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Hypertension. 2009;53:1000-1007; originally published online April 27, 2009;
doi: 10.1161/HYPERTENSIONAHA.108.128520
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0194-911X. Online ISSN: 1524-4563

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Abstract—Recent studies have shown that the epithelial sodium channel (ENaC) is expressed in vascular tissue. However, the role that ENaC may play in the responses to vasoconstrictors and NO production has yet to be addressed. In this study, the contractile responses of perfused pressurized small-diameter rat mesenteric arteries to phenylephrine and serotonin were reduced by ENaC blockade with amiloride (75.1±3.2% and 16.9±2.3% of control values, respectively; $P<0.01$) that was dose dependent ($EC_{50}=88.9\pm1.6$ nmol/L). Incubation with benzamil, another ENaC blocker, had similar effects. α, β, and γ ENaC were identified in small-diameter rat mesenteric arteries using RT-PCR and Western blot with specific antibodies. In situ hybridization and immunohistochemistry localized ENaC expression to the *tunica media* and endothelium of small-diameter rat mesenteric arteries. Patch-clamp experiments demonstrated that primary cultures of mesenteric artery endothelial cells expressed amiloride-sensitive sodium currents. Mechanical ablation of the endothelium or inhibition of eNOS with N^{ω}-nitro-L-arginine inhibited the reduction in contractility caused by ENaC blockers. ENaC inhibitors increased eNOS phosphorylation (Ser 1177) and Akt phosphorylation (Ser 473). The presence of the phosphoinositide 3-kinase inhibitor LY294002 blunted Akt phosphorylation and eNOS phosphorylation and the decrease in the response to phenylephrine caused by blockers of ENaC, indicating that the phosphoinositide 3-kinase/Akt pathway was activated after ENaC inhibition. Finally, we observed that the effects of blockers of ENaC were flow dependent and that the vasodilatory response to shear stress was enhanced by ENaC blockade. Our results identify a previously unappreciated role for ENaC as a negative modulator of eNOS and NO production in resistance arteries. (***Hypertension*****. 2009;53:1000-1007.)**

Key Words: NO synthase ■ aldosterone ■ endothelium-derived factors

The epithelial sodium channel (ENaC) is present in distal nephron segments and the colonic epithelium, where it mediates electrogenic Na^+ influx across the apical membrane.[1–3] ENaC belongs to the degenerin/ENaC family of proteins and can be reversibly inhibited by the pyrazine diuretics, eg, amiloride and benzamil.[1–3] In rat epithelial tissue, ENaC is composed of 3 homologous subunits α, β, and γ.[4] Recent studies have shown that ENaC subunits are also present in arteries[5–7]; however, specific functions that may be attributed to vascular ENaC have only recently begun to be investigated.

Vascular smooth muscle cells from rat cerebral arteries and mouse kidney arteries express β and γ ENaC,[5,8] and the activation of ENaC present in vascular smooth muscle cells has been implicated in the vasoconstrictor response to elevated pressure.[9] On the other hand, several studies have indicated that ENaC may be present in vascular endothelium. ENaC α mRNA transcripts and protein were identified in cultured human umbilical vein endothelial cells,[6] and the activity of ENaC in human umbilical vein endothelial cell has been shown to mediate cell swelling[7,10] and plasma membrane stiffening.[11] Studies in oocytes have demonstrated that ENaC activity is modulated by laminar shear stress,[12] and it has been suggested that endothelial ENaC activity is inhibitory on NO production stimulated by shear stress.[11] However, the role of endothelial ENaC in arteries has yet to be investigated.

Here we analyzed the expression of ENaC in rat small-diameter rat mesenteric arteries (SDMAs) and rat endothelial cell primary culture. The role of ENaC in the contractile response to serotonin and phenylephrine was analyzed in isolated SDMAs in the presence of pharmacological ENaC inhibitors amiloride and benzamil. To address the relevance of the endothelium in the response to ENaC blockade, intact

Received December 24, 2008; first decision January 7, 2009; revision accepted March 31, 2009.

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Hypertension **is available at http://hyper.ahajournals.org** **DOI: 10.1161/HYPERTENSIONAHA.108.128520**

arteries and endothelium-denuded arteries were studied. Finally, we analyzed endothelial NO synthase (eNOS), the signaling pathways modulated by ENaC activity, and the role of ENaC in shear stress–induced vasodilation in SDMAs.

Methods

All of the methods are described in detail in the online data supplement at http://hyper.ahajournals.org.

Animals

Arteries were obtained from adult male (180 to 240 g) Sprague-Dawley rats. Protocol was approved by the ethics committee of the faculty of medicine at the Universidad de Chile, according to National Institutes of Health Guide for the Care and Use of the Laboratory Animals.

SDMA Isolation

Second-order mesenteric arteries were isolated and used for biochemical or functional studies.[13]

Contractility Studies

Arteries were cannulated, perfused, and pressurized, as described previously.[13,14] External artery diameter was determined by videomicroscopy. Vasodilatory responses to shear stress were measured according to Matrougui et al.[15] Isometric tension studies were performed according to Rahman et al.[16] Vasoconstrictors were tested after 10 minutes of incubation in the presence of inhibitors (ENaC or phosphoinositide 3-kinase [PI3K]) or vehicle (control). eNOS inhibitor was added 5 minutes before ENaC inhibitors and maintained throughout the experiment.

Fluorometric Determination of NO

NO production in perfused SDMA was estimated by 4-amino-5-metilamino-2′,7′-difluorofluorescein diacetate-AM fluorescence (Molecular Probes).

RT-PCR

Kidney and mesenteric arteries were processed for total RNA extraction,[14] and PCRs were carried out with specifics primers for rat ENaC subunits.

Western Blot Analysis for ENaC, eNOS, and Akt

Total protein fractions from SDMAs were prepared and used for Western blotting with specific antibodies.[13,17]

In Situ Hybridization

Tissue sections were hybridized with 400 pmol/mL of digoxigenin-labeled probes and developed with antibody anti–digoxigenin-alkaline phosphatase F′ab fragments (Roche).

Immunohistochemistry

SDMA 4-μm sections were incubated with anti-rat ENaC subunits, and the immunoreaction was visualized with 3,3-diamino-benzidine.

Primary Culture of Mesenteric Artery Endothelial Cells

Endothelial cells were isolated by collagenase digestion from rat mesenteric arteries as described previously.[18]

Patch-Clamp Studies

Standard whole-cell patch-clamp recordings in primary cultures of mesenteric artery endothelial cells were performed as described.[19]

Statistical Analysis

Values are expressed as mean±SE. Statistical comparisons were performed using 1-way ANOVA, ANOVA for repeated measurements (followed by Bonferroni-Dunn posthoc test), or unpaired Student *t* test when appropriate. The EC_{50} of the vasoconstrictor was determined for each curve using a nonlinear least-square fitting procedure for data from individual experiments (GraphPad Prism, GraphPad Software). Differences were considered statistically significant for $P<0.05$.

Results

The role of ENaC in the response of SDMAs to phenylephrine was studied in isolated perfused (15 μL/min) and pressurized arteries. Vasocontraction was detectable with 0.1 μmol/L of phenylephrine (Figure 1A) and reached maximum values at 100 μmol/L (E_{max}: 83.6±1.1 μmol/L; Figure 1B; please see video file in the online data supplement). The presence of 500 nmol/L of amiloride starting 10 minutes before phenylephrine decreased the E_{max} to 24.9% of the control value (Figure 1A and 1B; E_{max}: 22.6±0.7 μmol/L; n=6; $P<0.01$). Amiloride also increased the EC_{50} for phenylephrine (1.9±0.8 μmol/L in vehicle-treated arteries versus 6.3±0.4 μmol/L in amiloride-treated arteries; n=6; $P<0.01$).

In a separate set of experiments, we analyzed the dose-response curve of SDMA to amiloride, which was added 10 minutes before the stimulus with a maximal dose of phenylephrine. The maximal effect of amiloride was attained at 1 μmol/L with an EC_{50} of 88.9±1.6 nmol/L (please see http://hyper.ahajournals.org), consistent with ENaC inhibition.[1,2] In addition, an amiloride analog specific to the Na^+/H^+ antiporter (500 nmol/L of ethyl isopropyl amiloride) had no effect in the response of SDMAs to phenylephrine (please see the data supplement).

To test whether ENaC activity could also modulate the response to other vasoactive substances, the effect of amiloride on serotonin-induced constriction was tested (Figure 1C and 1D). Under control conditions, E_{max} was reached at a concentration of 100 μmol/L of serotonin (72.4±3.4 μm), and the EC_{50} of serotonin was 0.49±0.1 μmol/L. Similar to the response to phenylephrine, amiloride significantly reduced the contractile response to serotonin (E_{max}: 60.1±2.3 μm; n=6; $P<0.01$) and increased the EC_{50} to 2.9±0.3 μmol/L (n=6; $P<0.01$).

