

Lipopolysaccharide modifies amiloride-sensitive Na^+ transport processes across human airway cells: role of mitogen-activated protein kinases ERK 1/2 and 5

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Received: 5 May 2009 / Accepted: 14 August 2009 / Published online: 13 October 2009
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Abstract Bacterial lipopolysaccharides (LPS) are potent inducers of proinflammatory signaling pathways via the activation of nuclear factor-kappa B (NF- κ B) and mitogen-activated protein kinase (MAPK), causing changes in the processes that control lung fluid homeostasis and contributing to the pathogenesis of lung disease. In human H441 airway epithelial cells, incubation of cells with $15 \mu\text{g ml}^{-1}$ LPS caused a significant reduction in amiloride-sensitive I_{sc} from 15 ± 2 to $8 \pm 2 \mu\text{A cm}^{-2}$ ($p=0.01$, $n=13$) and a shift in IC_{50} amiloride of currents from 6.8×10^{-7} to 6.4×10^{-6} M. This effect was associated with a decrease in the activity of 5 pS, highly Na^+ selective, amiloride-sensitive $<1 \mu\text{M}$ channels (HSC) and an increase in the activity of ~ 18 pS, nonselective, amiloride-sensitive $>10 \mu\text{M}$ cation channels (NSC) in the apical membrane. LPS decreased αENaC mRNA and protein abundance, inferring that LPS inhibited αENaC gene expression. This correlated with the decrease in HSC activity, indicating that these channels, but not NSCs, were comprised of at least αENaC protein. LPS increased NF- κ B DNA binding activity and phosphorylation of extracellular signal-related kinase (ERK)1/2, but decreased phosphorylation of ERK5 in H441 cells. Pre-treatment of monolayers with PD98059 ($20 \mu\text{M}$) inhibited ERK1/2 phosphorylation, promoted phosphorylation of

ERK5, increased αENaC protein abundance, and reversed the effect of LPS on I_{sc} and the shift in amiloride sensitivity. Inhibitors of NF- κ B activation were without effect. Taken together, our data indicate that LPS acts via ERK signaling pathways to decrease αENaC transcription, reducing HSC/ αENaC channel abundance, activity, and transepithelial Na^+ transport in H441 airway epithelial cells.

Keywords Na^+ channel · Lung · Airway · Amiloride · LPS · ERK

Introduction

In lung disease states, particularly cystic fibrosis, *Pseudomonas aeruginosa* is a common opportunistic pathogen which can cause exacerbation of the existing condition. Such infections can lead to inflammation and damage to the epithelial barrier. This can result in increased fluid leak from the underlying tissues into the lumen of the conducting airways and the distal alveoli contributing to the formation of pulmonary edema. Evidence has indicated that bacterial and viral infections also modify the ion transport processes that control lung fluid homeostasis [30, 43]. These processes maintain the correct hydration of the luminal surface of the airway for optimal function of the mucociliary escalator as part of the airway defense against infection and the alveolar surface to maintain efficient gas exchange. The vectorial transport of Na^+ across the lung epithelium via amiloride-sensitive epithelial Na^+ channels drives the osmotic movement of fluid from the lumen to the interstitium. Two populations of amiloride-sensitive Na^+ -permeable cation channels have been described in the membrane of human H441 airway epithelial cells, alveolar type I and type II cells that could contribute to apical Na^+

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conductance (G_{Na^+}) [1, 8, 27, 28, 31] and these have been described as a highly Na^+ selective of 5 pS conductance channel that is inhibited by $<1 \mu\text{M}$ amiloride (HSC) and a nonselective cation channel of ~ 18 pS conductance that is inhibited by $>10 \mu\text{M}$ amiloride (NSC). The relative expression of these channels in alveolar type II are determined by culture conditions and the cellular abundance of protein subunits of the epithelial Na^+ channel (ENaC) [6, 27, 31]. The HSC is thought to comprise αENaC , βENaC , and γENaC in heterotrimeric combination, while the NSC has been reported to require only αENaC . As these channels are essential for the regulation of lung fluid homeostasis, then factors that alter their expression and function could potentially lead to a decrease in net fluid absorption and contribute to aberrant fluid homeostasis in the airway and the formation of pulmonary edema in the distal lung.

Lipopolysaccharide (LPS) present in the coat proteins of many bacterial pathogens including *P. aeruginosa* are potent inducers of the secretion and synthesis of proinflammatory cytokines such as $\text{TNF-}\alpha$, reactive oxygen species, and NO production via the activation of nuclear factor-kappa B (NF- κB) and mitogen-activated protein kinase (MAPK) signaling pathways [19, 20]. While the release of factors into the medium by LPS-stimulated alveolar macrophages has been shown to inhibit ENaC expression in fetal distal lung epithelial cells via a NO-dependent mechanism [13] and $\text{TNF-}\alpha$ has been demonstrated to decrease ENaC mRNA expression in adult rat alveolar epithelial cells [9, 10], the effects of LPS applied directly to airway epithelial cells have not been fully explored. In this paper, we have used the human H441 airway epithelial cell line, which has Clara cell-like properties, to show that LPS inhibits amiloride-sensitive Na^+ transport and alters the amiloride sensitivity of the monolayer by reducing the function of HSCs in the apical membrane. We have also used a combination of pharmacological and molecular techniques to characterize the role of NF- κB and the extracellular signal-regulated protein kinases 1, 2, and 5 (ERK1, ERK2, and ERK5) in these LPS-mediated effects.

Materials and methods

Cell culture

H441 cells (obtained from ATCC, USA) were grown in RPMI 1640 medium (Life Technologies, UK) supplemented with 2 mM L-glutamine, sodium pyruvate, 10% (v/v) fetal bovine serum (Immune Systems, UK) and antibiotics (penicillin/streptomycin; Life Technologies, UK). Cells were maintained at 37°C in humidified

atmospheric air +5% CO_2 and passaged twice weekly. To obtain resistive monolayers, H441 cells were seeded onto snapwell clear membranes (Costar, UK) at 1×10^6 cells per square centimeter and cultured overnight using the medium described above. The following day, the medium was replaced with RPMI 1640 medium including 4% dialyzed fetal bovine serum, 2 mM L-glutamine, sodium pyruvate, $5 \mu\text{g ml}^{-1}$ insulin, $5 \mu\text{g ml}^{-1}$ transferrin, 10 nM sodium selenite, 100 nM dexamethasone, and 10 nM triiodo-L-thyronine (T3) and the cells were cultured at air interface for 6–7 days. Cells with a transepithelial resistance (R_t) of $>200 \Omega \text{ cm}^{-2}$ were used in this study. Treatments were carried out using a strictly paired protocol. LPS from *P. aeruginosa* (Sigma, UK) was suspended in culture medium and used at a final concentration of $15 \mu\text{g ml}^{-1}$ in the basolateral compartment and 50 μl of LPS solution or normal medium was dropped carefully onto the apical surfaces of cells grown at air interface. This concentration of LPS evoked a maximal change in transcriptional activity of an NF- κB -driven reporter construct in these cells (data not shown) and was similar to that used in other lung studies [12]. Cells were pretreated with vehicle or MAPK inhibitor (PD98059) 20 μM (a concentration which effectively inhibited ERK1/2 phosphorylation in these cells) [34] or caffeic acid phenyl ester (CAPE) 25 $\mu\text{g ml}^{-1}$ in dimethyl sulfoxide [39] or sulfasalazine (SAS; Sigma, UK) 5 mM in culture medium [3, 21] for 30 min prior to the addition of LPS.

Functional studies

Snapwells supporting resistive monolayers of H441 cells were mounted in Ussing chambers and bathed with a physiological salt solution (in millimolars): NaCl, 117; NaHCO_3 , 25, KCl, 4.7; MgSO_4 , 1.2; KH_2PO_4 , 1.2; CaCl_2 , 2.5; D-glucose, 11; pH 7.3–7.4 when equilibrated with 5% CO_2 . The solution was maintained at 37°C and continuously circulated by bubbling with 21% O_2 +5% CO_2 premixed gas. The monolayers were firstly maintained under open circuit conditions, while the transepithelial potential difference (V_t) and resistance (R_t) were monitored and observed to reach a stable level. The cells were then short circuited by clamping V_t at 0 mV using a DVC-4000 voltage/current clamp (WPI, UK), and the current required to maintain this condition (I_{sc}) was measured and recorded using a PowerLab computer interface. The movement of cations from the apical to the basolateral side or anions from the basolateral to apical side of the monolayer is represented by positive values for I_{sc} . Amiloride hydrochloride was suspended in H_2O and used at concentrations ranging from 10^{-4} to 10^{-10} M. Ouabain was resuspended in 50:50 ethanol/ H_2O and used at a final concentration of 10^{-3} M.

