Comparative changes in the blood-brain barrier and cerebral infarction of SHR and WKY rats

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Hypertension is involved in the exacerbation of stroke. It is unclear how blood-brain barrier (BBB) tight-junction (TJ) and ion transporter proteins critical for maintaining brain homeostasis contribute to cerebral infarction during hypertension development. In the present study, we investigated cerebral infarct volume following permanent 4-h middle cerebral artery occlusion (MCAO) and characterized the expression of BBB TJ and ion transporter proteins in brain microvessels of spontaneously hypertensive rats (SHR) compared with age-matched Wistar-Kyoto (WKY) rats at 5 wk (prehypertension), 10 wk (early-stage hypertension), and 15 wk (late-stage hypertension) of age. Hypertensive SHR show increased infarct volume following MCAO compared with WKY control rats. BBB TJ and ion transporter proteins, known to contribute to edema and fluid volume changes in the brain, show differential protein expression patterns during hypertension development. Western blot analysis of TJ protein zonula occludens-2 (ZO-2) showed decreased expression, while ion transporter, Na+/H+ exchanger 1 (NHE-1), was markedly increased in hypertensive SHR. Expression of TJ proteins ZO-1, occludin, actin, Claudin-5, and Na+/K+−2Cl− cotransporter remain unaffected in SHR compared with control. Selective inhibition of NHE-1 using dimethylamiloride significantly attenuated ischemia-induced infarct volume in hypertensive SHR following MCAO, suggesting a novel role for NHE-1 in the brain in the regulation of ischemia-induced infarct volume in SHR.

Stroke is the third leading cause of death and a leading cause of long-term disability in the United States. Approximately 80% of these strokes are ischemic (1, 13). It has been reported that hypertension increases the occurrence and severity of ischemic stroke (44, 46), with infarct damage more frequently reported in hypertensive individuals (7, 34, 62, 64). These observations have also been made in the spontaneously hypertensive rat (SHR). SHR exhibit more severe neurological deficits and edema formation (63) and more frequently demonstrate diffuse and extensive cerebral infarct damage than normotensive animals following permanent focal cerebral ischemia (7, 15, 17). However, it remains unclear how elevated blood pressures are linked to increased cerebral infarct damage in hypertension.

The BBB is a metabolic and physical barrier that regulates passage of materials between the peripheral circulation and the central nervous system (31). Brain microvessels forming the BBB are lined with specialized endothelial cells that interact with astroglia, pericytes, and neurons to form a “neurovascular unit” (28, 47). Regulation of the brain microenvironment by the BBB is achieved by two main mechanisms: (1) tight junctions (TJ) which limit passage of hydrophilic molecules and 2) numerous transport and metabolic systems which maintain nutrient, fluid, and ion homeostasis (23, 24).

The TJ is a protein complex that limits paracellular diffusion and passage of immune cells from the blood to the central nervous system (12, 31, 42). The TJ consists of transmembrane proteins (occludin and claudins) that interact on adjacent endothelial cells to form a physical barrier to paracellular diffusion (19, 22, 29) and accessory proteins (zonula occludens family; ZO-1 and ZO-2) that anchor the transmembrane proteins to the cytoskeleton (4, 26, 31). Compromise of the TJ is a critical event in the progression of cerebral ischemia and ensuing edema formation (6).

The BBB also maintains the appropriate ionic composition of brain interstitial fluid for proper neuronal function. In brain microvessels endothelial cells, a number of transporters and channels are expressed, including ion channels (K+ and Ca2+ channels), primary active transporters (Na+/K+−ATPase), secondary active transporters [Na+/H+ exchanger (NHE) and Na+/K+−2Cl− cotransporter (NKCC)], efflux transporters (multi-drug resistance), and aquaporins (21). These proteins regulate transcellular transport across the BBB and have distinct localization to either the luminal [NKCC (54)] or abluminal [Na+/K+−ATPase (10) and aquaporin-4 (3)] membrane of the endothelial cell (21, 24). Although NHE has also been reported to be on the abluminal BBB membrane (9, 14), it remains unclear whether luminal localization of this exchange system occurs as well. Nevertheless, alterations in BBB ion transporter function can lead to edema formation, a potentially lethal complication of brain infarct damage.

