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Lipopolysaccharide increases Na^+,K^+-pump, but not ENaC, expression in guinea-pig airway epithelium

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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, α_1 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 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 α_1 Na^+,K^+-ATPase transcription were not affected. The expressions of α-, β-, and γ-ENaC and α_1 Na^+,K^+-pump from the 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 α_1 subunit and the α_1, α_2, and α_3 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.

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1. Introduction

Na^+ is transported across the airway epithelium by the epithelial Na^+ channel (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^+ hyper-absorption 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.

In previous investigations we found that the systemic administration of lipopolysaccharide (LPS) hyperpolarized the tracheal epithelium of the guinea pig (Johnston et al., 2004) by increasing the activities of the epithelial Na^+ channel and Na^+,K^+-pump (Dodrill and Fedan, 2010). 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.

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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 $\Delta\Delta C_t$ 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′-TCAGAGGGCTCCCTCAAAG-3′ (forward) and 5′-CGCTGTTGAAGTCACAGGAC-3′ (reverse) with probe 117; αENaC: guinea pig 5′-CAAGGGAGCCCTGAGAGTT-3′ (forward) and 5′-ACTCAGGAGGTCCCAGACG-3′ (reverse) with probe 92; α_1 Na^+,K^+-pump: rat 5′-GGAGAGCGTGTGCTAGGTTT-3′ (forward) and 5′-AAGCCTTCGGGAAACTGTTC-3′ (reverse) with probe 29; cyclooxygenase (COX)-2: guinea pig 5′-CTCGGCCAGACGCTATTTT-3′ (forward) and 5′-CCTTTCAAGGAGAATGGTGCT-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′-CACCACCAGCAGGAGCTT-3′ (reverse) with probe 65; tumor necrosis factor (TNF)-α: guinea pig 5′-GTCTCCTACCCG-GAAAAGGT-3′ (forward) and 5′-CTCCTTCTGGCAGGGACTCT-3′ (reverse) with probe 53; and interleukin (IL)-1β: guinea pig 5′-GGCAGACCGTCT-CACTCATC-3′ (forward) and 5′-ATGTGCAAGGAGCCAGCTT-3′ (reverse) with probe 73. Due to the unavailability of guinea-pig mRNA sequences, the α_1 Na^+,K^+-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; Woollhead and Baines, 2006). Using rat sequences to design primers for the α_1, α_2, α_3, α_4, β_1, β_2, β_3, and β_4 subunits of the Na^+,K^+-pump was only successful for the α_1 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 120 V 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 (Acros; Geel, Belgium) and the lanes cut apart. Membranes were blocked with 5% BSA/tween-20 0.05% for 1 h with rocking and washed 3 times with tris-buffered saline/tween-20 (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 (Fujifilm; 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 1:2000 dilution and detected with streptavidin-linked anti-rabbit secondary antibody (7074 Cell Signaling; Beverly, MA) diluted 1:1000. The Na^+,K^+-ATPase was probed using monoclonal antibodies against α_1 Na^+,K^+-ATPase (05-369 Millipore; Billerica, MA) diluted 1:500 and pan-α (α_1, α_2, and α_3) Na^+,K^+-ATPase (ab2871 Abcam; Cambridge, UK) diluted 1:1000 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 1:4000 and detected with anti-rabbit (7074 Cell Signaling) diluted to 1:2000.

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 $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, and 5.7 mM glucose) bubbled with 95% O_2, 5% CO_2. 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 $\Delta\Delta C_t$ method and significance was determined using Student's t-test. Western blot data were analyzed using the unpaired

