Epithelial Sodium Channel Stiffens the Vascular Endothelium In Vitro and in Liddle Mice

Pia Jeggle, Chiara Callies, Antoine Tarjus, Celine Fassot, Johannes Fels, Hans Oberleithner, Frederic Jaisser, Kristina Kusche-Vihrog

Abstract—Liddle syndrome, an inherited form of hypertension, is caused by gain-of-function mutations in the epithelial Na⁺ channel (ENaC), the principal mediator of Na⁺ reabsorption in the kidney. Accordingly, the disease pathology was ascribed to a primary renal mechanism. Whether this is the sole responsible mechanism, however, remains uncertain as dysregulation of ENaC in other tissues may also be involved. Previous work indicates that ENaC in the vascular endothelium is crucial for the regulation of cellular mechanics and thus vascular function. The hormone aldosterone has been shown to concomitantly increase ENaC surface expression and stiffness of the cell cortex in vascular endothelial cells. The latter entails a reduced release of the vasodilator nitric oxide, which eventually leads to an increase in vascular tone and blood pressure. Using atomic force microscopy, we have found a direct correlation between ENaC surface expression and the formation of cortical stiffness in endothelial cells. Stable knockdown of αENaC in endothelial cells evoked a reduced channel surface density and a lower cortical stiffness compared with the mock control. In turn, an increased αENaC expression induced an elevated cortical stiffness. More importantly, using ex vivo preparations from a mouse model for Liddle syndrome, we show that this disorder evokes enhanced ENaC expression and increased cortical stiffness in vascular endothelial cells in situ. We conclude that ENaC in the vascular endothelium determines cellular mechanics and hence might participate in the control of vascular function. (Hypertension. 2013;61:1053-1059.)

Online Data Supplement

Key Words: aldosterone ■ atomic force microscopy ■ epithelial sodium channel ■ hypertension ■ Liddle syndrome ■ vascular endothelium

The vascular endothelium is a key player in the control of vascular tone as it synthesizes and secretes vasoactive substances. A reduced bioavailability of the vasodilator nitric oxide (NO) in the vessel wall leads to impaired vasodilation and presents the hallmark of endothelial dysfunction.1 Proper endothelial function depends on the mechanical rigidity of the endothelial cortex. This structural layer just beneath the plasma membrane primarily consists of an actin mesh and is proximal to the blood stream. By virtue of this strategic position, it is responsible for determining cell shape and it is the first cytoskeletal component responding to mechanical stimuli such as shear stress.2 An increase in mechanical stiffness of the cell cortex diminishes the endogenous release of NO3 and renders the cell less responsive to shear stress exerted by the blood stream—a condition best described as the stiff endothelial cell syndrome.4 One factor that stiffens the endothelial cortex and leads to a reduced NO secretion is the mineralocorticoid hormone aldosterone.5,6 A more classic understanding of aldosterone action is the control of epithelial Na⁺ channel (ENaC) expression and insertion into the plasma membrane via mineralocorticoid receptor (MR)–associated pathways, not only in the epithelia of kidney, colon, and lung,7 but also in the vascular endothelium.8–11 These effects can be reversed by the ENaC-specific functional blocker amiloride or be prevented by spironolactone, a competitive MR antagonist.8 Aldosterone thus exerts a dual effect on the endothelium; it increases endothelial cortical stiffness and induces a rise in ENaC surface abundance. This strongly suggests that ENaC itself determines the mechanical properties of endothelial cells and thus potentially NO release and vascular tone. The ENaC is a heteromultimer consisting of partially homologous subunits α, β, and γ,12 whereof the α-subunit is necessary for proper channel function and insertion into the plasma membrane.13,14 In vascular endothelial cells, knockdown of αENaC causes a near absence of detectable ENaC currents.15 ENaC has been shown to participate in the control of human blood pressure by the elucidation of the pathogenesis of Liddle syndrome.
syndrome. In this disease, mutations in the ENaC β- or γ-subunit lead to channel gain-of-function via an increase in surface density and activity of the channel. The molecular pathways underlying the pathogenesis of Liddle syndrome and other genetic forms of hypertension have so far been attributed to a primary renal abnormality in Na+ handling. Whether this mechanism alone is sufficient to explain the disease pathology is, however, questionable; it has been proposed that dysregulation of vascular tone contributes to the development and maintenance of hypertension, independent of actions resulting from an increased blood pressure.