The incubation of SDMA in the presence of benzamil, another ENaC blocker, also reduced the contractile response to phenylephrine. As shown in Figure 1E, the E_{max} to phenylephrine decreased (25.3% of control value; n=6; $P<0.01$), and the EC_{50} shifted to the right (4.0±0.1 μmol/L; $P<0.01$). Contraction elicited by serotonin (Figure 1F) was also affected by benzamil, as shown by a shift to the right of the dose-response curve to serotonin (EC_{50}: 1.8±0.2 μmol/L; n=6; $P<0.01$). As in the case of amiloride, the dose-response curve to benzamil suggested ENaC blockade, with an EC_{50} of 8.2±0.2 nmol/L (please see the data supplement).

To assess expression of ENaC subunits in SDMAs, total RNA samples obtained from isolated arteries were analyzed by RT-PCR. Using primers specific for α, β, and γ ENaCs, all 3 of the ENaC subunit transcripts were detected (Figure 2A). Consistent with the RT-PCR results, α, β, and γ ENaC transcripts were detected in endothelial cell mesenteric arteries by in situ hybridization (Figure 2B). Labeling for all 3 of the subunits was also observed in smooth muscle cells, although with lower intensity for β ENaC. The presence of α,

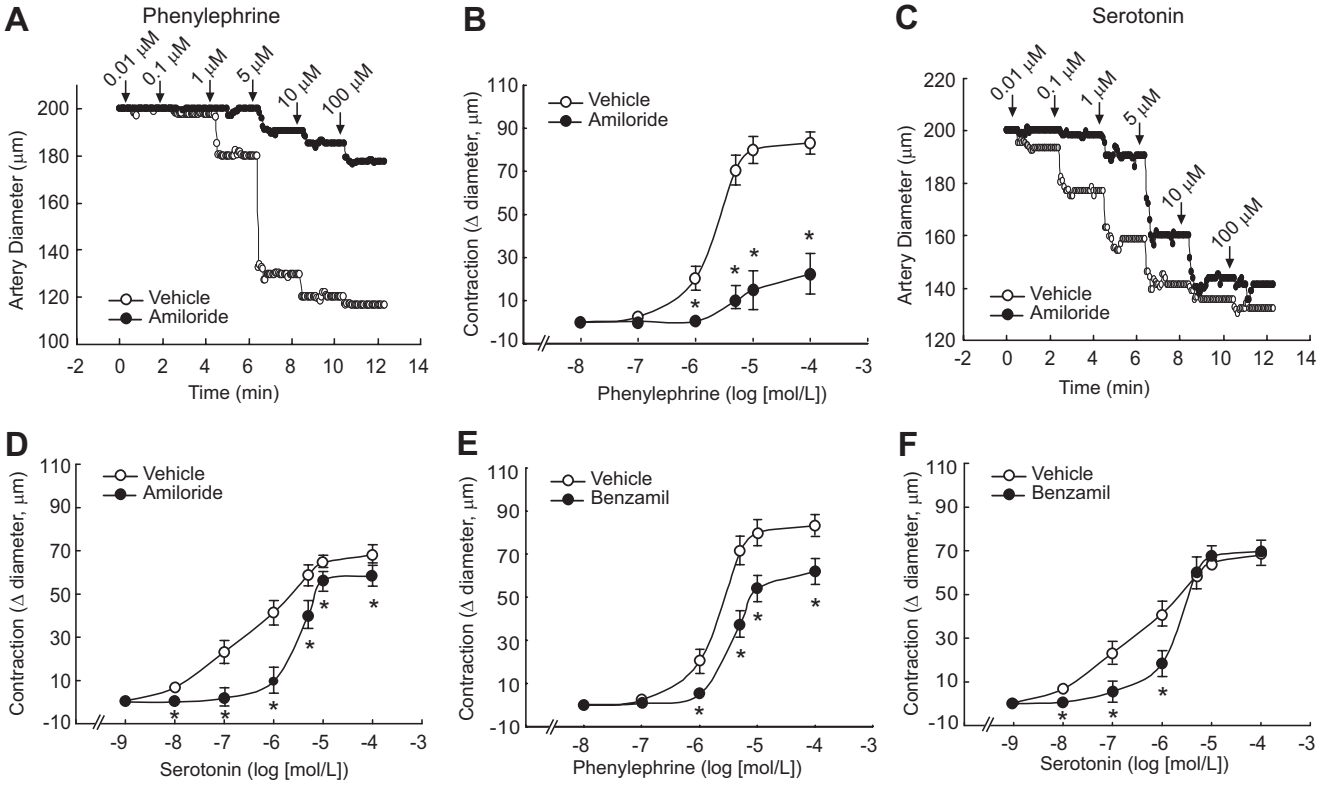

Figure 1. ENaC blockers decrease the contractile response to phenylephrine and serotonin. Representative concentration-response curves to phenylephrine (A) or serotonin (C). Videomicroscopy images were recorded every 4 seconds to measure arterial diameter. Small-diameter mesenteric arteries were incubated in the presence of 500 nmol/L of amiloride (●) or vehicle (○) 10 minutes before the addition of increasing concentrations of vasoconstrictor (added every 2 minutes). Dose-response curves for phenylephrine (B) or serotonin (D). Concentration-response curves for phenylephrine (E) or serotonin (F) in arteries incubated in the presence of vehicle (○) or 1 μmol/L of benzamil (10 minutes before serotonin; ●). Circles represent mean±SEM of the difference in diameter; *$P<0.01$; n=6.

β, and γ ENaC proteins in small diameter arteries was confirmed by Western blot analysis with anti-ENaC–specific antibodies (Figure 2C). Finally, immunohistochemical analysis of SDMA sections identified expression of α, β, and γ ENaC proteins in mesenteric endothelium and vascular smooth muscle cell (please see the data supplement).

The decrease in the contractile response observed on ENaC inhibition could have been attributed to the inhibition of the

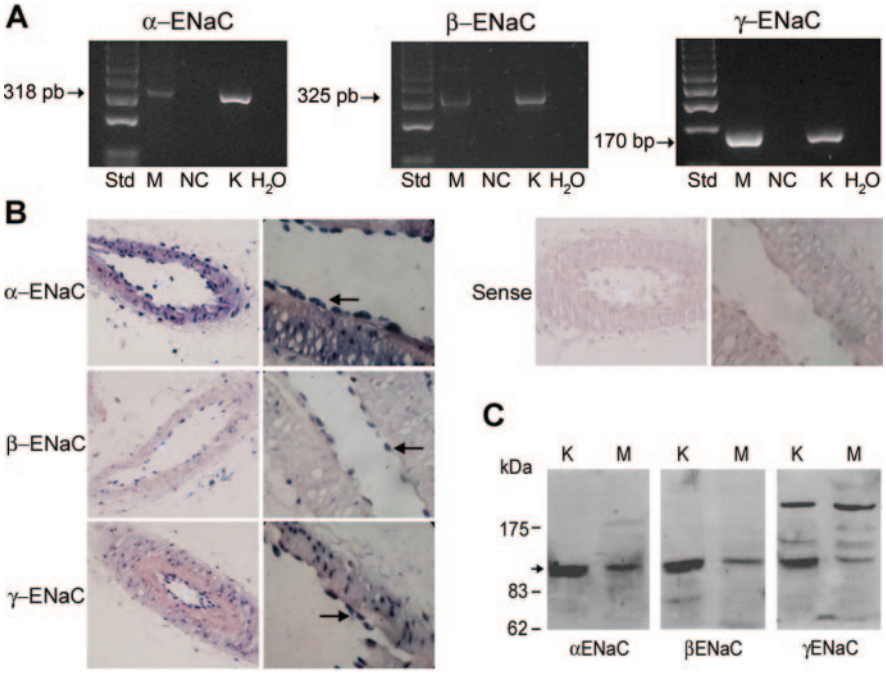

Figure 2. Expression of ENaC subunits in rat resistance arteries. Total RNA was extracted from small-diameter mesenteric arteries (M) and used for RT-PCR experiments. A, Agarose gel electrophoresis of PCR products. Positive control corresponds with PCR using kidney cDNA (K) as a template. Nontranscribed total RNA (NC) and water were used as negative controls. Products of the expected size were obtained when using α, β, and γ ENaC-specific primers (indicated by arrows) with mesenteric artery and kidney cDNAs. B, In situ hybridization to detect α, β, and γ ENaC mRNA in mesenteric arteries. Bright-field micrographs are shown at ×40 and ×100 magnification. Sections of mesenteric arteries were hybridized using the different dUTP probes, as indicated. C, Total protein homogenates from kidney (positive control) and small-diameter mesenteric artery were separated by SDS-PAGE. Western blot analysis with anti-α, anti-β, and anti-γ ENaC demonstrated the presence of the 3 ENaC subunits in the mesenteric artery.