Brain edema initially involves a net uptake of Na+ and water from blood into brain across an intact BBB (52, 54) along with astrocyte swelling (38, 66). During these initial stages, passive permeability to tracers such as α-aminoisobutyric acid remain intact indicating no breach in TJ integrity (11). As ischemia progresses, BBB disruption results in vasogenic edema forma-

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tion, which further contributes to damage (5, 41, 59). Few studies have investigated the effect of hypertension on critical proteins involved in both structural and transport maintenance of the BBB. Interestingly, stimulation of BBB NKCC by factors present during ischemia suggests the involvement of the cotransporter with increased brain Na\(^+\) uptake in brain edema (36, 53). Another ion transporter of interest is NHE-1, which is responsible for regulating cytoplasmic pH and cell fluid volume and whose activity is increased in SHR (37, 43, 55). These transporters are expressed at the BBB (58) and may play a significant role in Na\(^+\) regulation.

The aim of this study was to investigate molecular changes in the BBB associated with the development of hypertension and whether these changes correlate to increased infarct damage in ischemic stroke. This was accomplished by comparison of infarct volume following permanent middle cerebral artery occlusion (MCAO) at prehypertension (5 wk), early-stage (10 wk), and later-stage (15 wk) hypertension development in SHR compared with age-matched normotensive WKY. Expression of BBB TJ and ion transporter proteins was also investigated at these time points. Finally, the effect of NHE-1 inhibition on ischemia-induced infarct volume was assessed in hypertensive SHR (15 wk). Understanding and characterizing the role of these molecules at the BBB will lead to targeting potential therapeutics to improve the clinical outcome of stroke patients.

**MATERIALS AND METHODS**

**Materials.** The protease inhibitor (Complete Mini tablet; 10 ml) used in all buffers was purchased from Roche Biochemicals (St. Louis, MO). Nitrocellulose and Tris/HCl Criterion gels were purchased from Bio-Rad Laboratories (Hercules, CA). Western Lightning Chemiluminescence Reagent Plus was purchased from New England Nuclear/PerkinElmer Life Sciences (Boston, MA). Mouse anti-claudin-5, rabbit anti-occludin, rabbit anti-ZO-1, and -2, and anti-mouse peroxidase conjugated secondary antibody (used only with T4 antibody) were purchased from Zymed Laboratories (South San Francisco, CA). Mouse anti-actin was purchased from Sigma (St. Louis, MO). Mouse anti-NHE-1 was purchased from BD Transduction Laboratories (San Jose, CA). T4 monoclonal antibody (recognizes the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter protein) was obtained from the University of Iowa Developmental Studies Hybridoma Bank (Iowa City, IA). Anti-mouse and anti-rabbit peroxidase conjugated secondary antibodies were obtained from Amersham Biosciences (Piscataway, NJ). All other chemicals and supplies were purchased from Sigma.

**Animals and treatments.** All protocols used in this study were approved by the University of Arizona Institutional Animal Care and Use Committee and abide by National Institutes of Health Guidelines. Male WKY rats and SHR were purchased from Taconic Farms (Germantown, NY) and housed under standard 12:12-h light-dark conditions and received food and water ad libitum. All rats were handled frequently/daily for 1 wk prior to entering the experimental protocol to minimize stress and ensure consistency. Because hypertension development in SHR is dependent on age rather than size, we matched age rather than weight in our experimental paradigm to control for the weight-based strain variations. The treatment groups investigated consisted of comparing normotensive (WKY) vs. hypertensive (SHR) age-matched strains at 5 wk (prehypertension), 10 wk (early-stage hypertension), and 15 wk (late-stage hypertension) of age. The study groups were subjected to either sham or permanent 4-h MCAO, or cerebral microvessel protein was isolated for Western blot analyses. The treatment groups were subjected to either sham or permanent 4-h MCAO, or cerebral microvessel protein was isolated for Western blot analyses.

**Noninvasive blood pressure measurements.** Blood pressure measurements were taken using a noninvasive tail cuff blood pressure system following acclimation and stabilization in the restrainer (NIBP-1; Columbus Instruments, Columbus, OH). Once restrained, the conscious rat was placed under the tail warmer to dilate arterial vessels in the tail to obtain a sufficient pulse wave for measurement. Systolic, diastolic, and mean blood pressures (SBP, DBP, MBP, respectively) were recorded for each rat four times and averaged.