In the present work, we therefore analyzed the role of ENaC in the occurrence of endothelial cell stiffness and hence endothelial cell function. Using atomic force microscopy (AFM) nano-indentation measurements, endothelial cells with genetically altered ENaC abundance, namely knockdown and high-level expression of αENaC, were probed for their cellular stiffness. In situ endothelial cells of ex vivo preparations from a mouse model for Liddle syndrome were also analyzed in this study. This model provides a unique, more physiological, example of altered ENaC surface expression and helps in elucidating an endothelial contribution to the disease, which has been so far neglected.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich unless otherwise specified.

Cell and Tissue Culture

Human endothelial EA.hy 926 cells (kindly provided by Cora-Jean S. Edgell, University of North Carolina, Chapel Hill, NC) were grown in culture as described elsewhere. For experiments, cells were seeded on either glass bottom dishes (Ø=4 cm, Wilco Wells) or thin glass coverslips (Ø=12–15 mm) and used after reaching confluence (48–72 hours). Aortae preparations, fixed on glass coverslips using Cell-Tak (BD Biosciences), were cultured as previously described. Chemicals were added to the medium as adequate. Aldosterone (d-aldosterone), spironolactone (ICN Biochemicals), and amiloride were used at final concentrations of 0.1 to 1.0 nmol/L, 100 nmol/L, and 1 µmol/L, respectively. Aldosterone and spironolactone were applied as adequate. It has been shown that ENaC’s activity and Na+ uptake are both inhibited by spironolactone.

Stable Knockdown of αENaC in Endothelial Cells Using RNAi

EA.hy 926 cells were independently stably transfected with 2 different sequence-specific siRNAs (19 nt target sequence; specified in the online-only Data Supplement) against αENaC (SCNN1A; genebank: NM_001038.4) using the pSUPER RNAi system (OligoEngine) according to the manufacturer’s instruction. Selection of positive clones was achieved by adding 1 µg/ml puromycin to the culture medium. For the mock control, a cell line harboring an empty vector was used. On elimination of all untransfected cells, a single positive clone was isolated and grown to confluency.

High-Level Expression of αENaC in Endothelial Cells

For high-level expression of αENaC in EA.hy 926 cells, transient transfections were performed with 5-µg plasmid DNA (pcDNA3.1 αENaC–eGFP) per 30 mm diameter dish using FuGENE 6 transfection reagent (Roche) according to the manufacturer’s guidelines. The plasmid pcDNA3.1 αENaC–eGFP, a kind gift from Dr. Ryszard Grygorczyk (Université de Montréal, Canada), encodes for a functional αENaC–eGFP fusion protein, which also serves as a reporter for proper protein expression.

Animal and Ethical Approval for Studies

We used an established mouse model for Liddle syndrome, kindly provided by E. Hummel and B. Rossier, University of Lausanne, Switzerland and corresponding wild-type (WT) mice (species: C57/Bl6; Mus musculus). The study was conducted at the U872, team 1 (Paris, France), and all experiments were executed in accordance with the institutional guidelines and the recommendations for the care and use of laboratory animals put forward by the French Ministry of Agriculture (Agreement no. A75-06-12). Further specifications and primers for genotyping are listed in the online-only Data Supplement.

Dissection and Preparation of Mice Aortae

The first ≈1.5 cm of aortae, from heart to diaphragm, was dissected from the mice (killed by cervical dislocation) and stored at 4°C in a modified solution (0.25 mM Ca2+) of solution 8. Further preparations were conducted within 3 to 4 days as recently described and illustrated in Figure S1 in the online-only Data Supplement.

Immunostaining

αENaC abundance solely on the upper endothelial cell surface (facing the medium) was detected and quantified via quantum dot (QD)-based immunofluorescence. Endothelial cells (in vitro and in situ) were fixed and stained as described elsewhere and briefly summarized in the online Data Supplement. Negative controls were established by incubating cells solely with the secondary antibody.

AFM Measurements

Endothelial cell stiffness was determined using an AFM nano-indentation technique. Major components and basic principles of this method are illustrated in Figure S1B and S1C and described in the online Data Supplement. Stiffness measurements were conducted with a scanning probe microscope (MultiMode SPM, Bruker) or an AFM integrated into an inverted fluorescence microscope (Catalyst AFM, Bruker; combined with DMI 6000B, Leica). The latter system enabled simultaneous stiffness and fluorescence measurements.

Data Analysis and Statistics

The QD-based immunofluorescence was quantified by counting QD/1000 µm2 of cell surface using ImageJ software (National Institutes of Health, Bethesda, MD). Images were taken in 3 different sections of the endothelial monolayer, and all 3 images were analyzed simultaneously. QD background levels (QD detected in negative controls) were subtracted from the results. Obtained AFM data were collected with the NanoScope V8.10 software (Bruker), and stiffness values were calculated using the Protein Unfolding and Nano-Indentation Analysis Software (PUNIAS; http://site.viola.fr/punias). Each data point represents the stiffness of a single cell (mean of 5–8 stiffness values). Significance of difference in each data set was determined using 1-way ANOVA. When ANOVA detected significant differences between groups, post hoc comparisons were made by Student t test (**P<0.05, ***P<0.01, ****P<0.001). The different populations all followed a normal distribution. All specified data were calculated as mean (±SE of the mean) and are displayed as box plots: mean (square), median (horizontal line), 25th and 75th percentile (box), 10th and 90th percentile (wiskers).

