Systemic lipopolysaccharide stimulates airway transepithelial Na+ transport by increasing ENaC and Na+,K+ -pump activity

Michael W. Dodrill
West Virginia University

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Systemic lipopolysaccharide stimulates airway transepithelial Na\(^+\) transport by increasing ENaC and Na\(^+\),K\(^+\)-pump activity

Michael W. Dodrill

Dissertation submitted to the
School of Pharmacy
at West Virginia University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Pharmaceutical and Pharmacological Sciences

Jeffrey S. Fedan, Ph.D., Chair
Patrick S. Callery, Ph.D.
Jason D. Huber, Ph.D.
S. Jamal Mustafa, Ph.D.
James M. Antonini, Ph.D.

Department of Basic Pharmaceutical Sciences

Morgantown, West Virginia
2010

Keywords: Endotoxin; Airway Epithelium; Na\(^+\) Transport; Transepithelial Voltage; Epithelial Sodium Channel (ENaC); Sodium, Potassium ATPase (Na\(^+\),K\(^+\)-ATPase)
ABSTRACT

Systemic lipopolysaccharide stimulates airway transepithelial Na⁺ transport by increasing ENaC and Na⁺,K⁺-pump activity

Michael W. Dodrill

Our laboratory found that systemic administration of lipopolysaccharide (LPS; 4 mg/kg) hyperpolarized the transepithelial potential difference (Vt) of tracheal epithelium in the isolated, perfused trachea (IPT) of the guinea pig 18 h after injection. We hypothesized that LPS stimulates the transepithelial movement of Na⁺ via the epithelial sodium channel (ENaC)/Na⁺,K⁺-pump axis, leading to hyperpolarization of Vt. LPS increased the Vt response to amiloride, i.e., offset the effect of LPS, indicating that Na⁺ transport was increased. The functional activity of ENaC was measured in the IPT after short-circuiting the Na⁺,K⁺-pump with basolateral amphotericin B. LPS had no effect on the hyperpolarization response to apical trypsin 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 Vt 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. Using qPCR, the effects of LPS on the transcription of αENaC, α₁ Na⁺,K⁺-pump, COX-2, eNOS, iNOS, IL-1β, and TNF-α were measured at 3 and 18 h. In the epithelium, LPS increased the transcription of COX-2, IL-1β, and, to nonsignificant extent, TNF-α at 3 h, but not at 18 h. In alveolar macrophages, TNF-α, and, to a nonsignificant extent, COX-2 and IL-1β were up-regulated at 3 h, but not at 18 h. Even though LPS stimulated the transcription of some genes, αENaC and α₁ Na⁺,K⁺-ATPase transcription were not affected. The expression of α-, β-, and γ-ENaC and α₁ Na⁺,K⁺-pump from tracheal epithelium and kidney cortex/medulla were investigated by western blotting. All three ENaC subunits were detected as cleavage fragments, yet LPS had no effect on their expression. LPS increased the expression of the α₁ subunit and the α₁-, α₂-, and α₃-subunits, collectively, of the Na⁺,K⁺-pump. 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. ENaC activation by LPS is not accomplished via a change in ENaC regulation involving proteolytic cleavage. LPS increases Na⁺ transport downstream of the genetic level, in part, by stimulating the expression of the Na⁺,K⁺-pump.
Dedication

To my family, friends, and colleagues.
Acknowledgements

I wish to thank Jeffrey Fedan, my advisor, and my graduate committee, Patrick Callery, Jason Huber, S. Jamal Mustafa and James Antonini for their guidance, Janet Thompson for her technical assistance, Murali Rao and Terence Meighan for their support with PCR, Donald Beezhold for help with western blotting, and Yi Jing, Ugur Burcin Ismailoglu, Dovenia Ponnath, Eric Zaccone and Michael Shimko for additional support.
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Chapter 1:

Introduction, Research Plan, and Methods
1.1: Introduction

1.1.1: Lipopolysaccharide

Lipopolysaccharide (LPS) is a component of gram-negative bacteria that is toxic to the mammalian host during infection. LPS is a component of the outer membrane of gram-negative bacteria that helps to maintain its integrity and protect it from certain kinds of chemical attack (Liebers et al., 2008). It comprises a multi-sugar chain linked to a membrane-bound lipid A portion (Fig. 1-1) (Liebers et al., 2008). A gram-negative infection of the lung produces large amounts of LPS, which exacerbates the symptoms of allergy (Ulevitch and Tobias, 1995) and cause attacks in some types of asthma (Held and Uhlig, 2000). LPS is thought to be a causative agent for byssinosis (Smith et al., 1993; Nakládalová, 2000), endotoxin-induced inflammation, and lung injury triggers acute respiratory distress syndrome (Held and Uhlig, 2000).

LPS triggers the inflammatory responses of many mammalian cell types, including the airway epithelium, through its interaction with toll-like receptors (TLR)-2 and 4 (Fig. 1-2). An infection of the host by gram-negative bacteria produces copious amounts of LPS, which triggers many cell types to produce cytokines and reactive oxygen and nitrogen species by interacting with TLR-4 and activating the transcription factor, NF-κB (Basu and Fenton, 2004; Liang et al., 2007). The TLR-4-mediated activation of alveolar macrophages by LPS stimulates the cell to produce COX-2, IL-1β, and TNF-α (Kamachi et al., 2007). The interaction of LPS with TLR necessitates LPS binding protein which circulates in the bloodstream (Ulevitch and Tobias, 1995). Both TLR-4 and 2 are expressed in the human airway epithelium (Hertz et al., 2003; Guillot et al., 2004; Sha et al., 2004), and cytokine production is triggered in the mouse airway epithelium through the activation of NF-κB (Guillot et al., 2004). In the human lung, LPS upregulates the expression of TLR-4 and 2 (Saito et al., 2005), activates p38 (Roos-Engstrand et al., 2005), and upregulates extracellular signal-regulated kinases (ERK)1/2 and c-Jun N-terminal kinases (Jnk) (Guillot et al., 2004).
Many inflammatory mediators regulate airway epithelial Na\(^+\) transport. Tumor necrosis factor (TNF)-\(\alpha\) downregulates ENaC activity in the airway epithelium by decreasing the transcription of the \(\alpha\)ENaC subunit (Fukuda et al., 2001; Dagenais et al., 2004). Interleukin (IL)-4 has been demonstrated to favor the hydration of the airway surface liquid by decreasing \(\gamma\)ENaC transcription and ENaC activity and increasing the transcription and activity of the cystic fibrosis transmembrane regulator (CFTR) (Galietta et al., 2002). TGF-\(\beta\) increases the current across rat alveolar epithelial cells by increasing the expression of the \(\alpha_1\) and \(\beta_1\) subunits of the Na\(^+\),K\(^+\)-pump, but has no effect on \(\alpha\)ENaC expression (Willis et al., 2003). IL-1\(\beta\) increases amiloride-sensitive Na\(^+\) transport across the fetal guinea-pig lung epithelium by increasing the expression of ENaC and Na\(^+\),K\(^+\)-pump subunits (Ye et al., 2004). LPS induces the production of nitric oxide (NO) by increasing the expression of inducible nitric oxide synthase (iNOS) in the lung (Pier, 2007). The i.p. injection of mice with LPS has been shown to induce the expression of NF-\(\kappa\)B-mediated NO production in the lung by interacting with caveolin (Garrean et al., 2006). In addition to LPS, the cytokines IL-1\(\beta\) and TNF-\(\alpha\) also stimulate the production of the prostaglandin synthase, cyclooxygenase (COX)-2 (Kamachi et al., 2007).

LPS changes the reactivity of the airway smooth muscle to the contractile agent methacholine (MCh) and hyperosmolar solutions. The muscarinic agonist methacholine stimulates contraction when applied basolaterally to the airway smooth muscle (Fedan and Frazer, 1992). The guinea-pig airway epithelium acts as an osmosensor to increases in airway surface liquid osmolarity (Fedan et al., 2004a). When the epithelium is challenged with a hyperosmolar solution, such as NaCl, it stimulates relaxation of the airway smooth muscle via the release of epithelium-derived relaxing factor (EpDRF). EpDRF, a non-nitric oxide, non-prostanoid, yet unidentified substance which is thought to be one vehicle of regulation of airway tone by the epithelium, is thought to be dysregulated in airway constrictive diseases such as exercise-induced asthma (Fedan et al., 2004b). Our laboratory has studied the effects of LPS (4 mg/kg; i.p.) on the reactivity of the airways to MCh and hyperosmolar challenge. LPS reduced the reactivity of the airways to MCh in vivo (Johnston et al., 2004). Using the isolated, perfused trachea preparation (IPT) to measure transepithelial voltage (\(V_t\)) in vitro, our laboratory found that the response to MCh was
biphasic; \( V_t \) hyperpolarized, then depolarized with increasing concentrations of MCh (Johnston et al., 2004). LPS increased the hyperpolarization portion, but did not affect the depolarization portion of the response (Johnston et al., 2004). LPS also potentiated the depolarization response to hypertonicity evoked using NaCl as osmolyte (Johnston et al., 2004). Thus, LPS modifies the bioelectric activity of the epithelium in response to agents that affect smooth muscle tone. Our laboratory studied the roles of LPS and cytokines \textit{in vitro} on the reactivity of the smooth muscle to MCh and hyperosmolar challenge (Ismailoglu et al., 2009). Tracheas were excised, incubated with LPS, IL-1\( \beta \), IL-4, IL-13, IFN-\( \gamma \), and TNF-\( \alpha \) alone or in combination, and \( V_t \) was measured. \textit{In vitro} LPS and cytokines mimicked the \textit{in vivo} effects of LPS on smooth muscle responses to MCh and hyperosmolar challenge, but initiated different \( V_t \) responses from those to LPS \textit{in vivo} (Ismailoglu et al., 2009).

\subsection*{1.1.2: \( \text{Na}^+ \) transport and lung function}

The transport of \( \text{Na}^+ \) across the airway epithelium is vital for the maintenance of normal lung function. \( \text{Na}^+ \) is transported across the airway epithelium from the luminal to the basolateral compartment via ENaC in the apical membrane and the \( \text{Na}^+,\text{K}^+ \)-pump in the basolateral membrane (Fig. 1-3) (Boucher, 1994a). The \( \text{Na}^+,\text{K}^+ \)-pump drives the transport of \( \text{Na}^+ \) by pumping three \( \text{Na}^+ \) ions out of the cell for every two \( \text{K}^+ \) ions in against their concentration gradients. The net movement of cations and Cl\(^-\) through the epithelial cells and paracellularly osmotically draws water from the airway surface liquid layer to the interstitium via the transcellular and paracellular routes and is an important regulator of the thickness of the airway surface liquid layer. According to the two-layer model of mucus innate defense, a layer of mucus that captures debris and bacteria rides atop the airway surface liquid (Randell and Boucher, 2006). The epithelial ciliated cells propel the mucus layer up the airways by stroking their cilia, located in the underlying surface liquid layer, against the mucus layer. The proper function of this mucociliary escalator is maintained through tight regulation of epithelial \( \text{Na}^+ \) transport (Fig. 1-4).

The vital importance of the ENaC/\( \text{Na}^+,\text{K}^+ \)-pump axis is demonstrated by its malfunction in several disease states. Its importance in clearing lung fluid is indicated by the immediate death of
newborn αENaC−/− knockout mice due to their failure to clear lung fluid (Hummler et al., 1996). ENaC is inhibited in the genetic diseases of cystic fibrosis and pseudohypoaldosteronism type I. In cystic fibrosis, Cl− cannot leave the cell across the apical membrane because the cystic fibrosis transmembrane regulator, a chloride channel, is genetically defective (Fig. 1-3) (Donaldson et al., 2006). In Liddle’s syndrome, the PY motifs of βENaC, which serve as the binding sites for the ubiquitin ligase Nedd4-2, are mutated, inhibiting the ubiquitination and turnover of ENaC (Snyder et al., 2004). The hyperactivity of ENaC in both cystic fibrosis and Liddle’s syndrome result in the depletion of the airway surface liquid, inhibition of the mucociliary escalator (Matthay et al., 2005), and mucus plugging of the airways (Livraghi et al., 2008). Due to the inability of cystic fibrotics to clear lung bacteria, most patients perish from fatal lung infection (Boucher, 1994b; Galietta et al., 2002). Another genetic disease, pseudohypoaldosteronism type I, results in hypoactivity of ENaC with lung edema and impaired gas exchange (Staub et al., 2000; Rossier, 2004; Snyder et al., 2004; Rossier and Stutts, 2009).

1.1.3: Regulation of ENaC activity

ENaC is found in the apical membrane of many epithelia including the airway epithelium (Anantharam and Palmer, 2007). The channel assembly is composed of three subunit types, α, β, and γ, that surround the pore. The stoichiometry of the channel is debated; 2α:1β:1γ (Anantharam and Palmer, 2007) and 3α:3β:3γ (Staruschenko et al., 2004) have been reported. Each subunit’s structure has an extracellular loop connecting two transmembrane domains with the N- and C-termini inside the cell (Fig. 1-5) (Hughey et al., 2007). Na+ permeation through ENaC is regulated by many, complicated mechanisms including open probability, the number of mature and near-silent channels in the membrane, protein abundance, localization, and channel kinetics and degradation. The open probability of the channel is stimulated by carboxymethylation of the β subunit (Rokaw et al., 1998). The ubiquitination of ENaC by the ubiquitin ligase, neuronal precursor cell expressed developmentally downregulated (Nedd)4-2 (Snyder et al., 2004) targets it for internalization and lysosomal degradation, decreasing its membrane expression (Staub et al., 1997; Snyder et al., 2004). The mineralocorticoid hormones, notably aldosterone, upregulate
ENaC activity through many pathways (Fig. 1-6) (Loffing et al., 2006). Steroids bound to the mineralocorticoid receptor upregulate ENaC transcription at its core promoter. The postnatal rise in cortisol has been found to increase αENaC transcription and amiloride-sensitive lung fluid volume in guinea pigs (Baines et al., 2000). Additionally, increased cAMP (Woollhead and Baines, 2006) and pH (Collier and Snyder, 2009) have also been found to upregulate ENaC activity.

The main intracellular factors which activate ENaC in the epithelium of the renal distal tubules are the pathways regulated via the serum- and glucocorticoid-induced kinase (Sgk)1 (Fig. 1-6). Aldosterone activates Sgk1 in the kidney by binding to the mineralocorticoid receptor (Snyder et al., 2004). Sgk1 activates ENaC through a number of pathways including the direct phosphorylation and inhibition of the ubiquitin ligase Nedd4-2 (Loffing et al., 2006). Nedd4-2 ubiquitinates the β-subunit of ENaC to target the channel for internalization and endosomal degradation (Staub et al., 1997; Raikwar and Thomas, 2008). The open probability of ENaC is also increased by carboxymethylation of the β-subunit of ENaC (Rokaw et al., 1998). Elevation of cAMP increases short-circuit current and ENaC activity in human lung epithelial monolayers (Mazzochi et al., 2006b; Woollhead and Baines, 2006; Woollhead et al., 2007) by inducing the trafficking of ENaC from sub-membrane vesicles to the membrane (Mazzochi et al., 2006a; Woollhead and Baines, 2006; Bhalla and Hallows, 2008). Phosphatidylinositides have been observed to activate ENaC through a direct interaction, raising the possibility that G-protein coupled receptors regulate ENaC activity (Pochynyuk et al., 2007).

The endogenous serine protease enzymes furin and prostasin have recently been discovered to activate ENaC in kidney epithelium (Masilamani et al., 1999; Hughey et al., 2004a, b) and are found in the lung (Vallet et al., 1997; Myerburg et al., 2008). Two furin cleavage sites in the α-subunit and one furin and one prostasin cleavage site in γ-subunit activate the channel by liberating an inhibitory sequence from the extracellular loop regions of these subunits (Bruns et al., 2007; Carattino et al., 2008). The regulation of ENaC activation by proteolysis is incompletely understood, but is thought to occur both intracellularly (furin) (Sheng et al., 2006) and extracellularly (prostasin) (Myerburg et al., 2008; Rossier and Stutts, 2009). The theory is that newly synthesized ENaC protein can be sequestered in actin-bound
sub-apical vesicles for later insertion into the apical membrane with both the sub-apical and apical pools of ENaC acting as substrates for proteolytic activation to increase channel open probability (Fig. 1-7). ENaC activity in the airways is regulated by the concentration of prostatin in the airway surface liquid, which is thought to be a mechanism by which the surface liquid is maintained at a stable thickness (Myerburg et al., 2008). The serine protease trypsin activates ENaC by cleaving the furin and prostatin cleavage sites and has been used experimentally to activate ENaC (Bengrine et al., 2007).

1.1.4: Regulation of Na⁺,K⁺-pump activity

The Na⁺,K⁺-pump drives the transepithelial transport of Na⁺, which is regulated via alteration of its expression and turnover (Geering, 2008). The activity of the pump is stimulated by increased intracellular Na⁺ (Mairbäurl et al., 2002). The increase in intracellular Na⁺ that occurs in the cystic fibrotic epithelium stimulates the expression of FXYD5, a γ subunit of the Na⁺,K⁺-pump, that stimulates the activity of the pump by associating with the α catalytic and the β chaperone subunits (Miller and Davis, 2008). Increased intracellular Na⁺ concentrations do not necessarily upregulate FXYD5 in the epithelium; using apical nystatin to permeabilize human bronchial epithelial cells in an effort to model cystic fibrosis increased the activity of the Na⁺,K⁺-pump without effect on the transcription of FXYD5 or the α₁ and β₁ subunits of the Na⁺,K⁺-pump (Livraghi et al., 2008). Several factors have been found to increase transepithelial Na⁺ transport by stimulating the transcription and expression of the Na⁺,K⁺-pump. Among the many pathways by which aldosterone increases the activity of the ENaC/Na⁺,K⁺-pump axis, it also upregulates the transcription of Na⁺,K⁺-pump (Horisberger and Rossier, 1992).