**Blood gas and blood chemistry measurements.** Rats were anesthetized with an intramuscular injection of rat cocktail (1 ml/kg) composed of acepromazine (0.6 mg/ml), ketamine (78.3 mg/ml), and xylazine (3.1 mg/ml). Blood gas and chemistry measurements were performed on blood collected from the right femoral artery. Alternatively, terminal blood gas measurements following permanent 4-h MCAO were obtained by exposing the descending aorta and collecting the arterial blood, using a blood collection set, into a heparinized Vacutainer (BD Biosciences).

**Cerebral blood flow measurements.** For the rats in the inhibition studies only, laser Doppler flowmetry (model MBF3D; Moor Instruments, Wilmington, DE) was used to verify occlusion of the MCA by monitoring local cortical microvascular perfusion in the primary ischemic zone of the cortex (7). Animals were placed in the prone position and the head was firmly immobilized in a stereotactic frame (Stoelting; Wood Dale, IL). A cranial window (~5 mm in diameter) was drilled through the skull leaving the dura intact above the cortical area receiving the blood supply from the MCA (centered at anterior/posterior = 0 mm and lateral = 3 mm from bregma with level skull (according to Ref. 57)). Two small holes were drilled into the skull and cranial screws were placed both anterior and posterior to the cranial window to aid in the stabilization of the mounted probe to the skull. Once a blood vessel was visualized, the laser Doppler probe (model P10S-TCG single-fiber probe; Moor Instruments) was mounted and permanently fixed with dental acrylic. Occlusion was confirmed using laser Doppler where cerebral blood flow (CBF) was continuously monitored before MCAO, during placement of the occluding filament at the MCA, and following occlusion. Measurement of flux (expressed as laser Doppler units) during and after occlusion was normalized to initial baseline values for each animal.

**MCAO.** Rats were anesthetized using rat cocktail as described above. Body temperature was monitored using a rectal temperature probe and maintained at 37°C using a heating pad (Stoelting Physiology Research Instruments, Wood Dale, IL). The intraluminal thread occlusion of the middle cerebral artery in the rat is a model based on that of Zia Longa et al. (69). Briefly, a 3-0 nylon monofilament (4-0 for 5-wk-old rats) was inserted retrograde into the left external carotid artery and advanced ~19 mm (14 mm for 5-wk-old rats) through the internal carotid artery to the origin of the MCA. Once the filament had been positioned, the neck incision was sutured and the rats were allowed to recover. All rats underwent postoperative neurological assessment at 1, 2, and 4 h following surgery. After a 4-h period of ischemia, the rats were reanesthetized by using an intramuscular injection of rat cocktail prior to decapitation.

**Neurological assessment.** All rats were evaluated at 1 and 2 h following surgery and scored at 4 h after sham or permanent 4-h MCAO. The rats were scored based on a method described in detail by Longa et al. (60) in four categories of behavior: 1) level of consciousness; 2) spontaneous circling; 3) front-limb paresis; and 4) front-limb paresis. Neurological function was graded on a scale of 0 to 3 for each category based on the level of neurological impairment with rats exhibiting no neurological deficits and showing normal behavior/no impairment received a score of 0, whereas severely impaired rats received a score of 3. The scores from each category were added together to yield a total neurological score. A total neurological score was assigned according to the criteria outlined and SHR were compared with age-matched control for differences in neurological impairment following surgery. For sham MCAO rats, a total neurological score >4 resulted in exclusion from the study. Of the sham rats used in this study, none exceeded the scoring criteria, and all were included. Rats that underwent permanent 4-h MCAO were excluded from the study if the total neurological score was <5.
or > 10. Following the scoring criteria for MCAO rats, <5% of all rats used exceeded the scoring criteria and were excluded from the study.

Brain infarct assessment. Following decapitation, rat brains were quickly removed and placed in ice-cold PBS and put in a −20°C freezer for 10 min. The brain was transferred to a rodent brain matrix (World Precision Instruments, Sarasota, FL) and sectioned at 2-mm intervals from the frontal pole to the occipital pole to yield seven coronal slices. The brain slices were then immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) and incubated at 37°C for 20 min, flipped after 10 min for consistent staining, and fixed in 2% paraformaldehyde overnight. The slices were scanned using a CanonScan FB 630P scanner (Canon Computer Systems, Costa Mesa, CA) and Adobe Photoshop software (Adobe, San Jose, CA). The images were converted to a digital format at a resolution of 300 dpi and analyzed for infarct area and infarct volume using Image-J analysis software (public domain software developed at the National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ij/image/). In each brain slice, the areas of noninfarcted gray matter (i.e., the areas with optical density greater than the threshold values) were measured by the image analysis system in each hemisphere. The infarct area (mm²) was expressed as a percentage area in each brain slice (Eq. 1). Brain infarct volume (mm³) was calculated by summing the infarct area (mm²) in each section and multiplying by the distance (mm) between sections for each hemisphere. The infarct volumes of the lesioned structures were expressed as a percentage volume of the structures in the control hemisphere (Eq. 2). This analysis was based on a semiautomated infarct volume method by Swanson et al. (65). Thus, for each structure the formula is as follows

