Liddle’s syndrome mutations increase Na\(^+\) transport through dual effects on epithelial Na\(^+\) channel surface expression and proteolytic cleavage

Kristin K. Knight, Diane R. Olson, Ruifeng Zhou, and Peter M. Snyder*

Departments of Internal Medicine and Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, IA 52242

Communicated by Michael J. Welsh, University of Iowa College of Medicine, Iowa City, IA, December 27, 2005 (received for review December 8, 2005)

Liddle’s syndrome, an inherited form of hypertension, is caused by mutations that delete or disrupt a C-terminal PY motif in the epithelial Na\(^+\) channel (ENaC). Previous work indicates that these mutations increase expression of ENaC at the cell surface by disrupting its binding to Nedd4-2, an E3 ubiquitin–protein ligase that targets ENaC for degradation. However, it remains uncertain whether this mechanism alone is responsible; increased activity of ENaC channels could also contribute to excessive Na\(^+\) transport in Liddle’s syndrome. ENaC activity is controlled in part by its cleavage state; proteolytic cleavage produces channels with a high open-state probability, whereas uncleaved channels are inactive. Here, we found that Liddle’s syndrome mutations have two distinct effects of ENaC surface expression, both of which contribute to increased Na\(^+\) transport. First, these mutations increased ENaC expression at the cell surface; second, they increased the fraction of ENaC at the cell surface that was cleaved (active). This disproportionate increase in cleavage was reproduced by expression of a dominant-negative Nedd4-2 or mutation of ENaC ubiquitination sites, interventions that disrupt ENaC endocytosis and lysosomal degradation. Conversely, overexpression of Nedd4-2 had the opposite effect, decreasing the fraction of cleaved ENaC at the cell surface. Thus, the data not only suggest that Nedd4-2 regulates expression of cleaved and uncleaved ENaC at the cell surface but also provide a mechanism by which Liddle’s syndrome mutations alter ENaC activity.

Results

Liddle’s Syndrome Mutations Increase ENaC Cleavage. To detect full-length and cleaved forms of αENaC, we transfected HEK293 cells with αENaC, βENaC, and γENaC; the α subunit contained a FLAG epitope at the C terminus. The total cellular pool of αENaC was detected by immunoprecipitation followed by Western blotting with anti-FLAG antibody. The majority of αENaC in this pool was full length (90 kDa), and there was a less abundant band at 65 kDa (Fig. 1A Upper). Mutation of two consensus sites for cleavage (14) abolished this band (Fig. 1A Upper), confirming that it represents the C-terminal cleavage fragment of αENaC.

To detect αENaC at the cell surface, we biotinylated cell surface proteins; biotinylated αENaC was isolated with neutravidin beads and detected by Western blot (anti-FLAG). We detected both full-length and cleaved αENaC at the cell surface (Fig. 1A Lower), consistent with previous data (18). However, in contrast to the total cellular pool, a larger fraction of αENaC at the cell surface was cleaved; the ratio of cleaved αENaC to full-length αENaC at the cell surface was 1:1 (Fig. 1A and C).

To test the effect of a Liddle’s syndrome mutation on cleavage, we introduced a mutation into βENaC that removes most of the PY motif (β_{R566X}). This mutation produced a significant increase in αENaC expressed at the cell surface (Fig. 1A Lower), consistent with previous work (4, 5). However, there was a disproportionately large increase in cleaved αENaC; the Liddle’s syndrome mutation increased surface expression of the cleaved form to a much greater extent than the full-length form of αENaC (Fig. 1A Lower and C). This mutation also increased the quantity of cleaved αENaC in the total cellular pool but had little effect on expression of full-length αENaC (Fig. 1A Upper). A missense mutation in the PY motif (β_{N620A}) produced a similar increase in surface expression of cleaved αENaC (data not shown).

Conflict of interest statement: No conflicts declared.

Abbreviation: ENaC, epithelial Na\(^+\) channel.

*To whom correspondence should be addressed: 371 John W. Eckstein Medical Research Building, University of Iowa College of Medicine, Iowa City, IA 52242. E-mail: peter-snyder@uiowa.edu.

© 2006 by The National Academy of Sciences of the USA
To test whether an equivalent truncation in γENaC also alters cleavage of αENaC, we transfected cells with γK576X along with α-FLAG and βENaC. Similar to the Liddle’s mutation in βENaC, this mutation increased surface expression of cleaved αENaC to a greater extent than full length (Fig. 1 B Lower and C). When we coexpressed βR566X and γK576X, there was an additional increase in cleaved αENaC at the cell surface (Fig. 1 B Lower and C). Thus, Liddle’s syndrome mutations in βENaC and γENaC not only increase cell surface expression of αENaC but also increase the fraction of α subunits that are proteolytically cleaved.