Single-cation channel currents were recorded from H441 cell monolayers with an AXOPatch 200B patch clamp amplifier (Axon Instruments, Union City, CA, USA) at room temperature (20–25°C) using the cell-attached configuration of the patch clamp technique as previously described [1]. To set the membrane potential at approximately 0 mV, H441 monolayer cells were perfused with a bathing solution containing (in millimolars): KCl, 145; CaCl₂, 1.8; MgCl₂, 2; glucose, 5.5; and HEPES, 10; pH to 7.4 with KOH. The standard patch pipette solution contained (in millimolars): NaCl, 145; CaCl₂, 1.8; MgCl₂, 2; glucose, 5.5; and HEPES, 10; pH to 7.4 with NaOH, and with this solution, patch pipette resistances were 10–15 MΩ. To investigate the amiloride sensitivity of cation channel activity, 1 or 10 μM amiloride was included in the standard patch pipette solution. Cation channel currents were recorded and analyzed using pCLAMP software (version 9.0, Axon Instruments) whereby channel activity was sampled at between 1 and 2 kHz and low-pass filtered between 100 and 200 Hz and then idealized channel openings were created using a 50% threshold method. Open times <3.332–6.664 ms (2× rise time of low-pass filter) were excluded to reduce the number of channel currents not reaching full amplitude. The amplitude of single-channel currents, open probability (NP_o), and permeability ratios of amiloride-sensitive channels in H441 cells were previously described by Albert et al. [1].

RNA isolation and Northern blotting

Cells were lysed directly in TRI reagent (Genetic Research Instrumentation, Baintree, UK). RNA was prepared according to the manufacturer's instructions. Total RNA was resuspended in RNase-free H₂O and quantified by analysis of optical density (OD) at 260 nm by UV spectrophotometry. For the northern analysis, 20 μg of denatured total RNA was loaded onto formaldehyde gels (1.0% agarose, 2.2 M formaldehyde in 3-(*N*-morpholino)propanesulfonic acid [MOPS] buffer), electrophoresed in MOPS buffer at 1 V cm⁻¹ for 3–4 h, then transferred to a nylon membrane (Amersham Biosciences, Bucks, UK) by an electrophoretic transfer system (Amersham) and cross-linked with an ultraviolet cross-linker (UVP, Upland, CA, USA) as previously described [18].

αENaC, βENaC, and γENaC probes were prepared by reverse transcription polymerase chain reaction. The sense and antisense primers correspond to bases 1,059–1,079 and 1,802–1,822 of rat αENaC (GenBank accession no. X70497), 880–900 and 1,232–1,252 of rat βENaC (GenBank accession no. X77932), and rat γENaC (GenBank accession no. X77933). An 18S oligonucleotide probe was used to correct for any loading variance between samples as previously described [18]. The αENaC cDNA was ampli-

fied by polymerase chain reaction and the resultant product was labeled with [α-³²P]dCTP by random priming with "Ready-to-Go" labeling beads (Amersham). Membranes were prehybridized at 42°C for 30 min in UltraHyb (Ambion, Abingdon, UK). Hybridizations were carried out overnight at 42°C. The blots were then washed to high stringency in 0.05× saline–sodium citrate + 0.1% sodium dodecyl sulfate (SDS) at 42°C and then were linearized by restriction digestion with *Sac*I and *Nco*I, respectively. Antisense riboprobes incorporating [α-³²P]CTP were generated from the linearized βENaC and γENaC cDNA templates using T7 RNA polymerase and SP6 polymerase, respectively. The 18S deoxyoligonucleotide probe terminal phosphate was replaced with ³²P-γATP using polynucleotide kinase (Promega, UK). Prehybridization, hybridization, and washes were carried out as described above but at a higher temperature of 68°C for these probes.

Western blots

For analysis of αENaC, phosphorylated ERK1, ERK2, ERK5, and β-actin abundance, protein was prepared from H441 cells by scraping cells into an ice-cold solution of tissue lysis buffer (100 mM Tris pH 6.8, 1 mM ethylenediaminetetraacetic acid pH 8.0, 10% *v/v* glycerol, and 10 μl ml⁻¹ protease inhibitor cocktail; Sigma, UK). Cells were disrupted by dounce homogenization and subjected to centrifugation (5 min, 250×*g*) to remove nuclei. Biotinylation and extraction of apical membrane proteins was performed as previously described [33, 49]. Nuclear protein was extracted as previously described [3]. For Western blotting, the supernatants from each preparation were heated to 94°C for 5 min in the presence of 100 mM DTT and 2% *w/v* SDS to denature the proteins. Samples containing 30 μg of protein were subjected to electrophoresis on SDS–polyacrylamide gels. Fractionated proteins were transferred to nitrocellulose membranes by electroblotting and immunostained with anti-αENaC antiserum N-terminal (Abcam, Cambridge, UK) or C-terminal (Santa Cruz Biotech, USA), phospho-ERK1/2 (New England Biolabs, USA) or phospho-ERK5 (Cell Signaling Technology, USA) using standard techniques. Anti-β-actin monoclonal antiserum (Sigma, UK) was used to assess protein loading. Immunostained proteins were visualized by enhanced chemiluminescence (Amersham ECL, Amersham, UK) and exposure to autoradiographic film. Electromobility shift assays were conducted using nuclear extracted protein or p50/p65 NF-κB protein (50 ng) and double-stranded oligonucleotide probes containing the human αENaC NF-κB consensus sequence 5'-ACACTTGG GACTCCCCCTT-3' or missense sequences as previously described [3]. Preincubation of nuclear extract with p50/p65 NF-κB antiserum (Cell Signaling Technology, USA) was used to confirm specificity of shift assay.

Data analysis

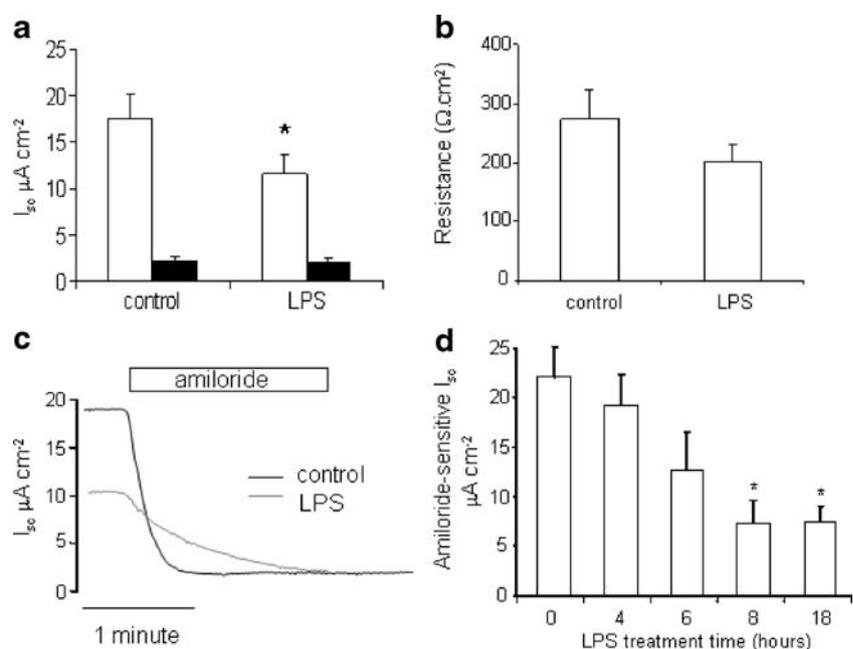
As spontaneous I_{sc} varied between batches of cells, controls and treatments were carried out on monolayers from batches of cells plated on the same day and grown for the same length of time under similar conditions. Results were analyzed using analysis of variance (ANOVA) with a post hoc Gabriel's test or Student's nonpaired or paired t tests where applicable. Values are presented as the mean \pm standard error of the mean (SEM); p values ≤ 0.05 were considered significant.

Results

Effect of LPS on amiloride-sensitive I_{sc}

We found that an 18-h treatment of H441 cells with $15 \mu\text{g ml}^{-1}$ LPS induced a significant reduction in amiloride-sensitive I_{sc} from 15.0 ± 2.0 to $8.0 \pm 2.0 \mu\text{A cm}^{-2}$ ($p=0.01$, $n=13$) but not amiloride-insensitive currents (Fig. 1a). Although there was an apparent reduction in resistance of the monolayers, this did not reach significance (Fig. 1b). We also noted that the inhibition of I_{sc} with $10 \mu\text{M}$ amiloride was slower in cells treated with LPS than in control cells (Fig. 1c). The effect of LPS on I_{sc} was significantly affected by treatment time (ANOVA, $p<0.01$, $n=4$). A decrease in I_{sc} was detectable after 4 h and was significantly reduced at 8 and 18 h after treatment with LPS ($p<0.05$, $n=4$; Fig. 1d). We did not observe any acute effects of LPS using continual I_{sc} measurements over a period of 1 h.