\[
\%\text{infarct} = 100 \times \left( \frac{A_c - A_i}{A_c} \right) \quad (1)
\]

\[
\%\text{total infarct} = 100 \times \left( \frac{V_c - V_i}{V_c} \right) \quad (2)
\]

where \( A_c \) is the area of normal gray matter in the control hemisphere, \( A_i \) is the area of normal gray matter in the ipsilateral (lesioned) hemisphere, \( V_c \) is the volume of the contralateral hemisphere and \( V_i \) is the volume of the ipsilateral hemisphere. This method is noted to minimize observer bias, is highly reproducible, and is unaffected by edema (65).

Isolation of rat cerebral microvessels. Cerebral microvessels were isolated from rat cortical gray matter for analysis of TJ and ion transporter protein expression. Rats were anesthetized, and the brain removed and immersed in ice-cold buffer A containing (in mM) 103 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 15 HEPES, pH 7.4, protease inhibitor. The meninges and choroid plexus were removed and immersed in ice-cold buffer A containing (in mM) 103 NaCl, 1.30 mm, lateral 1.50 mm) (57) a hole was drilled in the skull over the right lateral cerebroventricle. Intracerebroventricular injections of artificial cerebrospinal fluid (aCSF; 2 μl vehicle injection) or 5-(N,N-dimethylamino)jodohydrochloride (DMA; 10 mM in aCSF, 2 μl injection) were made via a blunt-tipped Hamilton microsyringe (26-gauge; 10 μl total volume) that was lowered into position through the hole (from skull −4.60 mm). At 1 min postinjection the syringe was removed, the hole was sealed with bone wax, and the wound site was cleaned and closed using stainless steel wound clips. Approximately 5 h after the intracerebroventricular injection (post-MCAO or sham), rats were killed and their brains removed. The brain was prepared for coronal slicing and TTC staining as previously described.

Functional inhibition of NHE-1. DMA, a selective inhibitor of NHE-1, was used to investigate the functional inhibition of NHE-1 on

Fig. 1. Profile of mean blood pressures (MBP) showing hypertension development in age-matched spontaneously hypertensive rats (SHR) compared with normotensive Wistar-Kyoto (WKY) rats at 4–25 wk of age. Boxed regions indicate the age and stage of development that are categorized as prehypertension (5 wk), early-stage (10 wk), and later-stage (15 wk) hypertension. Each point on the graph shows the mean ± SE for MBP (mm Hg) in SHR and WKY rats during development (n = 3 for each strain; n = 4 measurements per rat per time point).
ischemia-induced infarct volume. This investigation focused on the normotensive WKY controls and hypertensive SHR at 15 wk. Four study groups were examined and the groups consisted of the following: sham/aCSF vehicle (group I); sham/DMA (group II); MCAO/aCSF vehicle (group III); MCAO/DMA (group IV). Prior to anesthesia, each rat was weighed, and noninvasive blood pressure measurements were obtained. Following anesthesia (as previously described), blood pressures were again measured and the incision sites for MCAO and the skull were shaved, scrubbed, and prepped for surgery. Briefly, each rat underwent laser Doppler probe placement and CBF was continuously monitored. Once the probe was set, the site was again prepared for intracerebroventricular injection of either drug (DMA) or vehicle (aCSF) as previously described. Following intracerebroventricular injection, the wound site was closed and blood pressures were measured. The rat was prepped for MCAO surgery. Occlusion of the MCAO was verified by laser Doppler and the rat was allowed to recover. One hour post-MCAO surgery, blood pressures were again measured. Once the 4-h ischemic period was met, the rat was neurologically scored and then anesthetized. A terminal blood gas was obtained via the descending aorta to collect terminal blood ion chemistry. The rat was killed, and the brain was prepped for slicing, staining, and fixing of tissues as previously described.

