Impaired pressure-induced constriction in mouse middle cerebral arteries of ASIC2 knockout mice

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Gannon KP, VanLandingham LG, Jernigan NL, Grifoni SC, Hamilton G, Drummond HA. Impaired pressure-induced constriction in mouse middle cerebral arteries of ASIC2 knockout mice. Am J Physiol Heart Circ Physiol 294: H1793–H1803, 2008.—Recent studies from our laboratory demonstrated the importance of mechanosensitive epithelial Na⁺ channel (ENaC) proteins in pressure-induced constriction in renal and cerebral arteries. ENaC proteins are closely related to acid-sensing ion channel 2 (ASIC2), a protein known to be required for normal mechanotransduction in certain sensory neurons. However, the role of the ASIC2 protein in pressure-induced constriction has never been addressed. The goal of the current study was to investigate the role of ASIC2 proteins in pressure-induced, or myogenic, constriction in the mouse middle cerebral arteries (MCAs) from ASIC2 wild-type (+/+), heterozygous (+/−), and null (−/−) mice. Constrictor responses to KCl (20–80 mM) and phenylephrine (10−7–10−4 M) were not different among groups. However, vasoconstrictor responses to increases in intraluminal pressure (15–90 mmHg) were impaired in MCAs from ASIC2−/− and +/− mice. At 60 and 90 mmHg, MCAs from ASIC2−/+ mice generated 13.7 ± 2.1% and 15.8 ± 2.0% tone and ASIC2+/− mice generated 7.4 ± 2.8% and 12.5 ± 2.4% tone, respectively. Surprisingly, MCAs from ASIC2−/− mice generated 1.2 ± 2.2% and 3.9 ± 1.8% tone at 60 and 90 mmHg. The reason underlying the total loss of myogenic tone in the ASIC2−/− is not clear, although the loss of mechanosensitive β- and γ-ENaC proteins may be a contributing factor. These results demonstrate that normal ASIC2 expression is required for normal pressure-induced constriction in the MCA. Furthermore, ASIC2 may be involved in establishing the basal level of myogenic tone.

mechanotransduction; myogenic constriction; degenerin; acid-sensing ion channel

IN certain blood vessels, a mechanically mediated response, termed pressure-induced constriction (also referred to as myogenic constriction), contributes to vascular resistance. In this response, increases in intraluminal pressure initially cause vessel stretch, which is thought to activate a mechanosensor in the vascular smooth muscle cell (VSMC). The activation of the mechanosensor initiates a signaling cascade, leading to VSMC contraction and vasoconstriction. Although mechanisms of vasoconstriction are well established, the identity of the VSMC sensor is unclear. Previous studies from our laboratory have focused on the role of the epithelial Na⁺ channel (ENaC) proteins.

ENaC proteins are members of a larger family of proteins called the degenerin (DEG)/ENaC/acid-sensing ion channel (ASIC) family. A large body of evidence links proteins to mechanotransduction. Certain DEG/ENaC/ASIC proteins are expressed in mechanosensitive tissue in a diverse range of species (nematode, drosophila, and vertebrate), and the disruption of expression alters normal mechanosensory responses. Two groups of DEG/ENaC/ASIC proteins have been identified in mammals: ENaC and ASIC. Our laboratory has previously demonstrated a role for β- and γ-ENaC proteins in pressure-induced constrictor responses in renal and cerebral vessels (8, 15, 16). However, the role of the closely related ASIC proteins has never been addressed.

ASIC proteins are excellent candidates to form a mechanosensory channel in VSMCs. A number of ASIC channels have been identified: ASIC1 [also known as brain Na⁺ channel (BNaC or BNC)2], ASIC2 [BNC1 or BNaC1, also known as mammalian DEG (MDEG)], ASIC3 (dorsal root ASIC), ASIC4 (spinal cord ASIC), and brain liver intestine Na⁺ channel (17). ASIC proteins can interact to form homo- and heteromeric cation channels. ASIC proteins have been implicated in processes, including acid sensation, learning and memory, and mechanosensation (17, 20–22, 24, 30, 31). Several, but not all, studies support a role for ASIC2 in specific populations of visceral and peripheral mechanosensitive sensory neurons (22, 23). However, not all reports are consistent (6, 26).

A previous study by our laboratory demonstrated ASIC2 expression in cultured VSMCs; however, the importance of ASIC2 in pressure-induced constriction is unknown. Therefore, the goal of this study was to determine whether the DEG/ENaC protein, ASIC2, is required for pressure-induced vasoconstriction in cerebral vessels. To address this goal, we evaluated ASIC2 expression and agonist- and pressure-induced constriction using the ASIC2 mouse model (23). Our results demonstrate that normal ASIC2 expression is required for normal pressure-induced constrictor responses in the mouse middle cerebral artery.

METHODS

All protocols and procedures used in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center.

Knockout mouse model. ASIC2 knockout mice were used for all experiments. ASIC2−/+ mating pairs were generously provided by...
Michael J. Welsh and Margaret P. Price (University of Iowa). The generation of the knockout model, created by an insertion of a neomycin resistance cassette into a region coding for the second membrane-spanning domain, has been described previously (23). The knockout model demonstrates no differences in appearance, growth, size, temperature, fertility, or life span and showed no obvious behavioral abnormalities. The current study utilized all male mice 6–9 wk of age (mean, +/+; 6.7; +/-; 8.2; and +/-; 8.2 wk) and one female +/-–littermate (8.1 wk).