Lung Na⁺ transport is also affected by cytokines. TFG-1β increases the expression of the α₁ and β₁ Na⁺,K⁺-pump subunits, but without effect on αENaC protein, in rat alveolar type II cells (Willis et al., 2003). IL-1β increases the activities of both ENaC and the Na⁺,K⁺-pump by increasing the protein expressions of the α₁ and β₁ subunits of the Na⁺,K⁺-pump and the α and β subunits of ENaC in the fetal guinea-pig lung (Ye et al., 2004). This contrasts to our finding that in vitro incubation of the guinea-pig trachea with IL-1β decreased V₁ and increased R, (Ismailoglu et al., 2009).
Conditions used to model lung injury result in lung edema and inhibit lung gas exchange by inhibiting the transepithelial transport of Na⁺. Hypoxia and reactive oxygen species produced during inflammation in the lung stimulate the endocytosis of the Na⁺,K⁺-pump from the basolateral membrane via the serial activation of AMPK and PKC (Fig. 1-8) (Mairbäurl et al., 2002; Vadász et al., 2007; Woollhead et al., 2007; Gusarova et al., 2009). Other factors, including corticosteroids and growth factors, which aid in the resolving of edema and reepithelialization after injury, stimulate the trafficking of the Na⁺,K⁺-pump to the membrane (Fig. 1-9) (Vadász et al., 2007). Nitric oxide reduces the activity of the Na⁺,K⁺-pump in the guinea-pig kidney (Seven et al., 2005). Nitric oxide reduces the expression of α₁ Na⁺,K⁺-pump in the rat sciatic nerve (Liu and Sheu, 1997). This effect of LPS appears to be mediated by a direct interaction between LPS and the cell because it is inhibited by polymyxin B, which binds to the lipid A portion of LPS.

1.2: Hypotheses and research plan

1.2.1: Hypothesis 1: LPS increases the activities of ENaC and the Na⁺,K⁺-pump

Previous work done in our laboratory has found that systemically administered LPS modifies airway reactivity in vivo and the bioelectric activity of the epithelium of the guinea-pig airways. The regulation of airway smooth muscle tone by epithelium-derived relaxing factor (EpDRF) has been linked to bioelectric responses of the airway epithelium (Dortch-Carnes et al., 1999). Therefore, our laboratory investigated the effects of LPS on the bioelectric activity of the epithelium in vitro. LPS (4 mg/kg; i.p.) reduced the in vivo reactivity of the guinea-pig airways to the constricting agent, methacholine (Johnston et al., 2004). Additionally, 18 h post LPS injection, the depolarization response to hyperosmolar challenge with NaCl in the IPT was potentiated (Johnston et al., 2004). In the IPT, LPS also potentiated the hyperpolarization portion of the biphasic Vt response to methacholine, but had no effect on the reactivity of the smooth muscle to methacholine (Johnston et al., 2004). Also, the administration of LPS in vitro to
the IPT inhibited the contractile responses to methacholine and histamine (Fedan et al., 1995). This inhibition involved NO, which did not seem to play any role in the absence of LPS.

For this study, I hypothesized that systemic LPS hyperpolarizes the airway epithelium by increasing the transepithelial transport of Na\(^+\). LPS hyperpolarized \(V_t\) measured in the IPT (Johnston et al., 2004). Amiloride, an inhibitor of ENaC (Kleyman and Cragoe, 1988), was used to measure the role of Na\(^+\) transport in the LPS-induced hyperpolarization. The depolarization caused by amiloride, that is, the amiloride-sensitive \(V_t\), was larger in tracheas from LPS-treated animals compared to controls, indicating that LPS increases the activity of ENaC. These findings were replicated (Chapter 2) and inspired the hypothesis. Na\(^+\) from the luminal compartment passes via ENaC into the epithelial cell down the concentration gradient created by the removal of Na\(^+\) from the epithelium by the Na\(^+\),K\(^+\)-pump to the basolateral compartment. Since the activity of the pump is electrogenic, the lumen is left with a net-negative charge and transepithelial voltage (\(V_t\)) is increased (Boucher, 1994a, b). Transepithelial Na\(^+\) transport is tightly regulated to maintain the thickness of the airway surface liquid layer and proper function of the mucociliary escalator (Randell and Boucher, 2006). Indeed, ENaC hyperactivity in cystic fibrosis leads to depletion of the airway surface liquid, plugging of the airways, and usually death due to the inability to clear lung infection (Boucher, 1994a; Galietta et al., 2002). Thus, the effect of LPS from a systemic gram-negative lung infection to hyperpolarize the epithelium could imply interference with lung function. In this study, the effects of in vivo LPS on the function of ENaC and the Na\(^+\),K\(^+\)-pump were measured.

1.2.1.1: ENaC activity

The effect of LPS on the activity of ENaC was measured in the IPT (Chapter 2). The contribution of the Na\(^+\),K\(^+\)-pump was removed by permeabilizing the basolateral membrane with amphotericin B, a cation pore-forming antifungal agent. This permeabilization strategy had been used in the Ussing chamber to measure the effects of hypoxia on ENaC activity (Mairbäurl et al., 2002). Basolateral Na\(^+\) was replaced with the nonpermeant cation, NMDG\(^+\), simultaneously to permeabilization to produce a large and uniform
Na⁺ gradient across the apical membrane. Amiloride-sensitive $V_t$ was subsequently measured. The results indicate that LPS increases the activity of ENaC.

### 1.2.1.2: Na⁺,K⁺-pump activity

The Na⁺,K⁺-pump has been identified as the rate-limiting step for Na⁺ transport across the airway epithelium (Looney et al., 2005). Since ENaC activity is driven by the Na⁺ gradient, which is produced by the Na⁺,K⁺-pump, the effects of LPS on the activity of the Na⁺,K⁺-pump were measured. Two, different approaches were used; the first one involved permeabilization of the apical membrane in the IPT, and the second one involved loading the epithelium with Na⁺ in the Ussing chamber.

#### 1.2.1.2.1: Apical permeabilization

The activity of the Na⁺,K⁺-pump was measured in tracheas from saline- and LPS-treated animals in the IPT after removing the contribution of ENaC from $V_t$. This was done by short-circuiting the Na⁺,K⁺-pump via the permeabilization of the basolateral membrane with amphotericin B, a technique which had been used in the Ussing chamber to measure the effects of hypoxia and TNF-α on Na⁺,K⁺-pump activity (Mairbäurl et al., 2002; Dagenais et al., 2004). $V_t$ measured after permeabilization is produced by the pump and the magnitude of $V_t$ reflects its activity. The findings from these experiments indicated that LPS more than doubled the activity of the Na⁺,K⁺-pump.

#### 1.2.1.2.2: Na⁺ loading

Due to the importance of the finding that LPS increases Na⁺,K⁺-pump activity, it was confirmed using an alternative Na⁺-loading approach in the Ussing chamber (Chapter 2). In this experiment, the activity of the Na⁺,K⁺-pump was inhibited by removing K⁺ from the modified Krebs-Henseleit solution (MKH) of the apical and basolateral baths. The magnitude of the depolarization during inhibition of the Na⁺,K⁺-pump reflected its activity. K⁺ was restored to both MKH baths and the magnitude of repolarization on resumption of Na⁺,K⁺-pump was measured. Both parameters were greater in tracheas
from LPS-treated animals compared to controls, confirming the finding that LPS increases the activity of
the Na⁺,K⁺-pump.

1.2.1.3: ENaC activation by channel activating proteases

The role of channel activating proteases in the activation of ENaC by LPS was investigated
(Chapter 2). ENaC could be activated by LPS through several mechanisms including increased
expression, open probability, or trafficking to the membrane. We hypothesized that LPS increases the
activation of ENaC by increasing its activation by endogenous channel activating proteases. The serine
protease, trypsin, activates ENaC by cleaving the same sites as furin and prostasin. If LPS triggers the
activation of ENaC by channel activating proteases, we hypothesized that the pool of near-silent,
uncleaved ENaC in the apical membrane would be smaller and render the tracheas from LPS-treated
animals less responsive to trypsin. This hypothesis was investigated in the Ussing chamber by measuring
the Vᵢ responses to exogenous trypsin and by measuring the effects of trypsin on amiloride-sensitive Vᵢ.

1.2.1.3.1: Trypsin effects on amiloride-sensitive Vᵢ

The Vᵢ responses to trypsin in the Ussing chamber were compared between tracheas from saline-
and LPS-treated animals (Chapter 2). Additionally, the Vᵢ responses in the absence and presence of
trypsin were compared to determine the effect of trypsin on amiloride-sensitive Vᵢ and ENaC activity. If
LPS increased the activity of ENaC via channel activating proteases, we expected to measure a larger Vᵢ
response in tracheas from LPS-treated animals. The Vᵢ responses to trypsin were not different between
treatment groups even though amiloride-sensitive Vᵢ was larger in tracheas from LPS-treated animals in
the absence and presence of trypsin. Thus, LPS does not increase the activation of ENaC via proteolytic
cleavage.

1.2.1.3.2: Trypsin effects on Vᵢ
The $V_t$ responses to apical trypsin in tracheas from saline- and LPS-treated animals were also investigated in the Ussing chamber without previously adding amiloride and washing it out. We had observed that the addition of apical trypsin to a trachea from an untreated animal in the Ussing chamber increased both $V_t$ and $R_t$. Thus, we considered the possibility that the increase in $V_t$ could be caused by an increase in $R_t$. We hypothesized that if the trypsin-induced increase in $V_t$ were due to ion transport, $R_t$ should be increased to a proportionately smaller degree than $V_t$. Furthermore, if LPS increased ENaC activity via channel activating proteases, the effect of trypsin on ion transport should be smaller in tracheas from LPS-treated animals compared to controls. The results indicated that $V_t$ and $R_t$ were increased to the same proportion and support the conclusion that LPS has no affect on the activation of ENaC by channel activating proteases.

1.2.2: Hypothesis 2: LPS increases ENaC and Na$^+$,K$^+$-pump message

The effects of LPS on the transcription of ENaC and the Na$^+$,K$^+$-pump, as well as a selection of inflammatory genes, were investigated by quantitative, real-time polymerase chain reaction (qPCR; Chapter 3). The rate of transcription of ENaC has been demonstrated to be an important determinant of ENaC activity and plays a critical role in the absorption of lung fluid (Dickie et al., 2000; Xu et al., 2007). The core promoter of ENaC is activated by the steroid aldosterone (Horisberger and Rossier, 1992; Ma et al., 2004) and glucocorticoids such as dexamethasone (Dagenais et al., 2006; Husted et al., 2007; Xu et al., 2007), which lead to greater ENaC activity (Snyder et al., 2004; Dagenais et al., 2006; Husted et al., 2007). Other factors such as cigarette smoke (Xu et al., 2007) and LPS-activated macrophages (Dickie et al., 2000) have been demonstrated to inhibit ENaC transcription.

The effects of LPS on the transcription of a selection of cytokines and inflammatory mediators were also measured by qPCR. Many cytokines have been found to affect Na$^+$ transport including IL-4 (Galietta et al., 2002), TNF-$\alpha$ (Dagenais et al., 2004), which inhibit ENaC transcription and activity, and IL-1$\beta$, which increases the activities of ENaC and the Na$^+$,K$^+$-pump in fetal guinea pigs (Ye et al., 2004). TNF-$\alpha$ has been found to inhibit the up-regulation of ENaC by dexamethasone (Dagenais et al., 2006).
NO influences lung ion transport by inhibiting the activity of the Na\(^+\),K\(^+\)-pump (Seven et al., 2005), decreasing the open probability of ENaC via increased production of cGMP (Matalon et al., 2003; Eaton et al., 2008) and activating Cl\(^-\) channels (Duta et al., 2006).

Activation of the prostaglandin synthase COX-2 leads to the production of prostaglandin (PG) E\(_2\). PGE\(_2\) increases the open probability of ENaC (Wang et al., 2009), and has been reported to stimulate the transcription and activity of Na\(^+\),K\(^+\)-ATPase (Matlhagela and Taub, 2006), even though inhibition of the Na\(^+\),K\(^+\)-pump has also been reported (Kreydiyyeh et al., 2007; Oliveira et al., 2009). LPS induces smooth muscle relaxation in the mouse IPT by inducing the transcription of COX-2 via TLR-4 and NF-\(\kappa\)B (Balzary and Cocks, 2006). In addition to LPS (Held and Uhlig, 2000; Balzary and Cocks, 2006), ozone (Nakano et al., 2000) and ovalbumin (Oguma et al., 2002) also stimulate the transcription and activity of COX-2. Using the guinea-pig model, our laboratory has found that the hyperpolarization of the airway epithelium by LPS is inhibited by indomethacin, albeit to a lesser degree than by amiloride, suggesting that prostaglandins also play a role in the hyperpolarizing effect of LPS (Johnston et al., 2004).

The effects of LPS on the rates of transcription of \(\alpha\)ENaC, \(\alpha_1\) Na\(^+\),K\(^+\)-ATPase, COX-2, eNOS, IL-1\(\beta\), iNOS, and TNF-\(\alpha\) were measured by qPCR (Chapter 3). Transcription was measured in samples taken at 3 and 18 h post-injection of LPS or saline because each cytokine’s expression follows a different time-course and magnitude of up-regulation when stimulated. Readings were also made on alveolar macrophage as positive controls. The findings revealed that even though LPS increased the transcription of several inflammatory-response genes, no increase in the transcription of ENaC or Na\(^+\),K\(^+\)-ATPase was observed. No evidence was found that LPS regulates ENaC or the Na\(^+\),K\(^+\)-pump at the level of transcription.

### 1.2.3: Hypothesis 3: LPS increases ENaC and Na\(^+\),K\(^+\)-pump expression

As mentioned above, we found that LPS increased the functional activity of ENaC and of the Na\(^+\),K\(^+\)-pump in the tracheal epithelium of the guinea pig (Dodrill and Fedan, 2010). As described in the literature review, the activities of ENaC and the Na\(^+\),K\(^+\)-pump are regulated at the level of protein
expression. We hypothesized that the LPS-induced increase in Na\(^+\) transport is mediated by a stimulation of the expression of ENaC and the Na\(^+\),K\(^+\)-ATPase. As described in Chapter 3, we investigated the effects of LPS on the expression of ENaC and the Na\(^+\),K\(^+\)-ATPase. This investigation revealed that LPS did not affect the expression or cleavage of ENaC, supporting our earlier finding using exogenous trypsin that activating proteases are not involved in ENaC activation by LPS. LPS increased the expression of the Na\(^+\),K\(^+\)-pump. This finding indicates LPS activates the transepithelial transport of Na\(^+\), in part, by increasing the expression of the Na\(^+\),K\(^+\)-pump.

1.2.3.1: ENaC protein expression

The effects of LPS on ENaC expression were investigated by western blotting. Polyclonal antibodies against \(\alpha\)-, \(\beta\)-, and \(\gamma\)-ENaC were used to probe ENaC in whole-cell epithelium homogenates 18 h post-injection. We hypothesized that an effect of LPS on ENaC abundance would be detected as an increase in band intensity. Many commercially-available antibodies were tested until ones were identified which recognized guinea-pig ENaC. Indeed, the literature we found describing western blotting of guinea-pig ENaC in native tissues used custom-made antibodies (Mackler et al., 1998; Ramírez-Gil et al., 1998). The \(\beta\)-, and \(\gamma\)-ENaC subunits where detected in their full-length form, while \(\alpha\)ENaC was found a high molecular-weight complexed form. Additionally, each of the three subunits was detected in lower molecular-weight bands suggesting proteolytic cleavage. There was no difference in abundance of any of these bands between saline- and LS-treated animals. Thus, no effect of LPS on the abundance, cleavage, or other biochemical modification was detected.

1.2.3.2: Na\(^+\),K\(^+\)-pump protein expression

We found that LPS increased the activity of the Na\(^+\),K\(^+\)-pump (Chapter 2), yet no effect on \(\alpha_1\) Na\(^+\),K\(^+\)-ATPase message was detected. Due to the difficulties identifying working qPCR assays, we were only able to investigate the \(\alpha_1\) subunit, and it is possible that the transcription of other subunits could have been stimulated. As described in the literature review, several factors have been identified that upregulate
Na\textsuperscript{+},K\textsuperscript{+}-ATPase expression without an effect on transcription. We hypothesized that LPS increased Na\textsuperscript{+},K\textsuperscript{+}-pump activity by increasing its protein abundance. Monoclonal antibodies against the $\alpha_1$ subunit and pan-$\alpha$ ($\alpha_1$, $\alpha_2$, and $\alpha_3$ as a group) were used to probe protein abundances in western blots. Both $\alpha_1$ subunit and pan-$\alpha$ abundance were increased in the epithelium from LPS-treated animals. This finding supports on a molecular level the increase in Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity that was observed in the IPT.

1.2.3.3: Investigation of ENaC trafficking

LPS increased the activity of ENaC, but had no effect on its transcription, abundance, or cleavage. Thus, we hypothesized that LPS could increase the activity of ENaC by stimulating its trafficking from sub-membrane stores to the apical membrane. To investigate this hypothesis, we undertook apical cell surface protein isolation using biotinylation and probed for ENaC by western blotting as described in Chapter 3. The resulting bands were too faint to quantify densitometrically. Despite exhaustive efforts to improve the protein yield of the isolation protocol, the signals remained too weak. We concluded that the apical membrane pool of ENaC could not be probed because it is too small, given the limited amount of protein available from guinea-pig tracheal epithelium.

1.3: Methods of approach and experimental design

Specific methods are given in Chapters 2 and 3.