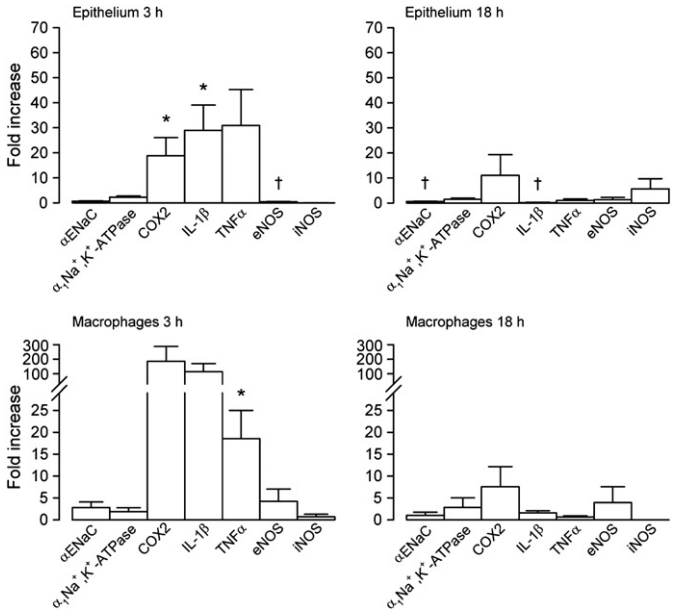

Fig. 1. LPS has no effect on the transcription of ENaC or Na^+,K^+-ATPase, but up-regulates some inflammatory genes. The effects of LPS on the transcription of αENaC, α_1 Na^+,K^+-ATPase, COX-2, IL-1β, TNF-α, eNOS, and iNOS were determined using qPCR. Tracheal epithelium (upper panels) and alveolar macrophages (lower panels) were isolated at 3 and 18 h after the injection of saline or LPS. *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.

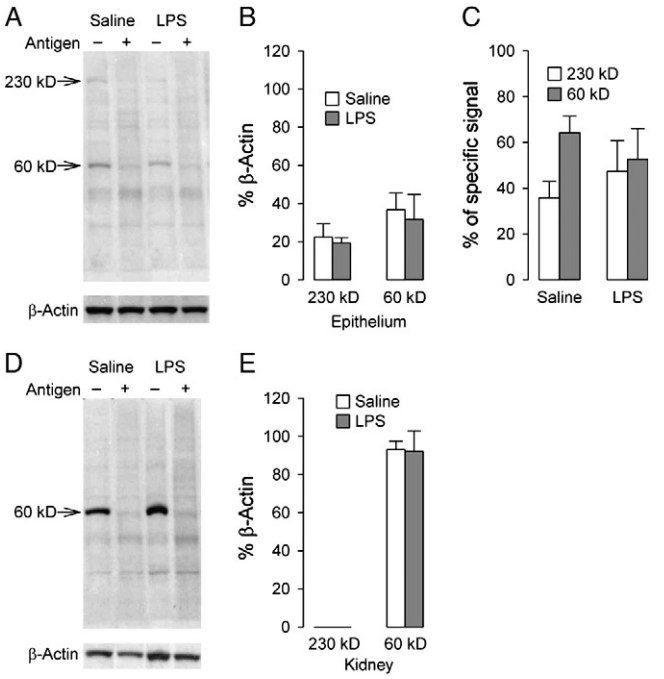

Fig. 2. LPS has no effect on the expression of αENaC in the epithelium and kidney. Tracheal epithelium (A, B, and C) and kidney (D and E) samples were collected from saline- and LPS-treated animals 18 h post-injection and prepared for western blotting. A, To identify specific labeling by the polyclonal antibody, identically loaded lanes were probed with antibody which was blocked by pre-adsorption with its antigen peptide (blocking peptide; Antigen +). B, Relative intensities of the 230 and 60 kDa bands in epithelia of saline- and LPS-treated animals. 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. D, The blocking peptide revealed that the antibody detected one, 60 kD band in the kidney from saline- and LPS-treated animals. E, Relative intensities of the 60 kD band between kidney tissue from saline- and LPS-treated animals. 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: $n = 3$.

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 α_1- 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 αENaC and α_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-κB 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-1β, TNF-α and other cytokines through NF-κB (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

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                                                                                    Antigen
Human       1    -------------------------MEGNKLEEQDSSPPQSTPGLMKGNKREEQGLGPEPAAPQQPTAEEEALIEFHRSY  55
Guinea pig  1    -------------------------MKGDELKEQGPLPPQPLQGPLKGDKCEQPGLGPEPTAPQQHTEEEEALTEFHRSY  55
Rat         1    MLDHTRAPELNIDLDLHASNSPKGSMKGNQFKEQDPCPPQPMQGLGKGDKREEQGLGPEPSAPRQPTEEEEALIEFHRSY  80
Mouse       1    MLDHTRAPELNLDLDLDVSNSPKGSMKGNNFKEQDLCPPLPMQGLGKGDKREEQALGPEPSEPRQPTEEEEALIEFHRSY  80

