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Lipopolysaccharide hyperpolarizes the guinea-pig airway epithelium by increasing the activities of the epithelial Na^+ channel and the Na^+,K^+-pump

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Abstract

Earlier, we found that systemic administration of lipopolysaccharide (LPS; 4 mg/kg) hyperpolarized the transepithelial potential difference (V_t) of tracheal epithelium in the isolated, perfused trachea (IPT) of the guinea pig 18 h after injection. As well, LPS increased the hyperpolarization component of the response to basolateral methacholine, and potentiated the epithelium-derived relaxing factor (EpDRF)-mediated relaxation responses to hyperosmolar solutions applied to the apical membrane. We hypothesized that LPS stimulates the transepithelial movement of Na^+ via the epithelial sodium channel (ENaC)/Na^+,K^+-pump axis, leading to hyperpolarization of V_t. LPS increased the V_t-depolarizing response to amiloride (10 μM), i.e., offset the effect of LPS, indicating that Na^+ transport activity was increased. The functional activity of ENaC was measured in the IPT after short-circuiting the Na^+,K^+-pump with basolateral amphotericin B (7.5 μM). LPS had no effect on the hyperpolarization response to apical trypsin (100 U/ml) in the Ussing chamber, indicating that channel activating proteases are not involved in the LPS-induced activation of ENaC. To assess Na^+,K^+-pump activity in the IPT, ENaC was short-circuited with apical amphotericin B. The greater V_t in the presence of amphotericin B in tracheas from LPS-treated animals compared to controls revealed that LPS increased Na^+,K^+-pump activity. This finding was confirmed in the Ussing chamber by inhibiting the Na^+,K^+-pump via extracellular K^+ removal, loading the epithelium with Na^+, and observing a greater hyperpolarization response to K^+ restoration. Taken together, the findings of this study reveal that LPS hyperpolarizes the airway epithelium by increasing the activities of ENaC and the Na^+,K^+-pump.

Keywords: Endotoxin, lung, airway epithelium, Na^+ transport, transepithelial voltage

Introduction

The airway epithelium forms a barrier between the airway lumen and the lung. It performs many functions, one of which is the regulation of the composition and depth of the airway surface liquid through the transepithelial transport of ions to maintain proper function of the mucociliary escalator (41). The epithelial transport of Na^+ is tightly regulated via the regulation of the open probability (48), membrane expression, and internalization (58) of the epithelial sodium channel (ENaC) and the turnover and membrane expression of the Na^+,K^+-pump (21). The regulation of ENaC activity is not fully understood, but factors which have been identified to regulate ENaC include IL-4 (19), TNF-α (11), IL-1β (67), aldosterone (38), the serine proteases furin and prostasin (51), the ubiquitin ligases neuronal precursor cell expressed developmentally down-regulated (Nedd)4-2 (56), cAMP (65), the thickness and osmolarity of the airway surface liquid (44), and pH (10). The transport of Na^+ in the lung is also up-regulated by catecholamines (1), steroids (2), thyroid hormone (5), and inhibited by hypoxia (40, 47, 68). The Na^+,K^+-pump, which is the rate-limiting step in transepithelial Na^+ transport (21), is regulated by aldosterone and nitric oxide (62).

LPS, which accumulates in the mammalian host upon infection with gram-negative bacteria, stimulates the expression of Toll-like receptors (TLR)2 and 4 in mouse lung epithelium (52) and the production of inflammatory cytokines via the TLR4-mediated activation of p38 (49), ERK1/2 and JNK in human alveolar epithelial cells (23). Both TLR4 and TLR2 are expressed in the human airway epithelium (23, 27, 54). The airway epithelium plays an important role in the immunological defense of the host by responding to and secreting inflammatory mediators (63). Many of these factors, including TNF-α (11, 18), IL-4 (19), TGF-β (64), IL-1β (67) and nitric oxide (15), regulate the rate of airway epithelial Na^+ transport.

Normal ENaC function is vital, and the severe consequences of improper ENaC function are manifested in cystic fibrosis, Liddle's syndrome, and pseudohypoaldosteronism type-I. Mice deficient in αENaC are unable to clear liquid from their lungs and die after birth (31). ENaC hyperactivity in

cystic fibrosis, due to defective Cl^- transport, and in Liddle's syndrome, resulting from the inability to ubiquitinate ENaC due to a mutated PY motif in βENaC, leads to a depletion of the airway surface liquid, plugging of the airways, and susceptibility to lung infection, while ENaC hypo-activity in pseudohypoaldosteronism type-I causes lung edema (50, 51, 56)

LPS modifies the reactivity of the airways to contractile and relaxing agents and affects the bioelectric behavior of the airway epithelium. Endotoxins cause attacks in some types of asthma (25), and are thought to be the causative agent for byssinosis (9). Lung injury from endotoxin-induced inflammation has also been identified as a trigger for acute respiratory distress syndrome (25). Earlier, we observed that administration of LPS *in vitro* to the guinea-pig isolated, perfused trachea (IPT) inhibited the contractile response to methacholine and histamine (17). These effects of LPS on the reactivity to methacholine and histamine involve nitric oxide, which does not seem to play any role in the absence of LPS (17). Systemic LPS potentiated, in the IPT, the relaxant effect of hyperosmolar solution applied apically to the epithelium, which is mediated by the release of epithelium-derived relaxing factor (EpDRF), but had no effect on the reactivity of the airway smooth muscle to methacholine (33). It has been suggested that the regulation of the airway smooth muscle tone by EpDRF is linked to epithelial ion transport (14). Indeed, systemic LPS increased basal V_t, increased the hyperpolarization component of the biphasic methacholine concentration-response curve for bioelectrical responses to methacholine, and potentiated hypertonic NaCl-induced depolarization (33). The effects of systemic LPS require the presence of LPS binding protein, needed for the activation of TLR4 by LPS (61). Therefore, we earlier investigated the *in vitro* effects of LPS and cytokines on the reactivity of the airway smooth muscle and epithelium to methacholine and hyperosmolarity in the absence of LPS binding protein in the IPT and the Ussing chamber (32). Incubating tracheas with LPS, IL-1β, IL-4, IL-13, IFN-γ, and TNF-α alone or in combination caused effects similar to those that *in vivo* LPS treatment had on mechanical responses of the airways to methacholine and hyperosmolar solutions, but had varying effects on V_t and bioelectric responses of the epithelium that did not entirely reproduce the effects of LPS given *in vivo* (32).

In this study, we investigated the effects of systemically administered LPS on Na^+ transport in the guinea-pig airway epithelium. The effects of LPS on ENaC and Na^+,K^+-pump function were investigated using the IPT and the Ussing chamber preparations. We found that LPS hyperpolarizes the airway epithelium, and that the effect of amiloride on V_t is larger in the epithelium from LPS-treated animals compared to controls (33), i.e., amiloride offsets the effect of LPS on V_t. Therefore, we hypothesized that LPS hyperpolarizes the epithelium by increasing the transepithelial transport of Na^+. Using the IPT, we measured the effects of LPS on the activities of ENaC and the Na^+,K^+-pump in the epithelium. LPS increased the activities of both ENaC and the Na^+,K^+-pump. Additionally, the effects of LPS on the activity of the Na^+,K^+-pump were measured in the Ussing chamber using a Na^+-loading technique, and the increase in Na^+,K^+-pump activity by LPS was confirmed. The effects of LPS on the proteolytic activation of ENaC in the epithelium were measured using apical trypsin in the Ussing chamber. The findings indicated that LPS does not increase ENaC activation via proteolytic cleavage. Taken together, the results suggest that LPS increases airway epithelial Na^+ transport by increasing the activities of both ENaC and the Na^+,K^+-pump, and that the effect of LPS on ENaC activity does not involve proteolytic activation.

Materials and Methods

Animals. These studies were conducted in facilities accredited fully by the Association for the Assessment and Accreditation of Laboratory Animal Care International and the research protocol was approved by the Institutional Animal Care and Use Committee. Male Hartley guinea pigs (Crl:Ha 600-700 g) from Charles River Laboratories (Wilmington, MA), monitored free of endogenous viral pathogens, parasites, and bacteria, were used in all experiments. The animals were acclimated before use and were housed in filtered ventilated cages on Alpha-Dri virgin cellulose chips and hardwood Beta chips as bedding, provided HEPA-filtered air, Teklad 7906 diet and tap water *ad libitum*, under controlled light cycle (12 h light) and temperature (22-25 °C) conditions. The animals were anesthetized with sodium pentobarbital (65 mg/kg, i.p.) and sacrificed by thoracotomy and bleeding before removing the trachea.

