Dietary salt enhances benzamil-sensitive component of myogenic constriction in mesenteric arteries

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Submitted 16 May 2007; accepted in final form 13 November 2007

Jernigan NL, LaMarca B, Speed J, Galmiche L, Granger JP, Drummond HA. Dietary salt enhances benzamil-sensitive component of myogenic constriction in mesenteric arteries. Am J Physiol Heart Circ Physiol 294: H409–H420, 2008. First published November 16, 2007; doi:10.1152/ajpheart.00571.2007.—Recent work from our laboratory indicates that epithelial Na\(^+\) channel (ENaC) function plays an important role in modulating myogenic vascular reactivity. Increases in dietary sodium are known to affect vascular reactivity. Although previous studies have demonstrated that dietary salt intake regulates ENaC expression and activity in epithelial tissue, the importance of dietary salt on ENaC expression in vascular smooth muscle cells (VSMCs) and its role in myogenic constriction is unknown. Therefore, the goal of the present study was to determine whether dietary salt modulates ENaC expression and function in myogenic vasoconstriction. To accomplish this goal, we examined ENaC expression in freshly dispersed VSMCs and pressure-induced vasoconstrictor responses in isolated mesenteric resistance arteries from normotensive Sprague-Dawley rats fed a normal-salt (NS; 0.4% NaCl) or high-salt (HS; 8% NaCl for 2 wk) diet. VSMCs from the mesenteric arteries of NS-fed animals express α-, β-, and γ-ENaC. The HS diet reduced whole cell α- and γ-ENaC and induced a pronounced translocation of β-ENaC from intracellular regions toward the VSMC membrane (−336 nm). Associated with this change in expression was a change in the importance of ENaC in pressure-induced constriction. Pressure-induced constriction in NS-fed animals was insensitive to ENaC inhibition with 1 μM benzamil, suggesting that ENaC proteins do not contribute to myogenic constriction in mesenteric arteries under NS intake. In contrast, ENaC inhibition blocked pressure-induced constriction in HS-fed animals. These data suggest that dietary sodium regulates ENaC expression and the quantitative importance of the vascular ENaC signaling pathway contributing to myogenic constriction.

METHODOLOGY

Experimental groups. Male Sprague-Dawley rats (275–375 g body wt; Harlan) were divided into two groups for each experiment: NS diet (0.4% NaCl) and HS diet (8% NaCl for 2 wk). Animals (1/cage) were housed in a temperature-controlled room (23°C) with a 12-h light-dark cycle. All protocols and procedures employed in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center.

