus 0.97 ± 0.29 for β rENaC; 1.00 ± 0.00 versus 0.69 ± 0.21 for γ rENaC; $p > 0.05$; $n = 5$;

Figure 1. Direct LPS-stimulated AM coculture decreases DLEC rENaC mRNA levels. The level of α -, β -, and γ rENaC mRNA in DLEC cultured for 16 h in the presence or absence of AM with or without LPS is shown. Control monolayers were exposed to media only. Levels of 18S RNA were used to assess RNA integrity and loading. rENaC mRNA was significantly decreased in DLEC exposed to LPS-stimulated AM. These data are representative of three separate studies.

all densitometry were first normalized over 18S RNA, and then to the media control group). The rENaC mRNA levels were significantly decreased, however, in DLEC exposed to LPS-stimulated AM for 16 h in the direct coculture system ($p < 0.05$, Fig. 1). Similar results were obtained after 16 h of physically separated coculture (data not shown), implicating a role for a soluble factor(s) released from AM exposed to LPS. Studies were undertaken to examine changes in DLEC rENaC mRNA expression over time after both direct and physically separated coculture with LPS-stimulated AM. The level of α rENaC decreased after 8 h of indirect coculture (Fig. 2).

DLEC cytotoxicity. DLEC exposed to LPS-stimulated AM in physically separated coculture for 16 h showed no significant difference in [14 C]adenine release compared with cells exposed to medium alone ($30.3 \pm 1.2\%$ versus $37.0 \pm 4.0\%$ of total release resulting from Triton-X treatment; $p = \text{NS}$). Similarly, no evidence of DLEC cytotoxicity was seen 24 h after the removal of the medium conditioned by the activated AM ($41.1 \pm 1.0\%$ versus $47.3 \pm 4.5\%$ for control monolayers; $p > 0.05$).

Prevention of the decrease in DLEC rENaC mRNA levels by antioxidants. NAC was added to DLEC monolayers exposed to the cell-free supernatant of LPS-stimulated AM or direct coculture with activated AM. Sixteen-hour exposure of DLEC to stimulated AM supernatant resulted in decreased

Figure 2. Time course of changes in DLEC α rENaC mRNA levels resulting from physically separated coculture with LPS-stimulated AM. The level of α rENaC mRNA expression over time in DLEC physically separated from LPS-stimulated AM, as described above, is shown. α rENaC mRNA expression was consistently decreased after 8 h of coculture in the presence of LPS. These data are representative of four separate time course studies. Similar results were obtained for β - and γ rENaC (data not shown).

mRNA levels for α rENaC subunits, which was prevented by NAC (α rENaC, 1.00 ± 0.17 for media, 0.41 ± 0.13 for supernatant, and 1.63 ± 0.68 for NAC; β rENaC, 1.00 ± 0.28 for media, 0.66 ± 0.37 for supernatant, 2.69 ± 1.39 for NAC; γ rENaC, 1.00 ± 0.26 for media, 0.26 ± 0.13 for supernatant, and 1.17 ± 0.65 for NAC; $n = 4-6$; $p < 0.05$; all densitometry were first normalized to the 18S RNA and then to the media control; Fig. 3). Similar results were obtained in direct coculture experiments ($p < 0.05$; $n = 5$; data not shown). In contrast, DTT had no such protective effect ($p < 0.05$; data not shown).

Prevention of the LPS-stimulated AM-induced impairment of DLEC Na^+ transport by antioxidants. When DLEC were exposed to the supernatant of LPS-stimulated AM, there was a significant decrease in DLEC amiloride-sensitive I_{sc} , compared with DLEC exposed to medium alone (Fig. 4A). NAC reversed this impairment (Fig. 4A). Baseline bioelectric properties, as summarized in Table 1, were not different between experimental groups. Addition of DTT did not prevent the impairment of DLEC amiloride-sensitive I_{sc} resulting from 16 h of exposure to LPS-stimulated AM supernatant (Fig. 4B).