To test whether a Liddle’s syndrome mutation alters proteolytic cleavage of γENaC, we placed a FLAG epitope at its C terminus. Transfection of HEK293 cells with γ-FLAG (with wild-type α and βENaC) resulted in full-length (92 kDa) and cleaved (75 kDa) bands in the total cellular protein pool (Fig. 1D Upper) and at the cell surface (Fig. 1D Lower). Similar to αENaC, the ratio of cleaved γENaC to full-length γENaC at the cell surface was ~1:1. When we introduced a Liddle’s syndrome mutation in βENaC (βR566X), there was increased expression of full-length and cleaved γENaC at the cell surface, but the increase in the cleaved form was much greater than in the full-length form (Fig. 1D Lower).

Liddle’s Syndrome Mutations Decrease ENaC Stimulation by Trypsin. Cleaved ENaC channels at the cell surface conduct Na⁺, generating amiloride-sensitive current. In contrast, uncleaved channels are inactive but can be acutely cleaved/activated by treatment with trypsin (14, 17). This observation provides an electrophysiological strategy to quantitate the relative expression of cleaved and uncleaved channels at the cell surface. For example, under conditions that increase ENaC cleavage by endogenous proteases, fewer channels are available to be activated by trypsin.

Therefore, as a second strategy to test whether Liddle’s syndrome mutations alter ENaC cleavage, we expressed wild-type ENaC in Fischer rat thyroid epithelia and measured the amiloride-sensitive, short-circuit current as an assay of ENaC function. Trypsin increased amiloride-sensitive current (Fig. 2A). In Fig. 2C, we plotted the trypsin-induced increase in ENaC current relative to baseline ENaC current, which is a measure of the fraction of channels at the cell surface that are uncleaved. With wild-type ENaC, the trypsin-activated current was quantitatively similar to the baseline amiloride-sensitive current (before the addition of trypsin). Thus, this functional assay indicated that the ratio of cleaved channels to uncleaved channels at the cell surface was ~1:1 (Fig. 2C), similar to the biotinylation assay. When we introduced Liddle’s syndrome mutations into βENaC and γENaC, the baseline amiloride-sensitive (ENaC) current was increased compared with that of the wild type, but trypsin produced a much smaller fractional increase in ENaC current (Fig. 2B and C). Thus, at baseline, a larger proportion of channels at the cell surface was cleaved by endogenous proteases and, therefore, resistant to cleavage/activation by trypsin. Together with our biochemical data, these results indicate that Liddle’s syndrome mutations produce a disproportionate increase in the expression of proteolytically cleaved ENaC channels at the cell surface.

Nedd4-2 Increases ENaC Cleavage. Liddle’s syndrome mutations increase Na⁺ transport by disrupting Nedd4-2 binding to ENaC. We therefore asked whether Nedd4-2 alters expression of cleaved ENaC at the cell surface. We coexpressed α-FLAG, βENaC, and γENaC with Nedd4-2 or GFP (control) and detected biotinylated αENaC at the cell surface. Nedd4-2 decreased surface expression of full-length and cleaved αENaC (Fig. 3A). However, the decrease in cleaved αENaC was much greater than that in full-length αENaC. Thus, Nedd4-2 reduced the fraction of cleaved channels at the cell surface, opposite to the effect of Liddle’s syndrome mutations.

As a reciprocal strategy, we disrupted binding of endogenous

Fig. 2. Liddle’s syndrome mutations decrease ENaC activation by trypsin. (A and B) Representative short-circuit current traces in Fischer rat thyroid epithelia transfected with αENaC, βENaC (wild type or R566X), and γENaC (wild type or K576X) (0.33 μg each). Amiloride (10 μM) and trypsin (2 μg/ml) were added to apical bathing solution as indicated. (C) Plot of the trypsin-induced increase in amiloride-sensitive current relative to baseline amiloride-sensitive current (before trypsin) (n = 26–42; *, P < 1 × 10⁻¹⁷, t test).
Nedd4-2 to ENaC by using a dominant-negative Nedd4-2 construct that lacks the catalytic HECT domain (19). When co-transfected with ENaC, this construct increased surface expression of cleaved αENaC to a much greater extent than that of full-length αENaC (Fig. 3B). Together, these results suggest that Nedd4-2 not only controls ENaC expression at the cell surface but also regulates the relative distribution of channels between the cleaved pool and full-length pool.