Telemetric measurement of arterial pressure in conscious rats. To determine whether a HS diet increases blood pressure, arterial pressure was chronically monitored 1 wk before (NS diet) and then during a 2-wk HS diet in conscious rats by the use of a telemetry system. While the rats were under anesthesia with oxylfluorane, a flexible catheter attached to a radio transmitter (Data Sciences) was inserted in
and water. Blood pressures were recorded at 500 Hz for 20 s at 10-min intervals. The data were averaged in 60-min blocks for an analysis with arterial pressure averaged during 24-h periods for daily values. **Effect of HS diet on ENaC expression, localization, and translocation.** To determine whether the HS diet altered ENaC expression and localization, we used quantitative immunolabeling in freshly dispersed mesenteric artery VSMCs and Western blot analysis of isolated mesenteric arteries. To determine the effect of the HS diet on ENaC expression and localization, VSMCs were enzymatically dispersed and fixed in 4% paraformaldehyde, as described previously (3, 12). The samples were labeled with mouse anti-smooth muscle (SM) α-actin (1:200) and either rabbit anti-α-, β-, or γ-ENaC antibodies (1:100) (11, 12). To determine the specificity of α-ENaC immunolabeling in dissociated VSMCs, we preincubated the antibody samples with antigen (1:10; Chemicon) before staining the cells. SM α-actin was used to identify VSMCs, normalize immunofluorescence, and mark near-membrane regions. For these experiments, sample preparation, immunostaining, and image-collection conditions were strictly controlled. All samples were examined using a ×63 objective (numerical aperture (NA) = 1.32). The central region of each cell was used for analysis and confirmed by scanning at multiple Z steps (0.5 μm each) above and below the optical section of interest. **Total ENaC expression was calculated as the ratio of whole cell ENaC to SM α-actin immunofluorescence using Leica Microsystems software, and the fluorescence intensity was expressed in relative fluorescence units (RU).** Numerous VSMCs (n = 93 to 334 cells) were used for the analysis and were obtained from two to three different animals for each group. As noted, the conditions of sample preparation, processing, and analysis were strictly controlled. To determine colocalization with the near-membrane marker α-actin, several VSMCs (n = 25–30) were imaged at a higher magnification and further analyzed in each group. All images were collected using a ×63 objective (NA = 1.32) to yield a lateral resolution of 148 and 165 nm when excited at 488 and 543 laser, respectively. A 1024 × 1024 scan format and ×5 optical zoom provided a pixel size of 46 nm. The percentage of total ENaC signal localized to a region of interest defined by α-actin immunolabeling was used to quantify colocalization. To quantify ENaC translocation, we analyzed profile histograms obtained from the VSMCs mentioned above using Leica Microsystems software. The profile histogram plots relative fluorescence intensity as a function of distance along a profile or line. A fluorescence peak characterized all cells for ENaC and α-actin near the membrane. Fluorescence peaks were used to locate concentrated sites of α-actin and α-, β-, and γ-ENaC. Profile lines were randomly drawn along vertical, horizontal, and diagonal axes. The distance between the peak fluorescence signals for α-actin and ENaC along each of the three axes was averaged to obtain a single value for each cell. Thus each value was an average of six individual values. This value was termed peak distance and included 25 to 30 VSMCs/group. For this analysis, we assumed the α-actin localization was stable and quantitated ENaC translocation relative to α-actin. A positive value for peak distance indicated that the ENaC immunofluorescence peak was located extracellularly relative to α-actin. Conversely, a negative value for peak distance indicated that the ENaC immunofluorescence peak was located intracellularly relative to α-actin. Therefore, peak distance was used to quantify the magnitude and direction of ENaC translocation in the near-membrane region. This same approach was used to determine whether dietary salt altered α-actin localization below the VSMC membrane.

In addition to the high-resolution scans, we used Western blot analysis to determine whether the HS diet altered cytosolic- and membrane-associated ENaC. For these experiments, mesenteric arteries were dissected from the surrounding tissue from animals (n = 4/group), frozen in liquid nitrogen, and stored at −70°C. Cellular proteins were extracted from vessels using a membrane protein extraction kit (Biovision, Mountain View, CA) according to the manufacturer’s protocol. The kit allows for the purification of cytosolic and total cellular membrane fractions (organelle and plasma membrane). Vessels (25 mg) were homogenized in 250 μl homogenization buffer, and the membrane pellet was reconstituted in 100 μl × 2 Laemml buffer. With the use of standard electrophoresis procedures, 25 μl of the cytosol fraction and 15 μl of the total membrane fraction were heated at 95°C for 10 min, separated on 7.5% Tris•HCl criterion gels (Bio-Rad, Hercules, CA), transferred to nitrocellulose membranes, immunolabeled, and visualized as described previously (3, 9, 12). Western blots were labeled with rabbit anti-α-ENaC (1:500; Chemicon), rabbit anti-β-ENaC (1:1,000), and rabbit anti-γ-ENaC (1:1,000). A sheep anti-β-ENaC antibody (directed to the NH2 terminus) was also used (1:1,000) (2). The membrane was cut below the 50-kDa molecular mass marker and used to probe for the internal loading control β-actin (mouse anti-β-actin, 1:10,000; Abcam). For antigen-blockade studies, the antibodies were incubated with the appropriate antigen (1:10) overnight at 4°C and centrifuged to remove insoluble material before exposure to the membrane. Quantitative analysis protein expression was performed using Leica Odyssey software. ENaC fluorescence intensity was normalized to β-actin. Only the staining of bands that were interrupted by antigen blockade was used for quantitative analysis.