Results

αENaC Surface Abundance Is Reduced in Stable αENaC-Knockdown Cell Lines

We generated 2 stable endothelial αENaC-knockdown cell lines (sixENaC-A and sixENaC-B) using different siRNAs. Quantification of the knockdown was estimated by Western blotting (n=6); whole-cell αENaC expression was reduced by about 52% (sixENaC-A) and 28% (sixENaC-B) compared with mock cells (cells stably transfected with an empty vector), as illustrated in Figure S2A (original blot) and S2B (graph). To demonstrate that the following findings were not inherent to...
clone-specific effects, we analyzed both αENaC-knockdown cell lines. We detected a similar behavior in the 2 cell lines and decided to focus on the siENaC-A cells. Findings of the siENaC-B cells are presented in Figure S3. QD-based immunofluorescence for αENaC was performed to detect αENaC abundance on the upper surface of nonpermeabilized endothelial cells. The staining procedure was validated beforehand, using the exact same antibodies, on nonpermeabilized cortical collecting duct epithelial cells (Figure S4). Immunofluorescence data are presented as detected QD/1000 μm² because endothelial cells have an approximate size of ≈1000 μm².33

Relative to mock cells (90.4±2.33, QD/1000 μm²), αENaC surface abundance in siENaC-A cells was significantly lower (−22%; 70.8±1.96, QD/1000 μm²; P<0.001) in the presence of aldosterone (Figure 1). The MR antagonist spironolactone abolished this difference; it reduced the αENaC surface abundance significantly in both cell lines (Figure 1) to 55.6±1.90 QD/1000 μm² (mock; −38%; P<0.001) and 58.0±2.30 QD/1000 μm² (siENaC-A; −18%; P<0.001), compared with the respective aldosterone-stimulated control condition.

αENaC Surface Abundance Determines the Cortical Stiffness of Endothelial Cells: An AFM Approach

We further compared mock (normal αENaC surface expression) and siENaC (reduced αENaC surface expression) cells in terms of their cortical stiffness using AFM nano-indentation measurements. Cortical stiffness is defined here as the force that needs to be exerted to indent the cell cortex for a fixed distance and hence directly reflects the mechanical rigidity of the submembranous region. We analyzed the cell cortex because experiments aimed at modifying ENaC surface expression in endothelial cells were shown to coincide with alterations in this region, rather than whole cell rigidity.4 Again, we detected a similar behavior in the 2 cell lines and decided to focus on the siENaC-A cells and present siENaC-B data in Figure S3C. As illustrated in Figure 2, under aldosterone-treated conditions, siENaC-A cells displayed a significantly lower cortical cell stiffness (−29%; 1.4±0.01 pN/nm; P<0.001) than mock cells (1.4±0.01 pN/nm). Significant alterations in cell stiffness were also observed on secondary spironolactone or amiloride treatment (relative to aldosterone-treated conditions). Additional treatment with spironolactone significantly lowered stiffness of mock (−21%; 1.1±0.01 pN/nm; P<0.001) and also significantly further reduced stiffness of siENaC-A cells (−10%; 0.9±0.01 pN/nm; P<0.001). A similar result was detected on acute application of the ENaC blocker amiloride; stiffness of aldosterone-treated mock and siENaC cells was significantly diminished to 1.0±0.02 pN/nm (−29%; P<0.001) and 0.9±0.02 pN/nm (−10%; P<0.001), respectively.

Interestingly, we observed an additive effect of spironolactone and amiloride; simultaneous stimulation of aldosterone-treated cells with both drugs significantly (P<0.001) reduced cortical stiffness of mock cells (0.9±0.01 pN/nm; −36%) even beneath the stiffness levels reached under stimulation with the drugs individually (data not shown).

In addition, we analyzed whether an increased αENaC expression affected cell stiffness. Indeed, transfection of endothelial cells with a vector designed for high-level expression of a functional αENaC–eGFP fusion protein14 (Figure 3) resulted in a significant increase in cortical stiffness (+31%; 1.7±0.21 pN/nm; P<0.001) compared with surrounding, untransfected control cells (1.3±0.11 pN/nm). These results indicate that ENaC is indeed one factor that determines cortical endothelial stiffness.