1.3.1: Animal model

The organism used for all of the experiments in this project was the male Hartley guinea pig. The guinea pigs were treated by injection of LPS (4 mg/kg) from *Salmonella enterica* serotype *typhimurium* or a volume equivalent of saline. Animals were anaesthetized with sodium pentobarbital (65 mg/kg; i.p.) and bled 18 h post-injection. For qPCR, some samples were also taken at 3 h. For functional experiments, tracheas were excised, cleaned in a dish of MKH solution, and mounted on the perfusion holder or the Ussing chamber. For biochemical experiments, tracheas were excised, cleaned, cut along the smooth muscle band, and flattened for epithelial cell removal.
1.3.2: Functional experiments

Functional experiments were carried out to investigate the effects of the in vivo administration of LPS on the activities of ENaC and the Na⁺,K⁺-pump. Two techniques were used, the Ussing chamber and the IPT. Both techniques allow agents that were used to study ion transport to be added selectively to the apical or basolateral faces of the airway (Fig. 1-10). These experiments are described in Chapter 2.

1.3.2.1: Ussing chamber

The Ussing chamber was used to investigate the proteolytic activation of ENaC and the activity of the Na⁺,K⁺-pump. The Ussing chamber is a device to measure transepithelial bioelectric voltage or current across epithelial sheets (Fig. 1-11). In contrast to a simple organ bath, the basolateral and apical baths are isolated from each other by the trachea itself. As described in Chapter 2, the trachea was cleaned, cut along the smooth muscle band, flattened, and mounted between two hemi-chambers. Each hemi-chamber contained a separate apical or basolateral bath of MKH. Each bath was bubbled with 95% O₂/5% CO₂ to aerate and recirculate the solutions and maintained at 37 °C using a water bath. One set of voltage electrodes was placed in each bath was used to measure Vᵢ under open-circuit conditions. The other set was used to apply 5 µAmp current pulses. Rᵢ was calculated using the magnitude of the deflection in Vᵢ caused by the 5 µAmp pulse in Ohm’s law. The effects of LPS on the proteolytic activation of ENaC were investigated by measuring the effects of apical trypsin (100 U/ml) on basal Vᵢ and amiloride-sensitive Vᵢ.

The Ussing chamber was used to investigate the proteolytic activation of ENaC as described in Chapter 2. Firstly, the effects of trypsin on Vᵢ and Rᵢ were measured in tracheas from saline- and LPS-treated animals and compared. Secondly, the effects of trypsin on amiloride-sensitive Vᵢ were also investigated using the Ussing chamber. Apical amiloride (10 µM) was added and the depolarization of Vᵢ was measured. Amiloride was washed out by draining and replacing the baths with fresh MKH solution.
times. The hyperpolarization response to apical trypsin (100 U/ml) was measured, and the depolarization of $V_I$ to amiloride (10 µM) was measured in the presence of trypsin.

The Ussing chamber was also used to investigate the activity of the $\mathrm{Na}^+\mathrm{K}^+$-pump using a $\mathrm{Na}^+$-loading approach. This experiment took advantage of the fact that the $\mathrm{Na}^+\mathrm{K}^+$-pump has an absolute requirement for extracellular $\mathrm{K}^+$ to function. Thus, $\mathrm{K}^+$ was removed from the apical and basolateral baths to inhibit the activity of the pump. This was accomplished by draining the normal MKH from each bath and replacing it with K$^+$-free MKH which lacked KCl and KH$_2$PO$_4$, but contained 119 mM NaCl. When the depolarization from $\mathrm{Na}^+\mathrm{K}^+$-pump inhibition stabilized, pump activity was restored by restoring $\mathrm{K}^+$ to the basolateral, then the apical bath. KCl was restored by adding 6 mM KCl to each bath. At the end of the experiment, basolateral ouabain (10 µM) was added to inhibit the $\mathrm{Na}^+\mathrm{K}^+$-pump and confirm that it was active.

**1.3.2.2: Isolated, perfused trachea preparation**

The IPT, which was used for many of the experiments in Chapter 2, is a technique which can be used to study the electrical behavior of the epithelium and smooth muscle tone simultaneously. The trachea was excised, cleaned, and mounted on a perfusion holder (Fig. 1-12). The lumen of the trachea was perfused with a re-circulating bath of MKH at a rate of 20 ml/min. The holder was placed in a separate, extraluminal bath (Fig. 1-13). Agents could be added selectively to either the intraluminal (apical) or extraluminal (basolateral) baths. A set of electrodes in continuity with both baths was used to measure $V_I$ under open-circuit conditions. Indwelling cannula at the inlet and outlet ends of the trachea provided the necessary resistance for pressure measurement. Side holes in these cannulae were connected to a differential pressure transducer and could be used to detect changes in trachea diameter, even though such measurements were not taken for this project. Both baths were aerated with 95% O$_2$/ 5% CO$_2$ to maintain the viability of the tissue and maintained at 37 °C using a water bath.
The IPT was used to investigate the effects of LPS on the activities of ENaC and the Na\(^+\),K\(^+\)-pump, as described in Chapter 2. Both of these experiments used the cation pore-forming antifungal agent, amphotericin B (7.5 µM) to permeabilize the contralateral membrane, as follows. To measure the activity of ENaC, the Na\(^+\),K\(^+\)-pump was short-circuited by the addition of amphotericin B to the basolateral bath. The activity of ENaC relies on the Na\(^+\) gradient created by the removal of intracellular Na\(^+\) by the Na\(^+\),K\(^+\)-pump. Without this gradient, there would be no Na\(^+\) current. For this reason, the Na\(^+\) in the basolateral bath was replaced with the nonpermeant cation, NMDG\(^+\) at the same time as amphotericin B addition. This was accomplished by draining the basolateral bath and replacing it with reduced Na\(^+\) MKH already containing amphotericin B (7.5 µM). The reduced Na\(^+\) MKH was prepared by omitting NaCl and replacing it with pH-adjusted N-methyl-D-glucamine (NMDG)-Cl (113 mM). As described in the methods, apical amiloride (10 µM) was administered to measure amiloride-sensitive \(V_t\) with the basolateral membrane short-circuited. To confirm that the channel had been blocked, apical trypsin (100 U/ml) was added in the presence of amiloride.

To measure the activity of the Na\(^+\),K\(^+\)-pump in the IPT, a similar protocol using amphotericin B to permeabilize the contralateral membrane was used. Apical amiloride (10 µM) was added to measure amiloride-sensitive \(V_t\). Apical amphotericin B (7.5 µM) was administered to short-circuit the apical membrane. Unlike the basolateral permeabilization experiment investigating ENaC activity, the ion composition of the MKH solution on both sides of the trachea did not need to be modified because the Na\(^+\),K\(^+\)-pump is energy-dependent and pumps both Na\(^+\) and K\(^+\) against their respective gradients. Trypsin (100 U/ml) was added apically in the presence of amphotericin B.

1.3.3: Biochemical experiments

Biochemical experiments were performed to investigate the effects of LPS on the expression of ENaC and the Na\(^+\),K\(^+\)-pump. They used the same guinea-pig model and LPS injection protocol as the functional experiments, but the tissues were prepared differently. After dissection and removal, the
trachea was cleaned and the epithelium removed. The cells were homogenized and prepared according to the appropriate protocol for the technique used.

1.3.3.1: Quantitative, real-time PCR

The effects of LPS on the transcription of ENaC and the Na⁺,K⁺-ATPase were determined using qPCR. This technique is used to measure the relative amount of starting mRNA by measuring amplification of fluorescent signal in the exponential phase. This study used regular PCR equipment in combination with the Universal Probe Library system (Roche; Basel, Switzerland), which uses 165 pre-validated probes that can be used to detect nearly any transcript. This small number of probes is made possible by using nucleic acids containing a methylene bridge between the 2'-O and the 4'-C atoms, strengthening Watson-Crick base-pairing and requiring a probe only 8 base pairs in length. They thus provide the advantage over non-specific SYBR Green by providing the specificity of custom designed probes. Primers were designed using online Probe Finder software, which is an optimized version of Primer3 software. Upon entering the mRNA sequence of the target and choosing human as the reference organism, the software designed primers. The software predicted the specificity of the primers using in silico PCR and selected probes. The isolation of RNA, reverse transcription, and real-time PCR were accomplished using standard methods as described in Chapter 3.

1.3.3.2: Western blotting

Western blotting was employed to investigate the effects of LPS on the abundance and cleavage of ENaC and the Na⁺,K⁺-pump. Standard denaturing, SDS-page conditions were used as described in Chapter 3. 7.5% Tris-HCl gradient gels were chosen because they separated best in the molecular-weight range of 65-200 kD, where most of the targets were expected to be detected. Targets were probed using monoclonal and polyclonal antibodies. As described in Chapter 4, many antibodies were tested for a variety of targets before working ones were identified. New primary antibodies were tested in serial dilutions; the blots of each of these dilutions were then tested with serial dilutions of secondary antibody.
The combination of primary and secondary antibody dilutions that yielded the best contrast of signal to background were chosen for experiments. During experimentation, the antibody dilutions, protein loading, incubation times, reagents used, and other parameters were adjusted to optimize the clarity of the blots.

1.3.3.3: Cell-surface protein isolation

Cell surface protein isolation was attempted to study the effects of LPS on the apical membrane abundance of ENaC. These experiments are covered in Chapter 3. After removal of the trachea from the guinea pig, it was cleaned and cannulated. The lumen was filled with biotinylation solution and incubated overnight to biotinylate the surface proteins of the apical membrane. The biotin solution was quenched and the epithelium was lysed. Biotinylated protein was isolated from the lysate using a neutravidin-linked bead column. The proteins eluted from the column were immunoblotted for α-, β-, and γ-ENaC using standard western blotting. The miniscule amount of ENaC in the apical membrane rendered these experiments unsuccessful.
1.4: Bibliography


1.5: Figures
Fig. 1-1: Structure of LPS. LPS is an important component of the outer membrane of gram-negative bacteria that helps maintain the membrane’s structure and protects it from certain kinds of chemical attack. The molecule consists of a highly variable polysaccharide portion linked to a lipid A portion which is anchored in the membrane. The polysaccharide portion is responsible for immunogenicity via its interaction with TLRs, while the lipid A portion is responsible for its toxicity in the host. Image used with permission from Liebers et al (Liebers et al., 2008).
The activation of the macrophage inflammatory response by LPS has been widely studied. The immunogenicity of LPS is mediated by the interaction of its polysaccharide portion with TLR, which necessitates circulating LPS binding protein and CD14. This interaction activates a number of intracellular signaling pathways including the MAPKs, Jnk, and ERK which activate the transcription factor NF-κB. NF-κB enters the nucleus and promotes the transcription of a number of inflammatory mediators including cytokines IL-1β, IL-6, IL-10, TNF-α and many others. Image used with permission from Liebers et al (Liebers et al., 2008).
Fig. 1-3. ENaC hyperactivity dehydrates the airway surface liquid layer. The thickness of the airway surface liquid is maintained by the lung epithelium to sustain the mucociliary escalator. Na+ transport from the lumen to the basolateral compartment osmotically draws water with it. Na+ transport is driven by the activity of the Na+,K+-pump, which moves 3 Na+ ions out of the cell for every 2 K+ ions brought in against their respective concentration gradients. The reduction in intracellular Na+ creates the gradient for ENaC current. When ENaC is hyperactive, as in cystic fibrosis where Cl⁻ cannot be secreted because of genetically defective cystic fibrosis transmembrane regulator, increasing the electrochemical gradient for Na+ entry. This increase in intracellular Na+ concentration triggers the expression of FXYD5 in the lung epithelium, which upregulates Na+,K+-pump activity. The net increase in Na+ transport dehydrates the airway surface liquid and inhibits the mucociliary escalator. Image used with permission from Miller and Davis (Miller and Davis, 2008).
According to the two-layer model of lung mucociliary clearance, the mucus layer floats atop the airway surface liquid layer. Under normal conditions, the rate of mucociliary clearance is determined to be ≈60 µm/s. Under conditions that increase hydration of the airway surface, such as a decrease in transepithelial Na⁺ transport, mucus absorbs water from the airway surface liquid layer and the rate of mucociliary clearance increases to 100 µm/s. When the airway surface liquid is depleted, as happens when ENaC is hyperactive in cystic fibrosis, the mucus layer dries out and mucociliary movement is inhibited. This state renders the patient susceptible to lung infection as foreign matter can no longer be cleared from the lung. Image used with permission from Randell and Boucher (Randell and Boucher, 2006).
Fig. 1-5. Structure of an ENaC subunit. The ENaC assembly is constituted of three subunit types α, β, and γ that are similar in structure. Two membrane spanning domains, M1 and M2, are connected by an extracellular loop (ECL), while the N- and C-termini are located within the cytoplasm of the cell. The cleavage of the α and γ subunits by the serine proteases furin and prostasin releases an inhibitory from the extracellular loop region and fully activates the channel. The α subunit is cleaved by furin at two RXXR motifs spanning the inhibitory domain. The γ subunit is cleaved by furin at an RXXR site and by prostasin at an RKRK site that corresponds to the second RXXR site in αENaC. The β subunit, which lacks RXXR and RKRK domains at the corresponding locations, is not cleaved by furin and prostasin. The PY domain located at the C-terminal end is ubiquitinated by Nedd4-2. Image used with permission from Sheng et al (Sheng et al., 2006).
Fig. 1-6. **Cellular regulators of ENaC.** ENaC is regulated by many mechanisms within the cell. Aldosterone, which upregulates Na⁺ absorption in the kidney epithelium, binds to the mineralocorticoid receptor and enters the nucleus. Insulin and growth factors activate serum- and glucocorticoid-induced kinase (Sgk)1 via PI-3K and 3-phosphoinositide-dependent protein kinase (PDK)-2. Sgk1 also becomes activated through phosphorylation by with no lysine kinase (WNK)1 and PDK1. Sgk1 stimulates ENaC activity by phosphorylating the channel directly, and by Nedd4-2 by phosphorylation. Nedd4-2 ubiquitinates the channel and targets it for internalization. Reprinted, with permission, from the Annual Review of Physiology, Volume 68 ©2006 by Annual Reviews www.annualreviews.org (Loffing et al., 2006).
Proteolytic activation of ENaC. ENaC activity is regulated by the serine protease enzymes furin and prostasin in the airway epithelium. Furin and prostasin cleavage releases an inhibitory domain from the extracellular loop structures of α- and γ-ENaC, increasing its open probability (Pₒ). A, The colocalization model of ENaC activation proposes that furin is located intracellularly, while other membrane-bound and soluble serine proteases (SP) are located at the apical membrane. The serine proteases are regulated by the concentration of serine protease inhibitor (serpin). ENaC becomes cleaved as it traffics through the parts of the pathway were the proteases are localized. B, The other model, known as the sequential model includes the direct cleavage of ENaC as postulated by the colocalization model, but adds the indirect activation of proteases as a regulator of channel activating proteases. According to this model, a chain of serine proteases activate other proteases before they, in turn, activate ENaC. Reprinted, with permission, from the Annual Review of Physiology, Volume 71©2009 by Annual Reviews www.annualreviews.org (Rossier and Stutts, 2009).
Fig. 1-8. Endocytosis of the Na⁺,K⁺-pump. Many models and markers of lung injury induce the internalization of the Na⁺,K⁺-pump and lead to decreased epithelial Na⁺ transport. Some of these factors include alveolar hypo- and hyper-oxia, elevated coagulation factor thrombin (THR), ventilator-induced lung injury (VILI), hypercapnia and hypercapnic alkilosis, and ischemia-reperfusion injury. Image used with permission from Vadász et al (Vadász et al., 2007).
Fig. 1-9. Trafficking of Na⁺,K⁺-pump to the membrane. Several factors stimulate the trafficking of Na⁺,K⁺-pump from endosomal compartments to the membrane in the lung epithelium. Some of those factors include β-adrenoreceptor agonists, dopamine, corticosteroids, and growth factors. Growth factors, which promote re-epithelialization after lung injury, may play a central role in the resolution of edema. Image used with permission from Vadász et al (Vadász et al., 2007).
Fig. 1-10. Drugs used for functional experiments. The IPT and the Ussing chamber allow the administration of drugs selectively to the apical or basolateral faces of the tracheal epithelium while measuring $V_t$. Amiloride-sensitive $V_t$ was used as a measure of the activity of ENaC and was determined by administering the ENaC blocking agent amiloride (10 µM) apically. The cation pore forming antifungal agent, amphotericin (7.5 µM), was added to the apical face to short-circuit ENaC and cancel its contribution to $V_t$. In other experiments, amphotericin B (7.5 µM) was added to the basolaterally to short-circuit the Na$^+,K^+$-pump to remove its contribution to $V_t$. Ouabain (10 µM) was added to the basolateral membrane to inhibit the activity of the Na$^+,K^+$-pump.
The Ussing chamber supports the measurement of bioelectric activity across epithelial sheets. In the experiments done for this project, the trachea was removed from the saline- or LPS-treated animal 18 h post-injection, cleaned, cut along the smooth muscle band, and flattened. A, The trachea, represented by the vertical line, was mounted between two hemi-chambers containing separate apical and basolateral baths. Two sets of electrodes were placed in each bath. One set of voltage electrodes permitted the measurement of $V_t$ under open-circuit conditions. Another set of current electrodes was used to apply a 5 µA current across the trachea at regular time intervals. Image used with permission from Wills et al (Wills et al., 1996). B, The voltage deflections $\Delta V_t$ in response to the application of 5 µA current pulses permitted the calculation of $R_t$ using Ohm’s law ($V=I\cdot R$).
Fig. 1-12. Isolated, perfused trachea holder. This graphic represents a cross-sectional view of the isolated, perfused trachea mounted in the perfusion holder. The trachea, represented in orange, is mounted on the perfusion holder after being excised from the saline- or LPS-treated animal and cleaned. The lumen is perfused with a re-circulating bath of MKH at a constant rate of 20 ml/min. The holder is mounted in a separate, extraluminal bath. Electrodes placed in continuity with each bath permit the measurement of $V_t$. Indwelling cannula in the inlet and outlet ends of the lumen apply the necessary resistance for the low perfusion flow rate. Side holes in the cannula are connected to a differential pressure transducer and allow the inlet minus outlet pressure to be measured. Changes in the pressure differential reflect changes in the diameter of the trachea. No pressure data were taken for this project.
Fig. 1-13. Isolated, perfused trachea preparation apparatus. The extraluminal and intraluminal baths were separate from each other. Both baths were maintained at 37 °C and bubbled with 95% O₂/5% CO₂ to maintain the viability of the tissue. The intraluminal bath is recirculated by a pump through a bubble trap, heat exchanger, and the lumen of the trachea. A differential pressure transducer is used to measure inlet minus outlet pressure difference.
Chapter 2
Lipopolysaccharide hyperpolarizes the guinea-pig airway epithelium by increasing the activities of
the epithelial Na$^+$ channel and the Na$^+$,K$^+$-pump

Michael W. Dodrill$^{1,2}$ and Jeffrey S. Fedan$^{1,2}$

From the Department of Basic Pharmaceutical Sciences, School of Pharmacy, Robert C. Byrd Health
Sciences Center, West Virginia University$^1$, and Health Effects Laboratory Division, National Institute
for Occupational Safety and Health$^2$, Morgantown, WV 26505.