**Cannulation of rat mesenteric resistance arteries for dimensional analysis.** After a 2-wk HS diet, animals were anesthetized with oxyfluorane, and the mesentery was excised and placed in ice-cold physiological saline solution (PSS; pH adjusted to 7.4 with NaOH) containing (in mM) 130 NaCl, 4 KCl, 1.2 MgSO4, 4 NaHCO3, 1.8 CaCl2, 10 HEPES, 1.18 KH2PO4, 6 glucose, and 0.03 EDTA. Mesenteric resistance artery branches (diameter = 85.9 ± 8.7 μm at 100
mmHg) were dissected from surrounding tissue just before entering the gut wall. Vessels were studied in a vessel chamber (CH/1/SH; Living Systems, Burlington, VT) and analyzed using MetaMorph software (Universal Imaging, Downingtown, PA), as described previously (11, 12).

Effect of HS diet on myogenic vasoreactivity. A pressure-diameter curve was generated to determine the effect of a HS diet on myogenic reactivity in mesenteric resistance arteries by exposing the arteries to stepwise increases in intraluminal pressure from 25–150 mmHg (25 mmHg steps, 5 min each) under zero-flow conditions. To determine the importance of ENaC activity in myogenic constriction, intraluminal pressure was set to 100 mmHg and arteries were incubated for 30 min with the specific ENaC inhibitor benzamil (1 μM, bath and lumen) before repeating the pressure-diameter curve. This dose of benzamil maximally inhibits ENaC and has little effect on the activity.

Fig. 3. Translocation of β-ENaC in mesenteric VSMCs in NS and HS rats. A: representative images for α-actin (top, left; green), β-ENaC (bottom, left; red), and merged immunofluorescence in a representative mesenteric VSMC from a NS-fed (left) or HS-fed (right) animal. The line through the merged image indicates the profile line represented in B for each group. B: profile histogram of fluorescence intensity [relative units (RU)] across the cell for α-actin (green tracing) and β-ENaC (red tracing). Peaks are marked with a' and b' for corresponding orientation of line in A; the a' region has been expanded in C. The arrowheads denote the peak for α-actin (green arrowhead) and β-ENaC (red arrowhead). The distance between the peaks for β-ENaC and α-actin with NS and HS diets is summarized in D (n = 14–15 VSMCs/group). Data are means ± SE.
of other channels and transporters (14). In isolated renal arteries, 1 μM benzamil inhibits myogenic constriction without altering phenylephrine (PE)-induced constriction (12). Mesenteric resistance arteries were then incubated for 30 min in the presence of Ca^{2+}-free PSS (same as above PSS with 2 mM EGTA and without 1.8 mM CaCl_2) to determine the passive pressure-diameter curve. Myogenic constriction was calculated as the percent difference between the active (PSS) and passive (Ca^{2+}-free PSS) inner diameter at each pressure.

Effect of HS diet on KCl- and PE-induced vasoreactivity. To determine the effect of the HS diet on agonist-induced vasoconstriction, we assessed responses to the depolarizing stimulus KCl (80 mM) and the α₁-adrenergic receptor agonist PE (10^{-9} - 10^{-5} M) in rat mesenteric resistance arteries in NS and HS animals. Each vessel was allowed 20 min to reequilibrate before the next agonist was examined. KCl- and PE-induced vasoconstrictor responses were calculated as a percentage of baseline inner diameter.

Fig. 4. Translocation of α-ENaC in mesenteric VSMCs in NS and HS rats. A: representative images for α-actin (top, left; green), α-ENaC (bottom, left; red), and merged immunofluorescence in a representative mesenteric VSMC from a NS-fed (left) or HS-fed (right) animal. The line through the merged image indicates the profile line represented in B for each group. B: profile histogram of fluorescence intensity (RU) across the cell for α-actin (green tracing) and α-ENaC (red tracing). Peaks are marked with a’ and b’ for corresponding orientation of line in A; the a’ region has been expanded in C. The arrowheads denote the peak for α-actin (green arrowhead) and α-ENaC (red arrowhead). The distance between the peaks for α-ENaC and α-actin with NS and HS diets is summarized in D (n = 14–15 VSMCs/group). Data are means ± SE.
Statistics. All data are expressed as means ± SE. A t-test or two-way ANOVA with repeated measures was used where appropriate. Differences among groups were determined using the Student-Newman-Keuls test. Statistical significance was considered at \( P \leq 0.05 \).