ENaC Abundance Is Increased in Endothelial Cells From Aortae of Liddle Mice

As shown for kidney35 and colon,36 the phenotypic manifestations of Liddle syndrome are primarily ascribed to
gain-of-function of the channel owing to an increased ENaC surface density. In this study, αENaC levels on the surface of in situ vascular endothelial cells from ex vivo aorta preparations of WT mice and a mouse model for Liddle syndrome (Liddle) were determined using QD-based immunofluorescence (Figure 4A and 4B). We chose to probe the aortal endothelium because this provides an experimentally accessible model for our present work. Previous studies also state that endothelial function determined in the aorta also applies to smaller resistance vessels. Under aldosterone-treated conditions, endothelial cells of Liddle mouse aorta displayed a significantly higher αENaC surface abundance (+34%; 43.6±4.12 QD/1000 μm²; P=0.03) than endothelial cells of WT mouse aorta (32.6±2.64 QD/1000 μm²; Figure 4B). Endothelial cells from WT and Liddle mouse aorta also functionally responded toward additional ex vivo spironolactone treatment. ENaC surface levels were significantly reduced to 22.8±2.53 QD/1000 μm² (−30%; P=0.009) in cells from WT and to 28.7±2.06 QD/1000 μm² (−34%; P=0.002) in cells from Liddle mice. Under this condition, we detected no significant difference between WT and Liddle cells.

Cortical Stiffness Is Increased in Endothelial Cells From Aorta of Liddle Mice

Repeated recordings of in situ endothelial cortical stiffness in ex vivo aorta preparations revealed that under aldosterone-treated conditions the increased αENaC surface abundance present in Liddle endothelial cells led to a significant rise in endothelial cortical stiffness by 29% (2.2±0.17 pN/nm; P<0.001), compared with WT cells (1.7±0.08 pN/nm). As further illustrated in Figure 4C, significant alterations in cell stiffness were also observed on spironolactone or amiloride treatment (relative to aldosterone-treated conditions). Stiffness of WT cells was lowered to 1.4±0.07 pN/nm (−18%; P=0.01) and to 1.3±0.07 pN/nm (−24%; P=0.001) by spironolactone and amiloride, respectively. Stiffness of Liddle cells was reduced to 1.4±0.13 pN/nm (−36%; P<0.001) and to 1.6±0.08 pN/nm (−27%; P=0.003) on spironolactone and amiloride treatment, respectively. Under these conditions, we detected no significant difference between WT and Liddle endothelial cells. From these experiments we conclude that the ENaC mutation causing Liddle syndrome affects the endothelium, and that αENaC surface expression conveys a significant influence on the cortical stiffness of endothelial cells in situ.

Discussion

In our in vitro experiments, knockdown of αENaC expression led to lower αENaC surface abundance, which coincided with significant reductions in the cortical stiffness of vascular endothelial cells. Accordingly, high-level
expression of αENaC causes an elevated cortical stiffness. Moreover, the MR antagonist spironolactone concomitantly reduced αENaC surface expression and cortical stiffness in mock and sixENaC cells. Thus, our results strongly suggest that αENaC is one crucial determinant of this mechanical property and that MR activation plays a major role in this process.

Notably, we also found a significant reduction in cortical stiffness on treatment with the specific ENaC blocker amiloride. Hence, not merely the presence of the channel in the plasma membrane but also function of the channel plays a significant role in ENaC-mediated cortical stiffening.

The mechanistic basis how ENaC determines cortical stiffness, however, remains to be determined. It could rely on either the channel’s activity (ie, Na⁺ influx) or its colocalization with F-actin.⁷ Both of these factors raise the F-actin:G-actin ratio in the cortical cytoskeleton⁸,⁹ and thus increase cortical stiffness. Owing to the interdependency of the 2 mechanisms, it is conceivable that a combination of them might determine cortical stiffness.

In our experiments on in situ endothelial cells of ex vivo mice aorta preparations, we demonstrated for the first time that ENaC mutations causing Liddle syndrome induce an increased αENaC surface expression and a concomitantly elevated cortical stiffness in the endothelium. This finding further supports the notion that ENaC is a crucial determinant of endothelial cortical stiffness, in vitro and ex vivo. Because Liddle mice are normotensive when fed a normal salt diet (the case in this study), it can be excluded that the observed cortical stiffening relates to changes in blood pressure. Whether and how ENaC-mediated endothelial stiffening then affects vascular function remains to be investigated. A role for ENaC in the regulation of myogenic tone¹⁰ and shear-stress sensing¹¹ in mesenteric arteries has already been proposed. Future studies using gene-specific targeting strategies will help in elucidating the pathophysiological vascular role of endothelial ENaC.