Running head: LPS increases ENaC and Na$^+$,K$^+$-pump activities

Address correspondence to: Jeffrey S. Fedan, Ph.D.
1095 Willowdale Road
Morgantown, WV 26505
Tele: 304-285-5766
Fax: 304-285-6928
E-mail: jsf2@cdc.gov
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-circuitied 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-\(\alpha\) (11), IL-1\(\beta\) (67), aldosterone (38), the serine proteases furin and prostatasin (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-\(\alpha\) (11, 18), IL-4 (19), TGF-\(\beta\) (64), IL-1\(\beta\) (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 \(\alpha\)ENaC 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 Vt, 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 Vt 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 Vt (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% O2/5% CO2, 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% O2/5% CO2. 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 (ΔP), but such measurements were not taken for this study. Vt 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 cm\(^2\)) 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\(_2\)PO\(_4\), 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\(_2\)PO\(_4\), 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 \((-34.2 \pm 2.7 \text{ mV})\) compared to controls \((-5.5 \pm 0.7 \text{ mV})\) (Fig. 1). The ENaC blocker, amiloride (10 \(\mu\)M) (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 \(-4.5 \pm 0.9 \text{ mV}\) in saline-treated animals and \(-13.1 \pm 1.3 \text{ mV}\) in the LPS-treated group. The effect of amiloride, or \(\Delta V_t\), was significantly greater (Mann-Whitney rank sum test) in tracheas from LPS-treated animals \((21.1 \pm 2.8 \text{ mV})\) compared to saline-treated animals \((1.0 \pm 0.3 \text{ mV})\). 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 \(\mu\)M), 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 \(\mu\)M), 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 \(\alpha\)- and \(\gamma\)-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 Vᵢ. A greater Vᵢ response to trypsin would reflect a greater pool of near-silent ENaC. If LPS increases cleavage-mediated activation of ENaC, we hypothesized that the Vᵢ response to trypsin should be reduced compared to controls. LPS increased basal Vᵢ, as we found earlier, and basal equivalent transepithelial current (Iₑ) (Table 1), but did not affect the basal resistance of the epithelium. Trypsin increased Vᵢ and Rₑ, but did not affect Iₑ in tracheas from both control and LPS-treated animals. The trypsin-induced changes in Vᵢ and Rₑ (ΔVᵢ and ΔRₑ), and Iₑ, were, however, not different between the two groups. As well, the %ΔVᵢ/%ΔRₑ ratio was not different between the untreated and LPS-treated groups. These results indicate that the effects of LPS on the Vᵢ, Rₑ, and Iₑ responses to trypsin cannot be explained by an increase in transepithelial ion transport. The small increases in Rₑ 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 Vᵢ 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 Vᵢ 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\(_i\) 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\(_i\) (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\(_i\), whereupon V\(_i\) was decreased. Even though the V\(_i\) 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 \textit{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 occurred 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 $R_i$, 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 $R_i$ 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 Ca²⁺ and PKC activation. The activation of PAR receptors has been found to inhibit the Ca²⁺ 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 $R_i$ in the airway epithelium. Due to the lack of effect of ouabain on the trypsin-induced effect on $R_i$ in that study, the authors concluded that $R_i$ 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 $Na^+\cdotK^+$-pump (22), or increased ENaC activity could stimulate the $Na^+\cdotK^+$-pump by elevating intracellular $Na^+$ concentrations (42). Since amiloride-sensitive $V_t$ was increased by LPS irrespective of whether the $Na^+\cdotK^+$-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 $\alpha$-, $\beta$-, or $\gamma$-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 $\beta$ENaC, but without an effect on $\alpha$ENaC or $\alpha_1\ Na^+\cdotK^+$-ATPase transcription (46). In a similar study, the intratracheal instillation of $P.\ aeruginosa$ in mice reduced the transcription of $\alpha$ENaC, but had no effect on the transcription of $\beta$- or $\gamma$-ENaC or $\alpha_1\ Na^+\cdotK^+$-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
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 α₁ 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 $\alpha_1$ 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.
Acknowledgements

The authors wish to thank Janet Thompson for her technical assistance. The authors declare that they have no conflicts of interest. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health. Mention of brand name does not constitute product endorsement.
References


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**Figure Legends**

**Fig. 1. LPS increases ENaC activity in the airway epithelium.** The IPT was used to measure amiloride-sensitive $V_i$ in tracheas from saline- and LPS-treated guinea pigs. Basal $V_i$ was greater in tracheas from LPS-treated animals. Amiloride (10 µM) inhibited $V_i$ ($\Delta V_i$) in both treatment groups, but to a larger extent after LPS treatment. The effect of amiloride on $V_i$ 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 $V_i$ 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 $V_i$ 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 $V_i$ before $V_i$ stabilized was not different between the epithelia of tracheas from saline- and LPS-treated animals. B, The stable $V_i$ 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 $V_i$ response to amiloride was greater in tracheas from LPS-treated animals compared to control (*p < 0.05, Mann-Whitney rank sum test). D, $V_i$ in the presence of trypsın was not different between tracheas from saline- or LPS-treated animals. E, The $\Delta V_i$ response to trypsın 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 trypsın 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. Trypsın (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), trypsın (100 U/ml), and amiloride (10 µM) in the presence of trypsın are expressed as the change in $V_i$ 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 $V_i$ 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 $V_i$. Amphotericin B (AB; 7.5 µM) was added to the apical bath to short-circuit the apical membrane of the epithelium. Trypsın (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 $V_i$, 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 KH₂PO₄ 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).
Table 1: Effects of trypsin on $V_t$, $R_t$, and $I_{sc}$ in tracheas from untreated and LPS-treated animals.

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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; $R_t$, $\Omega \cdot \text{cm}^2$; $I_{sc}$, $\mu\text{A}/\text{cm}^2$. Untreated, $n = 5$; LPS, $n = 6$. 

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Chapter 2: Figures
Figure 1

Saline

-40
-30
-20
-10
0
Saline

LPS

-40
-30
-20
-10
0
+Amiloride

- Amiloride

**

***

V$_t$(mV)
Figure 2

$V_i$ (mV)

Saline

LPS

10 min

$\text{AB}$
Figure 3

- **Transient hyperpolarization after AB and Na\(^+\) removal**
- **Stable \(V_t\) after AB and Na\(^+\) removal**
- **Amiloride response**

**A**

\(\Delta V_t, (mV)\)

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**B**

\(V_t, (mV)\)

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**C**

\(\Delta V_t, (mV)\)

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**D**

\(V_t, (mV)\)

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**E**

\(\Delta V_t, (mV)\)

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Figure 4

Saline

LPS

10 min
Figure 5

- Amiloride
- Trypsin
- Amiloride + Trypsin

 `$\Delta V_t$ (mV)`: 

- Saline
- LPS

*Significant difference
Figure 6

- **A**: Graph showing the effect of Saline LPS on membrane potential (Vt) over time. The graph depicts the change in membrane potential with time, indicating a decrease after the application of LPS.

- **B**: Bar graph showing the membrane potential (Vt) for Saline and LPS in AB condition. The Saline LPS condition shows a significant decrease in Vt with a * symbol indicating statistical significance.

- **C**: Bar graph showing the membrane potential (Vt) for Saline and LPS in AB + trypsin condition. The Saline LPS condition shows a decrease in Vt with a * symbol indicating statistical significance.

- **D**: Bar graph showing the change in membrane potential (∆Vt) for Saline and LPS in AB + trypsin condition. The Saline LPS condition shows a decrease in ∆Vt.
Figure 7
Chapter 3
Lipopolysaccharide increases Na⁺,K⁺-pump, but not ENaC, expression in guinea-pig airway epithelium

Michael W. Dodrill¹,², Donald H. Beezhold², Terence Meighan², Michael L. Kashon², and Jeffrey S. Fedan¹,²

From the Department of Basic Pharmaceutical Sciences, School of Pharmacy, Robert C. Byrd Health Sciences Center, West Virginia University¹, and the Health Effects Laboratory Division, National Institute for Occupational Safety and Health², Morgantown, WV 26505.

Running head: LPS, hyperpolarization, and Na⁺ transport

Address correspondence to: Jeffrey S. Fedan, Ph.D.
1095 Willowdale Road
Morgantown, WV 26505
Phone: 304-285-5760
Fax: 304-285-6928
E-mail: jsf2@cdc.gov
Abstract

Earlier, we found in functional experiments that lipopolysaccharide (LPS; 4 mg/kg; i.p.) hyperpolarized the epithelium by stimulating the transepithelial transport of Na⁺ in guinea-pig tracheal epithelium. Epithelial sodium channel (ENaC) activity and Na⁺,K⁺-pump activity were increased. In this study, we hypothesized that LPS increases the expression of ENaC and the Na⁺,K⁺-pump in the epithelium and investigated the levels of transcription and protein abundance. Using qPCR, the effects of LPS on the transcription of αENaC, α₁ Na⁺,K⁺-pump, COX-2, eNOS, iNOS, IL-1β, and TNF-α were measured at 3 and 18 h. In the epithelium, LPS increased the transcription of COX-2, IL-1β, and, to a nonsignificant extent, TNF-α at 3h, but not at 18 h. In alveolar macrophages, TNF-α, and, to a nonsignificant extent, COX-2 and IL-1β were up-regulated at 3 h, but not at 18 h. Even though LPS stimulated the transcription of some genes, αENaC and α₁ Na⁺,K⁺-ATPase transcription were not affected. The expression of α-, β-, and γ-ENaC and α₁ Na⁺,K⁺-pump from tracheal epithelium and kidney cortex/medulla were investigated by western blotting. All three ENaC subunits were detected as cleavage fragments, yet LPS had no effect on their expression. LPS increased the expression of the α₁ subunit and the α₁-, α₂-, and α₃-subunits, collectively, of the Na⁺,K⁺-pump. Taken together, these data indicate that LPS increases Na⁺ transport downstream of the genetic level, in part, by stimulating the expression of the Na⁺,K⁺-pump.

Keywords: Endotoxin; lung; airway epithelium; epithelial sodium channel (ENaC); sodium, potassium ATPase (Na⁺,K⁺-ATPase)
1. Introduction

In a previous investigation we found that the systemic administration of lipopolysaccharide (LPS) hyperpolarized the tracheal epithelium of the guinea pig (Johnston et al., 2004). Amiloride, and to a lesser extent, indomethacin, inhibited this hyperpolarization and indicated that LPS increased the transport of Na\(^+\) across the epithelium. Indeed, we found that LPS increased the activities of both ENaC and the Na\(^+\),K\(^+\)-pump. Additionally, the lack of effect of LPS on the transepithelial voltage (V\(_t\)) response of the trachea to exogenous apical trypsin ruled out a role of channel activating proteases in the hyperpolarizing effect of LPS. The stimulation of Na\(^+\) transport appeared to involve pathways that stimulate both ENaC and the Na\(^+\),K\(^+\)-pump.

Na\(^+\) is transported across the airway epithelium by ENaC in the apical membrane and the Na\(^+\),K\(^+\)-pump in the basolateral membrane. The rate of Na\(^+\) transport regulates airway surface liquid hydration and is regulated tightly to ensure the proper movement of mucus via the mucociliary escalator (Randell and Boucher, 2006). The importance of the ENaC/Na\(^+\),K\(^+\)-pump axis is demonstrated by the immediate death of newborn αENaC\(^−/−\) knockout mice due to their failure to clear lung fluid (Hummler et al., 1996). The postnatal rise in cortisol has been found to increase αENaC transcription and amiloride-sensitive lung fluid volume in guinea pigs (Baines et al., 2000). The severe consequences of disrupted Na\(^+\) transport are further demonstrated in the genetic diseases, cystic fibrosis and Liddle’s syndrome, where Na\(^+\) hyperabsorption dehydrates the mucus and prevents the clearing of lung infection (Donaldson et al., 2006), and pseudohypoaldosteronism type I, where ENaC is inhibited and the resulting edema prevents normal gas exchange (Staub et al., 1997).

LPS is a constituent of the gram-negative bacterial outer membrane that helps maintain its integrity and protect it from certain kinds of chemical attack (Liebers et al., 2008). A lung infection by gram-negative bacteria results in an abundance of LPS, which, through its interaction
with TLR-4, activates the production of cytokines and reactive oxygen and nitrogen species via activation of the transcription factor NF-κB (Basu and Fenton, 2004; Liang et al., 2007) in the airway epithelium (Guillot et al., 2004) and up-regulation of TLR-4 and -2 expression (Saito et al., 2005). Gram-negative bacterial infection of the lung presents a potential for perturbation of airway Na⁺ transport and lung function.

The aim of the present study was to investigate the cellular responses of the airway epithelium to LPS under conditions that lead to an increase in Na⁺ transport and hyperpolarization. We hypothesized that LPS increases the transcription and/or expression of ENaC and/or the Na⁺,K⁺-pump. The effects of LPS on the rates of transcription of ENaC and the Na⁺,K⁺-ATPase were investigated by quantitative, real-time PCR. Even though LPS increased the transcription of several inflammatory-response genes, no effect on the transcription of ENaC or Na⁺,K⁺-ATPase was observed. The effects of LPS on the expression of the three ENaC subunits were investigated using immunoblots. Whereas each of the three subunits was found to be proteolytically cleaved, there was no difference in their expression levels between saline- and LPS-treated animals. However, using western blots, LPS was found to increase the expression of the Na⁺,K⁺-pump.

2. Methods

2.1. 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 (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 injected with LPS (4 mg/kg; i.p.) from Salmonella enterica serotype typhimurium (phenol-extracted) prepared in saline or a volume-equivalent of saline (controls). The animals were anesthetized with sodium pentobarbital (65 mg/kg, i.p.) and were sacrificed by thoracotomy and bleeding before removing the trachea, kidneys or performing bronchoalveolar lavage.

2.2. Quantitative, real-time PCR (qPCR)

Quantitative real-time PCR was carried out to measure the effects of LPS on the transcription of a selection of genes in tracheal epithelium and alveolar macrophages; macrophages were used as positive controls and for comparison purposes. After administering LPS or saline, animals were anesthetized 3 or 18 h post-injection. A cannula was inserted into the lower trachea through an incision; the upper trachea was then removed. Alveolar macrophages were collected by bronchoalveolar lavage involving washing the lungs 10 times with 5 ml phosphate buffered saline each time. The trachea was cleaned, and the epithelium was isolated. Epithelium and macrophages were lysed and the mRNA was isolated using the RNAqueous-4PCR kit (Ambion; Austin, TX). After the determination of the mass of RNA present in each sample by spectrophotometry, duplicate samples of 0.5 μg of RNA were denatured in the presence of 1 μl Oligo(dT)_{12-18} primer (Invitrogen; Carlsbad, CA) and reverse-transcribed in the presence of 4 μl 5× first strand buffer (Invitrogen), 2 μl of 0.1 M DTT (Invitrogen), 2 μl deoxynucleotide mix (Sigma-Aldrich; St. Louis, MO), and 1 μl Super Script II RNase H (Invitrogen). The duplicates of cDNA were diluted 1:100 for PCR. The cDNA duplicates were loaded with a master mix containing 12.5 μl Fast Start TaqMan Probe Master (Roche; Basel, Switzerland), 2.5 μl each of forward and reverse primer (200 μM) and 0.25 μl of the appropriate
probe from the Universal Probe Library (Roche; Basel, Switzerland). PCR was run using the 7500 Real-Time PCR System (Applied Biosystems; Foster City, CA) and data were analyzed by ΔΔC\text{t} analysis using GADPH as the normalizing gene.