Human       56   RELFEFFCNNTTIHGAIRLVCSQHNRMKTAFWAVLWLCTFGMMYWQFGLLFGEYFSYPVSLNINLNSDKLVFPAVTICTL  135
Guinea pig  56   RELFQFFCNNTTIHGAIRLVCSKHNRMKTAFWAVLWLCTFGMMYWQFALLFGEYFSYPVSLNINLNSDKLVFPAVTVCTL  135
Rat         81   RELFQFFCNNTTIHGAIRLVCSKHNRMKTAFWAVLWLCTFGMMYWQFALLFEEYLSYPVSLNINLNSDKLVFPAVTVCTL  160
Mouse       81   RELFQFFCNNTTIHGAIRLVCSKHNRMKTAFWAVLWLCTFGMMYWQFALLFEEYFSYPVSLNINLNSDKLVFPAVTVCTL  160
                                                                  Furin    Inhibitor          Furin
Human       136  NPYRYPEIKEELEELDRITEQTLFDLYKYSSFTTLVAGS--RSRRDLRGTLPHPLQRLRVPPPPHGARRARSVASSLRDN  213
Guinea pig  136  NPYRYKEIKEQLRELDRITQQTLFDLYNYNASSTLLAGA--RSRRSLADTLPYPLQRIPVQPEP--RRARSSDPSSVRDN  211
Rat         161  NPYRYTEIKEELEELDRITEQTLFDLYKYNSSYTRQAGARRRSSRDLLGAFPHPLQRLRTPPPPYSGRTARSGSSSVRDN  240
Mouse       161  NPYRYTEIKEDLEELDRITEQTLFDLYKYNSSYTRQAGGRRRSTRDLRGALPHPLQRLRTPPPPNPARSARSASSSVRDN  240

Human       214  NPQVDWKDWKIGFQLCNQNKSDCFYQTYSSGVDAVREWYRFHYINILSRLPETLPSLEEDTLGNFIFACRFNQVSCNQAN  293
Guinea pig  212  NPRVDRRDWRVGFQLCNQNKSDCFYQTSSSGVDGVREWYRFHYINILAQVADTSPSLEEEALGNFIFACRFNQAPCTQEN  291
Rat         241  NPQVDRKDWKIGFQLCNQNKSDCFYQTYSSGVDAVREWYRFHYINILSRLSDTSPALEEEALGNFIFTCRFNQAPCNQAN  320
Mouse       241  NPQVDRKDWKIGFQLCNQNKSDCFYQTYSSGVDAVREWYRFHYINILSRLPDTSPALEEEALGSFIFTCRFNQAPCNQAN  320

Human       294  YSHFHHPMYGNCYTFNDKNNSNLWMSSMPGINNGLSLMLRAEQNDFIPLLSTVTGARVMVHGQDEPAFMDDGGFNLRPGV  373
Guinea pig  292  YSHFHHPIYGNCYTFNNKNDSSLWMASMPGINNGLSLTLRTEQNDYIPLLSTVTGARVTVHGQDEPAFMDDGGFNLRPGV  371
Rat         321  YSKFHHPMYGNCYTFNDKNNSNLWMSSMPGVNNGLSLTLRTEQNDFIPLLSTVTGARVMVHGQDEPAFMDDGGFNLRPGV  400
Mouse       321  YSQFHHPMYGNCYTFNNKNNSNLWMSSMPGVNNGLSLTLRTEQNDFIPLLSTVTGARVMVHGQDEPAFMDDGGFNVRPGV  400
                                                                          Suspected cleavage site
Human       374  ETSISMRKETLDRLGGDYGDCTKNGSDVPVENLYPSKYTQQVCIHSCFQESMIKECGCAYIFYPRPQNVEYCDYRKHSSW  453
Guinea pig  372  ETSISMRKEALDRLGGSYGDCTQDGSDVPVQNLYPSKYTQQVCIHSCFQENMIKQCGCAYIFYPKPKGVQFCDYRKQSAW  451
Rat         401  ETSISMRKEALDSLGGNYGDCTENGSDVPVKNLYPSKYTQQVCIHSCFQENMIKKCGCAYIFYPKPKGVEFCDYRKQSSW  480
Mouse       401  ETSISMRKEALDSLGGNYGDCTENGSDVPVKNLYPSKYTQQVCIHSCFQENMIKKCGCAYIFYPKPKGVEFCDYLKQSSW  480