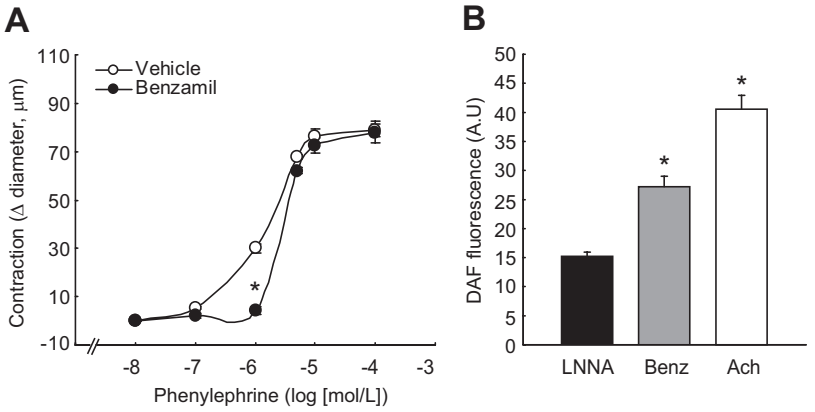

Figure 3. Role of the endothelium in the effect of benzamil on resistance arteries. A, Concentration-response curves of endothelium-denuded, small-diameter mesenteric arteries incubated in the presence of vehicle (○) or benzamil (●) and tested with increasing concentrations of phenylephrine. Circles represent mean±SEM; *$P<0.01$; n=6. B, Arterial NO production was estimated by 4-amino-5-methylamino-2′,7′-difluorescein fluorescence. Perfused and pressurized small-diameter arteries were treated with 100 μmol/L of L-NNA (■), 1 μmol/L of benzamil (▨), or 1 μmol/L of acetylcholine (Ach; positive control). Bars represent mean±SEM; *$P<0.05$; n=4.

channel in either endothelial and/or vascular smooth muscle cells. To distinguish between these possibilities, phenylephrine dose-response curves were measured for small diameter arteries after mechanical ablation of the endothelium. This procedure neither altered the dose-response curve to phenylephrine nor vasodilation in response to acetylcholine (please see the data supplement). However, the inhibitory action of benzamil on the phenylephrine concentration-response curve was almost undetectable in endothelium-denuded arteries (Figure 3A). Benzamil had no effect on E_{max}, and only vasoconstriction in response to low concentration of phenylephrine was reduced by the ENaC blocker. Similarly, amiloride had no effect on phenylephrine-evoked vasoconstriction after endothelium ablation (data not shown). These data suggest that reduced contractile response observed after blocking ENaC could be because of an endothelium-derived vasodilator.

To evaluate the possibility that NO might be involved in vasoconstriction, the NO-fluorescent indicator 4-amino-5-metilamino-2′,7′-difluorofluorescein diacetate-AM fluorescence was used in perfused arteries. After loading with the fluorophore, arteries were incubated either in the presence of benzamil and phenylephrine or the eNOS inhibitor N^{ω}-nitro-L-arginine (L-NNA) and phenylephrine. A significant increase in 4-amino-5-methylamino-2′,7′-difluorescein fluorescence was observed in benzamil-treated arteries (Figure 3B), consistent with the interpretation that NO was being produced after ENaC inhibition. In addition, the perfusion of endothelium-intact arteries with L-NNA (100 μmol/L) for 5 minutes before assessing the effect of amiloride or benzamil on the phenylephrine dose-response curves (Figure 4A and 4B) greatly diminished the effect of ENaC blockers. Similarly, L-NNA prevented the inhibitory effect of amiloride or benzamil on serotonin-induced contraction (Figure 4C and 4D).

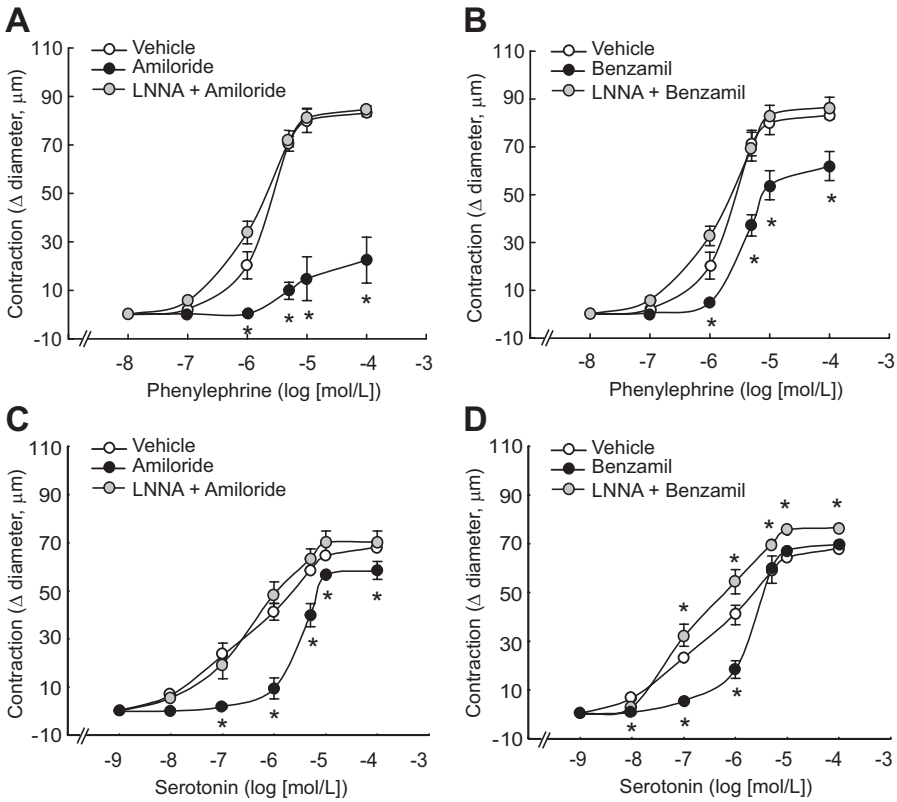

Figure 4. Inhibition of eNOS with L-NNA diminishes the effect of amiloride and benzamil on phenylephrine and serotonin-induced contraction and NO production. A, Arteries were incubated in the presence of 500 nmol/L of amiloride (●), 500 nmol/L of amiloride plus 100 μmol/L of L-NNA (◍), or vehicle (○) and tested with increasing concentrations of phenylephrine. B, Small-diameter mesenteric arteries were incubated in the presence of 1 μmol/L of benzamil (●), 1 μmol/L of benzamil plus 100 μmol/L of L-NNA (◍), or vehicle (○) and tested with increasing concentrations of phenylephrine. Similar effects were observed for the serotonin concentration-response curves in the presence of amiloride (C) or benzamil (D). Circles represent mean±SEM; *$P<0.01$; n=6.

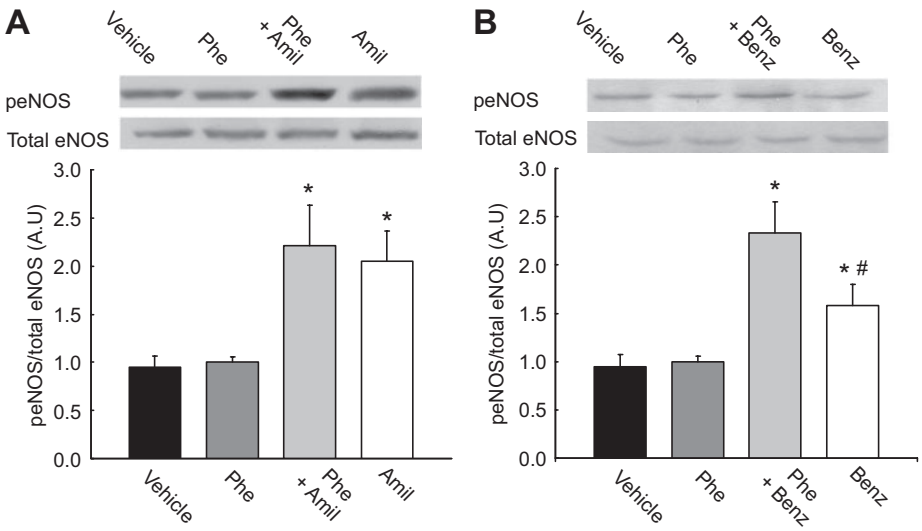

Figure 5. Benzamil and amiloride increase eNOS Ser 1177 phosphorylation. Small-diameter mesenteric arteries were incubated in the presence of 100 μmol/L of phenylephrine (Phe), 100 μmol/L of Phe plus 0.5 μmol/L of amiloride (Amil), 0.5 μmol/L of Amil, or vehicle (A) or 100 μmol/L of Phe, 100 μmol/L of Phe plus 1 μmol/L of benzamil (Benz), 1 μmol/L of Benz, or vehicle (B). After 10 minutes, total protein homogenates were prepared and analyzed by Western blot to detect peNOS and total eNOS. Top, Representative result. Bottom, mean±SEM of peNOS/total eNOS densitometry analysis; *$P<0.05$; n=4.

The presence of amiloride-sensitive Na^+ currents in the endothelium was studied by whole-cell patch clamp in primary cultures of mesenteric artery endothelial cells. Endothelial cell primary cultures expressed α, β, and γ ENaC transcripts, as demonstrated by RT-PCR (please see http://hyper.ahajournals.org). Currents elicited by voltage pulses between −80 and +80 mV are shown in the online Data Supplement. Using KCl-rich intracellular solution and an NaCl-rich extracellular solution, under this condition were recording inward and outward currents with a reversal potential of −16.7±3.2 mV (n=27). Replacing extracellular Na^+ with *N*-methyl-D-glucamine chloride markedly decreased inward currents (please see the data supplement) and shifted the reversal potential to approximately −22.8±2.6 mV (n=3). The addition of 500 nmol/L of amiloride to the medium led to an inhibition by ≈60% to 70% of the total current at −80 mV (please see http://hyper.ahajournals.org). The amiloride concentration-dependence studies showed an EC_{50} of 5.4±0.04 nmol/L of amiloride. These results support ENaC as being active in endothelial cells.