Statistical analysis. All values are presented as means ± SE. In each Western blot, each lane represents one animal and the total n values are indicated in the figure legends. All conditions were tested in at least triplicate. All statistical analyses were done using Sigma Stat software, version 2.0 (Systat Software, Point Richmond, CA). All data shown were analyzed using either two-way ANOVA followed by Tukey’s honestly significant difference post hoc analysis or Student’s t-test as indicated in the figure legends. P values < 0.05 were considered to indicate significant difference. Each F statistic is the mean square for the source of interest divided by mean square error, and the values are expressed as the degree of freedom for the numerator (age) and denominator (strain), as well as the total degree of freedom (mean – 1).

RESULTS

Physiological profile. The MBP of juvenile SHR and age-matched WKY controls were measured several times per week.
from 4–25 wk of age to chronicle a profile of spontaneous hypertension development. In Fig. 1, baseline MBP of SHR at 4–5 wk are within prehypertensive range (≈115 mmHg) and by 6–10 wk of age, gradual increases in MBP are observed compared with age-matched WKY rats. By week 10, SHR are exhibiting clinical signs of early-stage hypertension compared with normotensive WKY rats (MBP = 145 mmHg and 120 mmHg, respectively). Continued development of the SHR by 15 wk resulted in MBP in excess of 160 mmHg, while WKY controls remained in the normotensive range. Measurements from 15–25 wk of age show stabilization of MBP in both strains, with SHR and WKY rats exhibiting MBP of >160 mmHg and 130 mmHg, respectively. Furthermore, body and brain weights of SHR were significantly less than that of age-matched controls at all ages (Table 1).

Blood pressures. SBP, DBP, and MBP were also measured at 5, 10, and 15 wk of age in SHR and WKY rats (data not shown) and significantly increased with age (F1,179 = 101.859, P < 0.001; F2,179 = 90.613, P < 0.001; F3,179 = 133.908, P < 0.001), strain (F1,179 = 206.848, P < 0.001; F1,179 = 44.676, P < 0.001; F1,179 = 143.610, P < 0.001), and interactions between age and strain (F1,179 = 31.653, P < 0.001; F2,179 = 17.387, P < 0.001; F2,179 = 30.832, P < 0.001). Although SHR showed significant increases in SBP compared with age-matched controls in all age groups, DBP and MBP exhibited significant increases at only 10 and 15 wk. In comparison, WKY rats showed significant differences within strain in all age group interactions except between 10 and 15 wk in SBP, DBP, and MBP. Furthermore, there were significant differences in SBP, DBP, and MBP in all age groups within SHR.

Neurological profile, pattern of infarct, and brain infarct volume following MCAO. Rats were subjected to blood gas analysis following either sham or permanent 4-h MCAO. Blood gas parameters (pH, PCO2, PO2, hematocrit, Na+, K+, Ca2+, Cl−, SO2) showed no difference with age or between strains before or after sham or permanent 4-h MCAO and were within normal physiological range (data not shown). Additionally, all rats subjected to sham or permanent 4-h MCAO underwent neurological scoring. A significant increase in total neurological score was observed in all groups following
MCAO compared with sham MCAO within age-matched strain (Table 1). Following sham MCAO, there were no differences in neurological scores in SHR compared with age-matched controls. Following MCAO, 15-wk-old SHR demonstrated a slight decrease in the total neurological score compared with age-matched control.

Brain infarct size was compared among all groups in coronal brain slices stained by TTC. In Fig. 2, representative coronal brain slices show a typical pattern of infarct produced by permanent 4-h MCAO in SHR and age-matched WKY at 5, 10, and 15 wk. Although the total neurological score for 5-wk-old SHR indicated ischemic impairment (6.7 ± 0.2 vs. 1.8 ± 0.3, MCAO vs. sham, respectively; Table 1), the extent of the infarct damage in the coronal slices was less pronounced and was less consistent compared with age-matched control (Fig. 2). In hypertensive SHR (10 and 15 wk), the infarctions were typically restricted to the frontal and parietal areas with more enhanced and diffuse damage throughout the slices compared with normotensive, age-matched WKY. No cortical infarctions (data not shown) or neurological deficits (Table 1) were observed following sham surgery for all groups indicating ischemic tissue damage or neurological deficits. The percent total infarct (Fig. 3B) and infarction pattern (Fig. 3A) of 5-wk-old SHR showed no difference compared with age-matched WKY controls, whereas the percent total infarct of both 10- and 15-wk-old SHR demonstrated a 40% increase in infarct volume compared with age-matched WKY controls (Fig. 3, D and F, respectively). Furthermore, the infarction occupied most of the forebrain areas with significantly increased damage occurring in slices 1, 5–7 and show more diffuse damage in the posterior and extreme anterior portions of the forebrain compared with normotensive, age-matched slice controls (Fig. 3, C and E). These data suggest that the greatest increase in infarct volume in the SHR occurs at 10 and 15 wk of age, which correlate with development of spontaneous hypertension in this strain.