Human       454  GYCYYKLQVDFSSDHLGCFTKCRKPCSVTSYQLSAGYSRWPSVTSQEWVFQMLSRQNNYTVNNKRNGVAKVNIFFKELNY  533
Guinea pig  452  GYCYYKLQGAFSSDSLGCFNKCRKPCNVTIYKLSAGYSRWPSVTSQDWIFQMLSLQNNYTISNKRNGVANLNIYFKELNY  531
Rat         481  GYCYYKLQGAFSLDSLGCFSKCRKPCSVINYKLSAGYSRWPSVKSQDWIFEMLSLQNNYTINNKRNGVAKLNIFFKELNY  560
Mouse       481  GYCYYKLQAAFSLDSLGCFSKCRKPCSVTNYKLSAGYSRWPSVKSQDWIFEMLSLQNNYTINNKRNGVAKLNIFFKELNY  560

Human       534  KTNSESPSVTMVTLLSNLGSQWSLWFGSSVLSVVEMAELVFDLLVIMFLMLLRRFRSRYWSPGRGGRGAQEVASTLASSP  613
Guinea pig  532  RTNSESPSVTMVSLLSNLGSQWSLWFGSSVLSVVEMAEFMFDLLVITLLMLLRRFRSRYWSPGRGARAAREVACTPPPSL  611
Rat         561  KTNSESPSVTMVSLLSNLGSQWSLWFGSSVLSVVEMADVIFDLLVITLLMLLRRFRSRYWSPGRGARGAREVASTPASSF  640
Mouse       561  KTNSESPSVTMVSLLSNLGSQWSLWFGSSVLSVVEMAELIFDLLVITLIMLLHRFRSRYWSPGRGARGAREVASTPASSF  640

Human       614  PSHFCPHPMS--LSLSQPGPAPSPALTAPPPAYATLGPRPSPGGSAGASSSACPLGGP-  669
Guinea pig  612  PSRFCAHSAFP-------------TLTAPPPAYATLSACPPLQGLAGASSAACAPREP-  656
Rat         641  PSRFCPHPTSPPPSLPQQGMTPPLALTAPPPAYATLGPSAPPLDSAAPDCSACALA-AL  698
Mouse       641  PSRFCPHPTSPPPSLPQQGTTPPLALTAPPPAYATLGPSASPLDSAVPGSSACAPAMAL  699
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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 is 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).

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 α_1 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 ($2.0\times10^{-4}\pm6.5\times10^{-5}$-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 α_1 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, 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 Section 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 a 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.

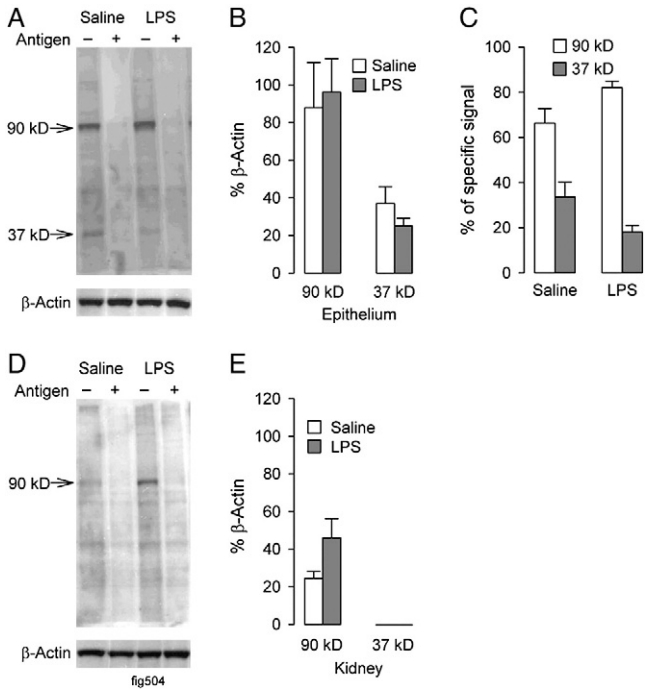

Fig. 4. LPS has no effect on the expression of βENaC in the epithelium and kidney. Tracheal epithelium and kidney samples were collected from saline- and LPS-treated animals 18 h post-injection and prepared for western blotting. A, To identify specific labeling by the polyclonal antibody, identically loaded lanes were probed with antibody which was blocked by pre-adsorption with its antigen peptide (blocking peptide; Antigen +). B, Relative intensities of the 90 and 37 kDa bands between the epithelia of saline- and LPS-treated animals. 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. D, The blocking peptide revealed that the antibody detected one, 90 kD band in the kidney from saline- and LPS-treated animals. E, Relative intensities of the 90 kD band between kidney tissue from saline- and LPS-treated animals. 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. Epithelium: $n = 3$ saline and LPS; kidney: $n = 4$ saline, $n = 3$ LPS.