Isolated, perfused trachea. The IPT, described previously (16, 43), can be used to measure simultaneously the effects of agents on airway diameter and V_t (14). Briefly, 18 h after the *in vivo* administration of LPS (4 mg/kg; i.p.) or a volume equivalent of saline, the animal was anesthetized and a 4.2 cm-section of trachea was excised, cleaned in a dish filled with modified Krebs-Henseleit (MKH) solution aerated with 95% O_2/5% CO_2, and mounted on a perfusion holder. The holder was placed in a bath of MKH (basolateral bath), while the lumen was perfused with MKH from a separate (apical) bath of MKH at a rate of 20 ml/min. Both baths were maintained at 37 °C and aerated with 95% O_2/5% CO_2. Agents could be added to either the apical or the basolateral bath. As described previously (14), indwelling cannulas inserted at the inlet and outlet ends of the tracheal lumen had side holes connected to the positive and negative sides, respectively, of a differential pressure transducer. Changes in the diameter of the trachea could be detected by measuring changes in the difference between the inlet minus the outlet pressures ($\triangle$P), but such measurements were not taken for this study. V_t was measured under open-circuit conditions using Ag/AgCl electrodes with 9% saline bridges in 4% agar. The apical electrode consisted of the side holes of the outlet cannula and

the basolateral electrode consisted of a J-tube placed in the basolateral bath 1 cm from the wall at the center of the trachea. Both electrodes were matched to within 2 mV and connected to a voltage/current clamp amplifier (DVC-1000; World Precision Instruments, Sarasota, Fl); the offset was adjusted to zero mV.

Ussing chamber. The Ussing chamber (CHM8 chamber; World Precision Instruments) was used to measure the effects of LPS-treatment and other agents on V_t and transepithelial resistance (R_t) of the tracheal epithelium. A 4-cm section of the trachea was excised from the animal, cleaned in MKH, cut along the smooth muscle band, and mounted over the aperture (0.125 cm²) between the hemi- chambers. The apical and basolateral faces of the trachea were each bathed with 5 ml of MKH at 37 °C and aerated with 95% O_2/5% CO_2. V_t and R_t were measured under open-circuit conditions using Ag/AgCl electrodes (0.9% NaCl in 4% agar). One pair of electrodes was used to measure voltage; the other was used to apply 5 μA current pulses for 5 s every 50 s to measure a voltage deflection for the calculation of R_t from Ohm's law. Each pair of electrodes was matched to less than 2 mV and the offset was adjusted to zero.

Materials. LPS from *Salmonella enterica* serotype typhimurium (phenol-extracted) was prepared in saline. MKH solution (pH 7.4, osmolarity of 281 ± 5 mosM and temperature of 37 °C) contained 113 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, and 5.7 mM glucose, and was saturated with 95% O_2/5% CO_2. K^+-free MKH lacked KCl and KH_2PO_4, but contained 119 mM NaCl. Reduced Na^+ MKH was prepared by omitting NaCl and replacing it with N-methyl-D-glucamine (NMDG)-Cl (113 mM). Amiloride and amphotericin B from *Streptomyces* were prepared in dimethyl sulfoxide (2.6 mM and 13.5 mM, respectively). Ouabain and trypsin type IX-S from porcine pancreas were dissolved in saline. All reagents were from Sigma-Aldrich (St. Louis, MO).

Data analysis. The unpaired or paired Student's t-tests were used where appropriate with a significance threshold of 5% (SigmaStat version 3.1; Systat Software, Inc.). Otherwise, the Wilcoxon

signed rank test or the Mann-Whitney rank sum test were used where appropriate. Error bars represent the standard errors of the mean. $p < 0.05$ was regarded as significant.

Results

LPS hyperpolarizes the airway epithelium by increasing the transport of Na^+. Basal V_t measured in the IPT was significantly greater in tracheas from LPS-treated animals (−34.2 ± 2.7 mV) compared to controls (−5.5 ± 0.7 mV) (Fig. 1). The ENaC blocker, amiloride (10 μM) (30), was added apically to determine the role of epithelial Na^+ transport in the hyperpolarization. Amiloride inhibited V_t in tracheas from both saline- and LPS-treated animals (Fig. 1). V_t in the presence of amiloride was −4.5 ± 0.9 mV in saline-treated animals and −13.1 ± 1.3 mV in the LPS-treated group. The effect of amiloride, or $\triangle V_t$, was significantly greater (Mann-Whitney rank sum test) in tracheas from LPS-treated animals (21.1 ± 2.8 mV) compared to saline-treated animals (1.0 ± 0.3 mV). These findings indicate that LPS hyperpolarizes the airway epithelium, at least in part, by increasing Na^+ transport via ENaC.

LPS increases the activity of ENaC. The main route for Na^+ transport across the airway epithelium is via ENaC in the apical membrane and the Na^+,K^+-pump in the basolateral membrane (6). LPS could increase transcellular Na^+ transport by increasing the open probability and/or abundance of ENaC (4) and/or by increasing the activity and/or abundance of the Na^+,K^+-pump (22). In order to measure the effects of LPS on ENaC activity in the IPT, the Na^+,K^+-pump was short-circuited with basolateral amphotericin B (7.5 μM), thereby removing the contribution of the Na^+,K^+-pump to V_t (Fig. 2). Concurrently with the administration of amphotericin B, Na^+ was replaced in the basolateral MKH with NMDG-Cl in order to establish a large Na^+ gradient across the apical membrane (Fig. 2). Consequently, any effect of LPS on the activity of ENaC would be measurable as a change in V_t. The response to NMDG-Cl/amphotericin B was complex and consisted of an initial hyperpolarization followed by a decrease to a steady state. The transient hyperpolarization was not different between treatment groups (Fig. 3A). To understand the transient hyperpolarization response, we investigated if it was due to permeabilization or sodium replacement. Nystatin (7.5 μM), a similar pore-forming agent, caused similar transient V_t responses after apical (n = 6) and basolateral (n = 1)

application in the Ussing chamber (data not shown). This effect was not observed when amphotericin B was added alone, but was caused by the removal of basolateral sodium whether before ($n = 2$), concurrently with ($n = 3$), or after ($n = 2$) the addition of amphotericin B (data not shown). Stable V_t was greater in the tracheas from LPS-treated animals, indicating that LPS increases the activity of ENaC (Fig. 3B).

To confirm the role of ENaC in the LPS-induced hyperpolarization under these conditions, apical amiloride (10 μM) was subsequently added. The effect of amiloride was greater in the tracheas from LPS-treated animals compared to controls (Fig. 3C), as it had been in the presence of normal basolateral MKH. Finally, apical trypsin (100 U/ml) was added to activate ENaC. This concentration is within the range reported by the literature that has been used to activate ENaC directly. Hughey *et al.* (28) and Sheng *et al.* (55) used a smaller concentration of 28.2 U/ml to activate ENaC expressed in *Xenopus* oocytes, while Bridges *et al.* (7) used a larger concentration of 336 U/ml. V_t in the presence of trypsin was not different between tracheas from control and LPS-treated guinea pigs (Fig. 3D). Trypsin also had little effect (Fig. 3E) in the presence of amiloride, demonstrating that amiloride had effectively inhibited ENaC. This finding further supports the conclusion that ENaC plays a major role in the LPS-induced hyperpolarization of the airway epithelium.

LPS does not affect the proteolytic activation of ENaC. The finding that LPS increases the activity of ENaC led us to ask if the mechanism involves increases in the proteolytic activation of ENaC. It has been recently discovered that endogenous serine proteases, including furin and prostasin, activate ENaC via the cleavage of an inhibitory domain from the channel's α- and γ-subunits (28, 30, 34). It is thought that the membrane-bound pool of ENaC exists in two states: an uncleaved, near-silent, immature state and a cleaved, activated or mature state (29). The regulation of these serine proteases is not fully understood, but it is thought that ENaC activity can be increased in renal and respiratory epithelia via protease activation or dis-inhibition, thereby shifting the balance of membrane ENaC from the near-silent state to the mature state (50, 51, 57). Trypsin has been used to activate ENaC in ENaC-transfected patch-clamped oocytes (3, 9, 10, 56), cystic fibrotic human

bronchial epithelial cells (7), human airway epithelial cells (13, 60), and increase amiloride-sensitive Na^+ transport in rat (60) and mouse trachea (57).