Hypertension development and expression of TJ and ion transporter proteins. The expression of ZO-1, ZO-2, occludin, actin, and claudin-5 were examined for changes in 5-, 10-, and 15-wk-old SHR and age-matched WKY rats. In 15-wk-old hypertensive SHR, expression of ZO-2 was significantly decreased compared with age-matched normotensive control (Table 2; Fig. 4). In contrast, ZO-1, occludin, actin, and claudin-5 showed no significant changes in protein expression among all treatment groups (Table 2). Expression levels of the ion transporter proteins, NKCC and NHE-1 in brain microvessels from SHR and WKY at 5, 10, and 15 wk were also evaluated. There were no alterations in NKCC expression among all groups (Table 2). However, a significant increase in protein expression was observed for NHE-1 in hypertensive 15-wk-old SHR compared with age-matched WKY rats (Table 2; Fig. 4). These results show differential regulation in the protein expression of NKCC and NHE-1 with established hypertension.

Inhibition of NHE-1 on ischemia-induced infarct volume. Figure 5A is a timeline illustrating the sequence of experimental events for all rats used in the inhibition of NHE-1 on ischemia-induced infarct volume in 15-wk-old SHR compared with age-matched WKY controls. Blood pressure measurements were obtained (designated I-IV on the timeline, corresponding to the groups) throughout the course of the experiment. No changes in blood pressures were observed with intracerebroventricular injection (n = 6 per group; data not shown). Figure 5B represents a laser Doppler trace showing CBF flux during intracerebroventricular injection of DMA (top trace) and the occlusion of the MCA (bottom trace). CBF flux measurements were determined immediately before

### Table 2. Effect of hypertension development on tight-junction (TJ) and ion transporter protein expression in brain microvessels of SHR compared to WKY rats at 5, 10, and 15 wks of age

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<tr>
<th></th>
<th>5wk</th>
<th>10wk</th>
<th>15wk</th>
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<tr>
<td><strong>TJ proteins, mol wt</strong></td>
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<td></td>
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<tr>
<td>ZO-1 (220 kDa)</td>
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<td>99.2±1.6</td>
<td>100±3.1</td>
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<tr>
<td>Actin (42 kDa)</td>
<td>100±7.2</td>
<td>101.1±8.5</td>
<td>100±3.1</td>
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<tr>
<td>Claudin-5 (22–24 kDa)</td>
<td>100±1.9</td>
<td>99.2±7.5</td>
<td>100±7.7</td>
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<tr>
<td><strong>Ion transporter proteins, mol wt</strong></td>
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</tr>
<tr>
<td>NKCC (145–202 kDa)</td>
<td>100±3.8</td>
<td>114.5±15.4</td>
<td>100±3.3</td>
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<tr>
<td>NHE-1 (92 kDa)</td>
<td>100±7.2</td>
<td>106.3±8.2</td>
<td>100±2.2</td>
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Data are presented as means ± SE % control of normalized optical density of SHR compared with normotensive, age-matched WKY control rats. ZO, zonula occludens. *P < 0.05 compared with age-matched WKY rats. All TJ proteins and Na⁺-K⁺-2Cl⁻ (NKCC) (n = 6 per treatment group); Na⁺/H⁺ exchanger 1 (NHE-1) (n = 12 per treatment group).
MCAO and after occlusion. All rats that underwent permanent 4-h MCAO in this study showed reduced CBF to an average of 25–35% preocclusion CBF. Intracerebroventricular injection had no effect on CBF, and sham rats showed no change in CBF (data not shown).