Effect of NAC and DTT on DLEC thiol levels. NAC treatment, but not DTT exposure, resulted in a significant increase in DLEC nonprotein and protein reduced sulfhydryl levels (Fig. 5).

Effect of NO synthase inhibitor on activated macrophage-induced decrease in DLEC mRNA levels. Because our laboratory had previously shown that activated AM modulate bioelectric properties of DLEC by an L-arginine-dependent mechanism (5), we incubated some DLEC with and without the NO synthase inhibitor, NAME, in the presence of activated AM. Incubation of DLEC with NAME did not significantly

Figure 3. NAC prevents LPS-stimulated AM supernatant (SUP)-induced decrease in rENaC mRNA levels. The level of α rENaC mRNA in DLEC exposed to the supernatant of LPS-stimulated AM for 16 h, with or without NAC, is shown. Control monolayers were exposed to medium alone. DLEC rENaC mRNA levels were significantly decreased as a result of exposure to AM supernatant, and this reduction was prevented by NAC. These data are representative of two separate studies.

Figure 4. NAC, but not DTT, prevents AM supernatant (SUP)-induced reduction of amiloride-sensitive I_{sc} . A, NAC prevented the reduction of DLEC amiloride-sensitive I_{sc} resulting from exposure to the supernatant of LPS-stimulated AM for 16 h. $n = 5-11$ monolayers per group, $*p < 0.001$ vs DLEC alone. B, DTT failed to modulate the impairment of DLEC I_{sc} resulting from exposure to the supernatant of LPS-stimulated AM. $n = 4-6$ samples per group, $*p < 0.001$ vs DLEC alone.

Table 1. DLEC baseline bioelectric properties

Variable	DLEC (n = 5)	DLEC + Sup (n = 5)	DLEC + Sup + NAC (n = 11)
Total I_{sc} ($\mu\text{A}/\text{cm}^2$)	4.32 ± 0.26	4.66 ± 1.02	4.85 ± 0.71
Resistance ($\Omega \cdot \text{cm}^2$)	1993 ± 391	2011 ± 805	2049 ± 495

Data are presented as mean $\pm$ SD; $p > 0.05$ for all groups. Sup, supernatant.

reverse the decrease of mRNA levels for any of the three subunits of ENaC by activated AM ($p > 0.05$; $n = 5$; data not shown).

DISCUSSION

The ability of the alveolar epithelium to maintain vectorial Na^+ transport is an important property of the alveolar-capillary barrier that helps maintain a fluid-free alveolar space. This study showed that a soluble factor(s) from LPS-stimulated AM significantly reduced DLEC α rENaC mRNA levels, as well as DLEC amiloride-sensitive Na^+ transport. We observed that the antioxidant NAC could significantly prevents both the reduction of DLEC rENaC mRNA levels and the impairment of DLEC Na^+ transport, resulting from exposure to LPS-

Figure 5. NAC, but not DTT, increases DLEC thiols. Nonprotein (NPSH) and protein sulphydryls (PSH) were increased in DLEC after exposure to NAC, whereas similar exposure to DTT resulted in no change in intracellular thiol levels.

stimulated AM supernatant. DTT had no such effects. NAC, but not DTT, significantly increased DLEC thiol levels, suggesting the effect of NAC is related to modulation of intracellular GSH levels, the most abundant intracellular thiol.

This effect on ENaC mRNA levels is unlikely to be related to cytotoxicity. This is shown by previous (5) and present [14 C]adenine release studies. Furthermore, it is demonstrated here that coculture does not initiate a process leading ultimately to cell death because no increase in [14 C]adenine release is seen in DLEC 24 h after the end of coculture. Previous experiments in our laboratory showed that physically separated coculture resulted in an increase in DLEC inducible NO synthase mRNA expression (17), and our present coculture experiments resulted in stable levels of 18S RNA. Therefore, the decrease in rENaC RNA levels cannot be explained by a general depression of DLEC RNA synthesis resulting from coculture.