To determine the effect of LPS on the balance of near-silent vs. mature ENaC, we administered apical trypsin (100 U/ml) in the Ussing chamber while measuring V_t. A greater V_t response to trypsin would reflect a greater pool of near-silent ENaC. If LPS increases cleavage-mediated activation of ENaC, we hypothesized that the V_t response to trypsin should be reduced compared to controls. LPS increased basal V_t , as we found earlier, and basal equivalent transepithelial current (I_{sc}) (Table 1), but did not affect the basal resistance of the epithelium. Trypsin increased V_t and R_t, but did not affect I_{sc}, in tracheas from both control and LPS-treated animals. The trypsin-induced changes in V_t and R_t ($\triangle V_t$ and $\triangle R_t$), and I_{sc}, were, however, not different between the two groups. As well, the $\%\triangle V_t/\%\triangle R_t$ ratio was not different between the untreated and LPS-treated groups. These results indicate that the effects of LPS on the V_t R_t, and I_{sc} responses to trypsin cannot be explained by an increase in transepithelial ion transport. The small increases in R_t by trypsin are surprising and are not understood at present, but have been observed by Swystun *et al* (60).

Greater abundance of mature ENaC after LPS should affect responses to amiloride after trypsin treatment. To investigate this possibility, apical amiloride was administered in the Ussing chamber to tracheas from saline- and LPS-treated animals before and after addition of trypsin (Fig. 4). Apical amiloride was added first to measure basal ENaC activity and washed out, apical trypsin was added to measure channel activation, and, lastly, apical amiloride was administered in the presence of trypsin. As in the IPT (Fig. 1), depolarization by amiloride was greater in the tracheas from LPS-treated animals compared to controls (Fig. 5), but the V_t responses to trypsin were not different (Fig. 5). This finding supports the evidence that the numbers of membrane-bound near-silent channels in the airway epithelium are not affected by LPS treatment. The V_t responses to apical amiloride in the presence of trypsin remained greater in tracheas from LPS-treated animals compared to controls (Fig. 5), indicating that ENaC activity remains elevated after the activation of the near-silent pool of ENaC.

The V_t responses to amiloride in the absence and presence of trypsin were not different from each other within both the saline- and LPS-treated groups, suggesting that the numbers of the membrane-bound near-silent ENaC substrates were small compared to the proteolytically activated pools. Taken together, these data suggest that trypsin had no significant effect on ENaC activity because most of the membrane-bound population of ENaC is in the proteolytically-activated state. Even if LPS would lead to activation or dis-inhibition of channel activating proteases, the number of membrane-bound near-silent ENaC appears to be sufficiently small for them to have no effect on V_t. Therefore, proteolytic activation of ENaC appears not to be involved in the hyperpolarizing effect of LPS in the epithelium, and the abundance and/or ratio of silent and active ENaC is seemingly not affected.

LPS increases Na^+,K^+-pump activity. The finding that LPS increases the activity of ENaC raised the question of whether LPS also affected Na^+,K^+-pump activity. Did increased ENaC activity and intracellular Na^+ concentrations stimulate the Na^+,K^+-pump, or did LPS stimulate the activity of the Na^+,K^+-pump? To address that question, the IPT was used to measure the contribution of the Na^+,K^+-pump to V_t in tracheas from saline- and LPS-treated animals after negating the involvement of ENaC. Amiloride was applied to the apical bath and apical amphotericin B (7.5 μM) was administered to short-circuit ENaC and remove its contribution to V_t (Fig. 6A). V_t at this point, due entirely to the Na^+,K^+-pump, was more than doubled in tracheas from the LPS-treated group compared to controls, indicating that LPS increases Na^+,K^+-pump turnover (Fig. 6B). Finally, apical trypsin was added to confirm that ENaC was inhibited by amiloride, short-circuited by amphotericin B, and did not contribute to V_t. Even though V_t in the presence of trypsin was doubled in the tracheas from LPS-treated animals compared to controls (Fig. 6C), there was no significant effect of trypsin on V_t (Fig. 6D). Taken together, these findings indicate that LPS increases the turnover of the Na^+,K^+-pump in addition to its effects on ENaC.

Additional evidence was sought to determine whether LPS increases Na^+,K^+-pump activity by adapting a Na^+-loading approach, used previously in rabbit arteriole myocytes (26), to measure tracheal epithelial Na^+,K^+-pump activity in the Ussing chamber. Because it has an absolute

requirement for extracellular K^+, the pump was inhibited by removing K^+ from the apical and basolateral MKH solution (Fig. 7A). Being electrogenic, the inhibition of the Na^+,K^+-pump resulted in a depolarization of the epithelium during accumulation of intracellular Na^+. The effect of K^+ removal on V_t was greater in tracheas from LPS-treated animals compared to controls (Fig. 7B), reflecting the greater contribution of pump current in the tracheas from LPS-treated animals. Basolateral K^+ was then restored to the MKH solution to stimulate pump activity and the epithelium repolarized (Fig. 7A). Apical K^+ was subsequently restored and had no effect on V_t (Fig. 7A). The repolarization response after basolateral and apical K^+ restoration was greater in the tracheas from LPS-treated animals compared to controls (Fig. 7B). This finding provides additional evidence that LPS increases the activity of the Na^+,K^+-pump. Finally, the Na^+,K^+-pump inhibitor, ouabain (10 μM), was added basolaterally to confirm the contribution of the Na^+,K^+-pump to V_t, whereupon V_t was decreased. Even though the V_t responses to ouabain were not different between the tracheas from saline- and LPS-treated animals, they were, however, both as large as the responses to K^+ removal, indicating that pump activity was restored by K^+ restoration (Fig. 7B). Taken together, the results indicate that LPS increases the activity of the Na^+,K^+-pump.

Discussion

The first major finding of this study is that, 18 h after its systemic administration, LPS causes hyperpolarization of the tracheal epithelium by increasing the transport of Na^+. This was demonstrated by the greater inhibition of V_t by amiloride in the epithelia in IPT and Ussing experiments from LPS-treated animals compared to controls. The second major finding, using exogenous trypsin, was that LPS increased Na^+ transport in a manner that seems to be independent of proteolytic cleavage of ENaC. Third, two, different approaches for measuring the activity of the Na^+,K^+-pump revealed that it was increased by LPS. Taken together, these results indicate that LPS up-regulates the activities of both ENaC and the Na^+,K^+-pump.

LPS plays a role in airway responsiveness in several diseases which are marked by dysregulation of airway tone. Earlier, the effects of intraperitoneally-administered LPS on guinea-pig airway smooth muscle reactivity and V_t were investigated in the IPT and found that LPS reduced the reactivity to inhaled methacholine and potentiated the EpDRF-mediated relaxation responses to hyperosmolar solutions *in vitro* (33). These effects were associated with a potentiation of the hyperpolarization portion of the biphasic V_t response to methacholine and a potentiation of hyperosmolar NaCl-induced depolarization of the airway epithelium (33). Since the main route of transepithelial Na^+ transport is through the ENaC/Na^+,K^+-pump axis, this study investigated the effects of LPS on the activities of ENaC and the Na^+,K^+-pump in functional experiments.

Using amiloride, the role of Na^+ transport in the effects of LPS on V_t were determined in the IPT. Amiloride inhibited V_t in both treatment groups, yet inhibited V_t in tracheas from the LPS-treated group to a greater extent. The V_t response to Na^+ removal was different from what was predicted. In an earlier report of the permeabilization of the basolateral membranes of rat primary alveolar epithelial cell monolayers, a similar Na^+ removal maneuver (replacement of 116 mM of a total of 141 mM NaCl with 116 mM NMDG-Cl in the basolateral Ringer's solution) done concurrently with the administration of basolateral amphotericin B at the same concentration (7.5

μM) resulted in a lasting and stable increase in trans-monolayer current (40). In our experiment, Na^+ removal hyperpolarized the epithelium only transiently before V_t became depolarized (Fig. 2) and LPS had no affect on the magnitude of the transient hyperpolarization (Fig. 3A). Another study using apical nystatin, a similar pore-forming antifungal agent, to short-circuit ENaC in cultured human bronchial epithelial cell monolayers in the Ussing chamber without removing Na^+ reported a similar effect, i.e., a transient increase in short-circuit current before returning to baseline (37). The authors (37) concluded that the Na^+,K^+-pump decreased short-circuit current by clearing an intracellular Na^+ load. We tested nystatin (7.5 μM) and observed similar transient V_t responses. In contrast, however, the transient V_t responses did not occur upon the addition of amphotericin B without the removal of basolateral Na^+, but occured upon basolateral Na^+ removal whether Na^+ was removed before, concurrently with, or after the addition of amphotericin B. That is, this complex response was attributable to the replacement of Na^+ by NMDG, not to amphotericin B per se.