The genotypic analysis of offspring from heterozygous mating pairs was screened by polymerase chain reaction (PCR). Tail DNA was isolated using direct PCR (Tail; Viagen Biotech, Los Angeles, CA), and PCR reactions were performed with AccuPrime Supermix (Invitrogen, Carlsbad, CA). Oligonucleotide sequences for the wild-type (WT) allele were 5'-AGTCCTGCACGGTGGGAGCTTCTA-3' and 5'-GAAGAGGAAGGGAGCCATGATGAG-3'. Oligonucleotide sequences for the knockout allele were 5'-ATGTTTGGAAGTGGTTTGCCATTGG-3' and 5'-TGGATGTGGAATGTGTGCGA-3'. DNA specific to WT and knockout alleles was amplified using a Stratagene Robocycler under the following conditions. Samples were held at 94°C for 2 min, then cycled at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The amplified products were separated on agarose gels and visualized using ethidium bromide. A sample genotypic analysis is shown in Fig. 1.

The cerebral cortex was harvested from 5–7-wk-old mice and homogenized in 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 2x halat protease inhibitor cocktail (Pierce), and 1X EDTA. The samples were vortexed, sonicated, and incubated at room temperature for 30 min. The samples were centrifuged at 16,000 g for 10 min to separate Triton X-100-soluble (cytosolic) and -insoluble (membrane associated) fractions. Proteins were separated using standard gel electrophoresis on 7.5% or 15% Tris-HCl gels (Bio-Rad, Hercules, CA). The proteins were transferred to nitrocellulose. After being rinsed in PBS, membranes were blocked in Odyssey blocking buffer (Li-Cor Biosciences) and then incubated overnight with our rabbit anti-ASIC2a/b (1:1,000) or goat anti-

Western blot analysis. The cerebral cortex was harvested from ASIC2+/+, +/-, and -/- mice and homogenized in 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 2x halat protease inhibitor cocktail (Pierce), and 1X EDTA. The samples were vortexed, sonicated, and incubated at room temperature for 30 min. The samples were centrifuged at 16,000 g for 10 min to separate Triton X-100-soluble (cytosolic) and -insoluble (membrane associated) fractions. Proteins were separated using standard gel electrophoresis on 7.5% or 15% Tris-HCl gels (Bio-Rad, Hercules, CA). The proteins were transferred to nitrocellulose. After being rinsed in PBS, membranes were blocked in Odyssey blocking buffer (Li-Cor Biosciences) and then incubated overnight with our rabbit anti-ASIC2a/b (1:1,000) or goat anti-

**Fig. 1.** Analysis of acid-sensing ion channel 2 (ASIC2) genotype by PCR from tail DNA. Typical results obtained from 2 different ASIC2 wild-type (WT; +/+), heterozygous (+/-), and homozygous null (-/-) mice (a and b, respectively). Arrowhead denotes the predicted size of the knockout (KO) and WT alleles. std, Standard.

**Fig. 2.** Characterization of ASIC2 expression in cerebral cortex of ASIC2 mice. A: RT-PCR detection of ASIC2 cerebral cortex mRNA samples from ASIC2+/+ and ASIC2-/- but not ASIC2+/– mice. B: Western blot analysis of ASIC2a/b protein from cerebral cortex. A band between 50 and 75 kDa was detected in Triton X-soluble (Tx-S) and -insoluble (Tx-I) fractions in cerebral cortex samples from ASIC2+/+ mice (arrowhead). The intensity of the corresponding band was less intense in the ASIC2–/- and absent in the ASIC2–/– cortex lysates. β-actin was used as a loading control. A nonspecific band was also detected in the Tx-I fraction near 75 kDa.

**Antibodies.** For immunolabeling studies, antibodies specific to the ASIC2a (MDEG1) and ASIC2b (MDEG2) splice variants were obtained from commercial sources (Alpha Diagnostic, San Antonio, TX). The ASIC2a and ASIC2b antibodies are directed to the NH2-terminal portion of the extracellular loop of the ASIC2a and ASIC2b splice variants. Additionally, an antibody that recognizes both splice variants was also developed. This antibody, named ASIC2a/b, was directed against the peptide sequence containing the COOH-terminal sequence [(498-511) CVPLQTALGTLEEIA] common to the two known ASIC2 splice variants (ASIC2a and ASIC2b). The peptide was conjugated to keyhole limpet hemocyanin-maleimide and raised in rabbits (Affinity Bioreagents, Golden, CO). The antibodies were affinity purified against the original antigen. The antibody specificity was tested by Western blot analysis in the cerebral cortex (Fig. 2) and immunolabeling in VSMCs in ASIC2 WT and knockout mice. For Western blot analysis and certain immunolabeling studies, a goat anti-ASIC2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), directed to the extreme NH2 terminus of ASIC2a, was also used. Antibodies to the COOH-terminal region of β- and γ-ENaC, generated in rabbits, were also used (8, 15, 16). As an internal control, we used mouse anti-smooth muscle α-actin (1:100; Sigma Chemicals, St. Louis, MO) and mouse anti-β-actin (1:2,000; Abcam, Cambridge, MA) for immunostaining and immunoblotting studies, respectively.