The Universal Probe Library system was used along with its online primer3 software (www.universalprobelibrary.com) to design primers (Eurofins MWG Operon; Huntsville, AL) and choose the probes. The assays used were: GAPDH: guinea pig 5′-TCAGAGGGCTCCCTCAAAAG-3′ (forward) and 5′-CGCTGTTGAAGTCACAGGAC-3′ (reverse) with probe 117; αENaC: guinea pig 5′-CAAGGAGCCCTGAGAGTT-3′ (forward) and 5′-ACTCAGAGGTCCCAGACG-3′ (reverse) with probe 92; α_{1} Na\textsuperscript{+},K\textsuperscript{+}-pump: rat 5′-GGAGAGCGGTGTGCTAGGTTT-3′ (forward) and 5′-AAGCCTTCCGGGAACTGTTC-3′ (reverse) with probe 29; cyclooxygenase (COX)-2: guinea pig 5′-CTCGGCCAGACGCTATTTT-3′ (forward) and 5′-CCTTCTAAGGAGATTGT-3′ (reverse) with probe 22; eNOS: guinea pig 5′-GACTTTCTGTGTGGGTGAGGA-3′ (forward) and 5′-GCATTTGGGGCTGAATATGT-3′ (reverse) with probe 70; inducible nitric oxide synthase (iNOS): guinea pig 5′-TCTCTGCATGGATCAGTACCA-3′ (forward) and 5′-CACCACCAGGAGCGTTCTATT-3′ (reverse) with probe 65; tumor necrosis factor (TNF)-α: guinea pig 5′-GTCTCCTACCCCGGAAAAGGT-3′ (forward) and 5′-CTCCTTCTGGCAGGGACTCT-3′ (reverse) with probe 53; and interleukin (IL)-1β: guinea pig 5′-GCCAGACCGTCTCACTC-3′ (forward) and 5′-ATGTGCAAGAGGCTCACTC-3′ (reverse) with probe 73. Due to the unavailability of guinea-pig mRNA sequences, the α_{1} Na\textsuperscript{+},K\textsuperscript{+}-pump assay was designed by substituting the corresponding sequence from rat. We had difficulty in constructing assays that yielded reliable signals during qPCR for some genes of interest. The guinea-pig αENaC subunit had been cloned previously (Schnizler et al., 2000), but the guinea-pig sequences for β- and γ-ENaC were unavailable. It is of great interest to understand whether LPS might alter expression of these subunits. Even though the substitution of rat sequences to design primers for guinea-pig targets has been successful on occasion (Yamada et al., 2005), it was unsuccessful in the primer design process for β-, and γ-
ENaC, which was not surprising since the sequence identity of guinea-pig and rat αENaC was reported to be 76% (Schnizler et al., 2000). The differences between guinea-pig and rat ENaC extend to their function in that human and guinea-pig αENaC, unlike in mice and rats, need basal cAMP to be trafficked to the membrane to be functional (Schnizler et al., 2000; Woolhead and Baines, 2006). Using rat sequences to design primers for the α1, α2, α3, α4, β1, β2, β3, and β4 subunits of the Na+,K+-pump was only successful for the α1 subunit. Thus, whether changes in the transcription of these subunits were initiated by LPS remains unknown. Other unsuccessful targets attempted using rat sequences were COX-1, IL-6, muscarinic receptors 1, 2, 3, and 5, and TLR-4. The bioelectric response of the epithelium to methacholine, a muscarinic agonist, is transformed after LPS (Fedan et al., 1995; Johnston et al., 2004), and the mechanism of this alteration remains an unanswered question (Fedan et al., 1995).

2.3. Western blotting

The effects of LPS on the expression of ENaC and the Na+,K+-pump were investigated in epithelial cell and kidney homogenates by western blotting under denaturing conditions. Animals were anesthetized 18 h after LPS injection for the collection of tissues. The tracheal epithelium was isolated and homogenized in RIPA lysis buffer (Santa Cruz Biotechnology; Santa Cruz, CA). As a positive control, ENaC from kidney was investigated using cross-sectional slices including cortex and medulla which were likewise prepared by homogenization in RIPA buffer. Protein concentration was measured using the BCA protein assay (Pierce; Rockford, IL) to normalize the loading of the wells to 20 µg each. Samples were prepared in 10 µl sample buffer and heated 5 min at 90 °C. Polyacrylamide gel electrophoresis was run using 7.5% tris-HCl ready gels (BioRad; Hercules, CA) in tris/glycine/sodium dodecyl sulfate buffer (BioRad) in the Protean running cell (BioRad) at 120V for 70 min. Protein was transferred to a nitrocellulose membrane (BioRad) in tris/glycine buffer (BioRad) at 30 V for 2 h. The membrane was dyed with Ponceau S.
Membranes were blocked with 5% BSA/tween-20 0.05% for 1 h with rocking and washed 3 times with tris-buffered saline (TBS)/tween-20 0.05%. Membranes were incubated with primary antibody diluted in BSA/tween-20 overnight with rocking at 4 °C, washed 3 times with TBS/tween-20, incubated with secondary antibody in BSA/tween-20 for 2 h with rocking at room temperature, and washed 5 times with TBS/tween. Streptavidin was activated by electrochemiluminescence (Amersham Biosciences; Amersham, UK) for 1 min and exposed to radiographic film (Fuji film; Minato, Japan). Membranes were stripped with Restore (Thermo Scientific; Rockford, IL) and reblotted for β-actin.

Primary and secondary antibodies were rigorously tested on guinea-pig epithelium and kidney samples at serial dilutions to optimize contrast of signal to background. The antibodies used were as follows. The ENaC subunits were probed with polyclonal antibodies from Genetex (San Antonio, TX): 23464 (αENaC), 22906 (βENaC), and 23468 (γENaC) at 1:2,000 dilution and detected with streptavidin-linked anti-rabbit secondary antibody (7074 Cell Signaling; Beverly, MA) diluted 1:1,000. The Na⁺,K⁺-ATPase was probed using monoclonal antibodies against α₁ Na⁺,K⁺-ATPase (05-369 Millipore; Billerica, MA) diluted 1:500 and pan-α (α₁, α₂, and α₃) Na⁺,K⁺-ATPase (ab2871 Abcam; Cambridge, UK) diluted 1:1,000 and detected with a streptavidin-linked anti-mouse secondary antibody (AP124P Millipore) diluted 1:64,000. After exposure and stripping, membranes were re-stained for β-actin (4967 Cell Signaling) diluted 1:4,000 and detected with anti-rabbit (7074 Cell Signaling) diluted to 1:2,000.

2.4. Apical membrane surface protein isolation

To investigate the effects of LPS on the apical membrane expression of ENaC, apical membrane proteins were isolated and western blotted. The Pierce (Rockford, IL) cell surface protein isolation kit was used to biotinylate the apical face of the epithelium in tracheas from saline- and LPS-treated animals. Tracheas were excised and cleaned in a dish containing modified...
Krebs-Henseleit solution (pH 7.4; osmolarity of 281 ± 5 mosM; temperature of 37 °C; contained 113 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, and 5.7 mM glucose) bubbled with 95% O₂, 5% CO₂. The tracheas were cannulated, the lumen filled with biotinylation solution and sealed, and rocked in modified Krebs-Henseleit solution overnight at 4 °C. After incubation with quenching solution, the epithelium was separated from the trachea and lysed in RIPA buffer. Biotinylated protein was isolated on a neutravidin bead-linked column, eluted, and separated by electrophoresis for western blotting. Due to the faint signals in western blots, in separate experiments we investigated total biotinylated protein in the epithelium. After treatment with biotinylation solution and quenching, the epithelium was lysed and prepared for western blotting without heating to prevent the breakage of the biotin-protein bond. Two methods were used to probe biotinylated proteins on the blots. In the first, the blot was incubated with peroxidase-conjugated streptavidin (Jackson ImmunoResearch; West Grove, PA) and visualized using electrochemiluminescence and exposure to radiographic film. In the second, the blot was incubated with alkaline phosphatase conjugated streptavidin (Jackson ImmunoResearch), which was activated by incubating with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (Promega; Rockford, IL).

2.5. Data analysis

Results are presented as means±S.E.M. qPCR was analyzed according to the ∆∆Ct method and significance was determined using Student’s t-test. Western blot data were analyzed using the unpaired and paired Student’s t-tests where appropriate (SigmaStat version 3.1; Systat Software, Inc., Chicago, IL). The western blot films were digitized and band intensities were determined using ImageJ software (http://rsb.info.nih.gov/ij/). Data were normalized as percent of the β-actin signal. The western blots for α₁- and pan-α-Na⁺,K⁺-pump were analyzed using the
mixed model analysis of variance with block as a random factor. \( p < 0.05 \) was regarded as significant.

3. Results

3.1. LPS does not affect the transcription of \( \alpha \)ENaC and \( \alpha_1 \) Na\(^+\),K\(^+\)-pump

Previous studies from our laboratory have indicated that LPS increases the functional activity of ENaC and the Na\(^+\),K\(^+\)-pump in airway epithelium. Therefore, qPCR was used to investigate whether this effect resulted from an increase in ENaC and/or Na\(^+\),K\(^+\)-ATPase transcription. The transport of Na\(^+\) through ENaC has been correlated with the rate of transcription of ENaC, identifying it as a critical regulator of ENaC activity (Xu et al., 2007). For example, in the kidney, aldosterone, via the mineralocorticoid receptor, increases Na\(^+\) reuptake by stimulating the transcription of ENaC and the Na\(^+\),K\(^+\)-pump (Horisberger and Rossier, 1992). Samples of alveolar macrophages were also prepared as positive controls to compare our results with other studies.

The effects of LPS on the rates of transcription of a selection of genes that respond to LPS were first measured; these have been reported to be activated via NF-\( \kappa \)B or to activate Na\(^+\) transport. TLR-4 and TLR-2, via activation of the MAP kinases, p38 and JNK, the main cellular mediators of the effects of LPS in the mammalian host, are expressed in the airway epithelium (Guillot et al., 2004; Saito et al., 2005). These pathways, in turn, stimulate the transcription of iNOS, IL-1\( \beta \), TNF-\( \alpha \) and other cytokines through NF-\( \kappa \)B (Adcock et al., 2006). We measured COX-2 transcription because our earlier observation that indomethacin also inhibited modestly the hyperpolarizing effect of LPS indicated a role of increased prostaglandin production by COX-1/2 (Johnston et al., 2004). Since NO has been linked with airway hyperreactivity (Jiang et al., 2006) and inhibition of the Na\(^+\),K\(^+\)-pump (Liu and Sheu, 1997; Seven et al., 2005), we measured
both eNOS and iNOS. IL-1β was measured because it has been observed to increase the expression of both the ENaC and the Na⁺,K⁺-pump (Ye et al., 2004). Finally, TNF-α was measured because it inhibits ENaC activity (Dagenais et al., 2004) and, like iNOS and IL-1β, it is activated by NF-κB. Samples were collected at both 3 h and 18 h because it was anticipated that inflammatory mediator transcription should be elevated at 3 h and because hyperpolarization had been characterized at 18 h after LPS injection (Johnston et al., 2004).

The qPCR results are presented in Fig. 1. Compared to controls, transcription of αENaC was not affected at 3 h (0.6±0.2-fold) and was significantly decreased at 18 h in the epithelium (0.6±0.1-fold); we regard these changes to be biologically unimportant. Transcription of the α₁ subunit of the Na⁺,K⁺-ATPase was not affected at 3 (2.3±0.5-fold) or 18 h (1.5±0.4-fold). This indicates that LPS does not increase epithelial Na⁺ transport via an increase in the transcription of αENaC or the Na⁺,K⁺-pump. LPS did increase transcription of other inflammatory genes in the epithelium. Genes regulated through NF-κB were up-regulated by LPS at 3 h (COX-2, 18.8±7.2-fold; IL-1β, 29.0±10.0-fold) except for iNOS (2.0×10⁻⁴±6.5×10⁻⁵-fold) and TNF-α (30.9±14.4-fold). The transcription of each of these genes returned to the control level by 18 h (COX-2, 11.0±8.3-fold; TNF-α, 2.8±2.1-fold; iNOS, 5.7±4.0-fold), with IL-1β being significantly lower than controls (0.2±0.1-fold). LPS significantly reduced eNOS transcription at 3 h (0.4±0.2-fold), but had no effect at 18 h (1.3±0.9-fold).

This pattern was generally reflected in macrophages (Fig. 1). The transcription of αENaC was not affected by LPS in those cells (3 h, 2.8±1.3-fold; 18 h, 1.0±0.7-fold), nor was there an effect on α₁ Na⁺,K⁺-pump transcription, at 3 (1.9±0.9-fold) or 18 h (2.8±2.2-fold). At 3 h, LPS increased the transcription of TNF-α (18.5±6.5-fold), COX-2 (185.2±102.0-fold) and IL-1β (115.0±54.7-fold) were also increased, although not significantly, at 3 h. The elevated transcription of all of these genes decreased to control by 18 h (COX-2, 7.6±4.6-fold; IL-1β, 1.6±0.5-fold; TNF-α, 0.6±0.3-fold). In macrophages, eNOS and iNOS were not affected by LPS.
at either 3 or 18 h (eNOS 3 h, 4.2±2.8-fold; eNOS 18 h, 3.9±3.6-fold; iNOS 3 h, 0.7±0.6-fold; and iNOS 18 h, 1.3±0.9-fold).

Thus, LPS stimulated the transcription of several inflammatory genes in epithelium and macrophages, but had no effect on transcription of ENaC or the Na⁺,K⁺-pump. LPS did stimulate, however, many of the same genes in epithelium as in macrophages. There being no transcriptional changes for ENaC and the Na⁺,K⁺-pump, the effects of LPS on Na⁺ transport by the epithelium may be concluded to result from changes downstream from the genetic level. Therefore, we investigated whether LPS stimulated ENaC and Na⁺,K⁺-pump protein expression or localization.

3.2. LPS does not affect the expression of ENaC

Previously we found using the isolated, perfused trachea preparation and the Ussing chamber that LPS increased the functional activity of ENaC and the Na⁺,K⁺-pump in epithelium. Absent a transcriptional change, this could result from several mechanisms, including an increase in the expression of ENaC, stimulating the trafficking of ENaC to the membrane from sub-membrane stores, increasing channel open probability by carboxymethylation (Rokaw et al., 1998) or proteolytic cleavage (Masilamani et al., 1999), down-regulation of ubiquitin-mediated internalization and degradation (Staub et al., 2000; Staub et al., 1997), or a change in modulation of the channel by a cellular mediator or regulator. Could LPS, therefore, increase Na⁺ transport by increasing the expression of ENaC even though we found no evidence that LPS affected the transcription of αENaC? To investigate this hypothesis, we investigated the α-, β-, and γ-subunits of ENaC using western blots in tracheal epithelial homogenates from saline- and LPS-treated animals.

Many commercially-available antibodies against α-, β-, and γ-ENaC, none of which were designed for guinea pigs, were tested as described in 2.3. until one working set was identified. To
identify the bands detected by specific binding of the antibody, identically loaded, otherwise identical paired lanes were probed with antibody that was previously adsorbed with its antigen peptide, i.e., its “blocking peptide.” Samples of kidney cortex/medulla homogenate from the same animals were also run as positive controls. In epithelial samples αENaC was identified among several bands at two molecular weights: 230 and 60 kD (Fig. 2A). A 60 kD band, but not a 230 kD αENaC band, was also detected in the kidney (Fig. 2D). In both tissues, no band was detected that corresponded to the ≈90 kD predicted molecular weight of full-length αENaC (Hughey et al., 2003). LPS had no effect on the relative expressions of the 230 and 60 kD bands in the epithelium (Fig. 2B) or the kidney (Fig. 2E), indicating that a change in αENaC expression is not involved in the stimulatory effect of LPS on Na⁺ transport. The 230 kD fragment may represent a multi-α-subunit complex (Ismailov et al., 1996; Rokaw et al., 1998), which is surprising considering that the samples were heated for 5 min in sample buffer and run under denaturing conditions. Since the antibody’s epitope was located at the N-terminal end of the antibody (Fig. 3), the 60 kD band represents an N-terminal fragment, likely a cleavage product of the full-length αENaC substrate. The percentages of specific signal measured in the 230 and 60 kD bands compared between the saline- and LPS-treated groups in the epithelium were not different (Fig. 2C), indicating that LPS had no effect on the sizes or relative abundances of cleaved and uncleaved α-subunits.

In the epithelium, βENaC was detected in two specific bands with molecular weights of 90 and 37 kD (Fig. 4A). The 90 kD molecular weight corresponds to the reported molecular weight of the full-length, uncleaved βENaC subunit (Hughey et al., 2004b). Since the epitope for the βENaC antibody aligns with a sequence located at the C-terminal end of the subunit’s structure (Fig. 5), the corresponding N-terminal fragment would be ≈53 kD in weight, assuming that βENaC is cleaved at only one location. In the kidney, however, only one 90 kD band was detected (Fig. 4D). LPS did not affect the relative intensities of either the 90 or 37 kD bands in the epithelium (Fig. 4C) or the 90 kD band in the kidney (Fig. 4E), indicating that an increase in βENaC abundance does not play a role in the effects of LPS on ENaC activity. The percentages
of specific βENaC signal in the 90 and 37 kD bands in the epithelium were not affected by LPS (Fig. 4C). These data indicate that, while βENaC in the epithelium was cleaved, LPS had no effect on its abundance or the sizes of the cleaved and uncleaved channel pools.

γENaC was detected in two, specific bands of 90 and 40 kD in both the airway epithelium (Fig. 6A) and the kidney (Fig. 6D). The 90 kD band corresponds to the reported molecular weight of the full-length, uncleaved γENaC subunit (Hughey et al., 2003). Since the epitope for the antibody is located at the C-terminal end of the γ-subunit structure (Fig. 7), the corresponding molecular weight of the N-terminal fragment would be ≈50 kD, assuming that the subunit was cleaved at one location. The relative expressions of both the 90 and 40 kD bands were not affected by LPS in the epithelium (Fig. 6B) or the kidney (Fig. 6E). The percentages of specific signal at both 90 and 40 kD were not affected by LPS in either the epithelium (Fig. 6C) or the kidney (Fig. 6F). Taken together, these data indicate that LPS had no effect on the total expression of α-, β-, and γ- ENaC protein or the extent of proteolytic cleavage of ENaC by LPS.

3.3. Apical surface isolation of ENaC.

The proportion of mature, cleaved ENaC in the membrane is thought to be small (Bhalla and Hallows, 2008), and it is possible that changes in membrane ENaC were, therefore, undetectable in homogenates after LPS. We also had found earlier that there was no difference in the hyperpolarization response to apical trypsin in tracheas from saline- and LPS-treated animals, providing functional evidence that LPS does not affect ENaC by altering the activities of channel activating proteases. We, therefore, tested the hypothesis that LPS stimulates ENaC activity by increasing its trafficking to the apical membrane or the activity of a small but physiologically important membrane pool using biotinylation.