RESULTS

Mean arterial pressure in Sprague-Dawley rats. Mean arterial pressure (MAP), measured by 24-h telemetric recordings, was unaltered with a HS diet (containing 114 ± 1 mmHg NS and 114 ± 1 mmHg HS; \( n = 5 \); Fig. 1). These data represent the average 24-h MAP for 7 days on a NS diet and 14 days following placement on a HS diet.

HS diet regulates ENaC expression in rat mesenteric VSMCs. To determine dietary salt regulation of ENaC expression in mesenteric VSMCs, we used quantitative immunolabeling. SM α-actin immunofluorescence, which was not dif-

Fig. 5. Translocation of γ-ENaC in mesenteric VSMCs in NS and HS rats. A: representative images for α-actin (top, left; green), γ-ENaC (bottom, left; red), and merged immunofluorescence in a representative mesenteric VSMC from a NS-fed (left) or HS-fed (right) animal. The line through the merged image indicates the profile line represented in B for each group. B: profile histogram of fluorescence intensity (RU) across the cell for α-actin (green tracing) and γ-ENaC (red tracing). Peaks are marked with a' and b' for corresponding orientation of line in A; the a' region has been expanded in C. The arrowheads denote the peak for α-actin (green arrowhead) and γ-ENaC (red arrowhead). The distance between the peaks for γ-ENaC and α-actin with NS and HS diets are summarized in D (\( n = 14–15 \) VSMCs/group). Data are means ± SE.
fere in VSMCs between NS (54.82 ± 1.14 RU; n = 831) and HS (54.74 ± 1.47 RU; n = 706) animals, was used to normalize ENaC expression and control for immunolabeling efficiency. Representative images and group data (93 to 334 cells/group) are shown in Fig. 2. Whole cell α-ENaC fluorescence levels were decreased by 48% (Fig. 2A). In previous investigations, we have been unable to detect α-ENaC immunolabeling in VSMCs dissociated from renal and cerebral arteries; we therefore performed antigen-blockade experiments to ensure the α-ENaC immunolabeling was specific (Fig. 2B). Whole cell β-ENaC was unchanged (Fig. 2C), and γ-ENaC levels were decreased by 20% (Fig. 2D). These findings indicate that VSMC ENaC-subunit expression is regulated in response to the HS diet.

**HS diet redistributes β-ENaC toward the membrane in mesenteric VSMCs.** We observed a dramatic shift in β-ENaC localization from the cytoplasm toward the membrane with a HS diet. To quantify this shift, we first evaluated near-membrane localization of ENaC proteins using α-actin colocalization (Fig. 2E). Under NS feeding, 19% of whole cell β-ENaC fluorescence was colocalized with α-actin. However, HS feeding increased β-ENaC colocalization with α-actin by nearly threefold to 58%. HS feeding did not alter the degree of colocalization of α- or γ-ENaC (44–46%) with α-actin. These findings suggest that most of the β-ENaC protein is located below the membrane and that a HS diet shifts, or translocates, β-ENaC from cytoplasmic pools toward membrane regions.