To gain further insight into the mechanisms underlying NO production after ENaC inhibition, we evaluated the possibility that eNOS was being activated by protein kinase B–dependent phosphorylation at serine 1177. Protein kinase B is activated through recruitment to the cellular membrane by PI3K lipid products and phosphorylation by 3′-phosphoinositide-dependent kinase 1. Western blotting experiments with total protein homogenates obtained from SDMAs showed that ENaC inhibition with amiloride or benzamil for 10 minutes produced a significant increase in eNOS phospho-Ser 1177 (Figure 5). The increase of eNOS phosphorylation at Ser 1177 was also observed when phenylephrine was present in the perfusion media. To evaluate Akt activation after ENaC blockade, the levels of phospho-Akt were determined. Incubation of arteries in the presence of phenylephrine plus benzamil caused a significant increase in phospho-Akt levels (Ser 473; Figure 6A) that was blocked in the presence of the LY294002, a PI3K inhibitor. Benzamil-induced eNOS phosphorylation was also blocked by LY294002 (Figure 6B).

On the basis of the results described above, we hypothesized that pharmacological inhibition of PI3K should also be effective in preventing reduced responsiveness to vasoconstrictors in the presence of ENaC blockers. As expected, the presence of LY294002 in the perfusion solution greatly reduced the ability of benzamil to inhibit phenylephrine-induced contraction (Figure 6C). In addition, LY294002 also prevented the increase in phenylephrine EC_{50} and the reduction in phenylephrine E_{max} caused by amiloride (Figure 6D).

ENaC has been implicated as part of flow sensor mechanisms,[11,12] and shear stress activates eNOS. We evaluated whether the reduction of intraluminal flow modulated the action of ENaC blockers in contractility evoked by phenylephrine. The magnitude of the effect of both amiloride and benzamil decreased at lower flow (Figure 7A), and in the absence of flow (isometric tension studies) we observed no significant effect of amiloride (Figure 7B). Finally, to test whether ENaC inhibition enhanced the vasodilatory response to shear stress, we measured flow-induced dilation in the presence of amiloride. Flow pressure-diameter relationships were established by step increases in flow (4 to 40 μL/min) to SDMAs. As shown in Figure 7C, external diameter increased when flow was raised, and this effect was increased by amiloride. The pressure-diameter relationships showed that, in amiloride-treated arteries, the diameter was significantly greater than in vehicle-treated arteries for similar pressures (Figure 7D).

Discussion

In the present study, we confirmed ENaC expression in SDMAs, and we found that ENaC blockers reduced the contractile response to phenylephrine and serotonin. The EC_{50} values obtained in dose-response experiments with

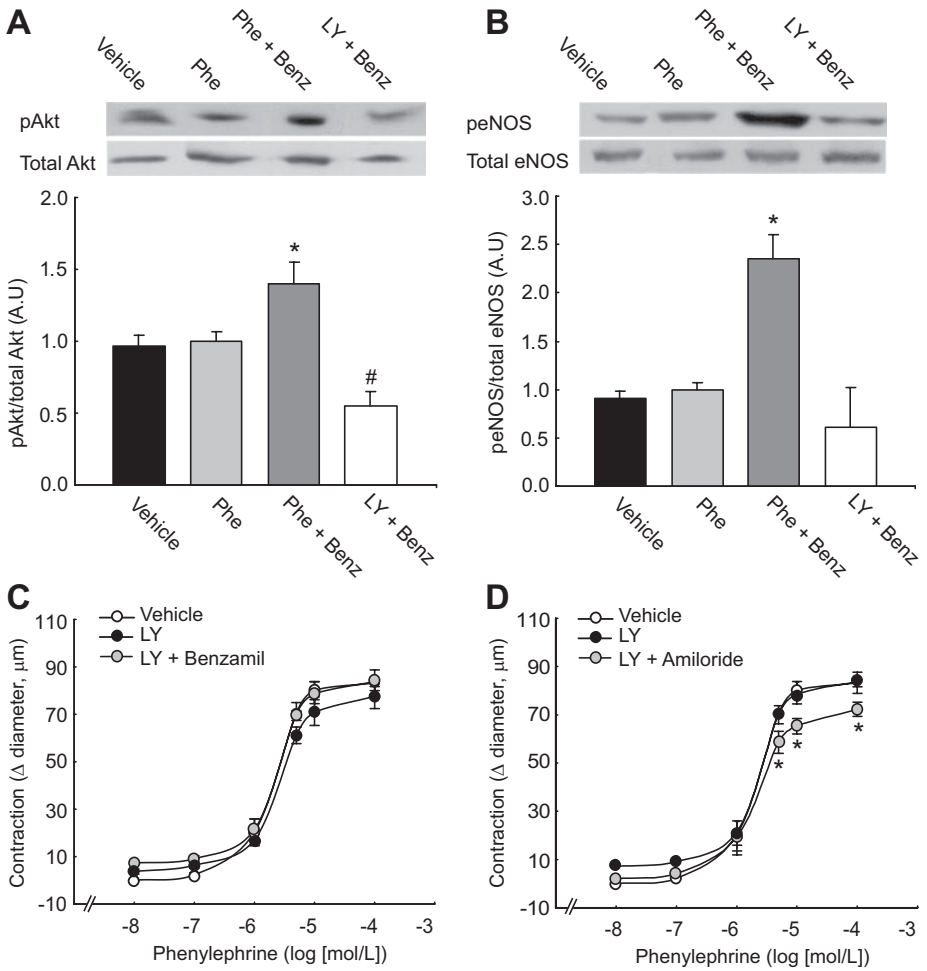

Figure 6. The PI3K/Akt pathway mediates the reduction of the contractility caused by ENaC blockers. SDMAs were incubated in the presence of 100 μmol/L of phenylephrine (Phe), 100 μmol/L of Phe plus 1 μmol/L of benzamil (Benz), 1 μmol/L of Benz plus LY294002, or vehicle. After 10 minutes, total protein homogenates were prepared and analyzed by Western blot to detect pAkt and total Akt (A). The same protein homogenates were used for peNOS and total eNOS measurements by Western blot (B). Top, Representative results. Bar graphs depict phosphorylated/total mean±SEM from 4 independent experiments. *$P<0.05$ vs vehicle, n=4. C, SDMAs were incubated in the presence of 10 μmol/L of LY294002 (●), 10 μmol/L of LY294002 plus 1 mmol/L of benzamil (◍), or vehicle (○). Control arteries were incubated in the presence of vehicle (○). D, Arteries were incubated in the presence of LY294002 compound (●) and tested in the presence of amiloride (◍). Circles represent mean±SEM from 6 independent experiments; *$P<0.05$ vs vehicle; n=5.

pharmacological ENaC inhibitors are similar to the published inhibition constant and IC_{50} values for ENaC in mammalian distal nephron segments and *Xenopus* oocytes expressing α, β, and γ ENaC (amiloride: 10 to 100 nmol/L; benzamil: 10 nmol/L).[1,2] In addition, an amiloride derivative (ethyl isopropyl amiloride) did not affect the contractile response to phenylephrine. In situ hybridization and immunohistochemistry studies showed α, β, and γ ENaC expression in the tunica media and endothelial cells of SDMAs. In addition, amiloride-sensitive sodium currents were detected in primary cultures of mesenteric artery endothelial cells. These data indicate expression of all 3 of the ENaC subunits in SDMAs that would form trimeric channels with biophysical properties similar to the channels present in kidney tubules.[1–3] However, our experiments also show that the efficacy of amiloride is higher than the efficacy of benzamil, as evidenced by the higher E_{max} observed in arteries treated with amiloride. This could be indicative of the presence of nonclassical ENaC channels.

In experiments with endothelium-denuded arteries, we observed a strong reduction in the ability of ENaC blockers to reduce the vasoconstriction. Benzamil treatment stimulated production of the NO, and pharmacological inhibition of eNOS greatly decreased the ability of amiloride and benzamil to reduce the response to vasoconstrictors. We conclude that reduced contractile responses observed on blocking ENaC are endothelium dependent and require eNOS activation, as well as NO production.