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ASIC2a (1:500) and mouse anti-β-actin (1:1,000) with 0.1% Tween 20. The membranes were rinsed in PBS plus 0.1% Tween 20 and then incubated with secondary antibodies (1:2,000, donkey anti-rabbit IR700 or donkey anti-goat IR700 and donkey anti-mouse IR800; 1 h). The membranes were rinsed in PBS and then examined using the Odyssey infrared imaging system.

Enzymatic dissociation of VSMCs. To dissociate VSMCs, mice were first anesthetized with isoflurane and transcardially flushed with cold Hank’s balanced salt solution (HBSS). The brain was removed, and cerebral vessels were dissected and placed in ice-cold HBSS. VSMCs were dissociated by incubating enzymatic digestion solution I (26 U/ml papain and 1 mg/ml dithioerythritol in HBSS) for 15 min at 37°C with occasional agitation. Vessels were then incubated in solution II (2 U/ml collagenase, 1 mg/ml soybean trypsin inhibitor, and 75 U/ml elastase in HBSS) for 12 min at 37°C. After digestion, vessel segments were centrifuged and rinsed with HBSS. VSMCs were released by gently triturating the tissue with a fire-polished Pasteur pipette and passing dissociated VSMCs through a 70-μm filter.

Immunolocalization in freshly dispersed VSMCs. To quantify ASIC2 expression and determine localization, freshly dissociated VSMCs were labeled using a technique previously published by our laboratory (7, 9, 10). For this approach, dissociated VSMCs are fixed in 4% paraformaldehyde for 10 min and then air-dried to charged glass slides at 37°C. Unless noted otherwise, all incubations were for 1 h at room temperature. To immunolabel the VSMCs, the prepared cells were rinsed in PBS and blocked in 5% normal donkey serum (NDS). The samples were then incubated with primary antibodies [rabbit anti-ASIC2a, anti-ASIC2b, anti-ASIC2a/b, anti-β-ENaC, or anti-γ-ENaC (1:100) and mouse anti-α-smooth muscle actin (1:100)] in 5% NDS overnight at 4°C and then rinsed in PBS. The samples were incubated with Cy3-conjugated donkey anti-rabbit (1:100; Jackson Immunologicals) and Cy5-conjugated donkey anti-mouse (1:100) for 1 h in 5% NDS. After a final rinse in PBS, the samples were covered with Gel Mount mounting media and coverslipped. Antibody localization was visualized using confocal microscopy (Leica TCS SP2). Importantly, all samples were collected, immunolabeled, and imaged side by side under identical conditions. ASIC2 labeling was

**Fig. 3.** ASIC2a and ASIC2b splice variants are expressed in cerebral vascular smooth muscle (SM) cells (VSMCs). Representative example of ASIC2a (A) and ASIC2b (B) immunolabeling in VSMCs isolated from cerebral vessels of ASIC2+/+ and ASIC2−/− littermates. ASIC2 (red) staining (top), smooth muscle α-actin VSMC marker (green; middle), and merged image (bottom) are shown. Note reduced or absence staining for ASIC2 variant in ASIC2−/− VSMCs.

**C:** representative example of ASIC2a/b immunolabeling in VSMCs isolated from cerebral vessels of ASIC2+/+, +/−, and −/− littermates. Immunostaining is shown with the rabbit anti-ASIC2 antibody directed to the COOH terminus common to ASIC2a and ASIC2b (top), smooth muscle α-actin (middle), and the merged image (bottom).

**D:** group data for ASIC2a/b immunolabeling normalized to α-actin (n = 15–22 VSMCs). ASIC2a/b protein expression is reduced in cerebral VSMCs from ASIC2+/+ and reduced further in ASIC2−/− mice. *P < 0.05, significantly different from ASIC2+/+; †P < 0.05, significantly different from ASIC2−/−. RFU, relative fluorescence units.
normalized to smooth muscle α-actin, an internal control. All images were prepared in Photoshop 6.0 (Adobe systems).

To determine colocalization of ASIC2 and ENaC, we used confocal microscopy. Dissociated VSMCs from a ASIC2+/− mouse were incubated with rabbit anti-ASIC2, rabbit anti-β-ENaC, or sheep anti-γ-ENaC antibodies and mouse anti-smooth muscle α-actin (1:100) in 5% NDS overnight at 4°C and then rinsed in PBS. For labeling with the ASIC2 and β-ENaC antibodies, we used a standard species conversion technique to convert rabbit anti-ASIC2 to goat by incubating samples with goat anti-rabbit monovalent Fab fragments (1:100) (10). For visualization of ASIC2 and β-ENaC, VSMCs were incubated with Cy5-conjugated donkey anti-mouse F(ab')2 (1:100), Alexa 488-conjugated donkey anti-goat IgG (1:1,000), and Cy3-conjugated donkey anti-rabbit F(ab')2 (1:100). For visualization of ASIC2 and γ-ENaC, samples were labeled with Cy5-conjugated donkey anti-mouse F(ab')2 (1:100), Cy3-conjugated donkey anti-rabbit F(ab')2 (1:100), and Alexa 488-conjugated donkey anti-sheep IgG (1:1,000). Staining was imaged using confocal microscopy. Experimental and control samples were examined under identical conditions, and images were prepared in Photoshop 6.0 (Adobe systems).