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. 4B) 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 affect 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

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Human       1    MHVKKYLLRGLHRLQRGPGYTYKELLVWYCDNTNTHGPKRIICEGPKKKAMWFLLTLLFAALVCWQWGIFIRTYLSWEVS  80
Guinea Pig  1    MHVKKYLLKGLHRLQKGPGYTYKELLVWYCNNTNTHGPKRIICEGPKKKAMWFLITLLFASLVCWQWSEFIKTYLNWEVT  80
Rat         1    MPVKKYLLKCLHRLQKGPGYTYKELLVWYCNNTNTHGPKRIICEGPKKKAMWFLLTLLFACLVCWQWGVFIQTYLSWEVS  80
Mouse       1    MPVKKYLLKCLHRLQKGPGYTYKELLVWYCNNTNTHGPKRIICEGPKKKAMWFLLTLLFACLVCWQWGVFIQTYLSWEVS  80

Human       81   VSLSVGFKTMDFPAVTICNASPFKYSKIKHLLKDLDELMEAVLERILAPELSHANATRNLNFSIWNHTPLVLIDERNPHH  160
Guinea Pig  81   VSLSLGFKTMDFPAVTICNASPFQYSKTKHLLMDLDELMKAVLERILAPEASYANTTSALNFTLWNNTPLVLIYEQDSSH  160
Rat         81   VSLSMGFKTMNFPAVTVCNSSPFQYSKVKHLLKDLYKLMEAVLDKILAPKSSHTNTTSTLNFTIWNHTPLVLIDERNPDH  160
Mouse       81   VSLSMGFKTMNFPAVTVCNSSPFQYSKVKHLLKDLDELMEAVLEKILAPEASHSNTTRTLNFTIWNHTPLVLIDERNPDH  160

Human       161  PMVLDLFGDNHNGLTS---SSASEKICNAHGCKMAMRLCSLNRTQCTFRNFTSATQALTEWYILQATNIFAQVPQQELVE  237
Guinea Pig  161  PVVLDLFENIPS--ASVGSNAAQGRTCSAPGCKLAMKLCSLNGTVCTFRNFTSATQAVTEWYALQATNIFSQVSRQELVE  238
Rat         161  PVVLNLFGDSHN---S--SNPAPGSTCNAQGCKVAMRLCSANGTVCTFRNFTSATQAVTEWYILQATNIFSQVLPQDLVG  235
Mouse       161  PVVLNLFGDSHN---S--SNPAPGSTCNAQGCKVAMRLCSANGTVCTLRNFTSATQAVTEWYILQATNIFSQVLPQDLVG  235

Human       238  MSYPGEQMILACLFGAEPCNYRNFTSIFYPHYGNCYIFNWGMTEKALPSANPGTEFGLKLILDIGQEDYVPFLASTAGVR  317
Guinea Pig  239  MGYSAEHMILACLFGTEPCSYRNFTSIFYPNYGNCYIFNWGMTEKALPSANPGAEFGLKLILDIDQEDYVPFLTSTAGAR  318
Rat         236  MGYAPDRIILACLFGTEPCSHRNFTPIFYPDYGNCYIFNWGMTEKALPSANPGTEFGLKLILDIGQEDYVPFLASTAGAR  315
Mouse       236  MGYAPDRIILACLFGTEPCSHRNFTPIFYPDYGNCYIFNWGMTEETLPSANPGTEFGLKLILDIGQEDYVPFLASTAGAR  315