Our studies revealed a significant increase of eNOS phosphorylation at Ser 1177 after ENaC inhibition, implying the PI3K/Akt pathway as the mechanism leading to eNOS phosphorylation. Pharmacological inhibition of PI3K with LY294002 was effective in reducing both Akt phosphorylation and eNOS phosphorylation. Moreover, PI3K inhibition was also effective in preventing the reduction in the contractile response to phenylephrine and serotonin caused by ENaC inhibitors. These results indicated that the endothelial PI3K/Akt pathway is necessary to evoke eNOS activation after

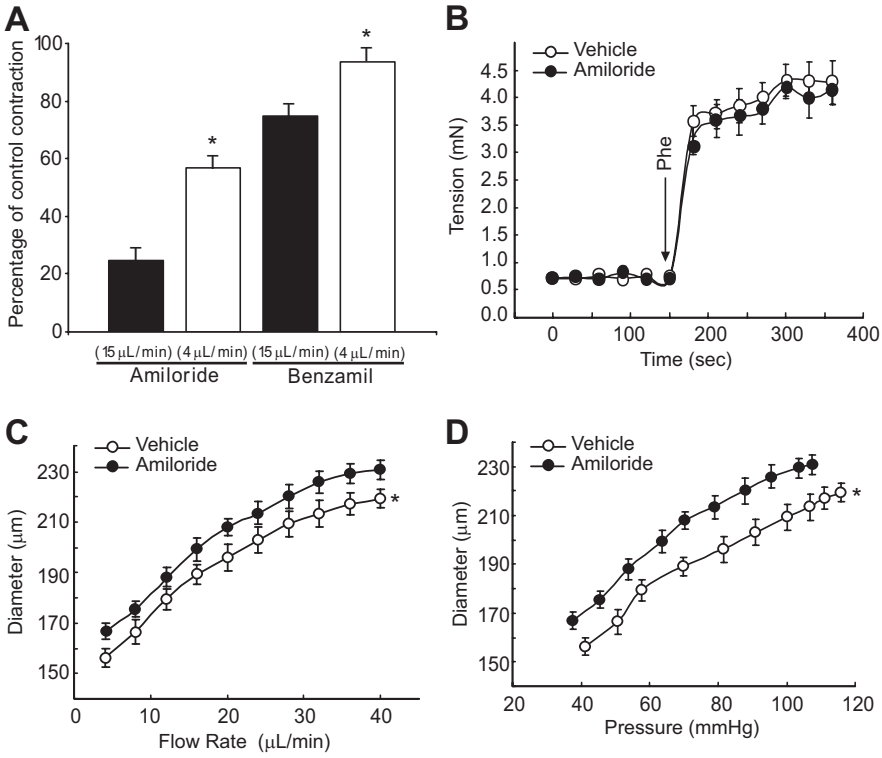

Figure 7. The response to ENaC blockers is flow dependent. A, SDMAs perfused with intraluminal flow of 15 μL/min or 4 μL/min; 500 nmol/L of amiloride or 1 μmol/L of benzamil was added for 10 minutes. Thereafter, 100 μmol/L of phenylephrine was added to the bath. Bars represent the average change in diameter±SE, expressed as the percentage of diameter change in the presence of vehicle (control: 100%) or ENaC blocker. B, Isometric tension studies (absence of flow) of SDMAs treated for 10 minutes with amiloride (●) or vehicle (○). Arrow indicates the addition of 100 μmol/L of phenylephrine. Changes in SDMA diameter (C) and pressure-diameter relationship (D) in response to step increments of perfusion flow (4 to 40 μL/min) in the presence of amiloride or vehicle. n=5; $P<0.05$ vs vehicle.

ENaC inhibition. Phosphorylation of eNOS at Ser 1177 enhances electron flux through the eNOS reductase domain by reducing calmodulin dissociation from the enzyme when calcium concentrations are low.[20] Therefore, ENaC blocking should decrease the dependence of eNOS activation on cytosolic calcium.

We observed that ENaC inhibition in SDMAs caused increased eNOS phosphorylation and NO production. Shear stress is the most potent physiological stimulus for NO production in endothelial cells.[21,22] The phosphorylation of eNOS at Ser 1177 by a sequential activation of PI3K and Akt pathway is one of the mechanisms by which shear stress can stimulate NO production.[23] In the present study, we observed that the action of ENaC blockers depended on intraluminal flow. In addition, vasodilatory response to shear stress was enhanced by amiloride. These observations support the notion that ENaC activity reduces endothelial PI3K/Akt activation by shear stress and subsequent phosphorylation of eNOS.[23]

Oberleithner et al,[11] using atomic force microscopy in human umbilical vein endothelial cells, demonstrated that small increments of extracellular [Na^+] under isotonic conditions increased cell volume and cell stiffening. These changes were amiloride sensitive, suggesting that ENaC mediated sodium influx. Furthermore, increased sodium influx resulted in decreased nitrite concentration in the culture medium of endothelial cells,[11] suggesting that ENaC activity could reduce endothelial NO production in response to shear stress and/or blood flow because of a loss of the cell's deformability. An alternative mechanism that may contribute to the effects of ENaC inhibitors in small-diameter mesenteric arteries is membrane hyperpolarization.[24] Endothelial cell membrane hyperpolarization is known to increase eNOS activity.[25] Under physiological conditions, ENaC in endothelial cells would have a depolarizing effect, acting as a negative modulator of eNOS activity in response to shear stress.

Perspectives

Our studies indicate that endothelial ENaC is a negative modulator of eNOS and vasodilation in response to shear stress. Endothelial cells express mineralocorticoid receptors,[26] and aldosterone decreases endothelial NO output.[27] Blocking of the mineralocorticoid receptor increases NO bioavailability and improves the impaired endothelial function in hypertension, atherosclerosis, myocardial infarction, and heart failure.[28–31] Aldosterone upregulates ENaC expression and activity in kidney collecting duct cells,[1–3] and increases the expression of α ENaC in human umbilical vein endothelial cells.[10] Therefore, in vivo, aldosterone may act directly in the endothelium by promoting ENaC expression and activity, contributing to endothelial dysfunction. Recently, mineralocorticoid receptor–dependent downregulation of eNOS phosphorylation (Ser 1177) in the endothelium has been reported.[27,31] Thus, further studies will be needed to clarify the relevance of endothelial ENaC in mediating the deleterious effects of aldosterone on eNOS.

Acknowledgments

We thank Dr Andrew Quest and Peter W. Murphy for their editorial advice, as well as Andrea Birkner her for technical assistance.

Sources of Funding

This work was supported by Fondo Nacional de Desarrollo Cientifico y Tecnológico grant 1050690, Fondo de Areas Prioritarias 15010006, and Millennium Nucleus on Immunology and Immunotherapy (P04/030-F).

Disclosures

None.

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Online Supplement

Endothelial ENaC Inhibition Activates eNOS via PI3K/Akt in Small Diameter Mesenteric Arteries

by

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Running Title: Endothelial ENaC inhibits eNOS

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Material and Methods

Small diameter mesenteric artery isolation: Arteries were isolated and used for biochemical or functional studies.[13] Rats received heparin (1250 UI intra-peritoneal injection). After euthanasia, the superior mesenteric artery was removed and placed in ice-cold (4°C) physiological Krebs-Ringer bicarbonate, containing (mmol/L): 120 NaCl, 4.2 KCl, 1.18 KH_2PO_4, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 25 Na_2CO_3, 5 D-glucose and 1.2 pyruvate, equilibrated with 95% O_2/5% CO_2 (pH 7.4). The tissue was cleaned of all adipose and connective tissue. Special care was taken to avoid stretching the tissue. The artery was cut into segments containing first and second order branches.

Vascular reactivity experiments: Arteries were cannulated, perfused and pressurized as previously described.[14] Superior mesenteric artery segments were placed in cold (4°C) modified Krebs-Ringer bicarbonate (KRB) containing (in mmol/L): 120 NaCl, 4.2 KCl, 1.18 KH_2PO_4, 1.2 $MgSO_4$, 1.3 CaCl2, 25 Na_2HCO_3, 5 D-glucose, 5 HEPES, and 1.2 pyruvate, equilibrated with 95%O_2/5%CO_2 (pH 7.4). Second order branches from the superior mesenteric artery were isolated and transferred to a 2 ml glass vial and kept at 4ºC. To measure vasoconstriction, arteries were perfused in a thermoregulated water chamber (37ºC BTC 9090 temperature controller, Brainchild Electronic Ltd) at constant pressure (60 mm Hg) under controlled flux. The selected artery was cannulated at both ends (MicroFil micropippette, WPI), and mounted in a thermostatically controlled 6-ml chamber. The perfusion flux varied from 12 to 15 µL/min (syringe pump SP101i, WPI). Intraluminal pressure (pressure transducer MLT0380/A, ADInstruments) was constantly monitored (PowerLab 4/30, ADinstruments). Tissues were superfused with a peristaltic pump at flow rate 2.5 mL/min (Dynamax RP-1, Rainin) with constantly gassed KRB. Arteries were allowed a 20 minutes equilibration period. In the present study 127 arteries were used, with an average basal diameter of 194.3±5.9 µm. Before starting the experiments, arterial viability was checked by adding phenylephrine (100 µmol/L, 2 minutes) to the superfusion. Endothelium integrity was tested by the subsequent addition of acetylcholine (1 µmol/L). Only arteries that showed sustained vasoconstriction after addition of phenylephrine and complete relaxation in the presence of acetylcholine were used. Thereafter, arteries were washed 3 times (superfusion exchange, 1 minute wash), followed by a 10-minute period of stabilization. In one series of experiments designed to analyze the effect of flow on the action of ENaC blockers, the flow was decreased to 4µL/min after the stabilization period, as indicated. Videomicroscopy images were acquired every 4 seconds using a zoom stereo-microscope (Olympus SZ61) coupled to a CCD camera (Moticam 2000, Motic China Group Co), and external artery diameter was determined for each image. For isometric force studies, SDMA were dissected and 2-mm-long artery segments were threaded onto two 40-µ stainless-steel wires and mounted in a wire myograph (TRN001 Kent Scientific Corporation Isometric Transducer, CT, USA) in a chamber filled with KRB at 37ºC. The chamber solution was gassed with 5% CO_2-95% O_2. After mounting the arteries, their internal circumference was normalized on the basis of the passive tension-length curve to a value that gives near maximal force development.[16] Before starting the experiments, arterial viability was checked by adding phenylephrine (100 µmol/L, 2