While they matched the molecular weights detected in the western blots of whole-cell homogenates (above), the resulting bands of biotinylated samples of epithelium were barely
visible to the naked eye, and could not be detected densitometrically (data not shown). These results could mean either that there is too little ENaC localized to the apical membrane to be reliably detected by this method and/or that cell-surface protein was inadequately isolated. To track ENaC through the membrane isolation process, we took samples of the whole cell homogenate, the pellet and supernatant after centrifugation according to the isolation protocol, and the wash-through and eluate from the neutravidin-linked bead column and probed for α-, β-, and γ- ENaC after western blotting (Fig. 8). ENaC was detected in the homogenate. After centrifugation, all ENaC was found in the supernatant with none settling in the pellet. Most of the ENaC washed through the column, with only a faint signal detected in the eluate. Thus, very little epithelial ENaC was biotinylated.

We feared that biotinylation could have been ineffective. To determine if surface proteins were biotinylated, we homogenized the epithelial cells after the apical biotinylation maneuver, and separated the proteins by western blot. All biotinylated protein was stained using two, separate approaches. Firstly, incubating the blot with streptavidin-linked HRP revealed many faint bands and two prominent ones of 122 and 68 kD, which did not correspond to α-, β-, and γ- ENaC blotted in identically loaded lanes on the same gel (data not shown). Secondly, the membrane was incubated sequentially with alkaline-phosphatase-conjugated streptavidin and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). This more sensitive technique revealed dozens of bands of various intensities (data not shown). These results demonstrate collectively that membrane proteins were, in fact, biotinylated, but the abundance of ENaC in the apical membrane surface was too small to be detected.

3.4. LPS increases the expression of the Na⁺,K⁺-pump.

Since the transport of Na⁺ across the epithelium is driven by the Na⁺,K⁺-pump (Geering, 2006), and we found that pump activity was increased after LPS, we reasoned that an increase in
the abundance of the \( \text{Na}^+ \),\( \text{K}^+ \)-pump could have led to potentiated \( \text{Na}^+ \) transport. To investigate this hypothesis, monoclonal antibodies were used to immunoblot the \( \alpha_1 \) subunit of the Na\(^+\),K\(^+\)-pump and pan-\( \alpha \) (\( \alpha_1 \), \( \alpha_2 \), and \( \alpha_3 \) subunits) in the epithelium from saline- and LPS-treated guinea pigs (Fig. 9). The expression of the \( \alpha_1 \) subunit was increased in the epithelium of animals from LPS-treated animals compared to saline-treated animals (Fig. 9B). Using the pan-\( \alpha \) antibody, the collective expression of \( \alpha_1 \), \( \alpha_2 \), and \( \alpha_3 \) was also observed to be increased in the epithelium from LPS-treated animals (Fig. 9D).

4. Discussion

We examined the hypothesis based on previous functional evidence that LPS increases the activity of ENaC and the Na\(^+\),K\(^+\)-pump in the tracheal epithelium by increasing the transcription and/or expression of ENaC and the Na\(^+\),K\(^+\)-pump. The first finding was that LPS did not affect \( \text{Na}^+ \) transport at the genetic level of regulation of ENaC and the Na\(^+\),K\(^+\)-pump. Secondly, LPS did not affect the transcription or cleavage of ENaC, in support of functional findings that activation of ENaC by LPS is not mediated by protease activation. Thirdly, LPS increased the expression of the Na\(^+\),K\(^+\)-pump. Lastly, some properties of ENaC and the Na\(^+\),K\(^+\)-pump in guinea-pig tracheal epithelium were newly characterized.

4.1. LPS does not affect \( \alpha \)ENaC transcription

The rate of \( \alpha \)ENaC transcription has been correlated to ENaC activity and plays a critical role in lung fluid absorption (Dickie et al., 2000; Horisberger and Rossier, 1992; Ma et al., 2004; Xu et al., 2007). LPS had essentially no effect on \( \alpha \)ENaC transcription at 3 and 18 h, suggesting that genetic regulation was not involved in hyperpolarization. This finding is to be treated with caution though, because changes in the transcription rate of \( \alpha \)ENaC does not necessarily
accompany changes in β- and γ-ENaC transcription (Pierre et al., 2007). IL-4, for example, decreases γ- and β-ENaC, but not αENaC, transcription in human bronchial epithelium (Galiotta et al., 2002).

4.2. LPS does not affect ENaC expression

In immunoblots each ENaC subunit was present as full-length, cleavage fragments, and/or multiple-subunit complexes. None of the bands were affected by LPS in the epithelium or the kidney. The 230 kD αENaC band, surprisingly appearing under denaturing conditions, may represent a complex of multiple α-subunits because it was not detected by the β- and γ-ENaC antibodies. A 180 kD (Ismailov et al., 1996) or 150 kD (Rokaw et al., 1998) αENaC band without a 90 kD band was blotted in ENaC-transfected and expressing oocytes under non-denaturing, but not denaturing, conditions. Another study detected a >600 kD band, probably the entire channel assembly, under non-denaturing conditions after co-expressing all three ENaC subunits (Staruschenko et al., 2004). αENaC has been found to be the main Na⁺-transporting channel in alveolar type II cells (Jain et al., 1999). In contrast to αENaC, the β and γ subunits were detected at 90 kD, corresponding to the molecular weight reported for full-length channel protein.

Furin and prostasin activate ENaC in kidney epithelium (Frindt et al., 2008; Hughey et al., 2004a, b; Masilamani et al., 1999) and are found in the lung (Myerburg et al., 2008; Vallet et al., 1997) but may be differently regulated there. Cleavage of two furin sites in the α-subunit and one furin and one prostasin site in γ-subunit activate channels by liberating inhibitory sequences (Bruns et al., 2007; Carattino et al., 2008). Cleavage is thought to occur both intracellularly (furin) (Sheng et al., 2006) and in the apical membrane (prostasin) (Myerburg et al., 2008; Rossier and Stutts, 2009).

Prostasin cleavage of γENaC yields a 75 kD C-terminal fragment (Hughey et al., 2004a), which is too large to explain the 40 kD fragment (Hughey et al., 2004a). Furin and prostasin do
not cleave βENaC (Hughey et al., 2004a), there are no RXXR or RKRK motifs in βENaC (Fig. 5), and the actions of these enzymes do not explain the 37 kD band. Based on the importance of the second disulfide bridge in ENaC function (Firsov et al., 1999), Rossier and Stutts (2009) proposed a putative cleavage site in that location for trypsin, chymotrypsin, elastase, and other channel activating proteases in α-, β- and γ-ENaC. Elastase in the lung increases ENaC activity (Caldwell et al., 2005; Harris et al., 2008; Planès et al., 2002). Indeed, the molecular weights of our β- (Fig. 5) and γ-ENaC (Fig. 7) C-terminal fragments indicate that cleavage occurs in the C-terminal area of the extracellular loop and may constitute evidence for that hypothesis. We are unaware of literature characterizing the cleavage products of elastase, kallikrein and other unknown proteases. The 37 kD αENaC band is similar to the 30 kD molecular weight reported for the N-terminal fragment of furin-cleaved αENaC (Hughey et al., 2004a). αENaC may also be cleaved at the putative site, but that would be masked by the N-terminal location of the antigen relative to the furin cleavage sites (Fig. 3).

The lack of effect of LPS on the levels of ENaC proteins and cleavage products does not support the hypothesis that LPS increases ENaC expression or protease activation of channels. Even though we found that LPS increases the activity of ENaC, we cannot distinguish among the epithelial cell types which are affected by LPS.

4.3.ENaC trafficking

Because LPS did not affect ENaC transcription and expression, we investigated whether LPS affected trafficking of ENaC from sub-membrane vesicles to the apical epithelial membrane. For example, the PKA-mediated production of cAMP increases ENaC activity in human lung epithelium by inducing the trafficking of ENaC (Bhalla and Hallows, 2008; Mazzochi et al., 2006a, b; Woollhead and Baines, 2006 and 2007). Using a similar biotinylation technique (Woollhead and Baines, 2006) in this study, the signals were too weak to be quantified.
densitometrically, but they could be seen by eye. These experiments showed that very little ENaC was localized to the membrane.

4.4. LPS increases Na\(^+\),K\(^-\)-pump expression but not transcription

We found earlier that LPS increases the functional activity of the Na\(^+\),K\(^+\)-pump. In agreement with this finding, this investigation revealed that expression of \(\alpha\) subunits was increased by LPS. The up-regulation may be predominantly on the \(\alpha_1\) subunit, as judged using the \(\alpha_1\) Na\(^+\),K\(^+\)-ATPase antibody, the signal being “diluted” by the \(\alpha_2\) and \(\alpha_3\) subunits detected by the pan-\(\alpha\) antibody. One study reported that, through increased NO levels, LPS reduces the expression of \(\alpha_1\) Na\(^+\),K\(^+\)-pump in the rat sciatic nerve, but is without effect on \(\alpha_2\) or \(\alpha_3\) subunits (Liu and Sheu, 1997). TGF-1\(\beta\) has been reported to increase the expression of the \(\alpha_1\) and \(\beta_1\) subunits in rat alveolar type II cells without affecting \(\alpha\)ENaC protein (Willis et al., 2003). The \(\alpha_1\) subunit has been found to be increased in the cystic fibrosis airway epithelium by FXYD5 (Miller and Davis, 2008), and it is convenient to speculate about FXYD5’s involvement in Na\(^+\),K\(^-\)-pump up-regulation by LPS.

4.5. Alternative pathways involved in ENaC and Na\(^+\),K\(^+\)-pump activity and regulation

Other pathways known to regulate ENaC activity could be involved and altered by LPS. Aldosterone activates Sgk1 (Snyder et al., 2004), which activates ENaC through inhibition of the ubiquitin ligase, Nedd4-2 (Loffing et al., 2006; Raikwar and Thomas, 2008; Staub et al., 1997) and increases the open probability of ENaC by carboxymethylating the \(\beta\)-subunit of ENaC (Rokaw et al., 1998). Phosphatidylinositides have been observed to activate ENaC, raising the possibility that G-protein coupled receptors may be involved (Pochynyuk et al., 2007). In the airway epithelium, glucocorticoids increase ENaC activity and increase the transcription of ENaC.
(Dagenais et al., 2006; Husted et al., 2007; Snyder et al., 2004). Other factors, such as the δENaC subunit (Ji et al., 2006), and cell swelling (Ma et al., 2004), also increase ENaC activity.

Functional studies in our laboratory showed that the COX-1/2 inhibitor, indomethacin, inhibited LPS-induced hyperpolarization of the tracheal epithelium, albeit to a lesser degree than amiloride (Johnston et al., 2004), indicating a role for prostanoids. Here we observed that COX-2 transcription was elevated by LPS at 3 h. LPS stimulates COX-2 transcription (Balzary and Cocks, 2006; Held and Uhlig, 2000) which causes smooth muscle relaxation in mouse trachea (Balzary and Cocks, 2006) and increases ENaC open probability in kidney (Wang et al., 2009) via PGE_{2}. Likewise, prostaglandins stimulate Na\(^+\),K\(^+\)-ATPase activity and transcription (Matlhagela and Taub, 2006). Both of these effects could contribute to hyperpolarization of the epithelium after LPS. However, inhibition of the Na\(^+\),K\(^+\)-pump by PGE\(_2\) also has been reported (Kreydiyyeh et al., 2007; Oliveira et al., 2009).

TNF-α inhibits ENaC activity (Dagenais et al., 2004) and transcription (Dagenais et al., 2006). IL-1β increases expression and activity of ENaC and the Na\(^+\),K\(^+\)-pump in fetal guinea pigs (Ye et al., 2004). The transcription of IL-1β and (nonsignificantly) TNF-α were activated in the epithelium and macrophages at 3 h, but not at the time of epithelial hyperpolarization (18 h), with IL-1β being decreased compared to the controls. This is consistent with the finding that pneumonia-induced TNF-α stimulates alveolar fluid clearance (Rezaiguia et al., 1997). In contrast, we found that incubation of guinea-pig trachea with IL-1β in vitro decreased transepithelial voltage and increased transepithelial resistance (Ismailoglu et al., 2009).

LPS has been demonstrated to increase iNOS and decrease cNOS activities in airways (Jiang et al., 2006; Seven et al., 2005; Tulić et al., 2001). NO decreases ENaC open probability through the production of cGMP (Eaton et al., 2008; Matalon et al., 2003) and inhibits the activity of the Na\(^+\),K\(^+\)-pump (Seven et al., 2005) by triggering its endocytosis and internalization from the basolateral membrane (Gusarova et al., 2009; Vadász et al., 2007). However, our earlier findings indicate that LPS increased, rather than decreased, Na\(^+\),K\(^+\)-pump activity and expression. We
found that eNOS transcription was decreased in the epithelium at 3 h but not at 18 h, and iNOS was not affected by LPS at 3 or 18 h. Neither eNOS nor iNOS were affected in macrophages 3 or 18 h post-injection. The small change was of doubtful biological significance, suggesting a marginal, if any, role of NO produced by the epithelium.

In conclusion, LPS has far-reaching effects on Na⁺ transport in airway epithelium. Its precise mechanisms of action will require exploration of many intracellular signaling pathways.
Acknowledgements

The authors wish to thank Janet Thompson for her technical assistance as well as Thomas R. Kleyman and Rebecca P. Hughey (University of Pittsburgh) for helpful discussions and insights. The authors declare that they have no conflicts of interest. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health. Mention of brand name does not constitute product endorsement.
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Fig. 1. LPS has no effect on the transcription of ENaC or Na\(^+\),K\(^+\)-ATPase, but up-regulates some inflammatory genes. The effects of LPS on the transcription of αENaC, α\(_1\) Na\(^+\),K\(^+\)-ATPase, COX-2, IL-1β, TNF-α, eNOS, and iNOS were determined using qPCR. Tracheal epithelium (upper panels) and alveolar macrophages (lower panels) were isolated at 3 and 18 h after the injection of saline or LPS. In the epithelium, COX-2 and IL-1β transcription, and to a nonsignificant degree, TNF-α, were elevated above controls, while eNOS was below controls at 3 h. The transcription of none of the genes measured were increased at 18 h, while αENaC and IL-1β were significantly lower than controls. In macrophages, TNF-α, and nonsignificantly, COX-2 and IL-1β, were elevated above controls at 3 h. The transcription of none of the genes were different from controls at 18 h. *Significantly greater than controls. †Significantly less than controls. Epithelium 3 h: \(n = 5\) saline and LPS for αENaC, COX-2, IL-1β, TNF-α, eNOS, and iNOS; \(n = 3\) saline and LPS for α\(_1\) Na\(^+\),K\(^+\)-ATPase. Epithelium 18 h: \(n = 8\) saline and LPS for αENaC, COX-2, and eNOS; \(n = 8\) for TNF-α saline; \(n = 7\) saline and LPS for α\(_1\) Na\(^+\),K\(^+\)-ATPase; \(n = 6\) LPS for IL-1β; \(n = 5\) saline IL-1β; \(n = 5\) LPS for TNF-α; \(n = 3\) saline for iNOS; and \(n = 2\) LPS for iNOS. Macrophages 3 h: \(n = 5\) saline and LPS for αENaC, COX-2, IL-1β, TNF-α, and eNOS; \(n = 3\) saline and LPS for α\(_1\) Na\(^+\),K\(^+\)-ATPase; and \(n = 2\) saline and LPS for iNOS. Macrophages 18 h: \(n = 3\) saline and LPS for αENaC, COX-2, IL-1β, TNF-α, and eNOS; and \(n = 2\) saline and LPS for α\(_1\) Na\(^+\),K\(^+\)-ATPase and iNOS.

Fig. 2. LPS has no effect on the expression of αENaC in the epithelium and kidney. Tracheal epithelium and kidney samples were collected from saline- and LPS-treated animals 18 h post-injection and prepared for western blotting. A, To identify specific labeling by the polyclonal antibody, identically loaded lanes were probed with antibody which was blocked by pre-adsorption with its antigen peptide (blocking peptide; Antigen +). 230 and 60 kD bands were
detected in the epithelium from both saline- and LPS-treated animals. B, The relative intensities of both bands were not different between the epithelia of saline- and LPS-treated animals, indicating that LPS had no effect on their expression. C, The β-actin-normalized results are presented here as the within-group distribution of signal intensity between the 230 and 60 kD αENaC bands of the epithelium. The distribution of signal between the bands was not different between saline- and LPS-treated animals, indicating that LPS did not affect the cleavage of αENaC. D, The blocking peptide revealed that the antibody detected one, 60 kD band in the kidney from saline- and LPS-treated animals. E, The relative intensities of the 60 kD band were not different between kidney tissue from saline- and LPS-treated animals. Taken together, LPS had no effect on the expression of αENaC in the tracheal epithelium or kidney tissue 18 h post-injection. The image contrast and brightness in A and D were adjusted for clarity. Please refer to Fig. S1 for the unmodified images, from which measurements were made. Saline: n = 4; LPS: n = 3.

**Fig. 3.** Structure of αENaC indicating the locations of the antigen and protease cleavage sites. The amino acid structure of human, guinea pig, rat, and mouse αENaC are shown. The antigen of the antibody used for western blotting is located near the N-terminal end. Furin cleavage at two RXXR motifs (Hughey et al., 2004a) liberates an inhibitory peptide (Carattino et al., 2008). A suspected protease cleavage site is located in the second disulfide bridge near the C-terminal end of the extracellular loop (Rossier and Stutts, 2009).