Apically-applied trypsin increased V_t and R_t, but had no effect on I_{sc} in tracheas from saline- and LPS-treated animals (Table 1). V_t was increased by the same proportion as R_t in both treatment groups, and trypsin had no effect on I_{sc}. These findings indicate that trypsin had no effect on the activity of ENaC and are compatible with the very small effect of amiloride on the tracheal epithelium from control animals (Fig. 1). We considered the possibility that protease activated receptors (PAR) may be involved in the effects of trypsin. Trypsin is an agonist of PARs, which stimulate an increase in the intracellular concentration of Ca^{2+} and PKC activation. The activation of PAR receptors has been found to inhibit the Ca^{2+} response of guinea-pig tracheal epithelial cells in culture to LPS (45). Small concentrations of trypsin (1-10 ng/ml) below those that cleave ENaC directly were found to activate ENaC via an indirect, receptor-mediated mechanism (3). However, PAR-2 overexpression or knockdown had no influence on the effects of trypsin in that study. Additionally, Swystun *et al.* (60) found that apical trypsin increased Na^+ and Cl^- transport, and I_{sc} and R_t in the airway epithelium. Due to the lack of effect of ouabain on the trypsin-induced effect on R_t in that study, the authors concluded that R_t was increased due to a decrease in paracellular conductance.

We are unaware of any literature linking PARs and ENaC and are left with a lack of understanding about why trypsin increase R_t until detailed future experiments can be performed.

The larger effect of amiloride on V_t could be explained two ways: ENaC activity could be stimulated by a reduction in intracellular Na^+ by the Na^+,K^+-pump (22), or increased ENaC activity could stimulate the Na^+,K^+-pump by elevating intracellular Na^+ concentrations (42). Since amiloride-sensitive V_t was increased by LPS irrespective of whether the Na^+,K^+-pump was short-circuited with amphotericin B or not, ENaC activity was stimulated by LPS independently of the intracellular Na^+ concentration. ENaC is regulated by many, complex mechanisms including open probability, the number of mature and near-silent channels in the membrane, protein abundance, localization, and channel kinetics and degradation. We have examined the effects of LPS on ENaC message and abundance (data not shown) and observed that it had no effect on the abundance or cleavage of the α-, β-, or γ-subunits of ENaC in the tracheal epithelium.

Several studies have investigated the effects of *P. aeruginosa* on the transcription or activity of ENaC. Intratracheal instillation of *P. aeruginosa* inhibits distal alveolar fluid clearance with a decrease in the transcription of βENaC, but without an effect on αENaC or α_1 Na^+,K^+-ATPase transcription (46). In a similar study, the intratracheal instillation of *P. aeruginosa* in mice reduced the transcription of αENaC, but had no effect on the transcription of β- or γ-ENaC or α_1 Na^+,K^+-ATPase (12). In another study, after dog bronchial epithelium was treated with a culture of *P. aeruginosa*, short circuit current, active Na^+ absorption, and Cl^- fluxes were decreased (59). In contrast to our findings with LPS, these studies, the results of which no doubt involve *P. aeruginosa* LPS, indicate that the infection of the lung with *P. aeruginosa* inhibits ENaC activity. They differ from our study, where we injected extracted LPS i.p., in that the bacterium was instilled into the lung. The instillation resulted in inflammation and edema, which were absent in the guinea-pig lung 18 h post-injection (33). LPS also inhibits transepithelial Na^+ transport *in vitro*. Amiloride-sensitive short-circuit current is inhibited by LPS in the mouse trachea in the Ussing chamber (35). Although the

ionic basis of the effect was not examined, we observed that incubation of guinea pig trachea *in vitro* with LPS reduced V_t (32).

Since the Na^+,K^+-pump has been identified as the rate-limiting step for Na^+ transport across the airway epithelium (39), another major emphasis of this study was to investigate the effects of LPS on its activity. The experimental design whereby the contribution of the Na^+,K^+-pump to V_t was measured in the IPT after short-circuiting ENaC using amphotericin B was adapted from one used in the Ussing chamber (11, 40). Using this technique to isolate Na^+,K^+-pump activity, V_t was twice as large in tracheas from LPS-treated animals compared to controls, indicating initially that LPS increases the activity of the Na^+,K^+-pump. Additional support for this notion was provided using the Na^+-loading approach that has been employed previously to measure pump activity in smooth muscle (8). The magnitude of the repolarization after loading the epithelium with Na^+ reflects the activity of the Na^+,K^+-pump and was increased by LPS.

The mechanisms through which LPS affects the activities of the Na^+,K^+-pump cannot be determined from the results of this study, but could involve the expression and/or abundance of the pump and/or its regulation. In our laboratory we have determined that the expression of the α_1 subunit of Na^+,K^+-ATPase is increased by LPS (data not shown). Several other factors that increase transepithelial Na^+ transport by increasing the activity of the Na^+,K^+-pump could contribute to the observed effects of LPS. FXYD5, found in the airway epithelium, belongs to the FXYD family of proteins which has been described as an auxiliary γ subunit of the Na^+,K^+-pump (22). Each isoform has a unique tissue distribution and regulatory effects on Na^+,K^+-pump activity (20). The airway epithelium responds to an increase in intracellular Na^+ by increasing the expression of FXYD5, which, in turn, binds to and increases the activity of the Na^+,K^+-pump (42). In the cystic fibrotic airway epithelium, the abnormally high intracellular Na^+ concentration stimulates the activity of the Na^+,K^+-pump by increasing the expression of FXYD5 (42). After the permeabilization of the apical membrane with amphotericin B in our experiments, the intracellular concentration of Na^+ in the epithelium of tracheas from both saline- and LPS-treated animals would approximate that of the

apical MKH bath, i.e., 119 mM. Therefore, it is convenient to speculate that LPS increases the expression of FXYD in the airway epithelium, which would increase both the response to intracellular Na^+ and the basal activity of the Na^+,K^+-pump.

We also earlier found that the cyclooxygenase inhibitor, indomethacin, inhibited V_t to a significantly greater extent in the tracheas from LPS-treated animals compared to controls (33). Even though the effect of indomethacin (33) was not as great as that of amiloride (Fig. 1), it indicates that cyclooxygenases also play a role in the LPS-induced hyperpolarization of the airway epithelium, suggesting a possible avenue of exploration in future experiments.

Hypoxia and reactive oxygen species have been found to reduce Na^+,K^+-pump activity. These factors, present in lung inflammation, cause edema by activating AMPK, which activates PKC, which, in turn, stimulates the endocytosis of the Na^+,K^+-pump from the basolateral membrane (24, 40, 62, 66). The LPS-stimulated up-regulation of nitric oxide synthases has been reported to reduce the activity of the Na^+,K^+-pump in the guinea-pig kidney (53) and to reduce the protein expression of the α_1 subunit of the Na^+,K^+-pump in the rat sciatic nerve (36). The effects of LPS appear to be mediated via an interaction between LPS and the cell because they were reversed in the latter study by polymyxin B, which binds to the lipid A portion of LPS. In our guinea pig experimental model, however, the effects of systemic LPS occur in the absence of inflammation in the alveoli, bronchioles, or bronchi (33), and, no doubt, reflect the concerted actions of proinflammatory mediators whose abundance is stimulated by LPS.

In conclusion, the systemic administration of LPS hyperpolarizes the airway epithelium, at least in part, by stimulating the transport of Na^+. LPS increases the activities of both ENaC and the Na^+,K^+-pump. The mechanisms through which Na^+ transport is increased cannot be determined from the present experiments, yet it seems that proteolytic activation of ENaC is not involved. In a separate investigation, we observed that the expression and abundance of Na^+,K^+-ATPase but not ENaC are increased following LPS treatment (data not shown).

Footnotes

The abbreviations used are: ENaC, epithelial sodium channel; LPS, lipopolysaccharide; R_t, transepithelial resistance; V_t, transepithelial voltage; I_{sc}, equivalent short circuit current; NMDG, N-methyl-D-glucamine; MKH, modified Krebs-Henseleit solution; EpDRF, epithelium-derived relaxing factor; PAR, protease-activated receptor.