Fig. 1 Effect of LPS on short circuit current (I_{sc}) across H441 cell monolayers. **a** Spontaneous I_{sc} before (white bars) and after the addition of $10 \mu\text{M}$ amiloride (black bars) in untreated H441 cells (control) and cells treated with LPS for 18 h (LPS). Data are shown as the mean \pm SEM. **b** Transepithelial resistance (R_t) of untreated and LPS-treated H441 cell monolayers. $*p<0.05$, significantly different from control, $n=13$. **c** Typical I_{sc} trace from untreated and LPS-treated H441 cell monolayers before and after the application of amiloride. **d** Effect of LPS treatment for 0–18 h on amiloride-sensitive I_{sc} . Data are shown as the mean \pm SEM. $*p<0.05$, significantly different from control, $n=4$ (ANOVA, post hoc Gabriel's test)



Effect of LPS on IC_{50} amiloride

The effect of different concentrations of amiloride on blockade of I_{sc} in cells treated with LPS was investigated. By constructing a concentration/response curve for both control and LPS-treated cells, we found that treatment with LPS significantly shifted the curve to the right. By fitting the Hill equation to our data, the EC_{50} for amiloride was calculated to be 6.8×10^{-7} M in control cells but significantly increased to 6.4×10^{-6} M in LPS-treated cells ($p<0.05$, $n=4$; Fig. 2).

Effect of LPS on channels in apical membrane

Over 90% of all cell-attached patches from the H441 cell apical membrane contained channels. In 55% of patches from untreated monolayers (four sets), only constitutively active cation channel currents that had a mean unitary amplitude of -0.64 ± 0.03 pA ($n=12$) at -100 mV and unitary conductance and reversal potential (E_r) values of about 5 pS and $+20$ mV, respectively, were displayed (Fig. 3a (i)). These properties were similar to those of highly Na^+ -selective cation channels (HSC) previously described in H441 cell monolayers [1]. The remaining 45% of control cell-attached patches exhibited both spontaneously active HSCs and cation channel currents with a larger mean amplitude of -1.77 ± 0.09 pA ($n=12$) at -100 mV and unitary conductance and E_r values of about 18 pS and 0 mV, respectively. These values were similar to those previously described for nonselective cation channels (NSC) in H441 cells (Fig. 3a (ii)). The I/V relationship

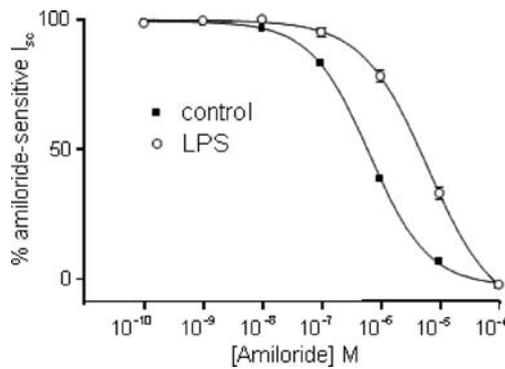


Fig. 2 Amiloride sensitivity of control and LPS-treated H441 cells. Graph of mean percentage inhibition of amiloride-sensitive I_{sc} plotted against concentration of amiloride for untreated cells (filled squares) and cells treated with LPS for 18 h (open circles; $n=4$). Fitting the Hill equation to the data indicated a shift in the curve to the right and a change in the EC_{50} for amiloride from 6.8×10^{-7} M in control cells to 6.4×10^{-6} M in LPS-treated cells

for HSCs and NSCs and the properties of channel currents using a patch pipette solution containing the less-permeant cation NMDG-Cl as the main cation charge carrier have been previously published [1]. These results indicate that H441 cell monolayers in the current study expressed functional constitutively active HSCs and NSCs at a similar frequency to previous data [1].

Treatment of H441 cells with LPS significantly increased the frequency of patches containing both constitutive HSCs and NSCs to 93% (13 out of 14 patches, $p < 0.05$, four sets of cell monolayers; Fig. 3b). LPS significantly increased the NP_o of cation channels in patches containing HSCs and NSCs by $\sim 100\%$ ($n=13$, $p < 0.05$) compared to control patches displaying HSCs and NSCs (Fig. 3c (i)). Treatment with LPS did not alter unitary conductance or E_r of HSCs or NSCs (data not shown). In addition, treatment with LPS significantly increased the number of NSC open

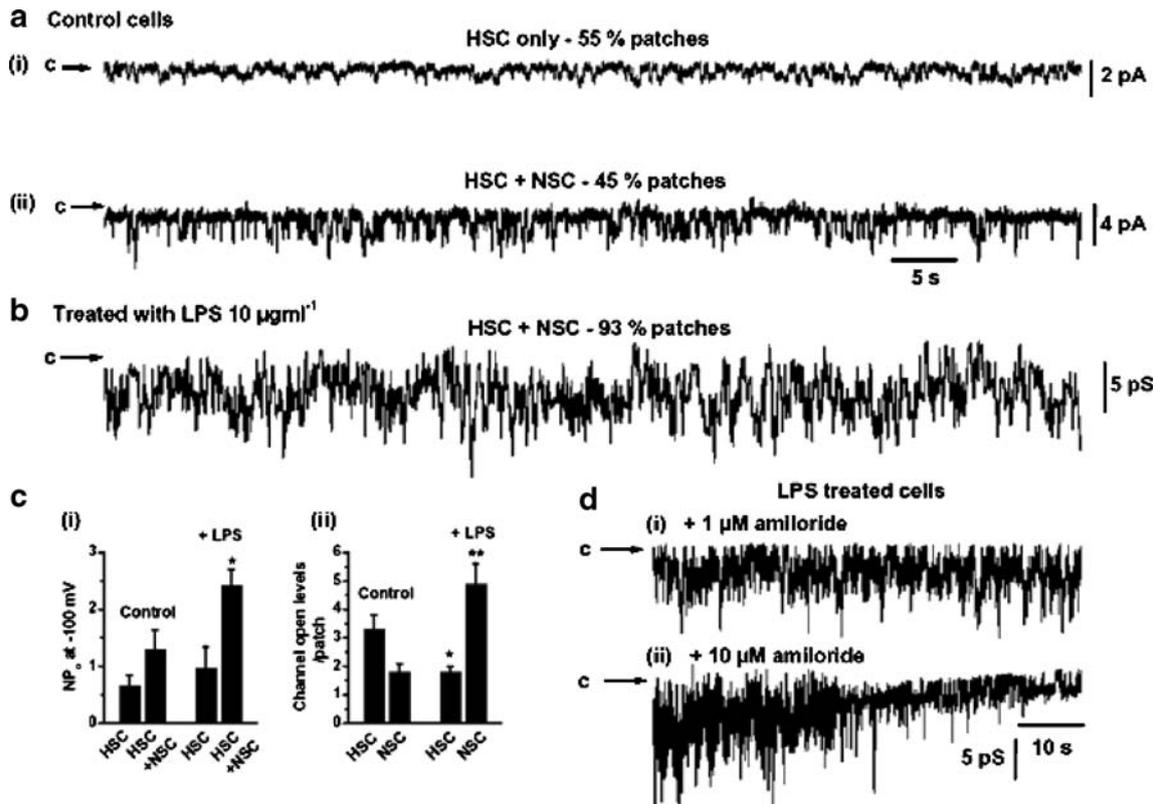


Fig. 3 Effect of LPS on constitutive cation channel activity in H441 cell monolayers. **a** Control cell-attached patches at -100 mV from H441 cell monolayers contained either only HSCs (55%; *i*) or both HSCs and NSCs (45%; *ii*) with similar properties and at similar frequencies as previously described (Albert et al. [1]). **b** Pretreatment of H441 cell monolayers with $15 \mu\text{g ml}^{-1}$ LPS for 18 h increased the frequency of cell-attached patches containing both HSC and NSC activity (93%). **c** Mean data from all cell-attached patches recorded showing that *i* LPS significantly increased NP_o value of constitutive

channel activity in patches containing both HSC + NSC ($n=13$, $p < 0.05$) and *ii* LPS significantly reduced the number of HSC open levels per patch in patches containing HSCs ($n=13$, $p < 0.05$; HSC) but increased the number of NSC open levels per patch in patches containing both HSC + NSC ($n=13$, $p < 0.01$; NSC). **d** *i* Inclusion of $1 \mu\text{M}$ amiloride in the patch pipette solution had no effect on NSC activity and *ii* $10 \mu\text{M}$ amiloride partially reduced NSC activity in cell-attached patches at -100 mV. Data are shown as the mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, significantly different from the control

levels per patch in patches containing HSCs and NSCs by approximately 300% ($n=13$, $p<0.01$) compared to control patches. However, the number of HSC channel open levels per patch was significantly reduced by about 50% ($n=13$, $p<0.05$; Fig. 3c (ii)).

To confirm that NSC activity present after LPS treatment was the same as previously described in untreated H441 cell monolayers, we investigated the effect of including the channel blocker amiloride in the patch pipette solution. Inclusion of 1 μM amiloride had no effect on NSC activity ($n=4$; Fig. 3d (i)) although it abolished HSC activity as previously described (data not shown; [1]). However, 10 μM amiloride in the patch pipette solution inhibited NSC activity by $67\pm 11\%$ at -100 mV ($n=4$), a value similar to that previously described in these cells [1] (Fig. 3d (ii)). These results provide clear evidence that LPS inhibits HSC activity and increases NSC activity.