Considering that an increased cortical stiffness entails a diminished NO release,¹²,¹⁶ our results may provide a mechanical link to the observation by Pérez et al.,⁴¹ namely that endothelial ENaC determines vasoconstriction by negatively modulating NO release in mesenteric arteries. Our findings might thus extend the pathophysiological role of ENaC in Liddle syndrome. The ENaC-mediated cortical stiffening and a subsequent reduced bioavailability of NO in the vessel wall (endothelial dysfunction) may result in cardiovascular disease such as hypertension. Whether altered ENaC function also participates in the pathology of more common forms of hypertension (eg, salt-sensitive, angiotensin II or endothelin-1–mediated hypertension) remains to be analyzed.

Consistent with observations by Liddle et al¹⁷ that amiloride effectively corrects the hypertension in Liddle syndrome by inhibiting the mutated ENaC, we also found significant reductions in cortical stiffness on amiloride application. In Liddle patients amiloride was only effective on a low Na⁺ diet, yet our results were obtained in the presence of moderate Na⁺ concentrations (140 mmol/L). This might point out differences between kidney and endothelium. Although spironolactone has proven to be ineffective in correcting the hypertension in Liddle syndrome,¹⁷ we observed distinct effects on the vascular endothelium: spironolactone significantly lowers αENaC surface expression and cortical stiffness in ex vivo WT and Liddle endothelial cells. This indicates that the low plasma aldosterone concentration present in Liddle syndrome⁴² and mimicked in our experiments (≈4 ng/dL) might still be high enough to affect the vascular endothelium, which again poses a discrepancy between kidney and endothelium.

Interestingly, a combined administration of spironolactone and amiloride led to a synergistic effect of the 2 drugs. This finding highlights the potential of the combined administration of amiloride and spironolactone as a new therapeutic endeavor suggested by Pratt et al.⁴³ It might aim at correcting both the high blood pressure and the endothelial dysfunction. The latter was found to often precede essential hypertension.⁴⁴

The pathology of Liddle syndrome might, therefore, relate not only to ENaC dysfunction in the kidney but also to improper functioning of this channel in the endothelium. This view is further supported by studies in Liddle patients: The severe hypertension could apparently be corrected by kidney transplantation in 5 cases,⁴²,⁴⁵ thus highlighting the important contribution of ENaC dysfunction in the distal nephron to the disease. This correction, however, was not complete or merely effective for a certain time span, which demonstrates that ENaC dysregulation in other tissues, possibly in the endothelium, also participates in the pathology of Liddle syndrome.

Perspectives

In this study, we report that ENaC plays a crucial role in vascular function as it determines cortical stiffness of vascular endothelial cells in vitro and ex vivo, which in turn controls NO production and thus vascular function. Regarding Liddle syndrome, this finding strongly suggests that improper functioning of ENaC not only in the kidney but also in the vascular endothelium contributes to the pathology of this disease. The findings of this work may also be considered relevant to the development of more general forms of hypertension. First, there are several genetic variations in ENaC, which could be linked to the development of more common forms of hypertension.⁴⁶,⁴⁷ Second, an endothelium-dependent dysregulation of vascular tone has already been considered relevant to the development of hypertension.⁴⁸,⁴⁹ On the basis of the findings of this study we propose that improper ENaC function is one of the factors causing such a dysregulation.

Acknowledgments

We thank Prof Hugh E. de Wardener (Imperial College, London) for valuable suggestions to the manuscript, E. Hummler and B. Rossier (University of Lausanne, Switzerland) for providing the mouse model for Liddle syndrome, and Marianne Wilhelmi and Anja Blanqué for the excellent work on the mice aorta preparations and immunostaining.

Sources of Funding

This work was supported by grants from the Deutsche Forschungsgemeinschaft (OB 63/17-1, Koselleck-OB 63/18, and
Disclosures

References


Action TD1002 for supporting networking activities.

1058

Hypertension May 2013

KU 14967/1–1), the “Innovative Medical Research” of the University of Muenster Medical School (KU 120808), the French National Research Agency (FJ ANR-09-BLAN-0156-01), the Leducq Foundation (FJ Transatlantic Network on Hypertension), and COST Action TD1002 for supporting networking activities.

None.


**Novelty and Significance**

**What Is New?**
- We have found a direct correlation between the surface expression of the epithelial Na\(^+\) channel (ENaC) and the regulation of cellular mechanics in endothelial cells.
- By the use of ex vivo preparations from a mouse model for Liddle syndrome, we show that the ENaC mutation causing the disease evokes enhanced expression of the ion channel and a decreased deformability of vascular endothelial cells.