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References

1. **Adir Y, Azzam ZS, Lecuona E, Leal S, Pesce L, Dumasius V, Bertorello AM, Factor P, Young JB, Ridge KM, and Sznajder JI**. Augmentation of endogenous dopamine production increases lung liquid clearance. *Am J Respir Crit Care Med* 169: 757-763, 2004.
2. **Barquin N, Ciccolella DE, Ridge KM, and Sznajder JI**. Dexamethasone upregulates the Na-K-ATPase in rat alveolar epithelial cells. *Am J Physiol* 273: L825-830, 1997.
3. **Bengrine A, Li J, Hamm LL, and Awayda MS**. Indirect activation of the epithelial Na^+ channel by trypsin. *J Biol Chem* 282: 26884-26896, 2007.
4. **Bhalla V, and Hallows KR**. Mechanisms of ENaC regulation and clinical implications. *J Am Soc Nephrol* 19: 1845-1854, 2008.
5. **Bhargava M, Runyon MR, Smirnov D, Lei J, Groppoli TJ, Mariash CN, Wangensteen OD, and Ingbar DH**. Triiodo-L-thyronine rapidly stimulates alveolar fluid clearance in normal and hyperoxia-injured lungs. *Am J Respir Crit Care Med* 178: 506-512, 2008.
6. **Boucher RC**. Human airway ion transport. Part one. *Am J Respir Crit Care Med* 150: 271-281, 1994.
7. **Bridges RJ, Newton BB, Pilewski JM, Devor DC, Poll CT, and Hall RL**. Na^+ transport in normal and CF human bronchial epithelial cells is inhibited by BAY 39-9437. *Am J Physiol Lung Cell Mol Physiol* 281: L16-23, 2001.
8. **Casteels R, Droogmans G, and Hendrickx H**. Membrane potential of smooth muscle cells in K-free solution. *J Physiol* 217: 281-295, 1971.
9. **Castellan RM, Olenchock SA, Kinsley KB, and Hankinson JL**. Inhaled endotoxin and decreased spirometric values. An exposure-response relation for cotton dust. *N Engl J Med* 317: 605-610, 1987.
10. **Collier DM, and Snyder PM**. Extracellular protons regulate human ENaC by modulating Na^+ self-inhibition. *J Biol Chem* 284: 792-798, 2009.

11. **Dagenais A, Fréchette R, Yamagata Y, Yamagata T, Carmel JF, Clermont ME, Brochiero E, Massé C, and Berthiaume Y**. Downregulation of ENaC activity and expression by TNF-α in alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 286: L301-311, 2004.

12. **Dagenais A, Gosselin D, Guilbault C, Radzioch D, and Berthiaume Y**. Modulation of epithelial sodium channel (ENaC) expression in mouse lung infected with Pseudomonas aeruginosa. *Respir Res* 6: 2, 2005.

13. **Donaldson SH, Hirsh A, Li DC, Holloway G, Chao J, Boucher RC, and Gabriel SE**. Regulation of the epithelial sodium channel by serine proteases in human airways. *J Biol Chem* 277: 8338-8345, 2002.

14. **Dortch-Carnes J, Van Scott MR, and Fedan JS**. Changes in smooth muscle tone during osmotic challenge in relation to epithelial bioelectric events in guinea pig isolated trachea. *J Pharmacol Exp Ther* 289: 911-917, 1999.

15. **Duszyk M**. Regulation of anion secretion by nitric oxide in human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 281: L450-457, 2001.

16. **Fedan JS, and Frazer DG**. Influence of epithelium on the reactivity of guinea pig isolated, perfused trachea to bronchoactive drugs. *J Pharmacol Exp Ther* 262: 741-750, 1992.

17. **Fedan JS, Warner TE, Yuan LX, Robinson V, and Frazer DG**. Nitric oxide synthase inhibitor and lipopolysaccharide effects on reactivity of guinea pig airways. *J Pharmacol Exp Ther* 272: 1141-1150, 1995.

18. **Fukuda N, Jayr C, Lazrak A, Wang Y, Lucas R, Matalon S, and Matthay MA**. Mechanisms of TNF-α stimulation of amiloride-sensitive sodium transport across alveolar epithelium. *Am J Physiol Lung Cell Mol Physiol* 280: L1258-1265, 2001.

19. **Galietta LJ, Pagesy P, Folli C, Caci E, Romio L, Costes B, Nicolis E, Cabrini G, Goossens M, Ravazzolo R, and Zegarra-Moran O**. IL-4 is a potent modulator of ion transport in the human bronchial epithelium in vitro. *J Immunol* 168: 839-845, 2002.

20. **Garty H, and Karlish SJ**. Role of FXYD proteins in ion transport. *Annu Rev Physiol* 68: 431-459, 2006.

21. **Geering K**. Functional roles of Na,K-ATPase subunits. *Curr Opin Nephrol Hypertens* 17: 526-532, 2008.

22. **Geering K**. FXYD proteins: new regulators of Na-K-ATPase. *Am J Physiol Renal Physiol* 290: F241-250, 2006.

23. **Guillot L, Medjane S, Le-Barillec K, Balloy V, Danel C, Chignard M, and Si-Tahar M**. Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like receptor 4 (TLR4)-dependent signaling pathways: evidence for an intracellular compartmentalization of TLR4. *J Biol Chem* 279: 2712-2718, 2004.

24. **Gusarova GA, Dada LA, Kelly AM, Brodie C, Witters LA, Chandel NS, and Sznajder JI**. α1-AMP-activated protein kinase regulates hypoxia-induced Na,K-ATPase endocytosis via direct phosphorylation of protein kinase Cζ. *Mol Cell Biol* 29: 3455-3464, 2009.

25. **Held HD, and Uhlig S**. Mechanisms of endotoxin-induced airway and pulmonary vascular hyperreactivity in mice. *Am J Respir Crit Care Med* 162: 1547-1552, 2000.

26. **Hendrickx H, and Casteels R**. Electrogenic sodium pump in arterial smooth muscle cells. *Pflügers Arch* 346: 299-306, 1974.

27. **Hertz CJ, Wu Q, Porter EM, Zhang YJ, Weismuller KH, Godowski PJ, Ganz T, Randell SH, and Modlin RL**. Activation of Toll-like receptor 2 on human tracheobronchial epithelial cells induces the antimicrobial peptide human beta defensin-2. *J Immunol* 171: 6820-6826, 2003.

28. **Hughey RP, Bruns JB, Kinlough CL, Harkleroad KL, Tong Q, Carattino MD, Johnson JP, Stockand JD, and Kleyman TR**. Epithelial sodium channels are activated by furin-dependent proteolysis. *J Biol Chem* 279: 18111-18114, 2004.

29. **Hughey RP, Bruns JB, Kinlough CL, and Kleyman TR**. Distinct pools of epithelial sodium channels are expressed at the plasma membrane. *J Biol Chem* 279: 48491-48494, 2004.

30. **Hughey RP, Mueller GM, Bruns JB, Kinlough CL, Poland PA, Harkleroad KL, Carattino MD, and Kleyman TR**. Maturation of the epithelial Na^+ channel involves proteolytic processing of the α- and γ-subunits. *J Biol Chem* 278: 37073-37082, 2003.

31. **Hummler E, Barker P, Gatzy J, Beermann F, Verdumo C, Schmidt A, Boucher R, and Rossier BC**. Early death due to defective neonatal lung liquid clearance in α-ENaC-deficient mice. *Nat Genet* 12: 325-328, 1996.

32. **Ismailoglu UB, Van Scott MR, and Fedan JS**. Effects of cytokines on mechanical and epithelial bioelectric responses to methacholine and hyperosmolarity in guinea-pig airways: an in vitro study. *Eur J Pharmacol* 612: 115-121, 2009.

33. **Johnston RA, Van Scott MR, Kommineni C, Millecchia LL, Dortch-Carnes J, and Fedan JS**. Hyperosmolar solution effects in guinea pig airways. IV. Lipopolysaccharide-induced alterations in airway reactivity and epithelial bioelectric responses to methacholine and hyperosmolarity. *J Pharmacol Exp Ther* 308: 37-46, 2004.

34. **Kleyman TR, Myerburg MM, and Hughey RP**. Regulation of ENaCs by proteases: An increasingly complex story. *Kidney Int* 70: 1391-1392, 2006.