Cannulation of mouse middle cerebral artery for analysis of myogenic tone. After anesthesia with isoflurane, animals (6–9 wk of age) were decapitated, and the brain was removed and placed in ice-cold physiological saline solution (PSS), pH adjusted to 7.4, with NaOH and 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 CaCl₂. Vasoconstrictor responses were calculated as a percentage of relative wall thickness normalized to smooth muscle α-actin, an internal control. Relative wall tension for ASIC2+/− and −/− animals was calculated compared with the wall tension in the ASIC2+/+ mice (100%). Wall thickness was calculated as (OD − ID)/2, where OD and ID represent outer and inner diameter (μm), respectively. To determine the percent change in arterial diameter for a given change in pressure, incremental distensibility was calculated as [(ΔID/ID × ΔPₐ)] × 100, where ΔID represents the change in inner diameter for each incremental change in intraluminal pressure (ΔPₐ) during Ca²⁺-free conditions. For calculation of circumferential stress (CSe), intraluminal pressure was converted from millimeters of mercury to newtons per square meter, where 1 mmHg = 1.334 × 10⁻⁵ N/m². CSe was calculated as CSe = (Pₐ × ID)/(2 wall thickness), and circumferential strain (CSa) was calculated as CSa = (ID − ID₀)/ID₀, where ID₀ represents the inner diameter at the lowest intraluminal pressure (15 mmHg) during Ca²⁺-free conditions.

Reverse transcriptase PCR. Reverse transcriptase (RT)-PCR was used to determine the expression of transcripts associated with the 5′ and 3′ end of ASIC2 mRNA in the cerebral cortex of ASIC2+/−, +/−, and −/− mice. Oligonucleotides for the 5′ end of the ASIC2a transcript were 5′-CGCGAACACTTCTACTTCCG-3′ and 5′-ATGCT-GAACATTCTTGTTCTGGAT-3′, whereas oligonucleotides for the 3′ end of ASIC2, which recognize the transcript sequence common to ASIC2a and ASIC2b, were 5′-TCCGAGAACATTCTCTTGGATG-3′ and 5′-GGTCTCTATCATGGCTGCTGCTCCTG-3′. Samples were heated at 94°C for 2 min, then cycled at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, for 35 cycles, and then held at 72°C for 5 min. PCR products were separated on agarose gels and visualized using ethidium bromide. PCR products were sequenced to confirm identity.

Statistics. All data are presented as means ± SE. A one-way or two-way ANOVA with repeated measures was used where appropriate (SigmaStat 3.0). Differences among groups were determined using the Student-Newman-Keuls post hoc analysis.

Myogenic vascular reactivity. Vessels were exposed to intraluminal pressure steps (5 min, 15 mmHg each) from 15 to 90 mmHg to evaluate myogenic reactivity. Images were collected at the end of each 5-min equilibration period to determine inner diameter. After the pressure steps, vessels were allowed to equilibrate for 30 min with Ca²⁺-free PSS containing (in mM) 130 NaCl, 4 KCl, 1.2 MgSO₄, 4 NaHCO₃, 10 HEPES, 1.18 KH₂PO₄, 6 glucose, 0.03 EDTA, and 2 EGTA, equilibrated to pH 7.4 with NaOH plus papaverine (10⁻⁵ M). The same pressure steps were repeated under Ca²⁺-free conditions to determine the passive diameter of each vessel. At each pressure step, myogenic tone in cannulated middle cerebral arteries was calculated as the difference between the diameter with and without calcium divided by the diameter without calcium.

Calculation of wall thickness, circumferential stress, and strain. Wall tension under Ca²⁺-containing conditions was calculated as inner radius multiplied by the intraluminal pressure. Relative wall tension for ASIC2+/− and −/− animals was calculated compared with the tension in the ASIC2+/+ mice (100%). Wall thickness was calculated as (OD − ID)/2, where OD and ID represent outer and inner diameter (μm), respectively. To determine the percent change in arterial diameter for a given change in pressure, incremental distensibility was calculated as [(ΔID/ID × ΔPₐ)] × 100, where ΔID represents the change in inner diameter for each incremental change in intraluminal pressure (ΔPₐ) during Ca²⁺-free conditions. For calculation of circumferential stress (CSe), intraluminal pressure was converted from millimeters of mercury to newtons per square meter, where 1 mmHg = 1.334 × 10⁻⁵ N/m². CSe was calculated as CSe = (Pₐ × ID)/(2 wall thickness), and circumferential strain (CSa) was calculated as CSa = (ID − ID₀)/ID₀, where ID₀ represents the inner diameter at the lowest intraluminal pressure (15 mmHg) during Ca²⁺-free conditions.