There is compelling evidence indicating the importance of ENaC in the clearance of fetal lung fluid at the time of birth. The expression of ENaC subunits is developmentally regulated in the rat whole lung and its cultured epithelial cells (14, 19). A recent study described the perinatal course of mice in which mouse α ENaC had been inactivated by gene targeting (16). All newborn knockout mice developed respiratory distress and died shortly after birth as a result of their inability to clear lung liquid, despite otherwise normal morphologic lung development. Whole lungs from these mice had significantly higher wet-to-dry weight ratios compared with wild-type littermates and showed a pattern similar to that seen with previous pharmacologic blockade of Na^+ channels in newborn guinea pigs (15).

In some experiments, ENaC RNA levels were also affected by macrophages that were not activated by endotoxin, probably because of the stress caused by the purification process, or low amount of endotoxin present in some of the reagents. However, rENaC mRNA levels for the non-LPS-treated AM cells were significantly higher than for those treated with endotoxin. Additionally, LPS alone seemed to have a dramatic effect on rENaC mRNA levels, although this was not significantly different from the media group. We believe that although these two treatments individually can affect rENaC mRNA levels, together their effect is additive.

In addition to examining the effect of LPS-stimulated AM products on DLEC rENaC expression, experiments were undertaken to define means to modulate these effects. These studies demonstrate that the thiol antioxidant NAC can block the reduction in DLEC rENaC mRNA levels resulting from exposure to soluble AM products. Further studies showed a similar effect on DLEC Na^+ transport, as NAC prevented the previously demonstrated (5) decrease in DLEC amiloride-sensitive I_{sc} resulting from exposure to the supernatant of LPS-stimulated AM. *In vivo* experimental studies have also suggested a protective effect of NAC in ALI (20), although benefit in patients with ARDS remains unproved (21).

NAC can act as an antioxidant both by a direct radical-scavenging effect (22) and through an increase in intracellular levels of the nonenzymatic antioxidant GSH (23). GSH is the most abundant intracellular thiol (24). NAC can replenish the intracellular GSH lost owing to its oxidation by ROS or peroxynitrite (25), an adduct of NO reactions with ROS, or alternatively modulate transcription factors such as NF- κ B and AP-1, which are known to be involved in cellular oxidative response (26). There are NF- κ B and activator protein-1 (AP-1) sites on the promoter of α ENaC (27).

In contrast to NAC, no specific membrane transport mechanisms exist for DTT, and DTT could not prevent the decrease in amiloride-sensitive I_{sc} . It has previously been demonstrated, and confirmed in the present studies, that NAC exposure results in a significant increase in DLEC thiol levels (28). As shown here, DTT treatment does not affect intracellular thiol concentrations in DLEC. Thus, NAC likely protects by an intracellular mechanism that depends on its ability to support DLEC production of GSH.

Although the NO synthase inhibitor NAME is able to prevent AM-induced decrease in DLEC Na^+ transport (5), in this study NAME was unable to confer any protection against AM-induced decrease in DLEC rENaC mRNA. This highlights the different mechanisms by which Na^+ transport is regulated in DLEC.

The relevance of these studies to ALI will require additional future investigation. These results do suggest cellular mechanisms that may underlie the clinical pulmonary derangements typical of ARDS. Matthay and Wiener-Kronish (3) have shown that a patient's ability to maintain alveolar fluid clearance is correlated to subsequent survival. The studies presented here support the notion that mediators released from AM in the setting of ALI, particularly arising from an infectious insult, may impair the ability of lung epithelium to transport Na^+ , and therefore fluid, through a number of mechanisms. These include effects even at the level of mRNA expression, influencing the most fundamental components of cell ion transport capability. A better understanding of these basic pathophysiologic processes will allow development of more rational, focused therapeutic strategies.

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