35. **Kunzelmann K, Sun J, Meanger J, King NJ, and Cook DI**. Inhibition of airway Na^+ transport by respiratory syncytial virus. *J Virol* 81: 3714-3720, 2007.

36. **Liu SH, and Sheu TJ**. The in vivo effect of lipopolysaccharide on Na^+,K^+-ATPase catalytic (α) subunit isoforms in rat sciatic nerve. *Neurosci Lett* 234: 166-168, 1997.

37. **Livraghi A, Mall M, Paradiso AM, Boucher RC, and Ribeiro CM**. Modelling dysregulated Na^+ absorption in airway epithelial cells with mucosal nystatin treatment. *Am J Respir Cell Mol Biol* 38: 423-434, 2008.

38. **Loffing J, Flores SY, and Staub O**. Sgk kinases and their role in epithelial transport. *Annu Rev Physiol* 68: 461-490, 2006.

39. **Looney MR, Sartori C, Chakraborty S, James PF, Lingrel JB, and Matthay MA**. Decreased expression of both the α_1- and α_2-subunits of the Na-K-ATPase reduces maximal alveolar epithelial fluid clearance. *Am J Physiol Lung Cell Mol Physiol* 289: L104-110, 2005.

40. **Mairbäurl H, Mayer K, Kim KJ, Borok Z, Bärtsch P, and Crandall ED**. Hypoxia decreases active Na transport across primary rat alveolar epithelial cell monolayers. *Am J Physiol Lung Cell Mol Physiol* 282: L659-665, 2002.

41. **Matthay MA, Robriquet L, and Fang X**. Alveolar epithelium: role in lung fluid balance and acute lung injury. *Proc Am Thorac Soc* 2: 206-213, 2005.

42. **Miller TJ, and Davis PB**. FXYD5 modulates Na^+ absorption and is increased in cystic fibrosis airway epithelia. *Am J Physiol Lung Cell Mol Physiol* 294: L654-664, 2008.

43. **Munakata M, Mitzner W, and Menkes H**. Osmotic stimuli induce epithelial-dependent relaxation in the guinea pig trachea. *J Appl Physiol* 64: 466-471, 1988.

44. **Myerburg MM, McKenna EE, Luke CJ, Frizzell RA, Kleyman TR, and Pilewski JM**. Prostasin expression is regulated by airway surface liquid volume and is increased in cystic fibrosis. *Am J Physiol Lung Cell Mol Physiol* 294: L932-941, 2008.

45. **Oshiro A, Otani H, Yagi Y, Fukuhara S, and Inagaki C**. Protease-activated receptor-2-mediated inhibition for Ca2+ response to lipopolysaccharide in Guinea pig tracheal epithelial cells. *Am J Respir Cell Mol Biol* 30: 886-892, 2004.

46. **Pierre M, Husson MO, Le Berre R, Desseyn JL, Galabert C, Beghin L, Beermann C, Dagenais A, Berthiaume Y, Cardinaud B, Barbry P, Gottrand F, and Guery BP**. Omega-3 polyunsaturated fatty acids improve host response in chronic *Pseudomonas aeruginosa* lung infection in mice. *Am J Physiol Lung Cell Mol Physiol* 292: L1422-1431, 2007.

47. **Planès C, Blot-Chabaud M, Matthay MA, Couette S, Uchida T, and Clerici C**. Hypoxia and β_2-agonists regulate cell surface expression of the epithelial sodium channel in native alveolar epithelial cells. *J Biol Chem* 277: 47318-47324, 2002.

48. **Rokaw MD, Wang JM, Edinger RS, Weisz OA, Hui D, Middleton P, Shlyonsky V, Berdiev BK, Ismailov I, Eaton DC, Benos DJ, and Johnson JP**. Carboxylmethylation of the β subunit of xENaC regulates channel activity. *J Biol Chem* 273: 28746-28751, 1998.

49. **Roos-Engstrand E, Wallin A, Bucht A, Pourazar J, Sandström T, and Blomberg A**. Increased expression of p38 MAPK in human bronchial epithelium after lipopolysaccharide exposure. *Eur Respir J* 25: 797-803, 2005.

50. **Rossier BC**. The epithelial sodium channel: activation by membrane-bound serine proteases. *Proc Am Thorac Soc* 1: 4-9, 2004.

51. **Rossier BC, and Stutts MJ**. Activation of the epithelial sodium channel (ENaC) by serine proteases. *Annu Rev Physiol* 71: 361-379, 2009.

52. **Saito T, Yamamoto T, Kazawa T, Gejyo H, and Naito M**. Expression of toll-like receptor 2 and 4 in lipopolysaccharide-induced lung injury in mouse. *Cell Tissue Res* 321: 75-88, 2005.

53. **Seven I, Türközkan N, and Çimen B**. The effects of nitric oxide synthesis on the Na^+,K^+-ATPase activity in guinea pig kidney exposed to lipopolysaccharides. *Mol Cell Biochem* 271: 107-112, 2005.

54. **Sha Q, Truong-Tran AQ, Plitt JR, Beck LA, and Schleimer RP**. Activation of airway epithelial cells by toll-like receptor agonists. *Am J Respir Cell Mol Biol* 31: 358-364, 2004.

55. **Sheng S, Carattino MD, Bruns JB, Hughey RP, and Kleyman TR**. Furin cleavage activates the epithelial Na^+ channel by relieving Na^+ self-inhibition. *Am J Physiol Renal Physiol* 290: F1488-1496, 2006.

56. **Snyder PM, Steines JC, and Olson DR**. Relative contribution of Nedd4 and Nedd4-2 to ENaC regulation in epithelia determined by RNA interference. *J Biol Chem* 279: 5042-5046, 2004.

57. **Song W, Wei S, Zhou Y, Lazrak A, Liu G, Londino JD, Squadrito GL, and Matalon S**. Inhibition of lung fluid clearance and epithelial Na^+ channels by chlorine, hypochlorous acid, and chloramines. *J Biol Chem* 285: 9716-9728, 2010.

58. **Staub O, Gautschi I, Ishikawa T, Breitschopf K, Ciechanover A, Schild L, and Rotin D**. Regulation of stability and function of the epithelial Na^+ channel (ENaC) by ubiquitination. *EMBO J* 16: 6325-6336, 1997.

59. **Stutts MJ, Schwab JH, Chen MG, Knowles MR, and Boucher RC**. Effects of Pseudomonas aeruginosa on bronchial epithelial ion transport. *Am Rev Respir Dis* 134: 17-21, 1986.

60. **Swystun V, Chen L, Factor P, Siroky B, Bell PD, and Matalon S**. Apical trypsin increases ion transport and resistance by a phospholipase C-dependent rise of Ca^{2+}. *Am J Physiol Lung Cell Mol Physiol* 288: L820-830, 2005.

61. **Ulevitch RJ, and Tobias PS**. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annu Rev Immunol* 13: 437-457, 1995.

62. **Vadász I, Raviv S, and Sznajder JI**. Alveolar epithelium and Na,K-ATPase in acute lung injury. *Intensive Care Med* 33: 1243-1251, 2007.

63. **Wang Y, Bai C, Li K, Adler KB, and Wang X**. Role of airway epithelial cells in development of asthma and allergic rhinitis. *Respir Med* 102: 949-955, 2008.

64. **Willis BC, Kim KJ, Li X, Liebler J, Crandall ED, and Borok Z**. Modulation of ion conductance and active transport by TGF-β1 in alveolar epithelial cell monolayers. *Am J Physiol Lung Cell Mol Physiol* 285: L1192-1200, 2003.

65. **Woollhead AM, and Baines DL**. Forskolin-induced cell shrinkage and apical translocation of functional enhanced green fluorescent protein-human αENaC in H441 lung epithelial cell monolayers. *J Biol Chem* 281: 5158-5168, 2006.

66. **Woollhead AM, Sivagnanasundaram J, Kalsi KK, Pucovsky V, Pellatt LJ, Scott JW, Mustard KJ, Hardie DG, and Baines DL**. Pharmacological activators of AMP-activated protein kinase have different effects on Na^+ transport processes across human lung epithelial cells. *Br J Pharmacol* 151: 1204-1215, 2007.

67. **Ye X, Acharya R, Herbert JB, Hamilton SE, and Folkesson HG**. IL-1β stimulates alveolar fluid absorption in fetal guinea pig lungs via the hypothalamus-pituitary-adrenal gland axis. *Am J Physiol Lung Cell Mol Physiol* 286: L756-766, 2004.