**What Is Relevant?**

The so far under-recognized endothelial ENaC is an important determinant of cell mechanics of the vascular endothelium and thus vascular function.

**Summary**
We conclude that ENaC in the vascular endothelium determines cellular mechanics and hence might participate in the control of vascular function.
ONLINE DATA SUPPLEMENT

EPITHELIAL NA⁺ CHANNEL STIFFENS THE VASCULAR ENDOTHELium IN
LIDDLE’S SYNDROME

Endothelial ENaC in Liddle’s syndrome

Pia Jeggle#, Chiara Callies#, Antoine Tarjus§, Celine Fassot§, Johannes Fels#, Hans Oberleithner#, Frederic Jaisser§° and Kristina Kusche-Vihrog#**

#Institute of Physiology II, University of Muenster, Robert-Koch-Straße 27b, 48149 Muenster, Germany

§INSERM U872 Team 1, Centre de Recherche des Cordeliers, Université René Descartes, Université Pierre et Marie Curie Paris, 15 rue de l’Ecole de Médecine, 75270 Paris cedex 06, France

°Shared senior authorship

*To whom correspondence should be addressed.

Address: Kristina Kusche-Vihrog, PhD
Institute of Physiology II
University of Muenster
Robert-Koch-Straße 27b
48149 Muenster, Germany
phone: 0049-251-83-55336
fax: 0049-251-83-55331
email: kusche@uni-muenster.de
EXPANDED MATERIALS AND METHODS
All chemicals were purchased from Sigma-Aldrich unless otherwise specified.

Cell culture
M-1 cortical collecting duct epithelial cells (CRL-2038; American Type Culture Collection (ATCC), Manassas, USA) were cultivated in T25 flasks using D-MEM/F-12 medium (ATCC) containing dexametasone (0.005 mM) and PC-1 primary cell serum-free medium (Lonza, Germany) containing L-glutamine (2 mM) as a 3:1 mixture supplemented with penicillin G (10,000 U/mL), streptomycin (10,000 µg/mL), NaCl (10 mM) and 5 % fetal bovine serum. After reaching confluence, cells were split using alfazyme and cultured at 37°C, 5 % CO₂ on filters (ThinCert™ tissue culture inserts, 12 well, 0.4 µm pore size; Greiner Bio-One GmbH). Aldosterone was added to the medium at a final concentration of 5 nM.

Animals
Animals were kept at the CEF (Centre d'Explorations Fonctionnelles of the Cordeliers Research Center) and maintained at a constant temperature and humidity in a light controlled room with a 12 hours light cycle. They had free access to food and tap water. Aortae preparations used in this study were obtained after killing the animals (n=13) by cervical dislocation.
Genotyping of the Liddle mice (mus musculus) was performed using the following primers: sens (5′-CTTCCAAGAGTTCAACTACCG-3′) and antisense (5′-TCTACCAGCTCAGCCACAGTG-3′)

Western Blot
Endothelial (siαENaC-A&-B, mock) and epithelial cells were grown to confluence and stimulated with aldosterone for ~48 h. Cells were lysed with ProteoExtract® Transmembrane Protein Extraction buffer (kit; Merck) supplemented with a protease inhibitor cocktail (Sigma-Aldrich) at 4 °C. About 25 µg of whole cell protein were separated on a 7.5% polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking (5% milk powder in TBS-T (50 mmol/L Tris HCl, 150 mmol/L NaCl, pH 7.6 + 0.1 % Tween-20), 1 h, 4°C) primary antibodies against human αENaC (rabbit; 1:500; ~75 kDa; Dianova) and GAPDH (mouse; 1:1000; ~36 kDa; Dianova) were applied over night at 4°C. Secondary antibodies anti-rabbit HRP (goat; 1:10.000, Dako) and anti-mouse POD (goat; 1:10.000; Dianova) were incubated for 2 h at room temperature (RT). Antibodies were diluted in TBS-T + 0.5 % milk power. The blots were developed using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) according to the manufacturer’s instructions. The anti-αENaC antibody (Dianova) was generated against a synthetic peptide corresponding to amino acids L(20)MKGNKREEQGLPPEPAAPQQPT(42) in the cytoplasmic region of human αENaC. The antibody detects two proteins in this study:
1. ~76 kDa, which corresponds to the molecular weight of full-length αENaC (isoform 1) of 75.7 kDa according to UniProtKB/Swiss-Prot databases and
previous publications by McDonald et al\(^1\) and Tucker et al\(^2\). Authors of the first paper predicted a size of 76 kDa for wild type human αENaC and observed a \(~74\) kDa protein. A 87 kDa protein represented glycosylated human αENaC, however, glycosylation of αENaC was shown to be unnecessary for proper trafficking to and insertion of the channel into the plasma membrane by Rotin et al\(^3\). In the latter publication, the authors also analyzed wild-type human αENaC and observed a \(77\pm2\) kDa protein.