Human       318  LMLHEQRSYPFIRDEGIYAMSGTETSIGVLVDKLQRMGEPYSPCTVNGSEVPVQNFYSDYNTTYSIQACLRSCFQDHMIR  397
Guinea Pig  319  LMLHEQRTYPFIKEEGIDAMSGTETSIGVLVDKLQRMGEPYSPCTMNGSDVAIRNLYSSYNTTYSSQACLHSCFQDHMIH  398
Rat         316  LMLHEQRTYPFIREEGIYAMAGTETSIGVLLDKLQGKGEPYSPCTMNGSDVAIQNLYSDYNTTYSIQACLHSCFQDHMIH  395
Mouse       316  LMLHEQRTYPFIREEGIYAMAGTETSIGVLVDKLQRKGEPYSPCTMNGSDVAIKNLYSVYNTTYSIQACLHSCFQDHMIR  395
                                Suspected cleavage site
Human       398  NCNCGHYLYPLPRGEKYCNNRDFPDWAHCYSDLQMSVAQRETCIGMCKESCNDTQYKMTISMADWPSEASEDWIFHVLSQ  477
Guinea Pig  399  NCSCGHFLYPLPPGARYCNNQDFPDWAYCYFNLWMSVTQRETCINMCKESCNDTQYTMTISMADWPSEASEDWIFHVLSQ  478
Rat         396  NCSCGHYLYPLPAGEKYCNNRDFPDWAYCYLSLQMSVVQRETCLSMCKESCNDTQYKMTISMADWPSEASEDWILHVLSQ  475
Mouse       396  NCSCGHYLYPLPEGEKYCNNRDFPDWAYCYLNLQMSVTQRETCLSMCKESCNDTQYKMTISMADWPSEASEDWILHVLSQ  475

Human       478  ERDQSTNITLSRKGIVKLNIYFQEFNYRTIEESAANNIVWLLSNLGGQFGFWMGGSVLCLIEFGEIIIDFVWITIIKLVA  557
Guinea Pig  479  ERDHSTNITLSRKGVVKLNIYFQEFNYRTIEESAANNIVWLLSNLGGQFGFWMGGSVLCLIEFGEIIIDFMWITIIKLVA  558
Rat         476  ERDQSSNITLSRKGIVKLNIYFQEFNYRTIEESPANNIVWLLSNLGGQFGFWMGGSVLCLIEFGEIIIDFIWITVIKLVA  555
Mouse       476  ERDQSSNITLSRKGIVKLNIYFQEFNYRTIEESPANNIVWLLSNLGGQFGFWMGGSVLCLIEFGEIIIDFIWITIIKLVA  555
                                                                                Antigen
Human       558  LAKSLRQRRAQASYAGPPPTVAELVEAHTNFGFQPDTAPRSPNTGPYPSEQALPIPGTPPPNYDSLRLQPLDVIESDSEG  637
Guinea Pig  559  FFKGLRQKQAQAAYTGPPPTVAELVEAHTNFGFQPDTTNPSPHVEAYPDEQTLPIPGTPPPNYDSLRLQPLDTIESDSEG  638
Rat         556  SCKGLRRRRPQRPYTGPPPTVAELVEAHTNCVFQPDTTSCRPNAEVYPDQQTLPIPGTPPPNYDSLRLQPLDTMESDSEV  635
Mouse       556  SCKGLRRRRPQAPYTGPPPTVAELVEAHTNFGFQPDTTSCRPHGEVYPDQQTLPIPGTPPPNYDSLRLQPLDTMESDSEV  635
                    Antigen
Human       638  DAI  640
Guinea Pig  639  DAI  641
Rat         636  EAI  638
Mouse       636  EAI  638
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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 is 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).