Because Nedd4-2 decreased the fraction of cleaved ENaC at the cell surface, we tested whether it would alter ENaC activation by trypsin. When ENaC was coexpressed with Nedd4-2, trypsin produced a larger increase in amiloride-sensitive current (relative to baseline amiloride-sensitive current) than when coexpressed with GFP (Fig. 3C). Thus, Nedd4-2 increased the fraction of channels at the cell surface that were susceptible to cleavage by trypsin (i.e., uncleaved). In contrast, expression of a dominant-negative Nedd4-2 construct decreased ENaC activation by trypsin (increased the fraction of cleaved channels), similar to Liddle’s syndrome mutations (Fig. 3C).

**Mutation of Ubiquitination Sites Increases ENaC Cleavage.** After binding to ENaC, Nedd4-2 is thought to catalyze ubiquitination of Lys residues located in the N terminus of αENaC and γENaC (20). We therefore tested whether mutation of these Lys residues would alter ENaC cleavage. In Fig. 3D, we mutated the γENaC Lys residues to Arg (γK,R) and detected biotinylated αENaC at the cell surface; these mutations increased surface expression of cleaved αENaC. Likewise, mutation of the N-terminal Lys residues in αENaC (αK,R) increased surface expression of cleaved γENaC (Fig. 3E). Moreover, coexpression of αK,R and γK,R (with wild-type β) decreased ENaC activation by trypsin (compared with wild-type ENaC) (Fig. 3F). These results suggest that the ubiquitination state of ENaC modulates the relative expression of cleaved and full-length ENaC at the cell surface.

**Discussion**

The data indicate that Liddle’s syndrome mutations have two distinct effects on ENaC surface expression, both contributing to increased Na⁺ current. First, the mutations increased the overall expression of ENaC at the cell surface. This result is consistent with previous work (4, 5). Second, and surprisingly, they increased the fraction of channels at the cell surface that were proteolytically cleaved. Because cleavage activates ENaC, increased cleavage provides a potential mechanism by which Liddle’s syndrome mutations might alter ENaC gating. Thus, the data may explain the previous observation that Liddle’s syndrome mutations increased Na⁺ current out of proportion to increased ENaC surface expression (5).

We hypothesize that the disproportionate increase in cleaved ENaC at the cell surface is an indirect consequence of defective ENaC trafficking in Liddle’s syndrome. Consistent with this hypothesis, we found that interventions that disrupt ENaC endocytosis and lysosomal degradation (dominant-negative Nedd4-2 and mutation of ENaC ubiquitination sites) reproduced the effect of Liddle’s syndrome mutations. Moreover, overexpression of Nedd4-2 produced the opposite effect, decreasing cleaved ENaC at the cell surface to a greater extent than full-length ENaC. We propose two potential mechanisms to explain how changes in ENaC trafficking could disproportionately alter surface expression of cleaved ENaC channels. First, it is possible that Nedd4-2 selectively removes cleaved (but not full-length) ENaC from the cell surface; perhaps cleavage increases the ability of Nedd4-2 to bind or ubiquitinate ENaC. Second, by reducing ENaC endocytosis, Liddle’s syndrome mutations might increase the time that ENaC is exposed to proteases at the cell surface (e.g., furin and CAP1-3), resulting in increased cleavage. In this scenario, increased cleavage results from an increase in ENaC residence time at the cell surface. Although less likely, we cannot exclude the possibility that Liddle’s syndrome mutations and Nedd4-2 alter the rate of ENaC cleavage at an intracellular location or alter delivery of cleaved channels to the cell surface.

How can we reconcile the current data with previous reports that Liddle’s syndrome mutations did not alter ENaC gating (4, 10, 12)? Recent work found that exogenous proteases (trypsin and neutrophil elastase) converted channels with a very low P₀ (“near silent”) into active channels but had little effect on channels that were active before protease treatment (17, 21). Using an independent measure of channel gating, Adebamiro et al. (22) found that inhibition of endogenous proteases (aprotinin) reduced the number of open channels at the plasma membrane but did not decrease P₀. Thus, by increasing the proportion of ENaC channels at the cell surface that are cleaved, we predict that Liddle’s syndrome mutations increase the fraction of near-silent channels that are converted to the active state. Consistent with this prediction, we found that Liddle’s syndrome mutations reduced the ability of trypsin to activate ENaC. However, because single-channel recordings are biased to detect active channels, it is not surprising that a change in the proportion of active (cleaved) and near silent (uncleaved) channels would be missed by this technique.