Effect of LPS on ENaC mRNA and abundance

To examine if the reduction of amiloride-sensitive current was mediated by a reduction in functional ENaC channels, the effect of LPS on mRNA levels was investigated in these cells. Single mRNA products were labeled with αENaC and βENaC probes corresponding to the predicted sizes of these products approximately 3.6 and 2.4 kb, respectively. Two mRNA products were determined with the γENaC probe of approximately 3.4 and 3.6 kb. We observed that αENaC mRNA abundance was reduced but did not observe similar changes in βENaC or γENaC mRNA abundance. These blots were repeated with similar results and the mean densitometry values of ENaC mRNA/18S ribosomal RNA are shown (Fig. 4).

Effect of LPS on NF- κB binding activity

Electromobility shift assays indicated that NF- κB protein (p50/p65) and nuclear extract from H441 cells inhibited the mobility of a αENaC NF- κB consensus binding sequence but not a missense sequence of similar length. This effect was abrogated by preincubation of nuclear protein with NF- κB p65 or p50 antiserum, indicating that H441 cells exhibited an endogenous NF- κB binding activity. Treatment with LPS increased NF- κB binding to the αENaC consensus sequence. The effect of LPS was inhibited by pretreatment with 5 mM SAS an inhibitor of I κB phosphorylation (Fig. 5a–c).

Effect of LPS on ERK1/2 and ERK5 phosphorylation

Treatment with LPS induced an increase in phosphorylated ERK1/2 and a decrease in phosphorylated ERK5

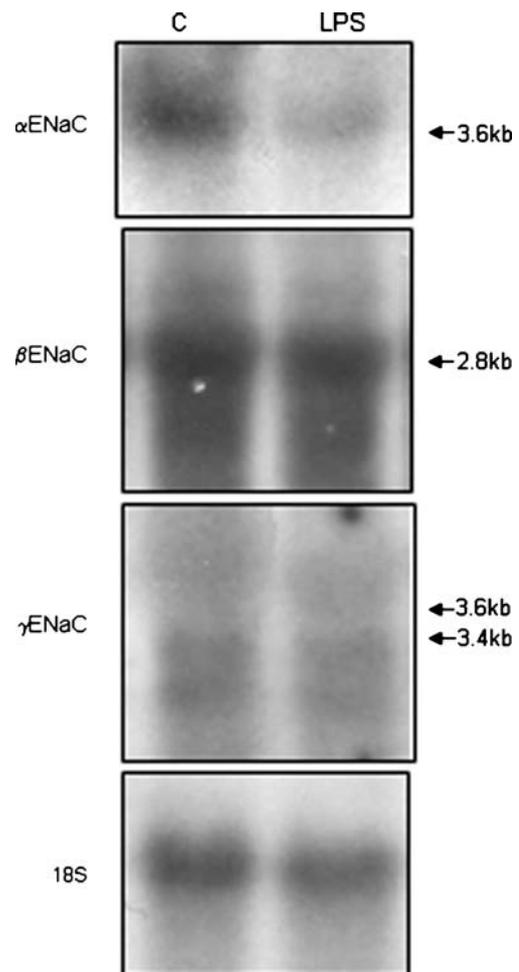


Fig. 4 Effect of LPS on ENaC subunit mRNA expression. **a** Northern blot of RNA extracted from untreated control (C) and LPS-treated (LPS) H441 cells showing labeled mRNA products corresponding to αENaC (~3.6 kb), βENaC (~2.4kb), and γENaC (~3.4 and ~3.6 kb). Corresponding 18S ribosomal RNA blots for each sample are also shown (18S). Blots were repeated with similar results ($n=2$)

when compared to β -actin control. Phosphorylated ERK1/2 proteins were detected after 10 min, reached maximal levels at 60–120 min ($p<0.05$, $n=3$), and although reduced, were still present after an overnight treatment with LPS although this did not quite reach significance ($p=0.06$, $n=3$; Fig. 6a, b). Pretreatment of cells with the MEK1 inhibitor PD98059 (20 μM) appeared to decrease basal ERK1/2 phosphorylation in unstimulated cells but, because levels were very low, we were unable to attribute significance to this by densitometry analysis. However, PD98059 increased levels of phosphorylated ERK5. Pretreatment with PD98059 inhibited the LPS-induced phosphorylation of ERK1/2 at 1 and 2 h and reversed the inhibitory effect of LPS on the phosphorylation of ERK5 (Fig. 7a, b).

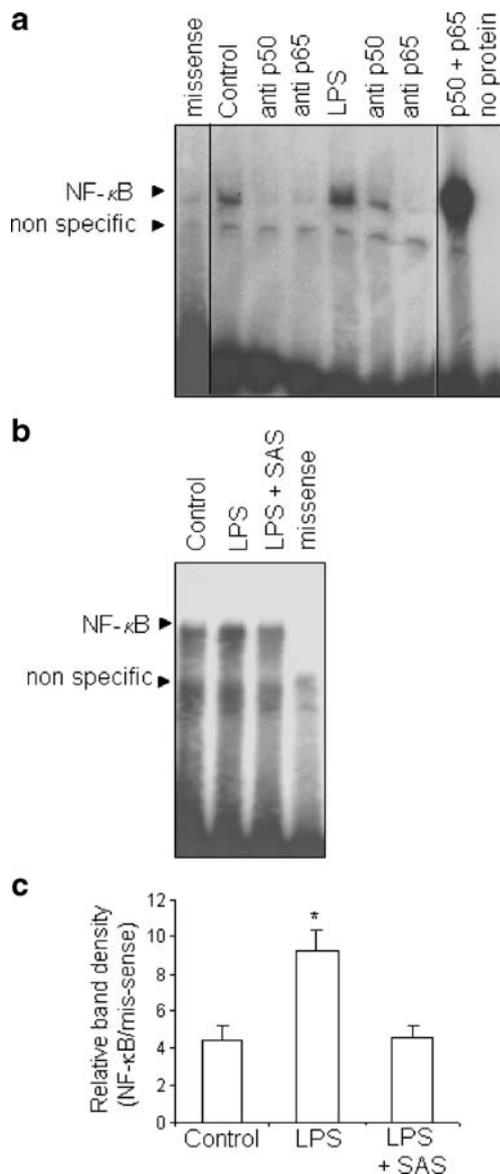


Fig. 5 Effect of LPS on NF- κ B binding activity in H441 cells. **a** Typical autoradiograph of an electromobility shift assay using a double-stranded missense oligonucleotide incubated with nuclear proteins extracted from control cells (*missense*) or NF- κ B consensus sequence from the human α ENaC promoter incubated with nuclear proteins extracted from control cells (*control*) or LPS-treated cells (*LPS*) or preincubated with anti-NF- κ B p50 antiserum (*anti p50*) or p65 antiserum (*anti p65*) prior to incubation with nuclear protein. Lanes showing NF- κ B consensus sequence incubated with purified p50+p65 NF- κ B protein or no protein are also shown. **b** Missense oligonucleotide (*missense*) or NF- κ B consensus sequence from the human α ENaC promoter incubated with nuclear proteins extracted from control cells (*control*) or LPS-treated cells (*LPS*) or cells pretreated with 5 mM SAS for 30 min prior to treatment with LPS (*LPS+SAS*). The position of NF- κ B shifted and nonspecific bands are indicated by arrows. **c** Graphical representation of data obtained from three electromobility shift assays quantified using densitometry analysis. Data are shown as the mean \pm SEM. * $p < 0.05$, significantly different from control

Role of NF- κ B, ERK1/2, and ERK5 in LPS-mediated effects on I_{sc}

In these experiments, amiloride-sensitive I_{sc} was reduced to $50 \pm 12\%$ of control by LPS alone. After pretreatment with 5 mM SAS (which inhibits I κ B kinase and activation of NF- κ B) or CAPE (which inhibits NF- κ B translocation to the nucleus), I_{sc} remained significantly lower than the control at $51 \pm 16\%$ and $63 \pm 8\%$, $p < 0.05$, $n = 4$ and $n = 3$, respectively (Fig. 8a). However, pretreatment of cells with 20 μ M PD98059 prevented the LPS-mediated decrease in amiloride-sensitive I_{sc} . Levels remained similar to untreated cells at $98 \pm 13\%$ of the control, $n = 5$ (Fig. 8a). There was a small apparent increase in amiloride-sensitive I_{sc} with PD98059 alone at $113 \pm 11\%$ but this did not reach significance ($n = 5$). Pretreatment with PD98059 also reversed the effect of the EC_{50} to 7.8×10^{-7} (Fig. 8b). These data imply that activation of ERK1/2 and/or inactivation of ERK5 are necessary for LPS-mediated inhibition of I_{sc} . The action of PD98059 alone on I_{sc} is most likely mediated via phosphorylation of ERK5 or inhibition of endogenous ERK1/2 phosphorylation (although we were unable to detect significant changes in ERK1/2 phosphorylation by PD98059 by Western blotting).