Fig. 4. ASIC2 genotype does not alter middle cerebral artery vasoconstriction to depolarization or α-adrenergic receptor activation. A: vasoconstrictor responses to increasing concentrations of KCl (4–80 mM) were unaffected by ASIC2 genotype. B: vasoconstrictor responses to increasing concentrations of phenylephrine (10⁻⁷–10⁻⁴ M) were unaffected by ASIC2 genotype (n = 7, 8, and 6 sample size or mice in ASIC2+/+, +/−, and −/− groups, respectively).
RESULTS

Expression of ASIC2 variants in cerebral artery VSMCs. To determine which ASIC2 splice variants are expressed in cerebral artery VSMCs, enzymatically dissociated VSMCs were immuno- labeled with antibodies specific for ASIC2a and ASIC2b. Representative images of ASIC2a and ASIC2b immunolocalization are shown in Fig. 3A. The splice variant-specific antibodies, obtained from commercial sources, were directed to a region near the NH2 terminus of ASIC2a and ASIC2b. Immunoreactivity was detected in VSMCs from WT mice and reduced or absent in ASIC2 null mice. There were several noteworthy findings. First, the localization pattern of ASIC2a at or near the surface membrane was suggested by the colocalization with α-actin, a near-membrane marker in freshly dissociated VSMCs, as well as the distribution in the cytoplasm. In contrast, ASIC2b staining was weaker but localized exclusively near the membrane. Second, some remaining signal for ASIC2a was present in ASIC2−/− VSMCs compared with the no-primary antibody control. In Fig. 3B, immunolabeling with an antibody directed to the extreme COOH terminus that recognizes both ASIC2a and ASIC2b variants was used to quantitatively compare total ASIC2 expression in cerebral

Fig. 5. Pressure-induced constriction in the middle cerebral artery is altered in ASIC2 mice. A–C: pressure-diameter responses under Ca2+-containing and Ca2+-free conditions in middle cerebral artery segments from ASIC2+/+ (n = 7; A), +/- (n = 8; B), and −/− (n = 6; C) animals. Under Ca2+-free conditions, vessels passively dilate in response to increases in intraluminal pressure to a similar extent for all genotypes. Under Ca2+-containing conditions, vessels from ASIC2+/+ and +/- mice constrict in response to increases in intraluminal pressure. In contrast, vessels from ASIC2−/− mice do not constrict with increases in intraluminal pressure. D: cerebral vessels from ASIC2−/− mice develop myogenic tone (MT) with increases in intraluminal pressure; however, they generate less tone at every pressure step compared with ASIC2+/+ vessels. With the use of 2-way ANOVA with repeated measures, the development of MT was significantly different for all genotypes at P < 0.05. Vessels from ASIC2+/+ mice fail to generate MT. E: same data as shown in D, but x-axis transformed to log value to generate a linear pressure-tone relationship. The corresponding slope and y-intercepts and their statistical significance are shown in F and G. Note the downward shift of the pressure-MT curve in the ASIC2−/− vessels and the flattening of the pressure-tone relationship in ASIC2+/− mice. *Significantly different from ASIC2+/+; †significantly different from ASIC2+/−. PSS, physiological saline solution.
VSMCs from ASIC2+/+, +/-, and −/− mice. A concentration-dependent effect of ASIC2 gene expression is seen in VSMCs from ASIC2 mice.

Agonist-induced constriction. Vasoconstrictor responses to KCl and PE were measured to determine whether the ASIC2 genotype altered general vasoconstrictor ability. As shown in Fig. 4, A and B, vasoconstrictor responses in middle cerebral artery segments to KCl [4 (control), 20, 40, and 80 mM] and PE (10−7–10−4 M) were not different among the ASIC2 genotypes.

Pressure-induced constriction. Changes in middle cerebral artery intraluminal diameter in response to increases in intraluminal pressure in ASIC2+/+, +/-, and −/− littersmates are shown in Fig. 5, A–C, respectively. Under Ca2+ free conditions (white symbols), arteries from all genotypes similarly dilate in response to an increase in luminal pressure. At 90 mmHg, the inner diameter was not different among groups (145 ± 6, 150 ± 4, and 152 ± 5 μm in ASIC2+/+, +/-, and −/− mice, respectively). The effect of ASIC2 genotype on the development of myogenic tone is shown in Fig. 5, D and E. The slope of the intraluminal pressure (log) versus myogenic tone relationship, an indicator of myogenic response sensitivity, is provided in Fig. 5F. The y-intercept of the intraluminal pressure (log) versus myogenic tone relationship is provided in Fig. 5G. There were two notable findings. First, when compared with the ASIC2+/+ group, there was a downward shift of the pressure-myogenic tone relationship in the ASIC2−/− (Fig. 5, D and E) group. At every pressure step, myogenic tone was significantly reduced in the ASIC2−/− group compared with the +/+ and −/− groups. This finding is supported by data in Fig. 5, F and G, which indicate no difference in the slope but a tendency toward a reduced y-intercept of the pressure (log)-myogenic tone relationship between ASIC2+/+ and −/− mice. Although the y-intercept tended to be reduced in the ASIC2−/− mice, the difference did not quite reach statistical difference (P = 0.121). The second notable finding was the near absence of myogenic response in the ASIC2−/− mice (Fig. 5, D and E); myogenic tone did not increase with increases in intraluminal pressure, and the slope of pressure (log)-tone relationship was not different from 0, the slope of a horizontal line.