minutes) to the superfusion. Endothelium integrity was tested by the subsequent addition of acetylcholine (1 μmol/L). Only arteries that showed sustained vasoconstriction after addition of phenylephrine and complete relaxation in the presence of acetylcholine were used. Thereafter, arteries were washed 3 times (superfusion exchange, 1 minute wash), followed by a 10-minute period of stabilization. Amiloride or vehicle were added for 5 minutes and the tension developed in response to 100 μmol/L phenylephrine was recorded. The same artery was tested in the presence of amiloride and after washing in the presence of vehicle. 50% of the arteries were treated with amiloride first and 50% were treated with vehicle first. To test if ENaC inhibition by amiloride enhanced the vasodilatory response to shear stress, flow-induced dilation was measured in cannulated, perfused and pressurized arteries, in the presence of amiloride 500 nmol/L or vehicle.[15] After the equilibration period, the arteries were incubated in the presence of amiloride or vehicle for 5 minutes (flow=15 μL/minute). Thereafter, the flow was decreased to 4 μL/minute and flow-pressure-diameter relationships were established by imposing step increases in flow (4 to 40 μL/min) to the mesenteric arteries. Each flow rate was maintained for 3 minutes so steady-state arterial diameters and intraluminal pressure were obtained. The arterial images and pressure were recorded continually during the experiments. Results from 5 independent experiments are presented as flow-diameter and pressure-diameter relationships. For NO measurements, cannulated arteries were loaded with 12.0 μmol/L of 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate-AM (DAF2-AM, Molecular Probes) for 30 minutes at room temperature, followed by 30 minutes at 37ºC in the dark. DAF2-AM was prepared as a stock solution in dimethylsulfoxide (DMSO) plus pluronic acid (2.5%), and final solvent concentration was 0.1% (vol/vol). After 20 minutes of washing, emitted fluorescence (510 nm) was acquired following excitation at 495 nm. Fluorescence was measured using an Eclipse E400 epifluorescence microscope with a FluorX10 water immersion objective (Nikon Corporation). Emitted fluorescence was registered with a cooled charge-coupled device video camera (Retiga 2000R Fast 1394, QImaging) and data obtained were processed using imaging software (IPLab Spectrum, Scanalytics).

Drug treatments: Benzamil, amiloride or vehicle were added 10 min before the addition of the vasoconstrictors (phenylephrine or serotonin), and were present throughout the experiment. eNOS was inhibited by 100 μmol/L of N-omega-nitro-L-arginine (LNNA), which was added 5 minutes before ENaC inhibitors and was present throughout the study. In some experiments LY294002 (10 μmol/L) was added with ENaC inhibitors, as indicated in the figures. Drugs were prepared as stock solutions and diluted in KRB to the desired concentration before use. ENaC inhibitors and LY294002 were dissolved in dimethylsulfoxide (DMSO) and diluted in KRB in the superfusion chamber (0.01% final concentration). The same final concentration of DMSO was added for control experiments. Vasoconstrictors were prepared in KRB as stock solutions before each experiment.

Preparation of tissue extracts: Total protein fractions from SDMA were prepared and used for Western blotting with specific antibodies.[13,15] Protein fractions were prepared from small-diameter arteries homogenized for 20 min in an ice-cold glass-glass homogenizer into 200 μl of Laemmli lysis buffer (10% glycerol, 6.25 mmol/L Tris-HCl, pH 6.8, 0.1% SDS, 5% β-mercaptoethanol and 0.01% bromophenol blue). Kidney was homogenized in a buffer Tris 0.1 mol/L, $MgCl_2$ 5 mmol/L pH 7.6 and protease inhibitor cocktail (Roche 11836153001). Samples were centrifuged to

5000xg for 10 minutes at 4°C, and supernatants were divided in aliquots stored at -20°C.

Western Blot analysis for eNOS, Akt, ENaC: Tissue extracts (40 μg protein) were separated by 7.5 % SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Pierce) using a Trans-blot unit (Bio-Rad Laboratories) for 1.5 hours at 115mA. Membranes were blocked with 5% (wt/vol) non-fat milk in TBS (pH 7.4) containing 0.1% (vol/vol) Tween 20 (TBST) for 1 hour at room temperature (25ºC). Then the membranes were incubated for 1 hour with the primary antibody, 1:500 antiphospho-eNOS (Santa Cruz Biotechnology) or 1:500 anti phospho-Akt (Cell Signaling Technology Inc.). After washing with TBST 1x 3 minutes and TBS 2x 3 minutes, blots were incubated for 1 hour at room temperature with horseradish peroxidase-linked secondary antibody (Calbiochem, Merck) at 1:5000 dilution, 5% wt/vol not fat milk TBST was used for all antibodies dilutions. Blots were washed 3x 5 minutes in TBST and 1x 10 minutes in TBS. For ENaC Western blots, protein extracts (30 μg) were separated by SDS-PAGE in 7.5% acrylamide gels, and transferred to nitrocellulose membrane 0.45 μm (Bio-Rad Laboratories, Hercules, CA, U.S.A) for 1.5 hour at 350 mA. Membranes were blocked with 8% non-fat milk in PBS-Tween 0.1% (PBST) overnight at 4ºC. Membranes were then incubated for 1 hr at room temperature with immunoaffinity purified rabbit polyclonal antibodies raised against short peptide sequences of the rat ENaC (kindly provided by Mark A Knepper), diluted 1:500 in PBST and subsequently washed 3x 15 minutes in PBST. Then blots were incubated 1hr at RT with secondary antibody anti-rabbit-HRP (Calbiochem) diluted 1:5000 in 5% non-fat milk PBST washed again with PBST, 3x 15 minutes. Specific binding was detected using enhanced chemiluminiscence (Amersham Biosciences) and exposed to CL-XPosure ™ Film (Pierce). Blots were quantified by laser-scanning densitometry.

RT-PCR: Total RNA was prepared from freshly isolated mesenteric arteries with Trizol (Invitrogen Corporation) as described.[14] RNA concentration was determined by spectrophotometry and RNA integrity was assessed by gel electrophoresis. RNA samples were treated with DNase I (DNAfree ™, Ambion). First strand synthesis from 0.2 μg total RNA primed by random hexamers was performed using the ImProm II™ Reverse Transcriptase System (Promega Corporation) according to the manufacturer´s instructions. Reactions were carried out using the following specific primer sets for α ENaC, sense´5 - tcctgcttc caggagaa cat-3´ antisense 5´- gagctttgc aactccgtt tc-3´ (which yield a 318 bp amplicon); β ENaC sense 5´- ctgcagtca tcggaactt ca-3´ antisense 5´- catggtgca gggactgta tg- 3´ (325 bp amplicon);γENaC :sense 5´- cctctgctg tggatcgcg ttcac-3´ antisense 5´cacagcact gtacttgta agggttgat a -3´ (170 bp amplicon). cDNAs were amplified with 2 U GoTaq® Flexi DNA Polymerase (Promega Corporation) PCR amplification was performed for 43 cycles using an ATC 401, Thermal Cycler, (NyxTechnik). For α , β and γ ENac the following thermal cycle profile: denaturing at 94 ºC for 30 s, primer annealing at 60 ºC for 30 s, and primer extension at 72 ºC for 30 s (44 cycles). PCR products (2 μL) were electrophoresed through a 1.5 % agarose gel and stained with GelStar® (Lonza Rockland Inc. ME, USA). The PCR products were sequenced (Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile.) and showed 100% identity with the ENaC sequences published in Genbank (Alfa ENaC: NM-031548.2; Beta ENaC: NM-012648.1; Gamma ENaC: NM-017046.1).

Tissue preparation. Kidneys and mesenteric arteries were fixed in 4% formalin overnight at room temperature, embedded in paraffin and sectioned in slices of 4 µm for *in situ* hybridization and immunohistochemical analysis.

Oligonucleotide digoxigenin (DIG) labeling. Oligonucleotides were labeled with DIG by the reaction with terminal transferase (400 U/µl, Roche) according to the manufacturer´s instructions. Briefly 100 pmol of oligonucleotide, 6.6 mmol/L CoCl, solution, 0.05 mmol/L DIG-ddUTP solution and buffer (0.1 mol/L Potassium cacodylate,12.5 mmol/L Tris-HCl, 0.125 mg/mL BSA) in a total volume of 15 µL of sterile water were combined with 1 µL of the enzyme on ice. The final concentrations of DIG-dUTP and dATP in the incubation mixture were 0.05 and 0.66 mmol/L, respectively. After incubation for 15 min at 37°C, the reaction was stopped by the addition of 1 µL stop solution (0.22 mol/L EDTA pH 7.2) in ice. The labeled oligonucleotide was purified by precipitation with 4 mol/L LiCl and absolute ethanol at -70°C overnight. Oligonucleotides were solubilized in sterile water at a final concentration of 1000 pmoles/mL. The reactions were carried out using specific sequence, αENaC´c5´ggcagaga gtttgtagtt gatcacacta caaggcttcβENaC 5´cacagtgggc ggcgggccag tgtagggtcg ctgtggccgc 3´; γENaC 5´cctggtacaa ctggtagtag caatacatcc agttggggtg 3´.