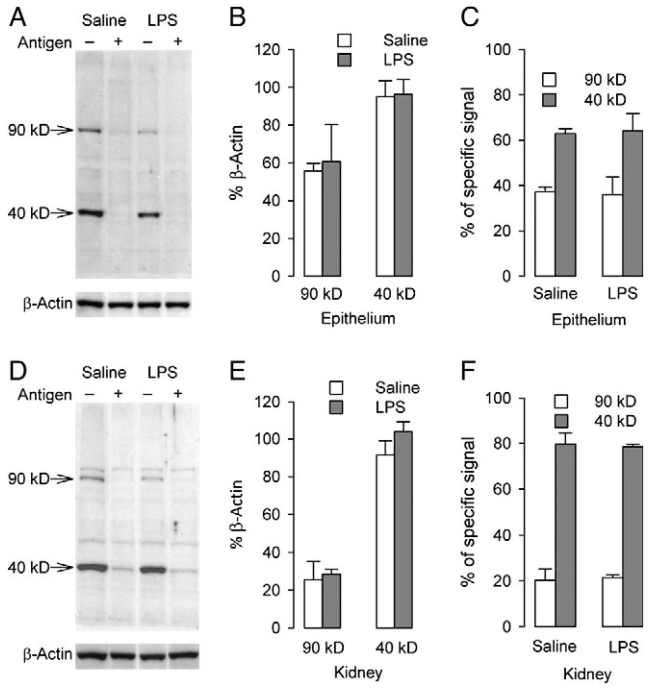

Fig. 6. LPS had no effect on the expression of γENaC in the epithelium and kidney. Tracheal epithelium and kidney samples were collected from saline- and LPS-treated animals 18 h post-injection and prepared for western blotting. A, To identify specific labeling by the polyclonal antibody, identically loaded lanes were probed with antibody which was blocked by pre-adsorption with its antigen peptide (blocking peptide; Antigen +). B, Relative intensities of 90 and 40 kDa bands between the epithelia of saline- and LPS-treated animals. C, The β-actin-normalized results are presented here as the within-group distribution of signal intensity between the 90 and 40 kD αENaC bands of the epithelium. 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, Relative intensities of the 90 and 40 kD bands 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 90 and 40 kD αENaC bands of the epithelium. 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: $n=3$.

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 (discussed earlier), 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 Na^+,K^+-pump could have led to potentiated Na^+ transport. To investigate this hypothesis, monoclonal antibodies were used to immunoblot the α_1 subunit of the Na^+,K^+-pump and pan-α (α_1, α_2, and α_3 subunits) in the epithelium from saline- and LPS-treated guinea pigs (Fig. 9). The expression of the α_1 subunit was increased in the epithelium of animals from LPS-treated animals compared to saline-treated animals (Fig. 9B). Using the pan-α antibody, the collective expression of α_1, α_2, and α_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 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 αENaC transcription

The rate of αENaC 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 αENaC 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 αENaC 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 (Galietta 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,