Role of ERK1/2 and ERK5 in LPS-mediated effects on aENaC protein

Using an N-terminal antiserum that we have previously shown to recognize GFP-tagged human α ENaC overexpressed in H441 cells, we identified a ~ 95 -kDa protein and a ~ 65 -kDa protease-cleaved α ENaC protein in total cell extracts [38, 41]. In addition, we detected a smaller protein of ~ 32 kDa which may also be a product of N-terminal furin cleavage (Fig. 9a). Consistent with the observations of others, in apical surface-biotinylated fractions, the ~ 65 -kDa protein was predominantly observed, indicating that α ENaC in these cells is also cleaved at the C terminus [41]. This may have been the result of incubating cells with fluid on the apical surface; a maneuver thought to activate protease cleavage [44]. Using a C-terminal antiserum, we also detected a ~ 65 -kDa protein in the biotinylated fractions consistent with N-terminal protease cleavage of α ENaC [23, 44] (Fig. 9b). Taken together, these data are consistent with the presence of active cleaved proteins in the cell membrane. Treatment with LPS significantly decreased the abundance of α ENaC proteins in total cell protein fractions ($p < 0.05$, $n = 3$), and this effect was mimicked in biotinylated fractions using either antiserum ($n = 3$). Pretreatment with PD98059 prevented the LPS-induced decreased in α ENaC abundance (Fig. 9a–c). No β -actin was detected in apical biotinylated fractions although it was present in

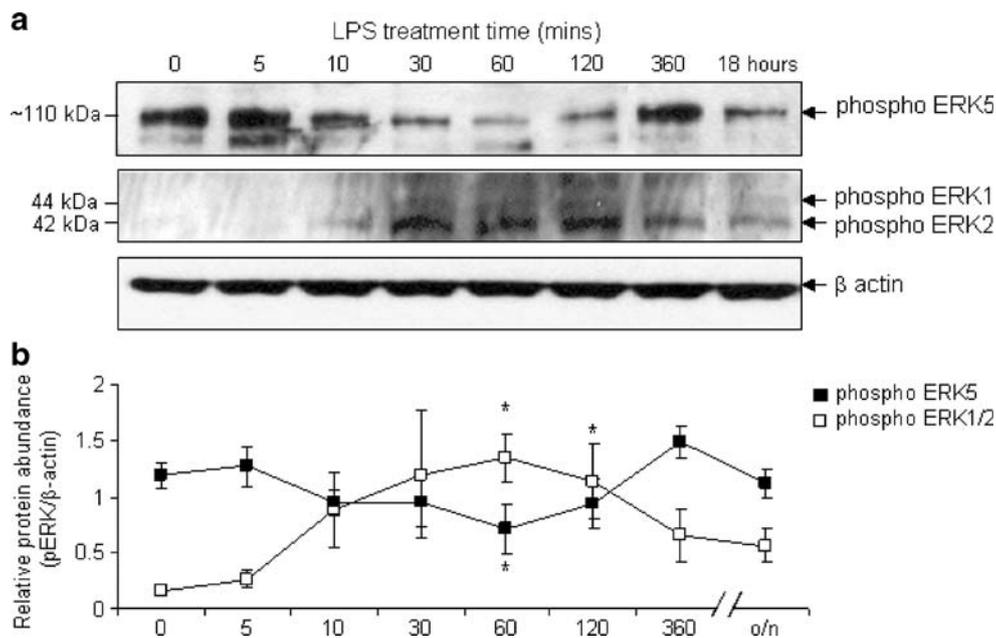


Fig. 6 Effect of LPS on ERK1/2 and ERK5 phosphorylation in H441 cells. **a** Typical Western blot of phosphorylated ERK1/2 (*open squares*) and ERK5 (*filled squares*) in protein extracted from H441 cells treated with LPS (*LPS*) for 0–360 min and 18 h. Blots were also immunostained with β -actin as a loading control. Protein products of 42 and 44 kDa corresponding to phospho-ERK1 and phospho-ERK2

and 110 kDa corresponding to ERK5 are indicated by *arrows*. Proteins of 42 kDa corresponding to β -actin were also immunostained in all samples. **b** Graphical representation of data obtained from three sets of Western blots quantified using densitometry analysis (phosphorylated ERK/ β -actin). Data are shown as the mean \pm SEM. * p <0.05, significantly different from control

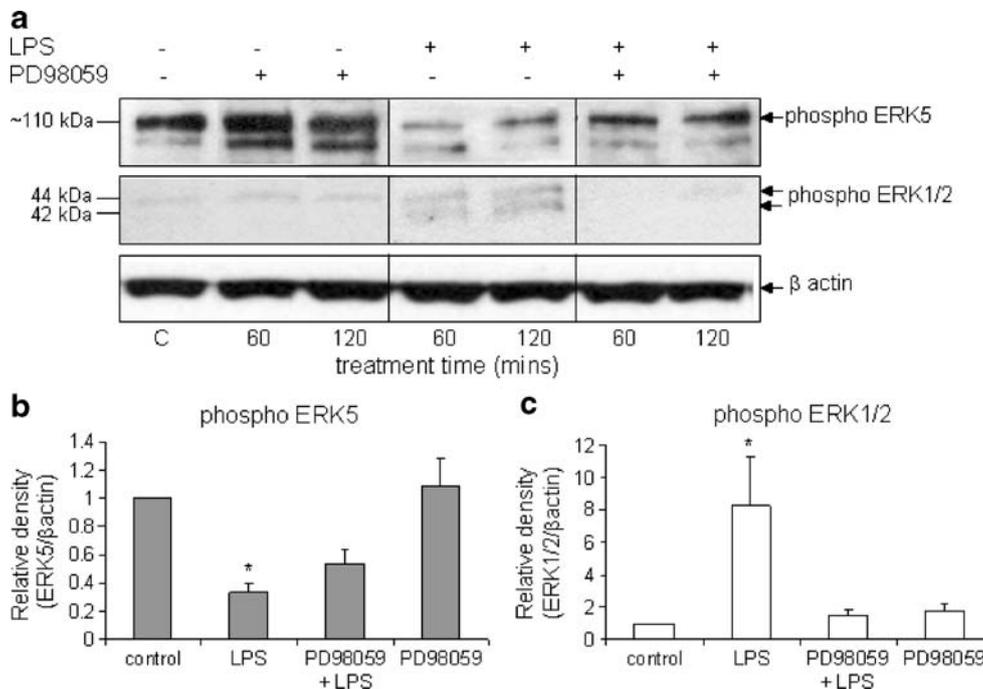


Fig. 7 Effect of PD98059 on ERK1/2 and ERK5 phosphorylation in H441 cells. **a** Typical Western blot of phosphorylated ERK1/2 and ERK5 in untreated H441 cells (*control*), after treatment with LPS (*LPS*), and after pretreatment with PD98059 for 30 min prior to the addition of LPS (*LPS*+*PD98059*). Protein products of ~42 and 44 kDa corresponding to phospho-ERK1 and phospho-ERK2 and ~110 kDa corresponding to phospho-ERK5 are indicated by *arrows*. ERK1/2 and ERK5 were immunostained using the same blots. Lanes shown in this

figure are from the same Western blot but have been digitally reordered for clarity. Because of the similarity in size, proteins of 42 kDa corresponding to β -actin were immunostained in the same samples run on separate gels to control for loading. **b** Graphical representation of data obtained from three sets of Western blots quantified using densitometry analysis of phosphorylated ERK5/ β -actin (*filled squares*) and **c** phosphorylated ERK1/2/ β -actin (*open squares*). Data are shown as the mean \pm SEM. * p <0.05, significantly different from control

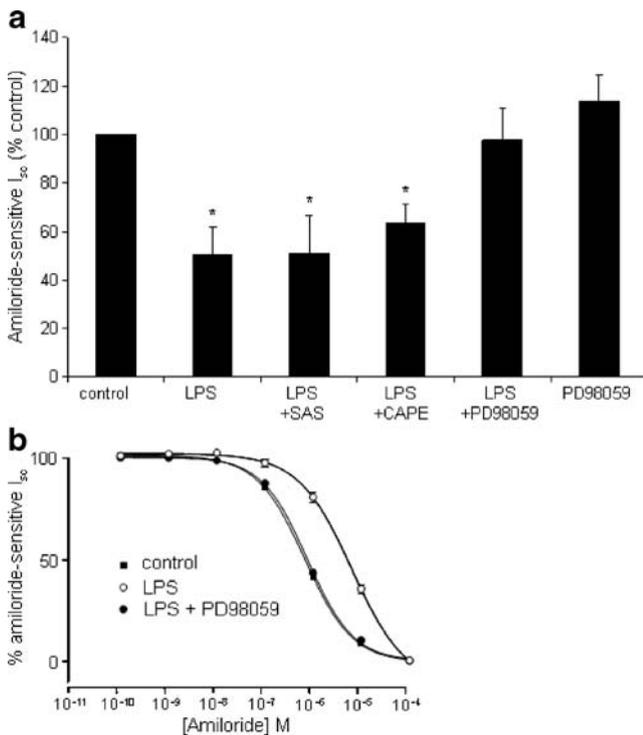


Fig. 8 Effect of inhibitors of NF- κ B activation and ERK1/2 phosphorylation on LPS-mediated suppression of amiloride-sensitive I_{sc} . **a** Amiloride-sensitive I_{sc} was measured by apical application of 10 μ M amiloride to monolayers of untreated cells (*control*) and cells treated with LPS (*LPS*) or pretreated with 5 mM SAS (*LPS+SAS*), 25 μ g/ml CAPE (*LPS+CAPE*), or 20 μ M PD98059 (*LPS+PD98059*) prior to LPS treatment or PD98059 alone (*PD98059*). Data are shown as the mean \pm SEM. * p <0.05, significantly different from control. **b** Graph of percentage inhibition of amiloride-sensitive I_{sc} plotted against the concentration of amiloride for untreated cells (*filled squares*; $n=4$) cells treated with LPS (*open circles*; $n=4$) or cells treated with PD98059 prior to the addition of LPS (*closed circles*; $n=3$). Fitting the Hill equation to the data indicated that the EC_{50} for amiloride in cells pretreated with PD98059 before LPS treatment was similar to control cells ($\sim 7 \times 10^{-7}$ M)

nonbound fractions from the same samples, showing that there was no contamination of apical surface membrane preparations with intracellular protein (Fig. 9c).