The existence of a pool of uncleaved channels at the cell surface suggests a mechanism by which Na⁺ transport could be regulated. This pool may function as a reservoir of quiescent channels that can be rapidly activated by proteases at the cell surface and in the extracellular environment. Our data suggest that the size of this pool is regulated by Nedd4-2, which decreases ENaC expression at the cell surface but increases the fraction of channels that are uncleaved and, therefore, available for acute activation by proteases. Discovery of additional pathways that control ENaC cleavage should provide new insights into the regulation of epithelial Na⁺ transport and the pathogenesis of hypertension.
Materials and Methods

cDNA Constructs. Human αENaC, βENaC, and γENaC in pMT3 were cloned as described in refs. 23 and 24. α-FLAG and γ-FLAG were generated by insertion of a FLAG epitope (DYKDDDDK) at the C terminus. Liddle’s syndrome mutations were generated in βENaC (R566X and Y620A) and γENaC (K576X) as described in ref. 4. αR (K22R and K25R), γκR (K6R, K8R, K10R, K12R, and K13R), and αC (where C is cleavage mutant; R175A, R177A, R178A, R181A, R201A, and R204A) were generated by site-directed mutagenesis (QuickChange; Stratagene). Nedd4-2 and dominant-negative Nedd4-2 (corresponding to amino acids 1 and 60–479 with Ser-221, Thr-246, and Ser-327 phosphorylation sites mutated to Ala) were generated as described in ref. 25.

Biochemistry. HEK293T cells were transfected with αENaC, βENaC, and γENaC (αENaC or γENaC contained FLAG epitope, as indicated in the figure legends) with or without Nedd4-2, dominant-negative Nedd4-2, or GFP by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Two days after transfection, the cells were transfected with cold PBS containing 1 mM CaCl2 and 1 mM MgCl2 (PBS-Ca/Mg), and then wash biotinylated proteins were biotinylated with 0.5 mg/ml sulfo-N-hydroxyssuccinimide-biotin (Pierce) for 30 min at 4°C. Excess biotin was quenched with 100 mM Gly in PBS-Ca/Mg for 20 min at 4°C. The cells were lysed in 1% Nonidet P-40/150 mM NaCl/50 mM Tris, pH 7.4, plus protease inhibitors (Sigma) at 4°C and then centrifuged at 16,110 g for 10 min to remove insoluble material. Biotinylated proteins were isolated by treading 1 mg of supernatant with 30 μM of neutravidin-agarose beads (Pierce) for 16 h at 4°C. After extensive washing, biotinylated proteins were eluted with SDS sample buffer (4% SDS/100 mM DTT/20% glycerol/100 mM Tris-HCl, pH 6.8) and separated by SDS/PAGE. After transfer to a nitrocellulose membrane and blocking in 5% BSA, biotinylated ENaC was detected by Western blot with anti-FLAG M2-peroxidase conjugate antibody (1:2,000; Sigma) and enhanced chemiluminescence (ECL Plus; Amersham Pharmacia BioSciences), and quantitated by densitometry (IMAGEJ). As a control for specificity, we did not detect biotinylation of Nedd4-2 or SGK (serum and glucocorticoid-regulated kinase), two cytoplasmic proteins.

To detect total αENaC or γENaC, 0.2 mg of cell lysate was immunoprecipitated with 30 μl of FLAG-agarose beads (Sigma) and detected by Western blot, as described above.

Electrophysiology. Fischer rat thyroid cells were grown on permeable filter supports as described in ref. 25. One day after seeding, cells were cotransfected with αENaC, βENaC, and γENaC (wild type or mutant, as indicated in the figure legends) with or without Nedd4-2, dominant-negative Nedd4-2, or GFP (control) by using TFX50 (Promega) as described in ref. 25. Na+ transport was measured under short-circuit conditions with modified Ussing chambers (Warner Instruments, Hamden, CT). The apical and basolateral surfaces were bathed in 135 mM NaCl/1.2 mM CaCl2/0.2 mM MgCl2/2.4 mM K-HPO4/0.6 mM KH2PO4/10 mM Hepes, pH 7.4, at 37°C. The amiloride-sensitive, short-circuit current was determined as the current difference with and without 10 μM amiloride in the apical bathing solution. To activate uncleaved ENaC channels, 2 μg/ml trypsin was added to the apical solution for 5 min. The amiloride-sensitive, short-circuit current was measured before and after treatment with trypsin.

We thank Kaela Kramer, Dan Collier, and Danielle Wentzlaff for assistance and the University of Iowa DNA Core Facility for its services. P.M.S. was supported by the National Institutes of Health.