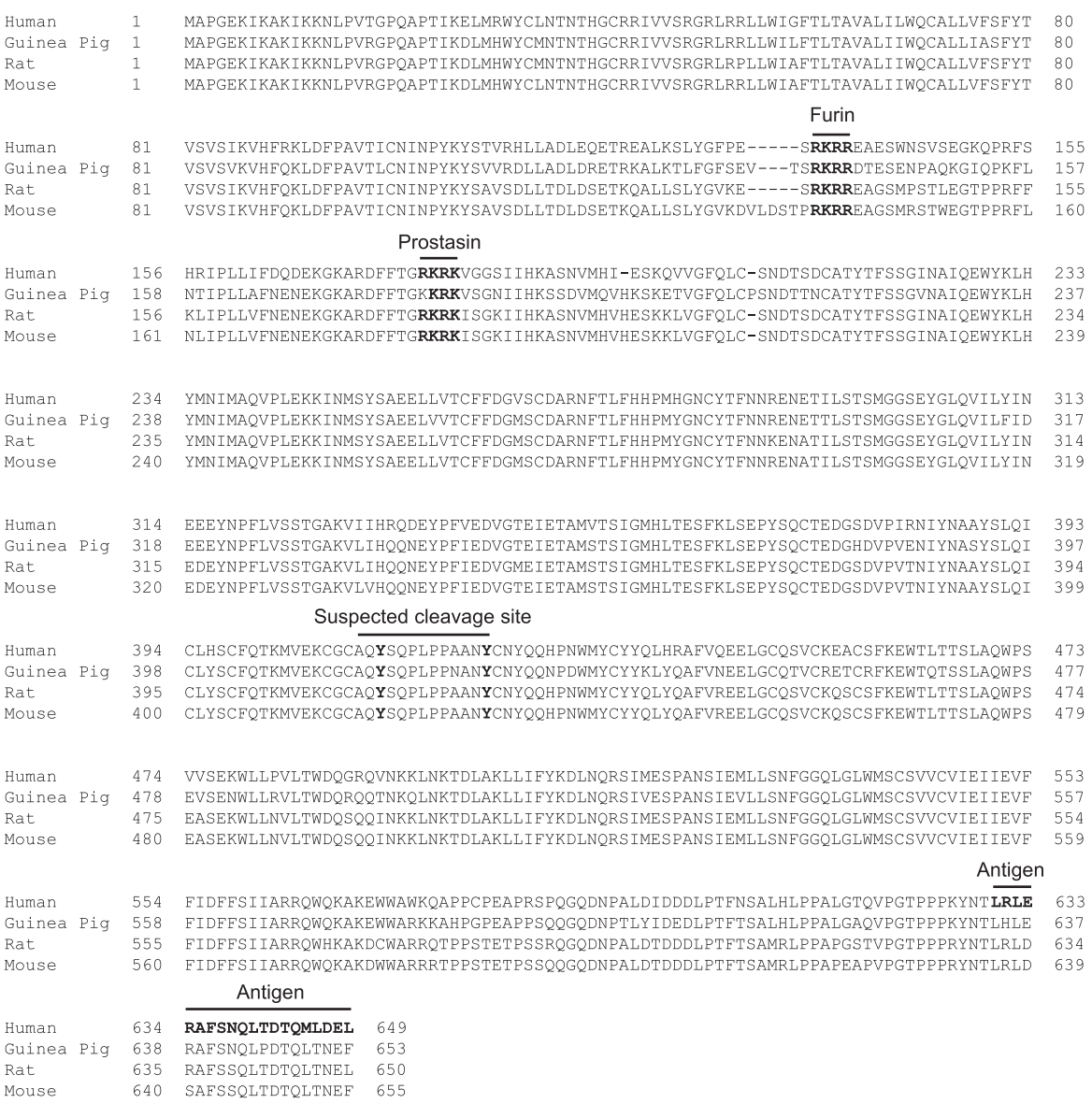

Fig. 7. Structure of γENaC indicating the locations of the antigen and protease cleavage sites. The amino acid structure of human, guinea pig, rat, and mouse γENaC are shown. The antigen of the antibody used for western blotting is located at the C-terminal end. Furin cleavage at an RXXR motif (Hughey 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).

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

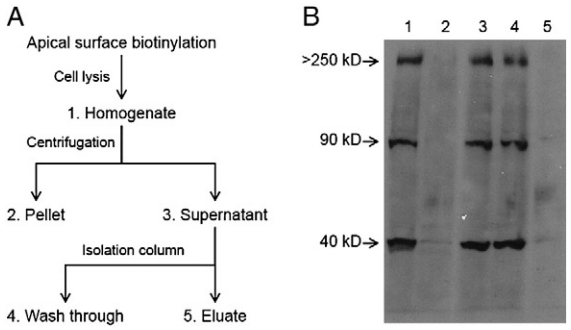

Fig. 8. Investigation of the effectiveness of the epithelial cell surface protein isolation protocol. The fate of ENaC was tracked through the cell surface protein biotinylation and isolation process by taking samples at each step and western blotting them. A, Flow diagram of the biotinylation and isolation 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.

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; Woollhead et al., 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.

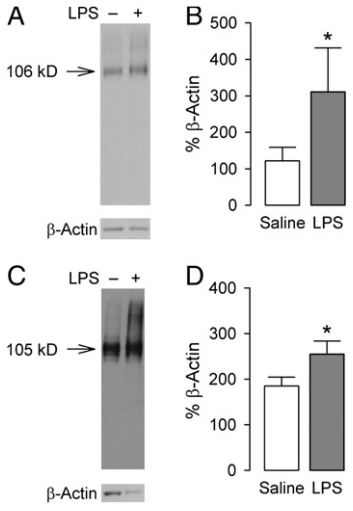

Fig. 9. LPS increased the expression of the Na^+,K^+-pump. The expression of the Na^+, K^+-pump was investigated by western blotting the epithelium of saline- and LPS-treated animals. A, A representative blot loaded with epithelial samples from a saline- and an LPS-treated animal blotted using an α_1 monoclonal antibody labeled a 106 kD band. B, Relative expression of the α_1 subunit of the Na^+,K^+-pump 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 (α_1, α_2, and α_3) antibody labeled a 105 kD band. D, Relative expression of pan-α Na^+,K^+-pump (α_1, α_2, and α_3) 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: $n = 10$.

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 α subunits was increased by LPS. The up-regulation may be predominantly on the α_1 subunit, as judged using the α_1 Na^+, K^+-ATPase antibody, the signal being "diluted" by the α_2 and α_3 subunits detected by the pan-α antibody. One study reported that, through increased NO levels, LPS reduces the expression of α_1 Na^+,K^+-pump in the rat sciatic nerve, but is without effect on α_2 or α_3 subunits (Liu and Sheu, 1997). TGF-1β has been reported to increase the expression of the α_1 and β_1 subunits in rat alveolar type II cells without affecting αENaC protein (Willis et al., 2003). The α_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 β 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.

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