2. \(~58\) kDa, which corresponds to a cleaved form of αENaC.

*Immunostaining*

αENaC abundance solely on the upper endothelial and epithelial cell surface (facing the medium) was detected and quantified via quantum dot (QD) based immunofluorescence.

Endothelial cells (*in vitro* and *in situ*) were fixed in a non-permeabilizing manner with 0.1 % glutaraldehyde. Fixed cells were successively blocked with 100 mM glycine and 10 % normal goat serum (NGS) at RT for 10 min and 30 min, respectively. The polyclonal anti-αENaC antibody (1:250; Santa Cruz) was applied to the cells for 1h at RT. For QD-staining, the QD655 goat F(ab’)\(_2\) anti-rabbit IgG conjugate secondary antibody (1:800; Invitrogen) was applied for 1 h at RT. The cells on glass coverslips were finally mounted onto microscope slides using fluorescence mounting medium (Dako). In between steps, cells were gently washed with PBS (in mM: 140 NaCl, 2 KCl, 4 Na\(_2\)HPO\(_4\), 1 KH\(_2\)PO\(_4\), pH 7.4). All antibodies were diluted in NGS. Auto-fluorescence of the cells, which accounts for fixation with glutaraldehyde, allows identification of cell boarders.

Epithelial cells were stained as follows: live cells seeded on filters were cooled to 4°C and blocked with HEPES buffer (in mM: 150 NaCl, 5 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 5 glucose, 10 HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4) containing 1 % BSA (blocking buffer) and 1 nM aldosterone for 1 h (4°C). The anti-αENaC antibody (1:250; Santa Cruz) was applied to the cells for 2 h (4°C) before washing the cells with blocking buffer. Cells were then fixed with HEPES buffer containing 4 % paraformaldehyde (dissolved right before fixation) for 15 min (4°C) and the QD655 goat F(ab’)\(_2\) anti-rabbit IgG conjugate secondary antibody (1:800; Invitrogen) was applied for 1 h at RT. Cells were subsequently permeabilized with 0.1 % triton X-100 in PBS for 1 min and stained with Oregon Green 488 phalloidin (1:75; Invitrogen) for 30 min (RT). Cells on filters were finally mounted onto microscope slides using fluorescence mounting medium supplemented with DAPI (4’,6-diamidino-2-phenylindole; Invitrogen). In between steps, cells were gently washed with PBS unless otherwise specified. All antibodies were diluted in blocking buffer.

Negative controls were established by incubating cells solely with the secondary antibody. Staining was verified via fluorescence microscopy (microscope: Leica DMI 6000B, Leica Microsystems; camera: CoolSNAP\(_{HQ}\), Photometrics).
Stable knockdown of αENaC in endothelial cells using RNAi

We used 60 nt oligonucleotides with 19 nt target sequence (metabion, Martinsried, Germany). The sense target sequence sense is highlighted in red, the antisense target sequence in blue. The resulting transcript folds back on itself to form a 19 base pair stem-loop structure. This is quickly cleaved in the cell to produce functional siRNA:

1. siαENaC A
forward: 5’-GATCCCCTGCTATCGCGACAGAACAATTCAAGAGATTGTTCTGTGCAGTAGCATTTTTTA-3’;
reverse: 3’-GGGACGATAGCGCTGTCTTGTTAAGTTCTCTAAACAAGACAGCGCTATCGTAAAAATTCGA-5’

2. siαENaC B
forward: 5’-GATCCCCCTTACACCGTCAACAACAAAGTTCAAGAGACTTGTTGTTGACGGTGTAATTTTTTA-3’;
reverse: 3’-GGGAATGTGGCAGTTGTTGTTCAAGTTCTCTGAACAAACACTGCCACATTAAAAATTCGA-5’