68. **Zhou G, Dada LA, and Sznajder JI**. Regulation of alveolar epithelial function by hypoxia. *Eur Respir J* 31: 1107-1113, 2008.

Figure Legends

Fig. 1. LPS increases ENaC activity in the airway epithelium. The IPT was used to measure amiloride-sensitive V_t in tracheas from saline- and LPS-treated guinea pigs. Basal V_t was greater in tracheas from LPS-treated animals. Amiloride (10 μM) inhibited V_t ($\triangle V_t$) in both treatment groups, but to a larger extent after LPS treatment. The effect of amiloride on V_t was greater in tracheas from LPS-treated animals compared to controls. Therefore, the LPS-induced hyperpolarization is due, in part, to an increase in Na^+ transport. Saline, $n = 6$; LPS, $n = 5$. *$p < 0.05$ compared with saline-treated controls (−amiloride; Wilcoxon signed rank test). **$p < 0.05$ compared with saline-treated controls (−amiloride). ***$p < 0.05$ compared with LPS-treated (−amiloride).

Fig. 2. LPS increases ENaC activity. Representative tracings from tracheas from a saline- and an LPS-treated animal demonstrate the experimental design. The contribution of the Na^+,K^+-pump to V_t was removed prior to measuring ENaC activity in the IPT by short-circuiting the basolateral membrane with amphotericin B (AB; 7.5 μM). In order to create a large gradient for Na^+ across the apical membrane, Na^+ was concurrently removed from the MKH of the basolateral bath by replacing the NaCl with NMDG-Cl (113 mM). The left arrows indicate an artifact due to turning off the preamplifier while the basolateral bath was drained and replaced with NMDG-Cl- and amphotericin B-containing MKH. The right arrows indicate the point of V_t measurement. Amiloride (A; 10 μM) and trypsin (T; 100 U/ml) were added to the apical bath. Saline, $n = 7$; LPS, $n = 5$.

Fig. 3. Summary of the effects of LPS on ENaC activity in the IPT preparation. See Figure 2 for the protocol of the experiments whose results are summarized in this figure. A, The maximum, transient hyperpolarization effect of basolateral Na^+ removal and amphotericin B addition on V_t before V_t stabilized was not different between the epithelia of tracheas from saline- and LPS-treated animals. B, The stable V_t response to Na^+ removal and amphotericin B addition was greater in

tracheas from LPS-treated animals compared to controls (*$p < 0.05$, t-test). C, The V_t response to amiloride was greater in tracheas from LPS-treated animals compared to control (*$p < 0.05$, Mann-Whitney rank sum test). D, V_t in the presence of trypsin was not different between tracheas from saline- or LPS-treated animals. E, The $\triangle V_t$ response to trypsin was not different between tracheas from saline- and LPS-treated animals (Mann-Whitney rank sum test). Saline, $n = 8$; LPS, $n = 5$.

Fig. 4. Representative tracings from tracheas from a saline-treated and an LPS-treated animal demonstrate the experimental design in which trypsin was used in the Ussing chamber to assess the effects of LPS on proteolytic activation of ENaC. Amiloride (A; 10 μM) was added to the apical bath to measure ENaC activity. W, Both the apical and basolateral baths were washed with fresh MKH. Trypsin (T; 100 U/ml) was added to the apical bath to activate ENaC.

Fig. 5. The stimulation of ENaC activity by LPS does not involve channel activating proteases. See Figure 5 for the protocol whose results are summarized in this figure. The effects of amiloride (10 μM), trypsin (100 U/ml), and amiloride (10 μM) in the presence of trypsin are expressed as the change in V_t after the administration of the agent. *$p < 0.05$ (t-test). Saline and LPS, $n = 11$.

Fig. 6. LPS stimulates the activity of the Na^+,K^+-pump in the IPT; amphotericin B approach. A, Representative tracings of V_t responses in tracheas from saline- and LPS-treated animals demonstrate the experimental design. Amiloride (A; 10 μM) was added to the apical bath to remove the contribution of ENaC from V_t. Amphotericin B (AB; 7.5 μM) was added to the apical bath to short-circuit the apical membrane of the epithelium. Trypsin (T; 100 U/ml) was added to the apical bath to verify that ENaC had been inhibited by amiloride and short-circuited by amphotericin B. B, The effect of amphotericin B on V_t, measured at the points indicated by the single-headed arrows in panel A, was greater in the epithelium of tracheas from LPS-treated animals compared to

controls. C, V_t measured in the presence of apical trypsin, at the points indicated by the double-headed arrows in panel A, was greater in tracheas from LPS-treated tracheas. D, The V_t response to the apical administration of trypsin (100 U/ml) was not different between groups. Since trypsin had no effect on V_t, the greater V_t was not due to ENaC. Saline, $n = 6$; LPS, $n = 5$. *$p < 0.05$ compared to saline-treated control (t-test).

Fig. 7. LPS stimulates the activity of the Na^+,K^+-pump in the Ussing chamber: Na^+-loading approach. A, Representative tracings demonstrate the Na^+-loading technique to measure the effects of LPS on the activity of the Na^+,K^+-pump in the Ussing chamber. At this compressed time scale, the upward deflections in V_t resulting from application of the current pulses are compressed together; thus, the lower edge of the signal represents V_t and the upper edge of the signal represents the deflections in V_t due to the applied current pulses. K^+-free, K^+ was removed from both the apical and basolateral baths by omitting KCl and KH_2PO_4 from the MKH solution and increasing the NaCl concentration to 119 mM. K^+ removal inhibited the Na^+,K^+-pump, Na^+ accumulated within the epithelium, and the epithelium depolarized. Arrows indicate noise in the signal during K^+ removal and NaCl addition. Basolateral K^+ was restored by readmitting KCl (B K^+) to the basolateral bath. Apical K^+ was restored by adding KCl (A K^+) to the apical bath. K^+ restoration allowed Na^+,K^+-pump activity to resume and the epithelium to repolarize. A greater V_t response to K^+ restoration was interpreted as indicative of increased Na^+,K^+-pump activity. Ouabain (O; 10 μM) was administered to the basolateral bath to confirm that the K^+-restoration maneuver restored the activity of the Na^+,K^+-pump. B, The effects of K^+ removal, K^+ restoration, and ouabain (10 μM) are expressed as the difference in V_t before and after the intervention. The effect of K^+ restoration takes into account responses to both the basolateral and apical additions of KCl, the latter of which were negligible. Saline and LPS, $n = 5$. *$p < 0.05$ (t-test).

Tables

Table 1. Effects of trypsin on V_t, R_t, and I_{sc} in tracheas from untreated and LPS-treated animals.

		Control	LPS
V_t			
	Basal	−3.4±0.9	−10.3±1.4
	Trypsin	−6.0±1.6	−13.8±2.3
	△	2.6±1.6	3.5±1.0
	%△	203±65	130±7
R_t			
	Basal	−93±21	−128.0±17
	Trypsin	−138±21	−166±25
	△	45±28	38±11
	%△	202±86	129±8
I_{sc}			
	Basal	39±9.3	84±14.8
	Trypsin	40±6.5	84.2±13.2
	△	1.4±3.3	−0.2±2.4
	%△	112.0±9.4	100.7±2.1
%△V_t/%△R_t		0.9±0.1	1.0±0.0

Table 1: Apical trypsin (100 U/ml) was added to tracheas from saline- and LPS-treated animals in the Ussing chamber. Percent changes, i.e., the ratios of the V_t, R_t, or I_{sc} values after trypsin addition over the values before, are the means of the individual percent changes from each animal. No significant differences were observed using the t-test. V_t, mV; R_t, Ω·cm²; I_{sc}, μA/cm². Untreated, n = 5; LPS, n = 6.