Discussion

In this paper, we have investigated the effect of LPS on transepithelial transport across human H441 airway epithelial cells. H441 cells form monolayers of moderate transepithelial resistance (R_t) of $\sim 300 \Omega$ in our culture conditions which is similar to that described for airway epithelial sheets [24]. LPS significantly decreased spontaneous transepithelial short circuit current (I_{sc}) in these cells and this effect was mediated by a reduction in the amiloride-sensitive but not the amiloride-insensitive component of I_{sc} . The change in I_{sc} was only measurable 4–6 h

post-treatment, inferring that LPS did not acutely modify the activity of the channels.

In I_{sc} experiments, LPS also increased the EC_{50} amiloride, indicating that channels in the apical membrane had a decreased affinity for amiloride. There are two amiloride-sensitive cation channels in the apical membrane of H441 cells that contribute to apical Na^+ conductance which have differing sensitivities to amiloride [1]. The HSC ENaC-like channel is inhibited by $<1 \mu$ M amiloride, while the NSC is inhibited by $>10 \mu$ M amiloride. In control cells, HSC activity predominated with channels observed in all patches, whereas NSCs were observed in only 45% of patches. These data were consistent with our previous observations and those in rat alveolar type II cells maintained under similar growth conditions [27]. Thus, in control cells, HSCs were the main population of channels contributing to I_{sc} across the monolayer, consistent with the calculated EC_{50} amiloride of 0.7 μ M. In LPS-treated cells, HSC activity decreased and NSC activity increased. As NSCs have a lower affinity for amiloride, their increased contribution to I_{sc} would explain the LPS induced shift in EC_{50} amiloride. Such shifts in channel populations have previously been described in response to changes in culture conditions but not in response to LPS [26, 27]. Interestingly, decreased HSC function correlated with reduced transepithelial Na^+ transport and would indicate that HSCs are the principal route of entry for Na^+ across the apical membrane of H441 cells. Furthermore, while our data indicate that NSCs also transport Na^+ , their functional significance and contribution to Na^+ transport in the airway requires further exploration.

Amiloride-sensitive channels with differing kinetics, Na^+/K^+ selectivity, and amiloride sensitivity can be a product of differential ENaC subunit combinations [16, 17]. In rat AII cells, the presence of α ENaC, β ENaC, and γ ENaC subunits favored the expression of HSCs with a low EC_{50} for amiloride [25], while NSCs were proposed to be a product of α ENaC alone or in dimeric combination with either β ENaC or γ ENaC [26]. The H441 cells in this study expressed α ENaC, β ENaC, and γ ENaC mRNA and α ENaC protein. We have previously shown that β ENaC and γ ENaC are also present in the membrane of these cells, consistent with HSC activity and the low EC_{50} for amiloride we observed in control monolayers [33, 44, 47, 49]. Work in *Xenopus* oocytes showed that α ENaC is necessary to produce functional channels at the cell surface and that maximal amiloride-sensitive current was achieved with a α ENaC, β ENaC, and γ ENaC combination and when the α and/or γ subunits are protease cleaved [6, 22, 44]. Our finding that α ENaC in the apical membrane was cleaved and that LPS decreased mRNA and abundance of cleaved and noncleaved protein would, therefore, support the observed decrease in the number of functional HSC

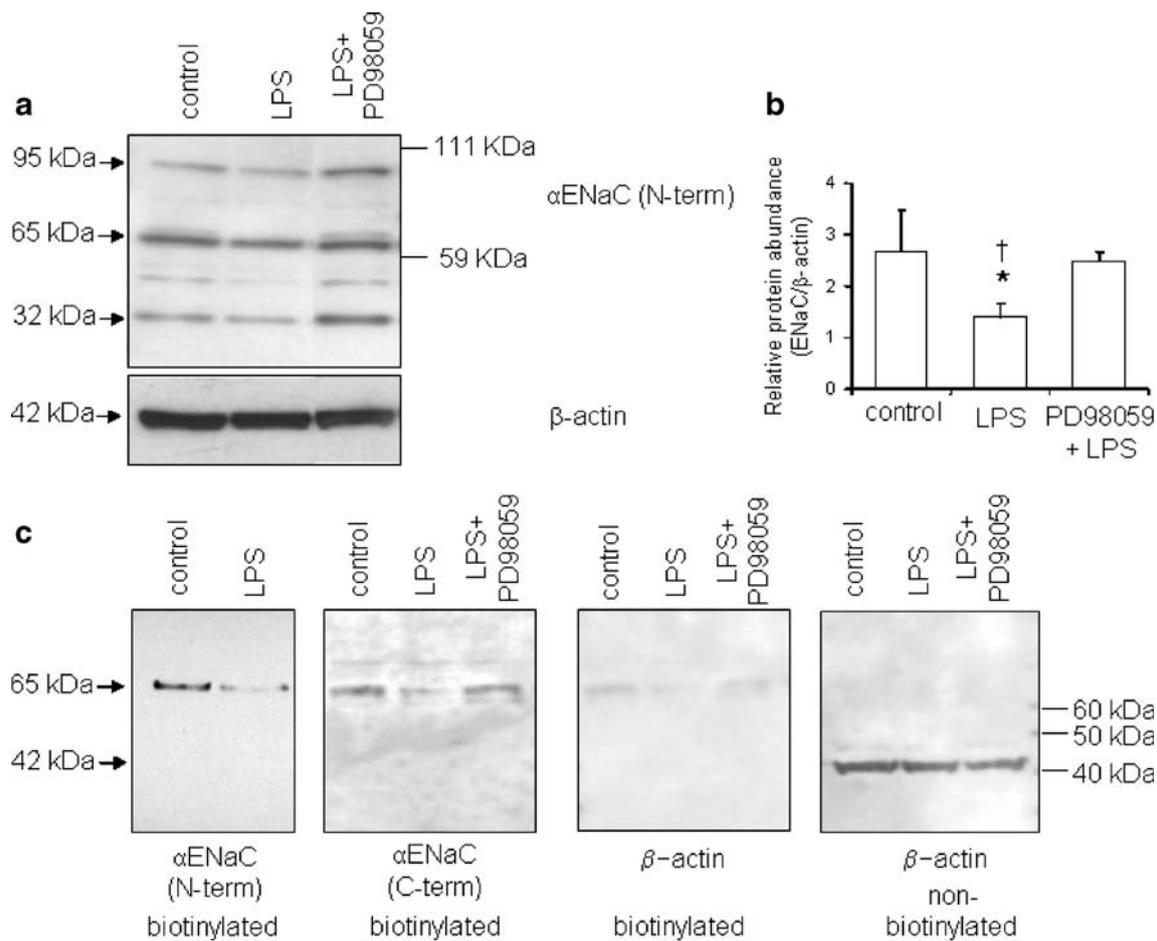


Fig. 9 Effect of LPS and PD98059 on α ENaC protein levels in H441 cells. **a** Typical Western blot of proteins extracted from untreated (*control*), LPS-treated (*LPS*), and PD98059-pretreated cells (*LPS+PD98059*) immunostained with anti- α ENaC and anti- β -actin antisera. Protein products of ~95, ~65, and ~32 kDa corresponding to full-length and putative protease-cleaved fragments of α ENaC using an N-terminal antiserum and 42 kDa corresponding to β -actin were immunostained in all samples. Lanes shown in this figure are from the same Western blot but the last lane has been digitally reordered for clarity. **b** Graph of relative abundance of α ENaC protein quantified by densitometry analysis (α ENaC/ β -actin). * $p < 0.05$, significantly different to from the control, $n = 4$; † $p < 0.05$, significantly different from cells pretreated with LPS, $n = 3$. **c** Western blot of apical surface biotinylated

proteins from untreated (*control*) or LPS-treated (*LPS*) H441 cell monolayers immunostained with N-terminal α ENaC antiserum or C-terminal antiserum. Protein products of ~65 kDa corresponding to protease-cleaved α ENaC were predominantly detected with either antiserum. To show that apical biotinylated protein was not contaminated with intracellular proteins, blots were washed and reprobed with anti β -actin monoclonal antiserum. No β -actin was detected in the apical biotinylated protein samples from the same blot as shown *second from left* although residual immunostaining of α ENaC is still present. β -Actin was present in the nonbound fraction from the same samples run on the same gel. The position of α ENaC and β -actin proteins are indicated by *arrows* and size markers by *lines*

channels and reduction in amiloride-sensitive I_{sc} . Similar findings have been described in alveolar epithelial cells and distal lung epithelial cells from the fetal rat where LPS inhibited ENaC mRNA expression and activity [12, 36].