Atomic Force Microscopy (AFM) Measurements

The spherical AFM tip (Ø = 10 µm; Novascan), mounted to a highly flexible cantilever (MLCT contact microlever, nominal spring constant: 0.01 N/m; Bruker) is gradually lowered onto the cell and indents the cell membrane upon contact. The resulting deflection of the flexible cantilever is measured via reflection of a laser beam from its gold-coated backside. Cantilever deflection can thus be plotted as a function of tip position along the z axis. In order to quantify cell stiffness, the obtained data is transformed into a force versus distance curve, using the cantilever’s spring constant and the optical cantilever sensitivity. The slope of this force-distance curve then directly reflects the force (in pico/nano Newton), here defined as stiffness, that has to be exerted to indent the cell for a certain distance. In figure 1B it is further illustrated that the first slope of a force-distance curve derived from a stiff cell is visibly steeper than the one found in soft, more deformable cells. Depending on the depth of indentation, two or more slopes can be identified in each curve. The first rather flat slope (indentation depth of 50-150 nm), ignoring the very soft endothelial glycocalyx, reflects the stiffness of the plasma membrane and the submembranous cortex, including the cortical cytoskeleton, whereas the later and steeper slope corresponds to cell center stiffness. All experiments in the present study focused on alterations in the mechanical properties of the cell cortex. Measurements were conducted in HEPES buffer (in mmol/L: 140 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 5 glucose, 10 HEPES (4-(2-hydroxyethyl)-1-piperazinedesulfonic acid)).
Data analysis and statistics
Western blots were quantitatively analyzed with the ImageJ software using the gel analysis tool. Calculated values for the protein of interest were corrected for values of the loading control (GAPDH). Significance of difference in the statistics was determined using ANOVA. All mentioned data were calculated as mean (±standard deviation).

REFERENCES

FIGURES AND SUPPORTING INFORMATION

A

I..

II..

III. [Image of a sample with a scale of 0.1 mm]

IV. [Image of a sample with a scale of 30 µm]

B

1. approach

2. contact

3. indent

Distance (nm)

Force (pN)

C

I. stiff cell

II. soft cell

Distance (nm)

Force (pN)
Fig. S1: Preparation of mice aortae and AFM nanoindentation measurements. (A)(I) Mice aortae were sliced into equally sized “rings” (II) By opening the “rings” with a single cut, the endothelium was exposed. (III) Dissected pieces of aortae were immobilized on glass. (IV) Immunostaining for the endothelium specific VE-cadherin shows a confluent monolayer of endothelial cells. (B) The spherical AFM tip approaches the cell as it is vertically lowered. Upon contact with the cell surface, the cantilever starts to bend and continues to do so as the cell is further indented. This deflection of the cantilever is detected by a laser beam reflected from the back of the cantilever. The slope of the cantilever deflection then enables assessment of cell stiffness. (C) Original tracings of force vs. distance curves obtained from (I) stiff and (II) soft cells. The first slope of the curve corresponds to the cell cortex, the second slope to the phase of the cell.
Fig. S2: Validation of siRNA-mediated αENaC-knockdown on the protein level. (A) Representative western blot of αENaC whole-cell expression in siαENaC-B (siB), siαENaC-A (siA) and mock endothelial cells and M-1 epithelial cells. The ~76 kDa band, corresponding to full-length αENaC, was detected in endothelial and epithelial cells. The ~58 kDa band corresponds to a cleaved form of αENaC, which is most likely endothelium-specific as there is no clear signal in the line of the M-1 epithelial cells. (B) Corresponding quantitative analysis of Western blot signal intensities (normalized to GAPDH loading control) of mock, siαENaC-A and siαENaC-B endothelial cells (N=6). Whole-cell αENaC expression was reduced by about 52% (siαENaC-A) and 28% (siαENaC-B) compared to mock cells (endothelial cells stably transfected with an empty vector). Values are a mean of the reduction in the 76 kDa and the 58 kDa protein band.
Fig. S3: αENaC surface abundance and cortical stiffness of a second αENaC-knockdown endothelial cell line. (A) QD-immunostaining for αENaC on the surface of siαENaC-B cells under aldosterone-treated conditions. (B) Staining quantification: αENaC surface density is significantly reduced in siαENaC-B (79.3±2.99 QD/1000μm²; -12%) cells compared to mock cells (90.4±2.33 QD/1000μm²) under aldosterone-treated conditions. Additional stimulation with spironolactone significantly decreases the αENaC surface abundance in both cell lines to 55.6±1.90 QD/1000μm² (-38%, mock) and 59.5±2.27 QD/1000μm² (-25%, siαENaC-B). Under spironolactone treatment, we detected no significant difference between the two cell lines. (C) siαENaC-B cells display a significantly lower cortical stiffness (1.17±0.01 pN/nm; -13%) than mock cells (1.35±0.01 pN/nm) under aldosterone-treated conditions. Additional application of amiloride leads to significant reductions in the cortical stiffness of mock cells (1.01±0.02 pN/nm; -25%) and siαENaC-B cells (0.84±0.01 pN/nm; -28%).
Fig. S4: Validation of QD-immunostaining for αENaC on the apical surface of epithelial cells. (A) QD-immunostaining for αENaC on the apical surface (focus plane) of M-1 cortical duct epithelial cells. (B) Control staining without primary antibody. Actin (phalloidin) and the nucleus (DAPI) were simultaneously stained.