**Fig. 4.** LPS has no effect on the expression of βENaC in the epithelium and kidney. Tracheal epithelium and kidney samples were collected from saline- and LPS-treated animals 18 h post-injection and prepared for western blotting. A, To identify specific labeling by the polyclonal antibody, identically loaded lanes were probed with antibody which was blocked by pre-adsorption with its antigen peptide (blocking peptide; Antigen +). 90 and 37 kD bands were
detected in the epithelium from both saline- and LPS-treated animals. B, The relative intensities of both bands were not different between the epithelia of saline- and LPS-treated animals, indicating that LPS had no effect on their expression. C, The β-actin-normalized results are presented here as the within-group distribution of signal intensity between the 90 and 37 kD βENaC bands of the epithelium. The distribution of signal between the bands was not different between saline- and LPS-treated animals, indicating that LPS did not affect the cleavage of βENaC. D, The blocking peptide revealed that the antibody detected one, 90 kD band in the kidney from saline- and LPS-treated animals. E, The relative intensities of the 90 kD band were not different between kidney tissue from saline- and LPS-treated animals. Taken together, LPS had no effect on the expression of βENaC in the tracheal epithelium or kidney tissue 18 h post-injection. The image contrast and brightness in A and D were adjusted for clarity. Please refer to Fig. S2 for the unmodified images, from which measurements were made. Epithelium: n = 3 saline and LPS; kidney: n = 4 saline, n = 3 LPS.

**Fig. 5.** Structure of βENaC indicating the locations of the antigen and protease cleavage sites. The amino acid structure of human, guinea pig, rat, and mouse βENaC are shown. The antigen of the antibody used for western blotting is located at the C-terminal end. A suspected protease cleavage site is located in the second disulfide bridge near the C-terminal end of the extracellular loop (Rossier and Stutts, 2009).

**Fig. 6.** LPS had no effect on the expression of γENaC in the epithelium and kidney. Tracheal epithelium and kidney samples were collected from saline- and LPS-treated animals 18 h post-injection and prepared for western blotting. A, To identify specific labeling by the polyclonal antibody, identically loaded lanes were probed with antibody which was blocked by pre-adsorption with its antigen peptide (blocking peptide; Antigen +). 90 and 40 kD bands were detected in the epithelium from both saline- and LPS-treated animals. B, The relative intensities
of both bands were not different between the epithelia of saline- and LPS-treated animals, indicating that LPS had no effect on their expression. C, The β-actin-normalized results are presented here as the within-group distribution of signal intensity between the 90 and 40 kD γENaC bands of the epithelium. The distribution of signal between the bands was not different between saline- and LPS-treated animals, indicating that LPS did not affect the cleavage of γENaC. D, The blocking peptide revealed that, like in the epithelium, 90 and 40 kD γENaC bands were detected in the kidney from saline- and LPS-treated animals. E, The relative intensities of the 90 and 40 kD bands were not different between kidney tissue from saline- and LPS-treated animals. F, The β-actin-normalized results are presented here as the within-group distribution of signal intensity between the 230 and 60 kD αENaC bands of the epithelium. The distribution of signal between the bands was not different between saline- and LPS-treated animals, indicating that LPS did not affect the cleavage of γENaC. Taken together, LPS had no effect on the expression of γENaC in the tracheal epithelium or kidney tissue 18 h post-injection. The image contrast and brightness in A and D were adjusted for clarity. Please refer to Fig. S3 for the unmodified images, from which measurements were made. Saline: n = 4; LPS: n = 3.

**Fig. 7.** Structure of γENaC indicating the locations of the antigen and protease cleavage sites. The amino acid structure of human, guinea pig, rat, and mouse γENaC are shown. The antigen of the antibody used for western blotting is located at the C-terminal end. Furin cleavage at an RXXR motif (Hughey et al., 2004a) and prostasin cleavage at an RKRK motif (Bruns et al., 2007) activates the channel. A suspected protease cleavage site is located in the second disulfide bridge near the C-terminal end of the extracellular loop (Rossier and Stutts, 2009).

**Fig. 8.** Investigation of the effectiveness of the epithelial cell surface protein isolation protocol. The fate of ENaC was tracked through the cell surface protein isolation process by taking samples at each step and western blotting them. A, Flow diagram of the biotinylation and isolation process...
with numbers indicating each step where a sample was taken. B, Western blot loaded with each of the samples numbered in A and blotted for γENaC (α- and β-ENaC not shown). ENaC is visible in the epithelial cell homogenate (1). During centrifugation, ENaC separated to the supernatant (3) with no more than a faint trace of the 40 kD band detected in the pellet (2). When passing the supernatant through the isolation column, most ENaC washed through the column (4). Only faint signals were detected in the eluate (5). In addition to the 90 and 40 kD γENaC bands, a third band was detected above the heaviest, 250 kD marker in the samples of the homogenate, supernatant, and wash-through. Since the samples were not homogenized in RIPA buffer in this experiment, the >250 kD band most likely represents the ENaC channel complex which was not dissociated. These findings reveal that very little of the epithelium’s ENaC is biotinylated, suggesting that ENaC is mostly localized within the cell.

**Fig. 9.** LPS increased the expression of the Na⁺,K⁺-pump. The expression of the Na⁺,K⁺-pump was investigated by western blotting the epithelium of saline- and LPS-treated animals. A, A representative blot loaded with epithelial samples from a saline- and an LPS-treated animal blotted using an α₁ monoclonal antibody labeled a 106 kD band. B, The expression of the α₁ subunit of the Na⁺,K⁺-pump was greater in the epithelium from LPS treated animals compared to saline treated controls. C, A representative blot loaded with epithelial samples from a saline- and an LPS-treated animal blotted using a pan-α Na⁺,K⁺-pump (α₁, α₂, and α₃) antibody labeled a 105 kD band. D, The expression of pan-α Na⁺,K⁺-pump (α₁, α₂, and α₃) was greater in the epithelium from LPS treated animals compared to saline treated controls. *Significantly different according to the mixed model analysis of variance with block as a random factor. Saline and LPS: n = 10.

**Fig. S1.** LPS has no effect on the expression of αENaC in the epithelium and kidney. This figure is identical to figure 2 except that the images of the western blots in A and D are unmodified and
their original contrast and brightness were not adjusted. Please refer to the legend for figure 2 for explanation of this figure.

**Fig. S2.** LPS has no effect on the expression of βENaC in the epithelium and kidney. This figure is identical to figure 4 except that the images of the western blots in A and D are unmodified and their original contrast and brightness were not adjusted. Please refer to the legend for figure 4 for explanation of this figure.

**Fig. S3.** LPS has no effect on the expression of γENaC in the epithelium and kidney. This figure is identical to figure 6 except that the images of the western blots in A and D are unmodified and their original contrast and brightness were not adjusted. Please refer to the legend for figure 6 for explanation of this figure.
Chapter 3: Figures
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Figure 2
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~131~
Figure 8

A

Apical surface biotinylation

Cell lysis

1. Homogenate

Centrifugation

2. Pellet
3. Supernatant

Isolation column

4. Wash through
5. Eluate

B

>250 kD

90 kD

40 kD

1 2 3 4 5
Figure 9

A

B

C

D

Saline LPS

Saline LPS

Percent β-Actin

Percent β-Actin

LPS − +

LPS − +

β-Actin

β-Actin

106 kD

105 kD

*
Supplementary Figure 1

A

Saline LPS

Antigen - + - +

230 kD

60 kD

β-Actin

D

Saline LPS

Antigen - + - +

60 kD

β-Actin
Supplementary Figure 2

A

Saline LPS

Antigen

- + - +

90 kD

37 kD

β-Actin

D

Saline LPS

Antigen

- + - +

90 kD

β-Actin
Supplementary Figure 3

A  
Saline | LPS
Antigen  -  +  -  +

90 kD ➔ ➔
40 kD ➔ ➔

β-Actin

D  
Saline | LPS
Antigen  -  +  -  +

90 kD ➔ ➔
40 kD ➔ ➔

β-Actin
Chapter 4:

Failed experiments
Primers for many targets were tested for qPCR. The guinea-pig mRNA sequences were not available on the Entrez Nucleotide database for many of the targets tested. For those targets where guinea-pig mRNA sequences were not available, the sequences from rat were substituted in the primer design process. All of the rat-based assays, with the exception of one for $\alpha_1$ Na$^+$/K$^+$-ATPase, were ineffective and failed to yield signals above baseline. This is not surprising because of the mRNA sequence differences between species. The sequence identity between rat and guinea-pig $\alpha$ENaC, for example, is $\approx$75% (Schnizler et al., 2000). Of all the targets for which primers were tested, working ones were identified that yielded signal for guinea-pig $\alpha$ENaC, $\alpha_1$ Na$^+$/K$^+$-ATPase, COX-2, IL-1$\beta$, TNF-\(\alpha\), eNOS, and iNOS. These experiments are covered in Chapter 3. The primers for all of these, with the exception of $\alpha_1$ Na$^+$/K$^+$-ATPase, were designed using available guinea-pig mRNA sequences.

Other targets for which we attempted unsuccessfully to design primers for were COX-1, muscarinic receptors 1, 2, 3, and 5, TLR-4, nNOS, and IL-6. The primers that failed to yield signal above baseline are listed in Table 4-1. As described in Chapter 1 and Chapter 3, we found that the non-specific COX-1/2 inhibitor, indomethacin inhibited $V_t$ to a greater extent in tracheas from LPS-treated animals compared to controls (Johnston et al., 2004). Even though we had observed, using working primers, that LPS increased the transcription of COX-2, the inducible form of COX, we sought to add to these findings by determining the relative rates of transcription of COX-1. The bioelectric response of the epithelium to methacholine, a muscarinic agonist, was transformed after the administration of LPS (Fedan et al., 1995; Johnston et al., 2004). The mechanism of this effect remains an unanswered question. We hypothesized that LPS may modify the expression of muscarinic receptors and tested primers for muscarinic receptors 1, 2, 3, and 5. TLR-4 and -2 expression and transcription in the mouse bronchial epithelium are stimulated by the inhalation of LPS (Saito et al., 2005). For this study, we asked how systemic LPS affects the transcription of TLR-4. As described in Chapter 1 and Chapter 3, NO regulates the activity of the Na$^+$/K$^+$-pump. The qPCR results using eNOS and iNOS primers revealed that LPS had little, if any, effect on
these two enzymes. In addition to eNOS and iNOS, we attempted to investigate neuronal NOS as well. Finally, we attempted to investigate IL-6 because, as also TNF-α, it is promoted by NF-κB and is used as a marker of lung inflammation (Roos-Engstrand et al., 2005; Pierre et al., 2007).

**Western blotting**

Chapter 3 describes western blotting investigating the effects of LPS on the expression of ENaC and the Na⁺,K⁺-pump. Those experiments were done with α-, β-, and γ-ENaC polyclonal antibodies and α1- and pan-α-Na⁺,K⁺-ATPase monoclonal antibodies. In parallel with the difficulties associated with the unavailability of guinea-pig mRNA sequences for designing PCR primers, we were faced with the unavailability of antibodies that were created for, or tested for, reactivity with guinea-pig antigens. In addition to ENaC and the Na⁺,K⁺-ATPase, we also tested antibodies with which to western blot COX-2, iNOS and TNF-α in order to evaluate the qPCR results described in Chapter 3. Commercially available monoclonal and polyclonal antibodies against these targets were tested by incubating identically loaded lanes with a dilution series of primary antibody. This dilution series blot was repeated several times and each repeat was incubated with a different serial dilution of secondary antibody. The combination of dilutions of primary and secondary antibody that yielded the best signal to background contrast was chosen for experiments.

The α-, β-, and γ-ENaC antibodies that were used in chapter 2 were the only ENaC antibodies that yielded good and consistent signal. The availability of the antigen peptides for these antibodies made possible the identification of the specific bands and permitted us to adjust the signal to background contrast. The two Na⁺,K⁺-ATPase antibodies used were both monoclonal. They exhibited minimal nonspecific binding and yielded clear signals which facilitated the refinement of antibody dilutions tremendously. The antibodies that failed our tests are listed in Table 4-2. The various COX-2 antibodies detected bands of different molecular weights in the same samples. We had no antigen peptides or other positive controls to determine which of those bands were due to specific binding or non-specific binding.
Therefore, we did not pursue the western blotting of COX-2 any further. The signals from iNOS and TNF-α were faint probably because of low concentrations of those targets in the epithelium.
Bibliography


Table 4-1: List of qPCR assays tested in guinea-pig airway epithelial samples.

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<th>Right Primer</th>
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The assays were designed using the Roche Universal Probe Library’s version of Primer3 software. In the transcript column, R = rat and GP = guinea pig.
Table 4-2: Antibodies tested, but not used.

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</tr>
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<tr>
<td>TNF-α</td>
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<td>Human</td>
<td>Monoclonal</td>
<td>Thermo Scientific</td>
<td>MS-410-P</td>
</tr>
</tbody>
</table>

The antibodies tested here were tested by incubating blots with serial dilutions of both primary and secondary antibody. Since they failed to yield clear, consistent signals in our tests, they were not used.
Chapter 5:

Discussion and conclusions
**Hypothesis 1: LPS increases ENaC and Na⁺,K⁺-pump activities**

Using the IPT to study the bioelectric behavior of the airway epithelium, we found that LPS hyperpolarizes the airway epithelium 18 h post-injection. As covered in Chapter 2, amiloride inhibited Vᵢ in tracheas from saline- and LPS-treated animals, but to a greater extent in the LPS-treated group. Thus, LPS hyperpolarizes the airway epithelium by increasing the transport of Na⁺ across the ENaC/Na⁺,K⁺-pump axis. The increase in Na⁺ transport by LPS could be explained by two possibilities; increased Na⁺,K⁺-pump activity could reduce the intracellular Na⁺ concentration, increasing Na⁺ current via ENaC (Geering 2006), or increased ENaC activity could raise intracellular Na⁺ concentrations, which would stimulate the activity of the Na⁺,K⁺-pump (Miller and Davis 2008).

**ENaC activity**

LPS increased amiloride-sensitive Vᵢ, indicating that LPS increased Na⁺ current via ENaC. To determine the effect of LPS on the activity of ENaC, ENaC activity was measured in the IPT from saline- and LPS-treated animals. To remove the contribution of the Na⁺,K⁺-pump to Vᵢ, the basolateral membrane was permeabilized with amphotericin B. At the same time, basolateral Na⁺ was replaced with NMDG⁺ to create a large and uniform Na⁺ gradient across the apical membrane. NMDG⁺ incited an unexpected, transient Vᵢ response discussed in Chapter 2. Nevertheless, Vᵢ was greater in tracheas from LPS-treated animals, indicating hyperpolarization due to greater Na⁺ transport. Indeed, the addition of apical amiloride revealed that amiloride-sensitive Vᵢ was larger in the LPS-treated group. The lack of effect of trypsin confirmed that ENaC had been blocked by amiloride. These results indicate that the activity of ENaC is increased by LPS. The increase in ENaC activity observed in this experiment was not due to an increase in intracellular Na⁺ concentrations, but an increase in ENaC activity. ENaC activity could be increased with an increase in abundance and/or open probability of the channel. Thus,
this project also investigated ENaC expression, proteolysis, and trafficking as possible mechanisms for increased ENaC activity. Since the basal activity of ENaC depends on the Na$^+$ gradient established by the Na$^+$/K$^+$-pump, the effects of LPS on its activity were also investigated.

**Na$^+$/K$^+$-pump activity**

We found that LPS more than doubles the rate of the Na$^+$/K$^+$-pump in the airway epithelium. This conclusion was supported by the results of separate, permeabilization and Na$^+$-loading approaches. The intracellular concentration of Na$^+$, which is reduced by the activity of the Na$^+$/K$^+$-pump, is a determinant of the rate of Na$^+$ entry via ENaC. In our experiments, even though an LPS-induced change in intracellular Na$^+$ concentrations may have contributed to the upregulation of Na$^+$/K$^+$-pump activity, the intracellular Na$^+$ concentration at the time $V_t$ was measured in the IPT was equal to the concentration in the apical bath (119 mM) in both control and LPS treatment groups. Likewise, in the Na$^+$-loading experiments, the hyperpolarization response to K$^+$ restoration was greater in the LPS-treated group even though the epithelia in both treatment groups had been nearly completely depolarized.

A recently discovered family of proteins, FXYD, has been identified as an auxiliary γ subunit that regulates the activity and expression of the Na$^+$/K$^+$-pump (Geering 2006). Each of the members of the FXYD family studied to date have unique tissue distributions and regulatory effects on Na$^+$/K$^+$-pump activity with FXYD5 identified in the lung epithelium (Garty and Karlish 2006). The increase in intracellular Na$^+$, which occurs in the cystic fibrotic airway epithelium, stimulates the expression of FXYD5 and could explain the net increase in transepithelial Na$^+$ transport in response to ENaC hyperactivity (Miller and Davis 2008). Because we detected no effect of LPS on Na$^+$/K$^+$-ATPase transcription, yet found that protein abundance and activity were increased, it is convenient to speculate that the stimulation of Na$^+$/K$^+$-pump activity could be mediated by FXYD5.
Role of CAPs in ENaC activation

The second major finding of this project was that channel activating proteases are not involved in the activation of ENaC by LPs. Channel activating proteases have recently been found to regulate the activity of ENaC. As described in Chapter 1, it is thought that ENaC is divided into two pools, an uncleaved, near silent one, and a cleaved, activated one. The active pool transports Na+, while the inactive pool could serve as substrate for activation intracellularly and at the apical membrane. Because LPS increased the activity of ENaC, we hypothesized that LPS increased its activation by channel activating proteases. This hypothesis was tested in the Ussing chamber by investigating the responsiveness of $V_t$ and $R_t$ to trypsin and by determining the effect of trypsin on amiloride-sensitive $V_t$. Additionally, biochemical results from western blotting supported the conclusion that channel activating proteases are not involved in LPS-induced ENaC activation.