Wall tension of middle cerebral artery segments in ASIC2 mice. The development of tension under Ca2+-containing conditions is shown in Fig. 6. Middle cerebral artery segments from ASIC2−/− mice develop significantly more absolute (Fig. 6A) and relative (Fig. 6B) tension than ASIC2+/+ mice.

Mechanical properties of middle cerebral artery segments in ASIC2 mice. Strain, stress, stress-strain relationship, and incremental distensibility were determined under passive (Ca2+ free) conditions to determine whether mechanical properties were altered by the ASIC2 genotype. As shown in Fig. 7, A–D, mechanical properties of the vessel wall were unchanged by the ASIC2 genotype. Additionally, the inner diameter, outer diameter, wall thickness, and wall thickness-to-lumen ratio were not different (Table 1).

Expression of β- and γ-ENaC subunits. We used quantitative immunolabeling to determine whether β- or γ-ENaC expression is altered in VSMCs of ASIC2+/, +/−, and −/− mice (Fig. 8). VSMC β- and γ-ENaC levels are reduced 50–60% in ASIC2+/− compared with +/+ mice. However, in the homozygous null animals, β- and γ-ENaC levels rebound to and above control values. Using immunolabeling, we determined that ASIC2 and β- and γ-ENaC localize to similar regions in VSMCs dissociated from ASIC2+/+ cerebral vessels (Fig. 8C). Furthermore, at least β-ENaC and ASIC2 colocalize in clusters on the surface of dissociated smooth muscle cells (Fig. 8D).

Expression of a truncated ASIC2 gene product. To determine whether truncated 5′ ASIC2 gene products were expressed in the ASIC2 model, we used RT-PCR and immunoblotting. We used RT-PCR with primers specific for 5′ and 3′ ends of ASIC2 mRNA to amplify ASIC2 transcripts from cerebral cortex RNA of ASIC2+/+, +/−, and −/− mice. We were unable to detect the transcript expression of the 3′ end of the ASIC2 transcript in the −/− animal. However, we were able to detect the expression of the 5′ end of ASIC2a splice variant in the −/− animal, suggesting that ASIC2 transcripts encoding the 5′ region are expressed in the ASIC2−/− mouse cerebral cortex (Fig. 9A). We then used immunoblotting to determine whether a truncated ASIC2 protein was expressed. We detected a fast-migrating protein (~12 kDa) in the cerebral cortex Triton X-100-soluble lysates from ASIC2+/− and −/− mice, using an antibody directed to the NH2 terminus of ASIC2a
The product was not detected in the cerebral cortex lysates from ASIC2+/− mice. Consistent with the increased expression of a NH2-terminal ASIC2a protein, the immunolabeling signal (with the same antibody) nearly doubled in the cerebral VSMCs in ASIC2+/− mice.

DISCUSSION

The myogenic response is a mechano-dependent response inherent to VSMCs and thought to be initiated by the activation of mechanosensitive ion channels in the vascular smooth muscle. Although the mechanisms underlying VSMC constriction are well established, the sensor of the mechanical stimulus is still unknown (11, 12). Members of the DEG/ENaC/ASIC protein family have been identified as evolutionarily conserved ion channels involved in mechanosensation (17, 28). Previous studies from our laboratory have demonstrated the importance of the ENaC proteins in the pressure-induced vasoconstriction of cerebral arteries; however, the role of the closely related ASIC proteins has not been determined (8). The aim of these studies was to determine whether ASIC2 is important in maintaining cerebral vasoconstriction of the middle cerebral artery. The major findings of this study are that 1) ASIC2a and ASIC2b are present in dissociated VSMCs and 2) ASIC2 is required for normal pressure-induced constrictor responses.

Table 1. Wall thickness and wall-to-lumen ratio

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<tr>
<th>ASIC2 Genotype</th>
<th>Wall thickness, μm</th>
<th>Wall-to-lumen ratio</th>
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<tr>
<td>+/+</td>
<td>17.43±1.43</td>
<td>0.21±0.02</td>
</tr>
<tr>
<td>+/-</td>
<td>14.94±1.48</td>
<td>0.21±0.04</td>
</tr>
<tr>
<td>−/−</td>
<td>15.33±1.46</td>
<td>0.26±0.06</td>
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ASIC2 expression in VSMCs. Two ASIC2 splice variants have been identified: ASIC2a and ASIC2b. The splice variants vary in their NH2-terminal sequence but share a common extracellular domain and COOH-terminal regions (25). ASIC2a and ASIC2b are often found colocalized in similar tissues, such as the brain, sour taste receptors, and dorsal root ganglion (3, 18, 29). Although we detected more ASIC2a immunolabeling signal, we are unable to determine whether this is due to higher expression levels or better antibody specificity of the ASIC2a antibody. ASIC2a can form an ion-conducting pore. Although ASIC2b does not conduct ions by itself, it modulates the activity of other ASIC channels (17, 18, 29). Thus it is likely that ASIC2a and ASIC2b may play similar roles in VSMCs.