To further investigate ENaC translocation, we generated profile histograms of fluorescence intensity using Leica software. This approach analyzes ENaC localization relative to α-actin. The result of the histogram analyses and representative images for β-, α-, and γ-ENaC are shown in Figs. 3, 4, and 5, respectively. Immunostaining for α-actin (green) and β-ENaC (red) in a representative VSMC with a sample profile line is shown in the merged image in Fig. 3A. The corresponding histogram plot of fluorescence intensity along the line drawn in Fig. 3A is shown in Fig. 3B. The fluorescence-intensity peaks in Fig. 3B, marked by a’ and b’ notation, correspond with the a’ and b’ points marked in Fig. 3A. Figure 3C shows the profile near region a’ on an expanded scale. The red and green arrowheads in Fig. 3C identify the locations of markers used to calculate distances between the fluorescence peaks. The mean distance between α-actin and ENaC fluorescence peaks, which was the average of two intersection points from three individual profile lines (n = 14 cells/group), is summarized in Fig. 3D. In NS-fed animals, peak β-ENaC fluorescence signals localized to −0.278 ± 0.025 μm (Fig. 3D) intracellularly relative to α-actin. After the HS diet, β-ENaC exhibited a remarkable shift in fluorescence peak position, shifting to an extracellular position (+0.066 ± 0.020 μm) relative to α-actin. This finding demonstrates that β-ENaC translocates toward the membrane with HS. Similar analyses were performed for α-ENaC and γ-ENaC localization (Figs. 4 and 5, respectively). In contrast to β-ENaC, α-ENaC and γ-ENaC do not undergo a dramatic translocation (Figs. 4D and 5D).

An inherent assumption in the histogram analyses is that the position of α-actin relative to the membrane does not change with dietary salt. To address this, we evaluated the distance between the VSMC membrane and peak α-actin fluorescence using the same histogram analysis (Fig. 6). Representative α-actin fluorescence and phase-contrast images are shown in Fig. 6A. Mean distances between peak α-actin fluorescence and the membrane are shown in Fig. 6B. These data demonstrate that α-actin localization below the plasma membrane is unaltered by dietary salt.

We used Western blot analysis of cytosolic and mixed membrane fractions of mesenteric artery homogenates as a secondary method to confirm immunostaining results and the redistribution of β-ENaC from the cytosol to the membrane. As shown in Fig. 7A, α-ENaC was detected only in the cytosol fraction and decreased significantly with HS feeding (~50%; n = 4 and P = 0.02). For β-ENaC (Fig. 7B), we found a tendency toward increased β-ENaC immunodetection of a high molecular mass protein (~250 kDa) in the total membrane fraction in mesenteric arteries from HS-fed animals using two different anti-β-ENaC antibodies (rabbit and sheep), suggesting the increased expression of an oligomeric β-ENaC (rabbit antibody, 47%; P = 0.25; and sheep antibody, 25%; P = 0.09). Cytosol γ-ENaC was unaltered by the HS diet; however, membrane-associated γ-ENaC tended to be reduced (~65 kDa, 14%, P = 0.226; and 75 kDa, 25%, P = 0.08). Thus the Western blot analysis results support the immunolocalization findings, demonstrating 1) the reduction of α-ENaC and γ-ENaC and 2) the redistribution of β-ENaC following the HS diet.
Fig. 7. HS diet increases β-ENaC localization in the membrane component of mesenteric vessels. Western blot analysis detection of α-, β-, and γ-ENaC and β-actin control in total membrane-associated and cytosol fractions of mesenteric vessel homogenates is shown on the left, and quantitative data are shown on the right (n = 4). The back-to-back arrow indicates that quantitation of band fluorescence intensity was performed because band labeling was reduced by Ag coincubation (D). A: HS diet led to a reduction in cytosol α-ENaC by ~50%. No α-ENaC was detected in the total membrane fraction. B: HS diet tended to reduce cytosol and increase in total membrane β-ENaC. When labeled with a different β-ENaC antibody (sheep anti-β-ENaC), a similar increase in membrane-associated β-ENaC was detected. C: HS diet tended to reduce cytosol and total membrane γ-ENaC by 14–25%. D: Ag-blockade studies conducted on cytosol and membrane fractions of mesenteric vessels. The presence and absence of Ag are indicated. Arrowheads identify bands present in tissue samples that had reduced labeling following Ag coincubation. MM, molecular mass; RFU, relative fluorescence units. *P < 0.05 denotes significant difference.
**HS diet increases importance of ENaC activity in myogenic constriction in mesenteric arteries.** Myogenic-constrictor responses were assessed as changes in inner diameter in response to increases in intraluminal pressure (Fig. 8). Mesenteric arteries from NS (Fig. 8A) and HS (Fig. 8B) groups constricted in response to increases in intraluminal pressure in Ca²⁺-containing PSS and distended passively in response to increases in intraluminal pressure in Ca²⁺-free PSS. ENaC inhibition with 1 μM benzamil did not change the pressure-diameter response compared with the vehicle response (Fig. 8A) in NS animals. However, ENaC inhibition reduced pressure-induced vasoconstriction in arteries from HS rats (Fig. 8B). As shown in Fig. 8C, although the total developed myogenic tone was similar between HS and NS animals (45–48% tone at 100 mmHg), HS animals developed a benzamil-sensitive component to myogenic constriction (Fig. 8, B and C). In HS-fed animals, ENaC blockade inhibited myogenic tone by ~37% (myogenic tone decreased from 48% to 30% tone at 100 mmHg). This finding indicates that ENaC activity contributes to myogenic constriction in HS- but not NS-fed rats.