***In situ* hybridization**. Deparaffinized and rehydrated sections were treated with 1 % pepsin (Pan Path) in 0.1 N HCl at 37°C for 30 min immediately before hybridization, rinsed in PBS and acetylated with 0.1 mol/L triethanolamine and 0.25% acetic anhydride at room temperature for 10 minutes. After rinsing the slices with PBS, sections were dehydrated in ethanol and air dried. The probe was denatured at 60°C for 3 minutes before being added to the hybridization solution (1X Denhardt's solution, 50% deionized formamide, 10 mg/mL yeast RNA, 10.8 µg/m denatured salmon sperm DNA, SSC 4X) at the final concentration of 400 pmol/mL. Approximately 50 µL of the solution were spread over the sections and hybridized for 16-20 h at 37°C in a humidified chamber. After hybridization, slides were rinsed with SSC 2X two times and with SSC 1X two times for five minutes. The sections were coated with blocking reagent (0.15 mol/L NaCl, 0.1 mol/L maleic acid pH 7.5 and blocking reagent (Roche) for 30 minutes and incubated with antibody anti-DIG-alkaline phosphatase F´ab fragments (Roche) dissolved in blocking reagent at room temperature for 2 hours. The sections were washed twice with washing buffer (0.15 mol/L NaCl, 0.1 mol/L maleic acid pH 7.5) for 5 minutes. Finally the sections were developed by incubation with substrate NBT/BCIP (Pan Path) overnight at room temperature. The sections were rinsed with water and then counterstained with neutral red. Before mounting (mounting media Merck) the sections were dehydrated in ethanol and treated with xylol.

Immunohistochemistry. Sections were deparaffinized and rehydrated in sequential gradients of ethanol and xylol. The slices were air dried and washed with PBS. The sections were treated with 0.5% hydrogen peroxide for 20 minutes to block peroxidase activity. Primary antibodies were applied at appropriate dilutions (1:100 anti-αENaC, 1:50 anti-βENaC and 1:50 anti-γENaC; kindly donated by Mark Knepper) at 25°C overnight in a humidified chamber. After rinsing the slices with PBS, secondary anti-rabbit antibody was incubated at 25°C for 2 hours. Immunoreaction was visualized with 3,3 diamino-benzidine (DAB) for 10 minutes. The slices were counterstained with hematoxylin and mounted in Permount.

Endothelial cell primary culture. Endothelial cells were isolated by enzymatic digestion from rat mesenteric arteries.[16] Freshly isolated mesenteric arteries from male Sprague-Dawley rats were used for enzymatic digestion (Collagenase type I, Worthington®; 50 minutes at 37°C). Endothelial cells were cultured in M-199 medium, supplemented with 20% FBS and 10 μg/mL of endothelial growth factor (Sigma). Cells were plated onto 24 x 50 mm microscope cover glasses (Marienfeld, Germany). After 4 hours the supernatant containing non-adherent cells was aspirated. New medium was added to each dish and adherent cells were cultured for 72 hours. Endothelial cell phenotype was checked analyzing Von Willebrand protein expression by immunofluorescence. Endothelial cells were used for RT-PCR experiments to detect ENaC subunits mRNAs and for patch clamp experiments.Total RNA extraction from endothelial cells was performed with Trizol (Invitrogen Corporation) as per manufacturer instructions. Total RNA quality was checked and 0.5 μg were used for RT-PCR reactions, as described above. PCR reactions for α, β and γ ENaC were performed with the same set of primers used with arterial and kidney tissue cDNAs.

Patch–clamp experiments: Standard whole cell patch clamp recordings were performed as described by Hamill *et al*.[17] Cells were mounted onto a Patch chamber (RC-28, Warner Instruments Corporation, USA). Patch pipettes were fabricated from thin-walled borosilicate glass capillary tubing (Harvard apparatus, Eden Bridge, Kent, UK.) using a Narishige puller (Japan) and had resistance of 3-5 MΩ). Whole-cell currents were recorded using an EPC-9 amplifier (Heka Elektronic, Lambrecht/ Pfalzt, Germany). The signal was analyzed off-line by means of the Pulse Tools software (Heka, Lambrecht / Pfalz, Germany). All measurements were made at room temperature. The bath solutions had the following composition (in mmol/L): 145 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 Na-HEPES, 10 glucose pH 7.4, and the pipette solution was (in mmol/L): 145 KCl, 5 NaCl, 10 Na-HEPES, 7.5glucose pH 7.4, unless stated otherwise. Cells were incubated in the presence of Amiloride (Sigma-Aldrich, Inc.) or vehicle for 10 minutes.

Results

Figure S 1

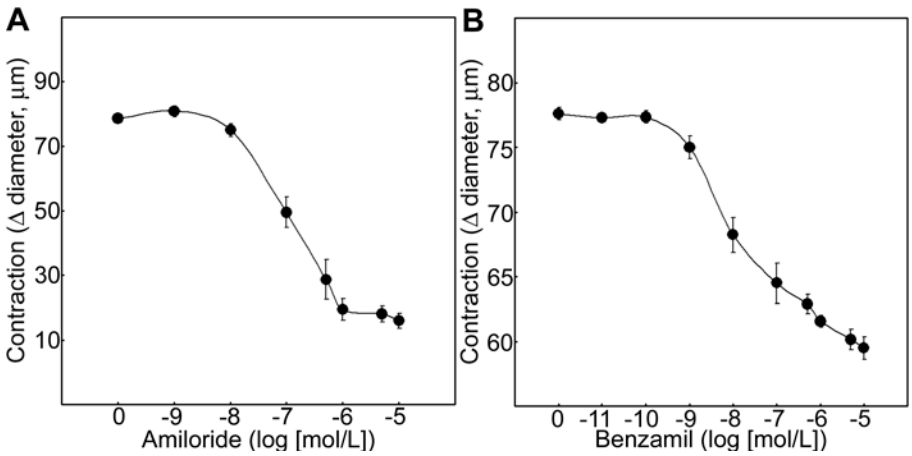

Figure S1. Dose response curves to amiloride and benzamil. SDMA were mounted in the perfusion chamber. After the equilibrium period, SDMA were incubated for 10 minutes in the presence of amiloride (A) or benzamil (B) at increasing concentrations (separate experiments each dose). Thereafter, 100 µmol/L phenylephrine was added and artery diameter was recorded during the second minute, when contraction reached a plateau. Each point in the curves represents the average diameter±SD of 4 independent experiments.

Figure S 2.

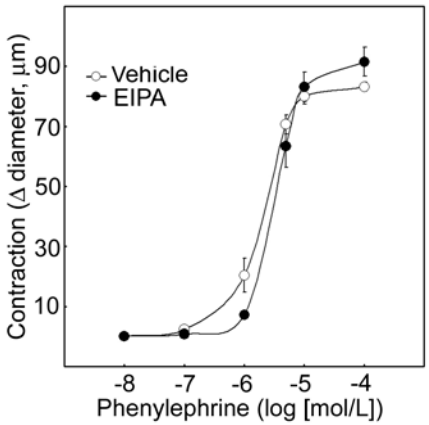

Figure S2. An amiloride analog, EIPA, did not affect SDMA response to phenylephrine. SDMA were perfused and pressurized. After 10 minutes of incubation in the presence of 500 nmol/L ethyl isopropyl amiloride (EIPA, black circles) or vehicle (white circles) increasing concentrations were added each 2 minutes. Each point represents the average diameter±SE of 4 independent experiments, NS.

Figure S 3.