The magnitude of the myogenic constrictor responses in the middle cerebral artery of WT mice (16% tone at 90 mmHg) is consistent with that of other recent publications (2, 4). In our current investigation, we found peak myogenic tone (90 mmHg) in the ASIC2+/− mice was inhibited by 40%. Other investigations have shown that mechanical activation of certain touch and visceral mechanoreceptor fibers was inhibited 20–50% in ASIC2+/− mice (22, 23). Thus the magnitude of the phenotypic difference between ASIC2+/− and −/− is comparable. ASIC2−/− mice appear normal, and preliminary reports suggest blood pressure is not different from ASIC2+/− mice; the phenotype of the ASIC2+/− has never been reported, so we are unable to determine whether the exaggerated phenotype in the ASIC2+/− is specific to pressure-induced constriction in VSMCs (23, 27).

ASIC2 genotype on myogenic tone. When compared with those from ASIC2+/− mice, middle cerebral arteries from ASIC2+/− mice had less tone at each intraluminal pressure step; however, the vessels still constricted to increases in intraluminal pressure. This finding suggests that although the baseline tone in the middle cerebral arteries was reduced,
Fig. 8. Mechanosensitive β-epithelial Na⁺ channel (β-ENaC) and γ-ENaC expression is altered in VSMCs of ASIC2+/+ and −/− mice. A: representative images of β-ENaC (top row, red), γ-ENaC (second row, blue), and α-actin (third row, green) immunolabeling in cerebral VSMCs from ASIC2+/+ and −/− mice. The merged image is shown at bottom. β- and γ-ENaC immunolabeling is reduced in cerebral VSMCs from ASIC2+/+ mice. B: group data for β- and γ-ENaC quantitative immunolabeling. ENaC immunolabeling was normalized to α-actin (n = 26–141 VSMCs/group). When compared with ASIC2+/+ VSMCs, β- and γ-ENaC immunolabeling was suppressed 40–60% in ASIC2−/− VSMCs. β-ENaC levels recovered to control levels, and γ-ENaC levels were upregulated twofold in ASIC2+/+ VSMCs. Compensatory upregulation of γ-ENaC may help preserve myogenic constrictor responses in ASIC2−/− mice. C–E: images are single optical sections taken through the middle (C and D) or surface (E) of VSMCs, demonstrating ASIC2 and ENaC localization in cerebral VSMCs from a ASIC2+/+ mouse. C: this optical section (×63 lens, ×5 optical zoom) shows SM α-actin (far left), ASIC2 (second left), β-ENaC (second right), and merged images (right). D: this optical section (×63 lens, ×10 optical zoom) shows SM α-actin (far left), ASIC2 (second left), β-ENaC (second right), and merged images (right). Images in C and D suggest ASIC2 and β- or γ-ENaC can be found in the same cell. E: image represents an optical section (×63 lens, ×20 optical zoom) taken at the cell surface of a different VSMC. ASIC2 (left, green), β-ENaC (middle, red), and merged images (right) are shown. Punctate staining patterns observed for ASIC2 and β-ENaC suggest the proteins are organized into clusters (arrowheads, right) near the VSMC surface. *Significantly different from ASIC2+/+ mice; †significantly different from ASIC2−/− mice.

the sensitivity of the myogenic response was not changed in the ASIC2−/− mice. To quantify this difference, we used linear regression of the pressure (log)-myogenic tone relationship and obtained the mean slope and y-intercept for ASIC2+/+, +/+ and −/− mice. The slope and the y-intercept were used assess the sensitivity and basal level of tone, respectively. The slope of the pressure (log) versus myogenic tone curve was not different in ASIC2+/+ versus −/− mice; however, the intercept tended to be lower in the ASIC2−/− mice. These data confirm our conclusion that the sensitivity of the myogenic response was not altered, but the basal tone was lower in middle cerebral arteries from ASIC2−/− mice. These findings suggest that although ASIC2 is involved in the myogenic response, ASIC2 by itself may not be a critical requirement in determining the ability of the middle cerebral artery to constrict in response to an increase in pressure; however, it is critical to setting baseline pressure-dependent tone.

Why is pressure-induced constriction abolished in middle cerebral arteries of ASIC2+/+ mice? Our finding that the myogenic response was abolished in cerebral vessels in...
ASIC2+/− mice was unexpected. We speculated that the expression of β- and/or γ-ENaC might be altered since previous investigations from our laboratory indicated that β- and γ-ENaC were required for pressure-induced constriction in renal vessels and that those of other laboratories indicate ASIC2 biochemically interacts with β- and γ-ENaC (15, 19). It is possible that the downregulation of β- and γ-ENaC in the ASIC2+/− mice may contribute to the loss of pressure-induced constriction. Similarly, the upregulation of γ-ENaC in the ASIC2−/− mice may help restore myogenic responsiveness and mask the importance of ASIC2. This compensatory upregulation of other DEG/ENaC proteins in the knockout might explain the lack of effect of the phenotypic difference in ASIC2−/− mice in certain studies (6, 26). Taken together, these findings suggest that β-ENaC, γ-ENaC, and ASIC2 interact directly or indirectly to form a heteromeric channel.