**Responses to KCl and PE were unaltered by HS diet.** After 2 wk on a HS diet, mesenteric artery responses to KCl and PE were unchanged. Depolarization-induced constriction, mediated by a high concentration of KCl, maximally constricted mesenteric resistance arteries similarly in NS (76 ± 2%) and HS (81 ± 2%) animals (Fig. 9A). There were no differences in the maximal PE-induced vasoconstriction (NS = 83 ± 2%, and HS = 84 ± 4%) or the half-maximal constriction (represented as −log EC₅₀) calculated from the average of each individual PE-concentration response curve (NS = −6.58 ± 0.08 and HS = −6.65 ± 0.10 M PE) (Fig. 9B) between groups. Vasoconstrictor responses to PE and KCl were repeated in the mesenteric arteries from HS-fed animals following benzamil exposure. Benzamil did not alter the maximal vasoconstriction to KCl (HS = 81 ± 2%, and HS with benzamil exposure = 79 ± 3%). Maximal PE-induced vasoconstriction (HS = 84 ± 4% and HS with benzamil exposure = 87 ± 5%) and half-maximal constriction (HS = −6.65 ± 0.10 and HS with benzamil exposure = −6.39 ± 0.12) were also unchanged following benzamil.

**DISCUSSION**

HS intake is associated with changes in the signaling mechanisms mediating mesenteric myogenic-constrictor responses (19–23, 27). Previous findings from our laboratory suggest β- and γ-ENaC proteins mediate pressure-induced constriction, possibly by acting as mechanosensors (3, 12). Although the importance of dietary salt in ENaC expression has been shown in epithelial tissue, the importance of dietary salt on the expression of ENaC proteins and their role in myogenic vascular reactivity is unknown (5, 8, 16–18, 26, 29). Here we report that a HS diet reduces α- and γ-ENaC expression and translocates β-ENaC toward the membrane of mesenteric VSMCs. These changes were associated with an increase in the benzamil sensitivity of myogenic-constrictor responses in mesenteric arteries, suggesting an increased dependence on benzamil-sensitive ENaC signaling.

**Novel ENaC expression pattern and importance in myogenic-constrictor responses in mesenteric arteries.** Recent studies from our laboratory demonstrate that β- and γ-ENaC proteins are expressed near the cell surface of renal and cerebral artery VSMCs (3, 11, 12). However, it is unknown whether ENaC proteins are expressed in another myogenically active bed, such as in the mesenteric circulation. In the current study, we found that mesenteric VSMCs have a unique pattern of ENaC-subunit expression. Unlike renal and cerebral artery VSMCs, mesenteric VSMCs expressed α-ENaC in addition to