Interestingly, the decreased abundance of α ENaC was inversely related to the frequency of observation and activity of NSCs. Our interpretation of this result is that NSCs are unlikely to be a product of α ENaC protein. In support of this notion, previous studies showed that antisense oligonucleotides to α ENaC, β ENaC, or γ ENaC decreased the observed frequency of HSCs but did not significantly affect the frequency of NSCs in AII cells

[27]. The identity of NSCs in H441 cells and how their observed frequency is increased by LPS, therefore, remains to be confirmed.

LPS has been shown to act through a number of cell signaling pathways to modulate gene expression (for a review, see [19]). We found that LPS increased binding of NF- κ B to a consensus sequence found in the promoter region of human α ENaC. Activation of MAP kinase pathways, phosphorylation of ERK1/2, and activation of NF- κ B have both been shown to modulate the transcription and activity of α ENaC in lung and renal epithelial cells [4, 11, 32, 45, 48]. Treatment with SAS, an inhibitor of I κ B

phosphorylation and NF- κ B binding activity, prevented LPS-mediated NF- κ B activation but did not reverse the suppressive effect of LPS on amiloride-sensitive currents in these cells. In addition, CAPE, which prevents translocation of NF- κ B to the nucleus, had no effect. In support of our findings, SAS did not reverse TNF- α suppression of α ENaC mRNA levels in type II epithelial cells [15].

LPS has been shown to stimulate both ERK1/2 and ERK5 in macrophages [51]. We found that LPS induced phosphorylation of ERK1/2 but decreased phosphorylation of ERK5. While we cannot rule out any effect of the treatments on ERK1/2 and ERK5 abundance, few roles for ERK5 have been described in the lung and the opposing changes in phosphorylation we have demonstrated infer that LPS differentially regulates these two kinases in H441 cells. Such differential regulation of ERK5 and ERK1/2 has been described in other tissues [7, 14] and ERK5 activation was shown to be enhanced when ERK1/2 signaling was suppressed in NIH3T3 cells [5]. Consistent with the findings of Kamakura et al., the MAP kinase inhibitor PD98059 prevented LPS-induced phosphorylation of ERK1/2 and the decrease in phosphorylation of ERK5, reversed the suppressive effect of LPS on amiloride-sensitive I_{sc} , increased α ENaC protein abundance, and restored amiloride sensitivity to that of untreated control cells [29]. Taken together, these data infer that inhibition of ERK1/2 phosphorylation and/or increased ERK5 phosphorylation were, at least in part, important mediators of the LPS effect on Na^+ transport.

We observed that LPS reduced α ENaC mRNA and decreased protein abundance in this study and inhibited transcriptional activity of the α ENaC promoter in A549 lung epithelial cells in a previous study [2]. Both ERK1/2 and ERK5 have been described to mediate changes in gene transcription in lung cells [42] and there is good evidence that transcriptional effects as a result of rapid ERK phosphorylation are maximal several hours later [37, 51]. ERK-sensitive pathways disrupted glucocorticoid transactivation of the α ENaC promoter in A549 cells [48] and decreased α -ENaC mRNA expression in rat parotid epithelial cells [50]. Furthermore, cross-talk between ERK and cAMP activation of CREB regulated α ENaC mRNA abundance in rat submandibular gland epithelial cells [37]. Thus, we propose that LPS inhibits α ENaC mRNA transcription by the activation of ERK1/2 and inhibition of ERK5. However, the pathways and the specific roles of ERK1/2 versus ERK5 in H441 cells remain to be defined.

Direct ERK2 phosphorylation of β and γ ENaC subunits has been shown to facilitate their interaction with the ubiquitin ligase Nedd4, promoting a decrease in functional channels at the membrane [46]. In addition, activation of ERK2 by progesterone or a vasopressin-induced factor (VIP32) downregulated ENaC activity in *Xenopus* oocytes

via a decrease in channel open probability (P_o) [35, 40]. If LPS activation of ERK1/2 mediated the effects on P_o , they would be expected to precede changes in transcription and α ENaC abundance. As we were unable to demonstrate any significant functional effects within 4 h, we do not think this is a likely route of LPS action. We cannot, however, rule out an action of endogenously active ERK1/2 on channel P_o . Neither do we exclude additional effects of LPS on mRNA and protein stability or the release of factors including TNF- α (a proinflammatory cytokine) which has also been shown to decrease ENaC mRNA and activity in lung epithelial cells [10, 12].

In conclusion, using a combination of techniques, we have demonstrated novel effects of LPS applied directly to human airway epithelial cells. We propose that LPS acts via ERK signaling pathways to decrease α ENaC transcription, reduce HSC/ENaC channel abundance, activity, and trans-epithelial Na^+ transport in H441 airway epithelial cells. Such changes could potentially lead to a decrease in net fluid absorption and contribute to the formation of pulmonary edema as a result of bacterial infection.

Acknowledgements The authors would like to thank Chong Tan and Susan Dunford for their contributions to this work and the Wellcome Trust (068674/Z/02) and BBSRC (BB/E013597/1) for their support.

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References

1. Albert AP, Woolthead AM, Mace OJ, Baines DL (2008) AICAR decreases the activity of two distinct amiloride-sensitive Na^+ -permeable channels in H441 human lung epithelial cell monolayers. *Am J Physiol* 295:L837–L848
2. Baines DL, Janes M (2003) Effect of LPS-induced NF- κ B activity on the transcriptional response of a 5' flanking region of the α ENaC gene. *FASEB J* 18 (abstract no. 329.326)
3. Baines DL, Janes M, Newman DP, Best OG (2002) Oxygen-evoked changes in transcriptional activity of the 5' flanking region of the human amiloride-sensitive sodium channel (α ENaC) gene: role of nuclear factor- κ B. *Biochem J* 364:537–545
4. Baines DL, Olver RE (2000) Transcriptional activity of α ENaC promoter in A549 cells maintained at different ambient O_2 concentrations. *FASEB J* 14:A336
5. Barros JC, Marshall CJ (2005) Activation of either ERK1/2 or ERK5 MAP kinase pathways can lead to disruption of the actin cytoskeleton. *J Cell Sci* 118:1663–1671
6. Canessa CM, Merillat A, Rossier BC (1994) Membrane topology of the epithelial sodium channel in intact cells. *Am J Physiol* 267: C1682–C1690
7. Cavanaugh JE, Ham J, Hetman M, Poser S, Yan C, Xia Z (2001) Differential regulation of mitogen-activated protein kinases ERK1/2 and ERK5 by neurotrophins, neuronal activity, and cAMP in neurons. *J Neurosci* 21:434–443