Why are β- and γ-ENaC downregulated in VSMCs from ASIC2+/− mice? The mechanism mediating the downregulation of β- and γ-ENaC is unknown; however, we considered the possibility that short, truncated NH2-terminal fragments of ASIC2 might suppress β- and γ-ENaC for several reasons. First, in preliminary studies for the current investigation, we observed considerable immunostaining for three different ASIC2a antibodies directed to the NH2 terminus/extracellular domain in the ASIC2+/− and −/− mouse cerebral cortex. The antibody is directed to the extreme NH2 terminus of ASIC2a. β-actin loading control (bottom) is shown. C: representative images (left) and quantitative group data (right) of ASIC2a (top) and α-actin (bottom) immunolabeling in cerebral VSMCs isolated from ASIC2+/+, +/−, and −/− littermates. The ASIC2a antibody used in C is the same as B. NH2-terminal ASIC2a expression, normalized for α-actin, was increased more than twofold in VSMCs from ASIC2−/− mice (n = 43–85 VSMCs/group). *P < 0.01, significantly different from ASIC2+/+ and +/− groups. NEO, neomycin resistance cassette.
the 5’ end could still be transcribed. Third, NH2-terminal truncations of DEG/ENaC proteins are known to suppress the expression of other interacting proteins (1, 13). Finally, ASIC2 biochemically interacts with ENaC proteins (5, 19). These four lines of evidence compelled us to determine whether truncated ASIC2 molecules are expressed in the ASIC2 mice. We detected a truncated ASIC2 transcript and protein (~12 kDa) using RT-PCR and Western blot analysis in the cerebral cortex from ASIC2+/− and −/− but not +/+ mice. Although these findings suggest the expression of a truncated ASIC2 molecule in the +/− and −/− mice, it is unknown whether this is responsible for the downregulation of β- and γ-ENaC in the ASIC2+/− VSMCs.

What is the importance of ASIC2 in the myogenic response? Because of the down- and upregulation of β-γ-ENaC in the ASIC2+/− and −/− mice, it is difficult to separate the importance of ASIC2 from β-γ-ENaC in pressure-induced constriction in the ASIC2 knockout model. In previous investigations, we have been able to use pharmacological and gene-silencing approaches [i.e., small-interfering RNA (siRNA)] to determine the importance of β-γ-ENaC proteins in pressure-induced constriction. Unfortunately, specific ASIC2 antagonists are not available, and the small magnitude of the myogenic response in mouse cerebral vessels coupled with the loss of responsiveness that occurs following the siRNA incubation period renders gene-silencing approaches prohibitive. Even though the sensitivity of the pressure-myogenic tone curve in the ASIC2−/− was unchanged, the curve was shifted downward (despite the increased expression of γ-ENaC), which suggests that ASIC2 participates in establishing basal levels of myogenic tone.

Although we do not understand the precise mechanisms leading to the loss of pressure-induced constriction in the middle cerebral arteries of ASIC2+/− mice, the ASIC2+/− mouse may be an excellent model to investigate the physiological importance of pressure-induced constriction. For example, this model can potentially be used to determine the importance of myogenic constriction in cerebral blood flow autoregulation or protection of the fragile cerebral microvasculature from pressure-related injury such as hypertension-induced edema or hemorrhagic stroke.

How do ASIC2 proteins participate in pressure-induced constriction? Previous findings from our laboratory have suggested that β- and γ-ENaC are components of a mechanosensor ion channel complex in VSMCs. The mechanosensor complex in VSMCs is probably similar to the mechanosensor formed by Caenorhabditis elegans DEG proteins (28). In this model, the mechanosensor consists of an ion channel pore anchored to the extracellular matrix, and cytoskeleton, either directly or via linking proteins. Mechanical force applied to the extracellular matrix modulates channel activity. We speculate that members of the ENaC/ASIC family form the ion channel pore, including ASIC2, β-, and γ-ENaC. Several lines of evidence suggest the potential for interaction between ASIC2 and other ENaC proteins. First, β- and γ-ENaC are expressed in similar VSMC populations. Second, in our current investigation, β- and γ-ENaC expression levels are altered in ASIC2 mice. Third, other investigators have shown ASIC2 interacts with β- and γ-ENaC (5, 19). Finally, the recent identification of the crystal structure of ASIC1 indicates a trimeric structure that may apply to other members of the DEG family (14). Thus we speculate ASIC2 may interact with β- and γ-ENaC to form the ion-conducting unit of a mechanosensor.

In summary, our data demonstrate that ASIC2a and ASIC2b proteins are expressed in middle cerebral artery VSMCs. The disruption of normal ASIC2 expression does not alter agonist- or depolarization-induced vasoconstriction. However, the disruption of ASIC2 expression alters expression of vascular smooth muscle ENaC proteins and pressure-induced vascular tone. These findings suggest that ASIC2 may interact with β- and γ-ENaC to form the ion-conducting unit of a mechanosensor that transduces pressure-induced vascular stretch into vasoconstriction and thereby play an important role in the local regulation of cerebral blood flow. The ASIC2 model may serve as a useful tool to advance our understanding of the importance of myogenic constriction in hyperperfusion/hypertension-related injury.

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