**Fig. 8. HS diet alters benzamil (Benz) sensitivity of the pressure-induced constrictor response in rat mesenteric resistance arteries.** Changes in vessel inner diameter to increases in intraluminal pressure during vehicle (active tone), following 30 min incubation with Benz (active tone), and following 30 min incubation with Ca²⁺-free physiological saline solution (PSS; passive tone) in mesenteric resistance arteries from NS (n = 5; A) and HS (n = 5; B) rats. C: percent myogenic vasoconstriction was calculated as the percent difference between the active and passive tone. Myogenic tone for NS + 1 μM Benz was not different than NS vehicle at any pressure step. Values are means ± SE. *P < 0.05 vs. control; †P < 0.05 vs. HS.
β- and γ-ENaC. It is unlikely that the α-ENaC staining was an artifact since coincubation with the α-ENaC antigen abolished all staining in mesenteric VSMCs and parallel experiments conducted in renal VSMCs demonstrated no detectable α-ENaC staining (data not shown). We also found that β-ENaC staining in cytoplasmic regions was more pronounced than previously observed in renal and cerebral VSMCs. This finding demonstrates that ENaC-subunit expression patterns are tissue specific.

In the current study, we used 1 μM benzamil to determine the importance of ENaC-mediated myogenic constriction. We chose benzamil because it is the most selective and specific ENaC inhibitor available. In heterologous expression systems, 1 μM benzamil maximally inhibits ENaC but has little effect on the activity of other channels and transporters (14). In isolated renal interlobar arteries, we have found that 1 μM benzamil inhibits myogenic constriction to an equivalent extent as β-ENaC and γ-ENaC gene-specific silencing approaches such as siRNA and dominant-negative approaches (11, 12). Furthermore, 1 μM benzamil does not alter agonist- or depolarization-induced constriction in the current experiments or previous experiments (12). Therefore, due to the high specificity of benzamil for ENaC and the ability to selectively block myogenic constriction, we used it as an indicator of ENaC-mediated myogenic constriction.

Unexpectedly, we did not observe an effect of 1 μM benzamil on pressure-induced constrictor responses in mesenteric arteries from NS-fed animals. This is in sharp contrast to previous studies in renal and cerebral arteries where 1 μM benzamil almost totally abolished pressure-induced constriction (3, 12). The reasons underlying the reduced benzamil sensitivity in mesenteric arteries are not clear. Reduced benzamil delivery to the target site in the vessels or in the presence of α-ENaC is a possible explanation. Another possibility is that the cytoplasmic localization of β-ENaC in mesenteric VSMCs may alter the channel composition and thus mediate the reduced benzamil sensitivity. It is important to point out that the lack of benzamil sensitivity in normal dietary salt does not necessarily imply that ENaCs are not involved in the pressure-induced constriction in mesenteric arteries in NS-fed animals. Rather, it may simply reflect a change in the stoichiometry of the mechanosensitive ion channel complex that renders the channel less sensitive to benzamil. Determining the importance of specific ENaC proteins in pressure-induced constriction will require the use of gene-silencing approaches and genetic models. Regardless of the reason, this observation is highly significant because it suggests that signaling mechanisms transducing pressure-induced constriction, i.e., the proteins that constitute mechanosensing protein complexes and their pharmacological inhibitors, may not be conserved among arteries of different circulatory beds.

High dietary salt regulates ENaC expression in VSMCs. This is the first investigation to determine the effect of dietary salt on vascular ENaC expression. Dietary salt is a known regulator of ENaC expression, although heterogeneity related to tissue- and subunit-specific regulation exists (5, 8, 16–18, 26). When examined on the whole cell level, the HS diet decreased α- and γ-ENaC and did not change β-ENaC immunofluorescence in VSMCs from mesenteric resistance arteries. These results were supported by Western blot analysis experiments (Fig. 7). The finding that the HS diet inhibits or does not change ENaC-subunit expression is consistent with other reports in epithelial tissues (8, 15, 18, 26, 29).

In addition to changes in the overall levels of ENaC-subunit expression, we found significant differences in the near membrane-associated levels of the β-ENaC subunit with a HS diet. With the use of α-actin colocalization, the fraction of β-ENaC fluorescence signal associated with the membrane marker was significantly increased for β-ENaC and unchanged for α- and γ-ENaC, suggesting that β-ENaC might be translocated from cytosolic to membrane-associated regions. It is important to recognize that the lack of difference in α- and γ-ENaC colocalization with α-actin may be due to a limitation in the optical resolution of our imaging system (148 and 165 nm), and it is possible that small changes in α- and/or γ-ENaC translocation are present but below detection sensitivity. Previous reports indicate that a HS diet typically leads to a decrease in apical membrane targeting (8, 16). Our finding that the HS diet increased membrane-associated localization of β-ENaC is a novel observation and may represent a response specific to VSMCs.