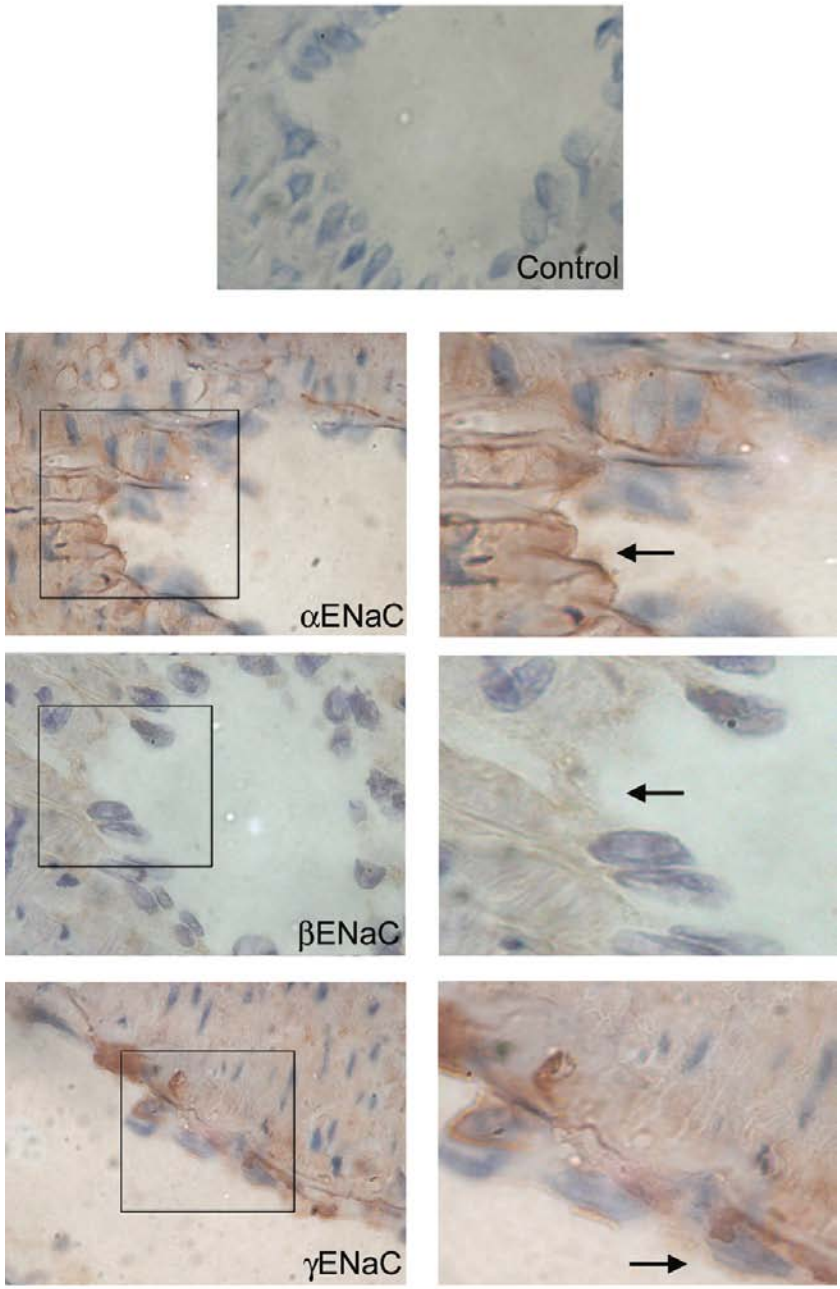

Figure S3. r ENaC immunohistochemistry in SDMA. Representative sections of SDMA showing immunohistochemistry for ENaC subunits (brown), counterstained with hematoxylin (blue, nuclei). The arrows in magnified section (x 200) indicate stain of ENaC subunits in endothelial cells. Control section was treated same way as the experimental, but without primary antibody.

Figure S 4.

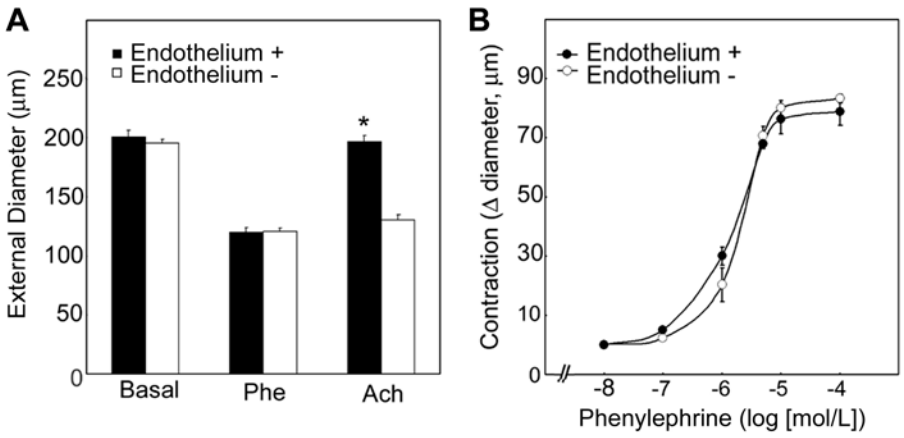

Figure S4. Endothelium ablation does not affect contractility. A, Arteries were pre-contracted with 100 µmol/L phenylephrine. In endothelium-intact arteries we observed 81.8±2.7 µm of vasodilation; endothelium-denuded arteries showed no significant vasodilation in response to Acetylcholine B, Concentration-response curves to phenylephrine of endothelium intact (black circles) and endothelium-denuded (white circles) arteries were similar, n=6, NS.

Figure S 5.

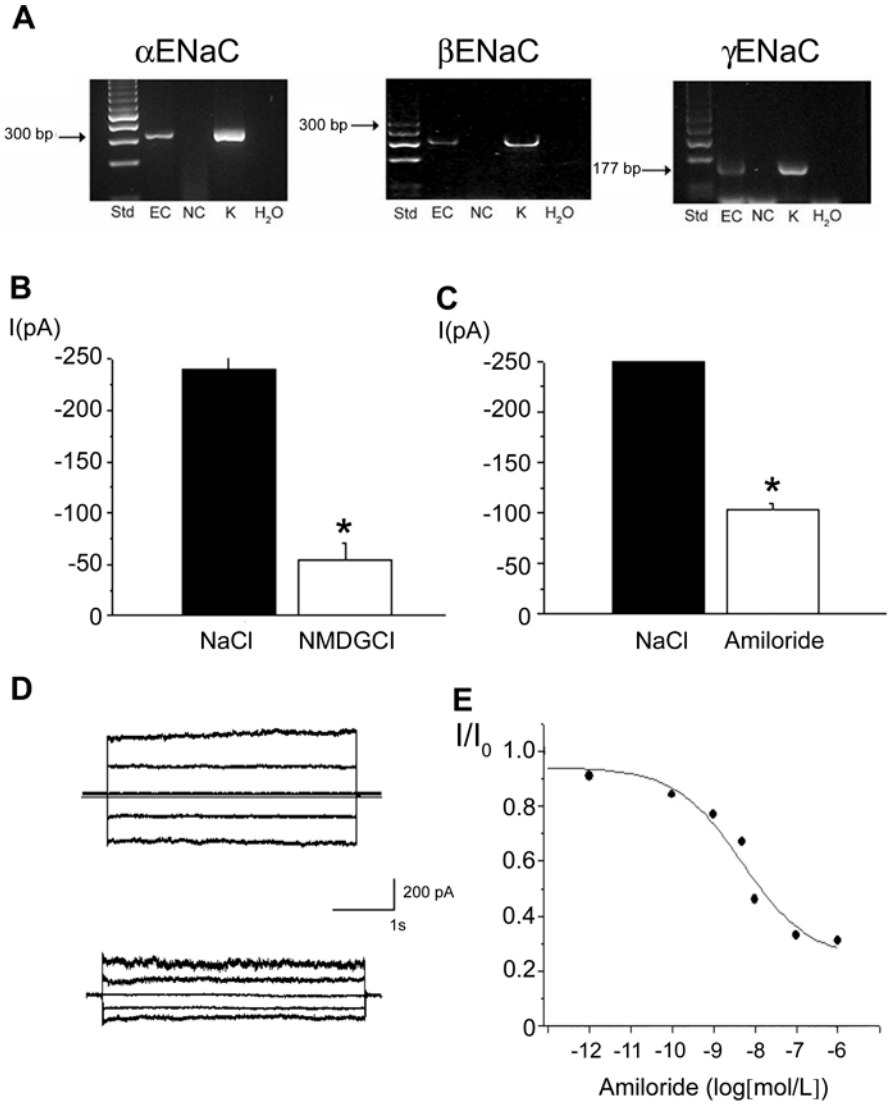

Figure S5. Cultured endothelial cells express ENaC and amiloride sensitive currents. A, Total RNA was extracted from cultured endothelial cells and used for RT-PCR experiments. The panels show agarose gel electrophoresis of PCR products. Positive control corresponds to PCR using kidney cDNA (K) as template. Non-transcribed total RNA (NC) and water were used as negative controls. Products of the expected size were obtained when using α, β and γ ENaC specific primers (indicated by arrows) with cDNAs from endothelial cells and kidney. B, Whole cell current recording were obtained using intracellular solution (in mmol/L) 145 KCl, 5 NaCl, 10 K-Hepes, 7.5 glucose (pH 7.4) and the extracellular solution (in mmol/L) 145 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 MgCl, 5 Na-Hepes, 10 glucose (pH 7.4). B, Effect on the inward current when the extracellular medium was replaced by a Na^+-free solution (n=3). The whole cell current at -80mV decreased from 239 ± 66 pA in control condition (black bar) to 54 ±16 pA in 145 N-methyl-D-glucamine chloride (NMDGCl) (white bar; *= P<0.05 *vs* control). C, The addition of 500 nmol/L amiloride caused a significant decrease of the inward currents with respect to control values. The whole cell current at -80 mV decreased from 266 ±8pA in control condition (black bar) to103±7 pA with amiloride (white bar; n=3, *= P<0.05 *vs* control). D, Typical whole cell currents recording obtained using a voltage pulse

protocol between -80mV and +80mV in 40 mV step with holding potential of 0 mV in control solutions (upper) and with amiloride 500 nmol/L (bottom). E, Concentration dependence of amiloride inhibition at -80mV. The current (I) was normalized relative to current amplitude in absence of amiloride (I_0) and expressed as I/I_0. Using these data, the calculated EC_{50} value was 5.4±0.04 nmol/L (n=4 separated determinations).