Fig. 1

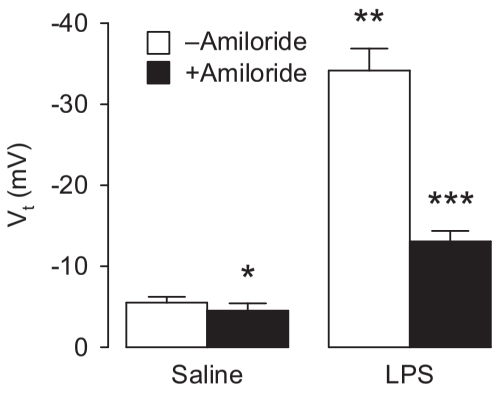


Fig. 2

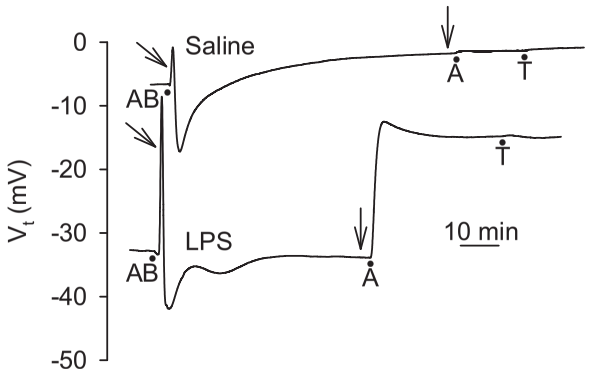


Fig. 3

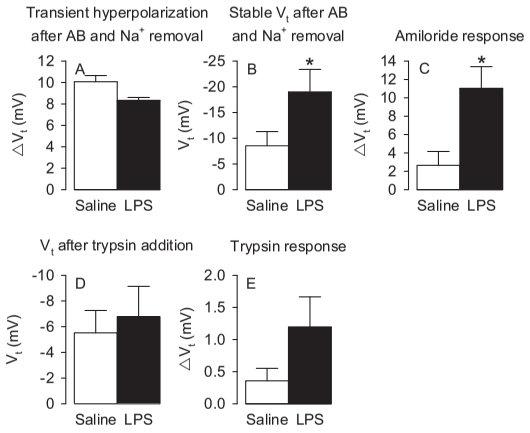

Transient hyperpolarization after AB and Na+ removal
Stable Vt after AB and Na+ removal
Amiloride response
A
B
C
*
*
ΔVt (mV)
Vt (mV)
ΔVt (mV)
12
10
8
6
4
2
0
-25
-20
-15
-10
-5
0
14
12
10
8
6
4
2
0
Saline LPS
Saline LPS
Saline LPS
Vt after trypsin addition
Trypsin response
D
E
Vt (mV)
ΔVt (mV)
-10
-8
-6
-4
-2
0
2.0
1.5
1.0
0.5
0.0
Saline LPS
Saline LPS

Fig. 4

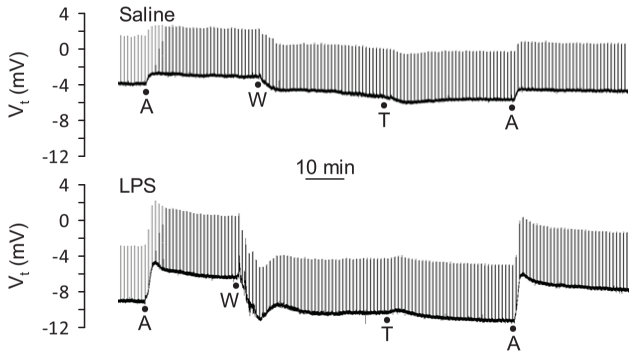


Fig. 5

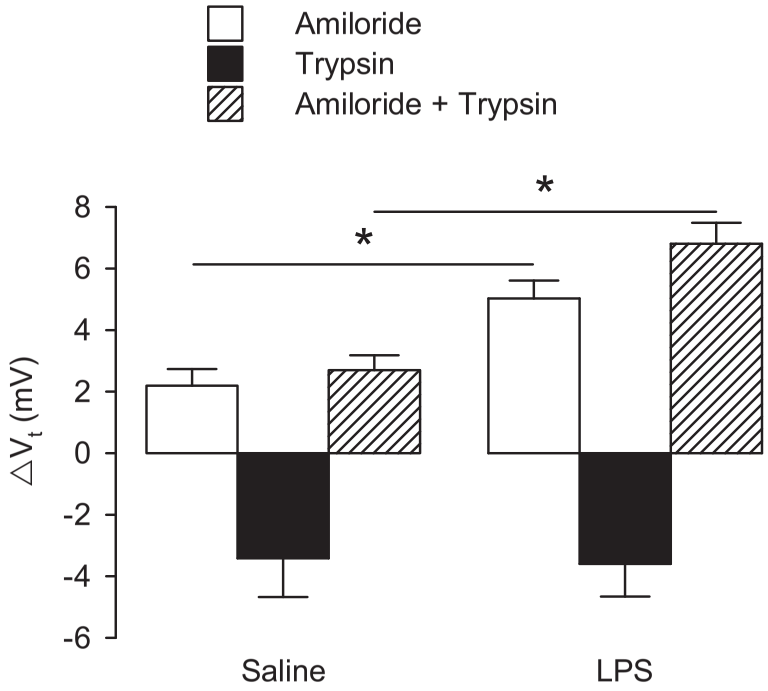

Amiloride
Trypsin
Amiloride + Trypsin
*
*
8
6
4
2
0
-2
-4
-6
△V_t (mV)
Saline
LPS

Fig. 6

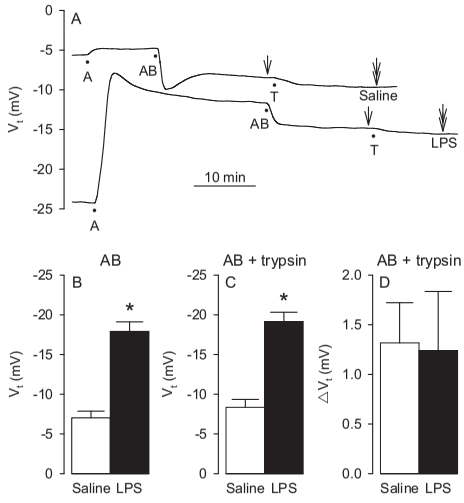
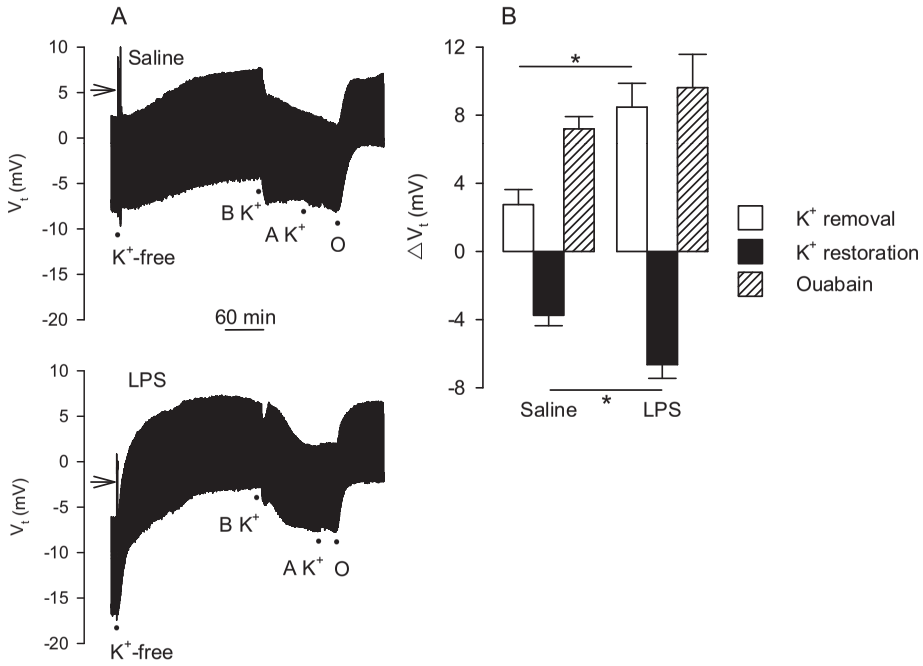

A
0
-5
-10
-15
-20
-25
V_t (mV)
A
AB
T
Saline
AB
T
LPS
10 min
A
AB
B
AB + trypsin
C
AB + trypsin
D
*
*
-25
-20
-15
-10
-5
0
2.0
1.5
1.0
0.5
0.0
$\triangle V_t$ (mV)
Saline LPS
Saline LPS
Saline LPS

Fig. 7


A
Saline
10
5
0
-5
-10
-15
-20
V_t (mV)
B K+
A K+
O
K+-free
60 min
LPS
B K+
A K+
O
K+-free
B
12
8
4
0
-4
-8
△V_t (mV)
*
*
Saline
LPS
K+ removal
K+ restoration
Ouabain