8. Clunes MT, Butt AG, Wilson SM (2004) A glucocorticoid-induced Na⁺ conductance in human airway epithelial cells identified by perforated patch recording. *J Physiol* 557:809–819
9. Dagenais A, Frechette R, Clermont ME, Masse C, Prive A, Brochiero E, Berthiaume Y (2006) Dexamethasone inhibits the action of TNF on ENaC expression and activity. *Am J Physiol* 291:L1220–L1231
10. Dagenais A, Frechette R, Yamagata Y, Yamagata T, Carmel J-F, Clermont M-E, Brochiero E, Masse C, Berthiaume Y (2004) Downregulation of ENaC activity and expression by TNF- α in alveolar epithelial cells. *Am J Physiol* 286:L301–L311
11. de Seigneux S, Leroy V, Ghzili H, Rousselot M, Nielsen S, Rossier BC, Martin PY, Feraïlle E (2008) NF- κ B inhibits sodium transport via down-regulation of SGK1 in renal collecting duct principal cells. *J Biol Chem* 283:25671–25681
12. Dickie AJ, Raffii B, Piovesan J, Davreux C, Ding J, Tanswell AK, Rotstein O, O’Brodivich H (2000) Preventing endotoxin-stimulated alveolar macrophages from decreasing epithelium Na⁺ channel (ENaC) mRNA levels and activity. *Pediatr Res* 48:305–310
13. Ding J, Dickie AJ, O’Brodivich H, Shintani Y, Raffii B, Hackman D, Marunaka Y, Rotstein OD (1998) Inhibition of amiloride-sensitive sodium channel activity in distal lung epithelial cells by nitric oxide. *Am J Physiol* 274:L378–L387
14. Dwivedi PP, Hii CS, Ferrante A, Tan J, Der CJ, Omdahl JL, Morris HA, May BK (2002) Role of MAP kinases in the 1, 25-dihydroxyvitamin D₃-induced transactivation of the rat cytochrome P450C₂₄ (CYP24) promoter. Specific functions for ERK1/ERK2 and ERK5. *J Biol Chem* 277:29643–29653
15. Frechette R, Yamagata T, Yamagata Y, Dagenais A, Berthiaume Y (2002) Role of NF- κ B in TNF-induced decrease of ENaC expression in alveolar epithelial cells. *FASEB J* 16:A413
16. Fyfe GK, Canessa CM (1998) Subunit composition determines the single channel kinetics of the epithelial sodium channel. *J Gen Physiol* 112:423–432
17. Fyfe GK, Zhang P, Canessa CM (1999) The second hydrophobic domain contributes to the kinetic properties of epithelial sodium channels. *J Biol Chem* 274:36415–36421
18. Gambling L, Dunford S, Wilson CA, McArdle HJ, Baines DL (2004) Estrogen and progesterone regulate α , β , and γ ENaC subunit mRNA levels in female rat kidney. *Kidney Int* 65:1–8
19. Guha M, Mackman N (2001) LPS induction of gene expression in human monocytes. *Cell Signal* 13:85–94
20. Guillot L, Medjane S, Le-Barillec K, Nballoy V, Danel C, Chignard M, Tahar M (2004) Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like receptor 4 (TRL4)-dependent signalling pathways: evidence for an intracellular compartmentalization of TLR4. *J Biol Chem* 279:2712–2718
21. Haddad JJ, Collett A, Land SC, Olver RE, Wilson SM (2001) NF- κ B blockade reduces the O₂-evoked rise in Na⁺ conductance in fetal alveolar cells. *Biochem Biophys Res Commun* 281:987–992
22. Harris M, Garcia-Caballero A, Stutts MJ, Firsov D, Rossier BC (2008) Preferential assembly of epithelial sodium channel (ENaC) subunits in *Xenopus* oocytes: role of furin-mediated endogenous proteolysis. *J Biol Chem* 283:7455–7463
23. Hughey RP, Bruns JB, Kinlough CL, Harkleroad KL, Tong Q, Carattino MD, Johnson JP, Stockand JD, Kleyman TR (2004) Epithelial sodium channels are activated by furin-dependent proteolysis. *J Biol Chem* 279:18111–18114
24. Inglis SK, Olver RE, Wilson SM (2000) Differential effects of UTP and ATP on ion transport in porcine tracheal epithelium. *Br J Pharmacol* 130:367–374
25. Itani OA, Aurbach SD, Husted RF, Volk KA, Ageloff S, Knepper MA, Stokes JB, Thomas CP (2002) Glucocorticoid-stimulated lung epithelial Na⁺ transport is associated with regulated ENaC and sgk1 expression. *Am J Physiol* 282:L631–L641
26. Jain L, Chen XJ, Malik B, Al-Khalili O, Eaton DC (1999) Antisense oligonucleotides against the alpha-subunit of ENaC decrease lung epithelial cation-channel activity. *Am J Physiol* 276: L1046–L1051
27. Jain L, Chen XJ, Ramosevac S, Brown LA, Eaton DC (2001) Expression of highly selective sodium channels in alveolar type II cells is determined by culture conditions. *Am J Physiol* 280: L646–L658
28. Johnson MD, Bao HF, Helms MN, Chen XJ, Tigue Z, Jain L, Dobbs LG, Eaton DC (2006) Functional ion channels in pulmonary alveolar type I cells support a role for type I cells in lung ion transport. *Proc Natl Acad Sci USA* 103:4964–4969
29. Kamakura S, Moriguchi T, Nishida E (1999) Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases. Identification and characterization of a signaling pathway to the nucleus. *J Biol Chem* 274:26563–26571
30. Kunzelmann K, Beesley AH, King NJ, Karupiah G, Young JA, Cook DI (2000) Influenza virus inhibits amiloride-sensitive Na⁺ channels in respiratory epithelia. *Proc Natl Acad Sci USA* 97:9827–9879
31. Lazrak A, Matalon S (2003) cAMP-induced changes of apical membrane potentials of confluent H441 monolayers. *Am J Physiol* 285:L443–L450
32. Lin HH, Zentner MD, Ho H-LL, Kim K-J, Ann DK (1999) The gene expression of the amiloride-sensitive epithelial sodium channel α -subunit is regulated by antagonistic effects between glucocorticoid hormone and Ras pathways in salivary epithelial cells. *J Biol Chem* 274:21544–21554
33. Mace OJ, Woolthead AM, Baines DL (2008) AICAR activates AMPK and alters PIP2 association with ENaC to inhibit Na⁺ transport in H441 lung epithelial cells. *J Physiol* 586:4541–4557
34. Miakotina OL, Goss KL, Snyder JM (2002) Insulin utilizes the PI 3-kinase pathway to inhibit SP-A gene expression in lung epithelial cells. *Respir Res* 3:27
35. Michlig S, Harris M, Loffing J, Rossier BC, Firsov D (2005) Progesterone down-regulates the open probability of the amiloride-sensitive epithelial sodium channel (ENaC) via a NEDD4-2 dependent mechanism. *J Biol Chem* 280:38264–38270
36. Morneau F, Dagenais A, Berthiaume Y (2002) cAMP modulates the effect of *Pseudomonas aeruginosa*-derived LPS on ENaC expression in alveolar epithelial cells. *FASEB J* 16:A412
37. Mustafa SB, Castro R, Falck AJ, Petershack JA, Henson BM, Mendoza YM, Choudary A, Seidner SR (2008) Protein kinase A and mitogen-activated protein kinase pathways mediate cAMP induction of alpha-epithelial Na⁺ channels (alpha-ENaC). *J Cell Physiol* 215:101–110
38. Myerburg MM, Butterworth MB, McKenna EE, Peters KW, Frizzell RA, Kleyman TR, Pilewski JM (2006) Airway surface liquid volume regulates ENaC by altering the serine protease-protease inhibitor balance: a mechanism for sodium hyperabsorption in cystic fibrosis. *J Biol Chem* 281:27942–27949
39. Natarajan K, Singh S, Burke TRJ, Grunberger D, Aggarwal BB (1996) Caffeic acid phenyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF- κ B. *Proc Natl Acad Sci USA* 93:9090–9095
40. Nicod M, Michlig S, Flahaut M, Salinas M, Fowler Jaeger N, Horisberger JD, Rossier BC, Firsov D (2002) A novel vasopressin-induced transcript promotes MAP kinase activation and ENaC downregulation. *EMBO J* 21:5109–5117
41. Planes C, Blot-Chabaud M, Matthey MA, Couette S, Uchida T, Clerici C (2002) Hypoxia and beta 2-agonists regulate cell surface expression of the epithelial sodium channel in native alveolar epithelial cells. *J Biol Chem* 277:47318–47324
42. Reddy SP, Adisheshaiah P, Shapiro P, Vuong H (2002) BMK1 (ERK5) regulates squamous differentiation marker SPRR1B

- transcription in Clara-like H441 cells. *Am J Respir Cell Mol Biol* 27:64–70
43. Rezaiguia S, Garat C, Delclaux C, Meignan M, Fleury J, Legrand P, Matthay MA, Jayr C (1997) Acute bacterial pneumonia in rats increases alveolar epithelial fluid clearance by a tumor necrosis factor- α -dependent mechanism. *J Clin Invest* 99:325–335
 44. Rossier BC, Stutts MJ (2009) Activation of the epithelial sodium channel (ENaC) by serine proteases. *Annu Rev Physiol* 71:361–379
 45. Roux J, Kawakatsu H, Gartland B, Pespeni M, Sheppard D, Matthay MA, Canessa CM, Pittet JF (2005) Interleukin-1 β decreases expression of the epithelial sodium channel α -subunit in alveolar epithelial cells via a p38 MAPK-dependent signaling pathway. *J Biol Chem* 280:18579–18589
 46. Shi H, Asher C, Chigaev A, Yung Y, Reuveny E, Seger R, Garty H (2002) Interactions of beta and gamma ENaC with Nedd4 can be facilitated by an ERK-mediated phosphorylation. *J Biol Chem* 277:13539–13547
 47. Thomas CP, Campbell JR, Wright PJ, Husted RF (2004) Cyclic AMP-stimulated Na⁺ transport in H441 distal lung epithelial cells: role of protein kinase A, phosphatidylinositol 3-kinase and sgk1. *Am J Physiol* 287:L843–L851
 48. Wang H-C, Zentner MD, Deng H-T, Kim K-J, Wu R, Yang P-C, Ann DK (2000) Oxidative stress disrupts glucocorticoid hormone-dependent transcription of the amiloride-sensitive epithelial sodium channel α -subunit in lung epithelial cells through ERK-dependent and thioredoxin-sensitive pathways. *J Biol Chem* 275:8600–8609
 49. Woollhead AM, Baines DL (2006) Forskolin-induced cell shrinkage and apical translocation of functional EGFP-human α ENaC in H441 lung epithelial cell monolayers. *J Biol Chem* 281:5158–5168
 50. Zentner MD, Lin HH, Wen X, Kim KJ, Ann DK (1998) The amiloride-sensitive epithelial sodium channel α -subunit is transcriptionally down-regulated in rat parotid cells by the extracellular signal-regulated protein kinase pathway. *J Biol Chem* 273:30770–30776
 51. Zhu W, Downey JS, Gu J, Di Padova F, Gram H, Han J (2000) Regulation of TNF expression by multiple mitogen-activated protein kinase pathways. *J Immunol* 164:6349–6358