LPS does not affect amiloride-sensitive $V_t$

We hypothesized that if LPS activated ENaC via channel activating proteases, the pool of near-silent, uncleaved ENaC would be reduced, rendering the tracheas from LPS-treated animals less sensitive to trypsin. Thus, the effect of trypsin on amiloride-sensitive $V_t$ should be smaller in tracheas from LPS-treated animals compared to controls. This hypothesis was investigated in the Ussing chamber using tracheas from saline- and LPS-treated animals, as described in Chapter 2. The results indicated that the amiloride-sensitive $V_t$ responses in the absence and presence of trypsin were greater in tracheas from LPS-treated animals compared to controls, indicating that ENaC activity was increased by LPS. Trypsin hyperpolarized $V_t$ in both treatment groups, but the response was not different between treatment groups. This indicates that the sizes of the near-silent, uncleaved pools of ENaC were the same and not affected by LPS. Trypsin also did not affect the $V_t$ response to amiloride in tracheas from either saline- or LPS-
treated animals. This indicates that ENaC activity was not affected by trypsin because the sizes of the near-silent pools of ENaC were very small in both treatment groups. Taken together, these results suggests that the systemic administration of LPS activates ENaC, but by a mechanism that does not involve channel activating proteases.

**Trypsin does not affect ion transport**

Even though the mechanism of LPS-induced ENaC activation does not seem to involve channel activating proteases, trypsin does activate ENaC. As covered in Chapter 2, the addition of apical trypsin in the Ussing chamber hyperpolarizes the epithelium. This hyperpolarization is consistent with activation of the channel by trypsin, but is inconsistent with the finding that trypsin does not affect amiloride-sensitive $V_t$. As $V_t$ hyperpolarized, the $V_t$ deflections to the applied current pulses increased in magnitude. To seek an explanation for these observations, we hypothesized that the hyperpolarization in $V_t$ by trypsin was brought about not by an increase in $Na^+$ transport, but by an increase in $R_t$. If the increase in $V_t$ was brought about by increased ion transport, $R_t$ should increase by a proportionately smaller amount, reflecting greater ion movement. This hypothesis was tested in the Ussing chamber by applying apical trypsin to tracheas from saline- and LPS-treated animals. The basal values of $V_t$ and $R_t$ were greater in tracheas from LPS-treated animals compared to controls. The addition of trypsin increased $V_t$ and $R_t$ in both treatment groups, with both parameters being larger in tracheas from LPS-treated animals. The proportionate effects of trypsin $V_t$ and $R_t$ were determined by calculating $\% \Delta V_t/\% \Delta R_t$. The values of $\% \Delta V_t/\% \Delta R_t$ were unity in tracheas from both saline- and LPS-treated animals, indicating that trypsin increased both parameters by the same proportions. Thus, the trypsin-induced increase in $V_t$ was due entirely to an increase in $R_t$, not an increase in ion transport. Even though the mechanism by which LPS increases ENaC activity cannot be determined from these experiments, proteolytic activation of ENaC by channel activating proteases is not involved.
Hypothesis 2: LPS increases ENaC and Na⁺,K⁺-pump transcription

The effects of LPS on the expression of αENaC and the α₁ subunit of the Na⁺,K⁺-pump were investigated using qPCR. Two, different approaches for measuring the activity of the Na⁺,K⁺-pump revealed that it was increased by LPS. We hypothesized that LPS increased the expression of ENaC and the Na⁺,K⁺-pump at the level of transcription. This hypothesis was addressed by collecting epithelium from saline- and LPS-treated animals and comparing the relative rates of transcription of target genes by qPCR.

We had great difficulty in constructing assays that yielded reliable signals during qPCR. The guinea-pig αENaC subunit had been cloned previously (Schnizler, Mastroberardino et al. 2000), but the guinea-pig sequences for β- and γ-ENaC were unavailable. It is of great interest to understand whether LPS might alter expression of these other subunits. Even though the substitution of rat sequences to design primers for guinea-pig targets has been successful on occasion (Yamada, Udagawa et al. 2005), it was unsuccessful in the primer design process for β-, and γ-ENaC, which was not surprising since the sequence identity of guinea-pig and rat αENaC was reported to be 76% (Schnizler, Mastroberardino et al. 2000). The differences between guinea-pig and rat ENaC extend to their function in that human and guinea-pig αENaC, unlike in mice and rats, need basal cAMP to be trafficked to the membrane to be functional (Schnizler, Mastroberardino et al. 2000; Woollhead and Baines 2006). Using rat sequences to design primers for the α₁, α₂, α₃, α₄, β₁, β₂, β₃, and β₄ subunits of the Na⁺,K⁺-pump was only successful for the α₁ subunit. Thus, whether changes in the transcription of these subunits were initiated by LPS remains unknown.

In this study, no effect of LPS on the transcription of αENaC or α₁ Na⁺,K⁺-pump at 3 or 18 h after LPS injection was detected, suggesting that a stimulation of the transcription of ENaC or the Na⁺,K⁺-pump was not involved in the LPS-induced hyperpolarization of the epithelium. This finding is to be treated with caution though, because changes in the transcription rate of the β
and γ subunits do not necessarily accompany changes to αENaC transcription. The intratracheal installation of *P. aeruginosa* into the mouse lung, which inhibited amiloride-sensitive Na⁺ transport in contrast to our findings, inhibited the transcription of βENaC with no effect on αENaC or α₁ Na⁺,K⁺-pump (Pierre, Husson et al. 2007). Additionally, IL-4 has been found to decrease γ-, and to a lesser degree, β-ENaC transcription without any affect on αENaC transcription in the human bronchial epithelium (Galiotta, Pagesy et al. 2002), while other factors such as the δENaC subunit (Ji, Su et al. 2006), and membrane stretch upon cell swelling (Ma, Al-Khalili et al. 2004), increase ENaC activity.

Earlier experiments done in our laboratory revealed that the non-specific COX1/2 inhibitor, indomethacin, inhibited V₄ to a greater extent in perfused tracheas from LPS-treated animals compared to controls (Johnston, Van Scott et al. 2004). Even though this inhibition was not as great as the effect of amiloride, this finding indicated that prostaglandins play a role in the LPS-induced hyperpolarization of airway epithelium. Indeed, COX-2 message was greater in the epithelium from LPS-treated animals compared to controls at 3 h. This finding suggests a possible avenue of exploration for future experiments.

NO, which is produced in large quantities by LPS activated macrophages (Zeidler and Castranova 2004), regulates ion transport in the airway epithelium. Epithelial anion secretion is increased by NO (Duszyk 2001). In the NO model of tissue injury, it has been found to decrease the activity of the Na⁺,K⁺-pump by triggering its endocytosis and internalization from the basolateral membrane of the airway epithelium (Vadász, Raviv et al. 2007; Gusarova, Dada et al. 2009). Because these reports are inconsistent with our findings that LPS stimulates Na⁺,K⁺-pump activity, and NO has been reported to be stimulated by LPS, we included eNOS and iNOS among the gene targets we investigated. The transcription of eNOS was decreased in the epithelium at 3 h post-injection, but not at 18 h and iNOS was not affected by LPS at 3 or 18 h. Neither eNOS nor iNOS transcription were affected in macrophages at either 3 or 18 h post-injection. Taken together, these data suggest that LPS has a marginal, if any, effect on the production of NO by the
epithelium and that stimuli of Na\(^+\),K\(^+\)-pump activity overcame any inhibitory effect NO may have had.

The effects of LPS on the transcription of two cytokines, TNF-\(\alpha\) and IL-1\(\beta\), were also investigated. The expression of both TNF-\(\alpha\) and IL-1\(\beta\) are activated by NF-\(\kappa\)B in macrophages (Takasuka, Matsuura et al. 1995). We asked the question if LPS activated these targets in the epithelium, a sign that NF-\(\kappa\)B may be activated. At 3 h post-injection, IL-1\(\beta\), and to a non-significant extent, TNF-\(\alpha\) were increased in the epithelium from LPS-treated animals compared to controls. In macrophages from LPS-treated animals, TNF-\(\alpha\), and, non-significantly, IL-1\(\beta\), were increased compared to controls. This data indicates that the transcription of these NF-\(\kappa\)B-regulated genes was stimulated by LPS. IL-1\(\beta\) has implications for epithelial Na\(^+\) transport because it has been found to increase \(\alpha\)- and \(\beta\)-ENaC and the \(\alpha_1\)- and \(\beta_1\)-Na\(^+\),K\(^+\)-pump protein in fetal guinea-pig lungs (Ye, Acharya et al. 2004).

**Hypothesis 3: LPS increases ENaC and Na\(^+\),K\(^+\)-pump expression**

**ENaC abundance**

In the functional experiments described above, we found that LPS increases the activity of ENaC in the airway epithelium. Our qPCR investigation determined that LPS had no effect on the transcription of \(\alpha\)ENaC. Therefore, we formulated the hypothesis that LPS increases the abundance of ENaC protein in the epithelium. To determine the effects of LPS on the expression of ENaC, \(\alpha\)-, \(\beta\)-, and \(\gamma\)-ENaC were western blotted. We found no commercially-available antibodies for guinea-pig ENaC. The studies that report western blotting of ENaC in native guinea-pig tissue in the literature we found used custom-made antibodies (Mackler, Kleyman et al. 1998; Ramírez-Gil, Trouvé et al. 1998). To identify antibodies that detect our guinea-pig targets, we tested many commercially-available antibodies. Only one set was successful and these were tested and optimized as described in Chapters 1 and 2.
The results revealed multiple bands for α-, β-, and γ-ENaC representing complexed, full-length, and fragmentary subunits. The abundance of none of these bands, however, was affected by LPS. αENaC was present in bands with molecular weights of 230 and 60 kD, but no band was detected corresponding to the ≈90 kD molecular weight reported for the full-length, uncleaved subunit. The 230 kD band most likely represents a complex of multiple αENaC subunits because it was not detected by the β- and γ-ENaC antibodies. Literature has reported 180 kD (Ismailov, Awayda et al. 1996) or 150 kD (Rokaw, Wang et al. 1998) αENaC bands in the absence of a 90 kD full-length band after separation in non-denaturing conditions, but not in the presence of denaturing dodecyl sulfate and dithiothreitol. Additionally, a >600 kD band, detected under non-denaturing conditions, has been reported (Staruschenko, Medina et al. 2004). Our 230 kD band is surprising because of the denaturing conditions that are expected to break apart protein-protein interactions. The 60 kD band represents an N-terminal fragment of the αENaC subunit because the antigen used to produce the antibody is located at the N-terminal end of the structure. This molecular weight is twice the molecular weight reported for the N-terminal fragment of furin-cleaved αENaC (Hughey, Bruns et al. 2004).

Both β- and γ-ENaC were detected in full-length and fragmentary forms. βENaC was detected at molecular weights of 90 and 37 kD and γENaC was detected at molecular weights of 90 and 40 kD. The antigens for both antibodies are located at the C-terminal ends of the subunits’ structures. The molecular weight of the γENaC subunit is reported to be 75 kD, while βENaC is reported not to be cleaved by furin or prostasin at all (Hughey, Bruns et al. 2004). Rossier and Stutts proposed putative cleavage sites for trypsin, chymotrypsin, elastase, and other channel activating proteases in the second disulfide bridges of the extracellular loop structures of each of the α-, β-, and γ-subunits of ENaC (Fig. 5-1) (Rossier and Stutts 2009). This hypothesis is based on the importance of the second disulfide bridge for ENaC function (Firsov, Robert-Nicoud et al. 1999). Elastase released in the lung by neutrophils has been reported to increase the activity of ENaC in the lung (Planès, Blot-Chabaud et al. 2002). Rossier and Stutts report that there is no
experimental evidence to support these hypothetical cleavage sites, but our results may constitute such evidence.

LPS had no effect on the expression of ENaC. The intensities of none of the bands of α-, β-, and γ-ENaC were different between the epithelium of saline- and LPS-treated animals. Thus, LPS does not increase the activity of ENaC by increasing its expression. Even though all three subunits were cleaved, LPS had no effect on the intensities of α-, β-, or γ-ENaC fragments, and had no effect on the distribution of signal among the full-length and fragmentary molecular weights. This lack of effect of LPS on ENaC cleavage is consistent with the Ussing chamber results where there was no difference in the effect of trypsin between treatment groups. This finding supports the conclusion that channel activating proteases are not involved in the stimulation of ENaC activity by LPS.

**ENaC trafficking**

Since LPS had no effect on the transcription or expression of ENaC, yet increased its activity, we hypothesized that LPS induced the trafficking of ENaC to the apical membrane from sub-membrane stores. The activation of protein kinase A has been found to induce the trafficking of intracellular ENaC to the apical membrane (Woollhead and Baines 2006). To investigate this hypothesis, the isolation of cell-surface proteins was attempted involving apical surface biotinylation, epithelium homogenation, and pulldown of biotinylated protein using a neutravidin bead-linked column. The eluate was then western blotted for ENaC. As described in Chapter 3, these experiments were unsuccessful because the resulting signals were too weak to be analyzed densitometrically. Determining the fate of ENaC through the surface protein isolation process revealed that ENaC passed through the column and was not biotinylated. Thus, the proportion of the whole cell’s ENaC located in the apical membrane is miniscule.
Na\textsuperscript{+},K\textsuperscript{+}-pump abundance.

The effects of LPS on the protein expression of the Na\textsuperscript{+},K\textsuperscript{+}-pump were determined because Na\textsuperscript{+},K\textsuperscript{+}-pump activity was increased by LPS. The epithelium from saline- and LPS-treated animals was western blotted. Two monoclonal antibodies were used, one for the \(\alpha_1\) subunit, and one pan-\(\alpha\) which recognized the \(\alpha_1\), \(\alpha_2\), and \(\alpha_3\) subunits as a group. The expression of \(\alpha_1\) and pan-\(\alpha\) Na\textsuperscript{+},K\textsuperscript{+}-pump subunits were significantly greater in the epithelium from LPS-treated animals compared to controls. Thus, the increase in Na\textsuperscript{+},K\textsuperscript{+}-pump function by LPS could be explained by a stimulation in its expression. As explained in Chapter 1, the expression of the Na\textsuperscript{+},K\textsuperscript{+}-pump is stimulated in the cystic fibrotic epithelium by FXYD5 in response to increased Na\textsuperscript{+} concentrations (Miller and Davis 2008), and it is convenient to speculate its involvement in the stimulation of Na\textsuperscript{+},K\textsuperscript{+}-pump expression by LPS.
Bibliography


Fig. 5-1. Locations of protease cleavage sites in α-, β-, and γ-ENaC. The locations of the serine protease cleavage sites for furin, prostasin, and elastase in α-, β- and γ-ENaC are indicated in along with the molecular weights of their cleavage fragments. Rossier and Stutts (2009) propose cleavage sites for trypsin, chymotripsin, elastase, and other channel activating proteases in the second disulfide bridge region near the second transmembrane-spanning domains of each of the three subunits. Image used with permission from Rossier and Stutts (2009).
CURRICULUM VITAE
Michael Dodrill

CONTACT
Name: Michael W. Dodrill
Address: Department of Basic Pharmaceutical Sciences
School of Pharmacy
Robert C. Byrd Health Sciences Center
West Virginia University
P.O. Box 9530
Morgantown, WV 26506-9530
Telephone: (304) 285-6182
Email: mdodrill@hsc.wvu.edu

EDUCATION
• Ph.D. in Pharmaceutical and Pharmacological Sciences
  o 2004–present, West Virginia University, Morgantown, WV
  o Mentor: Jeffrey S. Fedan, Ph.D.
• Master of Safety and Environmental Management
  o 2003–2004, West Virginia University
• Master of Occupational Safety and Occupational Health
  o 2002–2004, West Virginia University
  o Mentor: Steven E. Guffey, Ph.D., C.I.H.
  o Thesis title: Experimental Validation of the “Target Hood Static Pressure” Balancing Method for Exhaust Ventilation Systems
• Bachelor of Science in Biology
  o 1998–2002, West Virginia University

MEMBERSHIPS
• American Society for Pharmacology and Experimental Therapeutics 2005–present
• American Industrial Hygiene Society 2002–present
• βββ Biological Honors Society 2000–2002

AWARDS
• Basic Pharmaceutical Sciences Departmental Graduate Student Travel Award to attend the Experimental Biology 2008 national meeting
• American Society for Experimental Therapeutics Graduate Student Travel Award to attend the Experimental Biology 2008 national meeting
• Basic Pharmaceutical Sciences Departmental Graduate Student Travel Award to attend the Experimental Biology 2009 national meeting
• Basic Pharmaceutical Sciences Departmental Graduate Student Travel Award to attend the Experimental Biology 2010 national meeting
• American Society for Experimental Therapeutics Graduate Student Travel Award to attend the Experimental Biology 2010 national meeting
• Graduate Research Office Student travel award to attend the Experimental Biology 2010 national meeting
• Runner up best abstract award at the Oral Competition at the iSTCP Divisional Meeting held during the Experimental Biology 2010 national meeting
TEACHING

- West Virginia University: Moderator of problem-based learning exercises for pharmacy students (PHAR 701 Pharmaceutical Care Lab 1) 2006.
- West Virginia University: Lectured about anticoagulant drugs to undergraduate students in Drugs and Medicine (PHAR 449) 2008.

ARTICLES


ABSTRACTS

Dodrill, MW and Fedan, JS. 2010. Lipopolysaccharide (LPS) hyperpolarizes the guinea-pig tracheal epithelium (Epi) by increasing the activities of the epithelial sodium channel (ENaC) and the Na⁺,K⁺-pump. *FASEB J*.


INVITED PRESENTATIONS

Dodrill MW. Systemic lipopolysaccharide stimulates airway transepithelial Na⁺ transport by increasing ENaC and Na⁺,K⁺-pump activities. NIOSH HELD Pathology and Physiology Research Branch Seminar. April 6th, 2010.

Dodrill MW. Lipopolysaccharide hyperpolarizes the guinea-pig tracheal epithelium by increasing the activities of the epithelial sodium channel and the Na,K-pump. Experimental Biology National Meeting. April 27, 2010. Winner of the best abstract competition.

Dodrill MW. Are lipopolysaccharide-induced changes to airway epithelial sodium transport mediated by amiloride-sensitive sodium channels? NIOSH inflammation group seminar series. 2007.