Another noteworthy observation is the presence of a high molecular mass form (~250 kDa) of β-ENaC in the total membrane fraction. The detection of a high molecular mass...
form of β-ENaC is not a novel observation and has been reported in cultured VSMCs and other heterologous expression systems (9, 10). However, our observation that the high molecular mass form appears to be the predominant, or preferred, form of β-ENaC in endogenous tissue is novel. The presence of a high molecular mass form of β-ENaC suggests that an oligomeric form of β-ENaC may be targeted to membrane fractions.

Three lines of evidence suggest that the HS diet increased β-ENaC targeting to the surface membrane. First, the HS diet increased β-ENaC colocalization with the near-membrane marker SM α-actin. Second, the HS diet caused a shift in the location of the peak immunofluorescence of ~300 µm from the cytosol toward the membrane. Third, the HS diet tended to increase the amount of a high molecular mass form of β-ENaC in mixed membrane fractions. These findings are consistent with an increase in membrane localization. However, the mechanisms that increase β-ENaC targeting to the membrane are unknown.

Many, but not all, previous studies have shown that high dietary salt reduces pressure-induced constrictor responses (7, 19–23). In the current investigation dietary salt did not reduce pressure-induced constriction. The difference is most likely dependent on the duration of salt supplementation, which is 2 wk in the current investigation compared with 4–8 wk in prior studies. Vascular remodeling occurring over the longer period of salt intake may contribute to the altered pressure-induced constrictor responses observed in other investigations.

What is the significance of enhanced benzamil sensitivity of pressure-induced constriction following a HS diet? An obvious interpretation is that pressure-induced constriction may have an increased dependence on ENaC signaling with a HS diet. Shifts in signaling pathways, without affecting overall reactivity, have also been found by others (7, 25, 28). Alternatively, increased benzamil sensitivity may reflect a change in the composition of mechanosensitive channel complexes. In other words, ENaC subunits may still form mechanosensitive ion channel complexes in mesenteric VSMCs under a NS diet; however, differences in subunit abundance and localization with dietary salt may determine benzamil sensitivity. The changes in localization of β-ENaC (i.e., increased membrane expression) in mesenteric VSMCs with dietary salt are consistent with this explanation.

Dietary salt-induced changes in VSMC ENaC are likely mediated by the salt-sensitive renin-angiotensin system. Although angiotensin II is known to regulate ENaC expression in epithelial tissue, the direct effects of angiotensin II on ENaC expression in VSMCs have not been evaluated (17).

The role of ENaC proteins in mediating pressure-induced constrictor responses in mesenteric arteries and the importance of dietary salt in regulating SM expression of ENaC proteins have not been previously addressed. The results of this study suggest 1) the mechanisms underlying pressure-induced constrictor responses vary among circulatory beds and 2) the short-term increases in dietary salt enhance the importance of ENaC proteins in pressure-induced constrictor responses in mesenteric arteries. Increased salt intake is a known risk factor for hypertension, a cause of end-organ injury. Other investigators have suggested that myogenic constriction protects against pressure-induced injury (1, 24), thus determining how a HS diet alters myogenic signaling is a key step in understanding the mechanisms leading to end-organ damage in hypertension.

ACKNOWLEDGMENTS

We thank Kathy Cockrell and Angela Hoover for technical assistance and our laboratory colleague Samira Grifoni for assistance and discussion.

GRANTS

This work was supported by the National Heart, Lung, and Blood Institute Grants HL-082425 (to N. Jernigan), HL-071603 (to H. Drummond), HL-086996 (to H. Drummond), HL-51971 (to J. Granger, H. Drummond, and B. LaMarca), HL-33947 (to J. Granger), and HL-66662 (to B. LaMarca) and American Heart Association Grant AHA-0655305-B (to H. Drummond).

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