Following either sham or permanent 4-h MCAO, 15-wk-old SHR or WKY rats were subjected to terminal blood gas analysis and neurological scoring. All blood gas values were well within normal physiological limits (data not shown). No significant (NS) differences were observed in total neurological scoring of sham rats or MCAO rats (group I: sham + aCSF, WKY NS = 0.5 ± 0.0, SHR NS = 0.6 ± 0.1; group II: sham + DMA, WKY NS = 0.6 ± 0.1, SHR NS = 0.6 ± 0.1; group III: MCAO + aCSF, WKY NS = 8.3 ± 0.2, SHR NS = 8.3 ± 0.2; group IV: MCAO + DMA, WKY NS = 8.0 ± 0.3, SHR NS = 7.7 ± 0.2). Additionally, rats that underwent MCAO demonstrated significant neurological impairment, while sham rats did not. No cortical infarctions or neurological deficits were observed following sham surgery, indicating that the surgical procedure or administration of drug or vehicle did not contribute to ischemia-induced tissue damage or neurological deficits. Furthermore, DMA administration did not have
Fig. 6. Representative brain slices showing cerebral infarction in 7 coronal brain sections stained with 2% TTC following permanent 4-h MCAO in 15-wk-old age-matched SHR and WKY rats [group II; artificial cerebrospinal fluid (aCSF); group IV (+) DMA]. Each planar image corresponds to a 2-mm section surface. The unstained regions of the brain indicate ischemic infarction due to occlusion of the MCA. The last row indicates an observational difference in ~50% of the rats measured [group IV (+) DMA-treated SHR] where the infarct area is partially stained and appears pink in color, indicating possible tissue viability in the presence of DMA. Coronal slices were prepared for all groups (n = 6 per group).

Any significant effects on baseline CBF or MBP levels (data not shown).

Figure 6 shows representative coronal brain slices that illustrate a typical pattern of infarction produced by permanent 4-h MCAO in SHR and WKY at 15 wk (groups III and IV). When treated with aCSF, the pattern of infarction appears greater in SHR compared with WKY (group III). However, an overall attenuation in infarct volume was observed in SHR treated with DMA (group IV). Although infarct volume was attenuated in DMA-treated SHR, two different patterns of infarcted tissue were observed. In one portion of SHR investigated, the infarcts were small, focused lesions that remained unstained by TTC. Alternatively, some of the rats appeared to partially stain for TTC, and as a consequence, some of the infarcted area appeared pink in color. This is perhaps indicative of some viable tissue that survived within the lesioned area (see last row, Fig. 6). No ischemic tissue was detected in sham rats (groups I and II, data not shown).

Semiautomated analysis of infarct area in coronal brain slices (Fig. 7, A and B) shows the forebrain infarction profile following permanent 4-h MCAO. The infarction profile of 15-wk-old SHR injected with aCSF vehicle (group III) showed that the infarction occupied most of the forebrain region with significant increases in infarct size occurring in slices 1, 5–7 compared with normotensive, group-matched slice controls (Fig. 7A). However, DMA-treated SHR (group IV) showed attenuated infarct damage with a marked decrease in slice 2 compared with normotensive, group-matched slice controls (Fig. 7B).

Figure 7C shows the percent total infarct of 15-wk-old SHR and WKY rats treated with aCSF or DMA (group III or IV, respectively) following permanent 4-h MCAO. SHR treated with aCSF vehicle demonstrated a 40% increase in ischemia-induced infarct volume compared with group-matched WKY controls. Furthermore, a significant decrease of nearly threefold was observed in DMA-treated SHR compared with aCSF-treated SHR, indicating inhibition of NHE-1 attenuated ischemia-induced infarct volume. These data suggest that vehicle-treated SHR (group III) showed similar increase in infarction pattern and percent total infarct compared with WKY controls as was previously observed in hypertensive SHR (15 wk; Fig. 3, E–F). Moreover, upon inhibition with DMA (group IV), SHR show marked attenuation in total infarct volume compared with SHR treated with aCSF vehicle (Fig. 7C). Thus, these data suggest that inhibition of NHE-1 results in partial protection from ischemia-induced neuronal tissue damage following stroke in the SHR strain.

**DISCUSSION**

Ischemic stroke pathophysiology consists of a number of molecular events that contribute to brain injury, including nutrient deprivation, cellular excitotoxicity, reactive oxygen species formation, tissue acidosis, inflammation, and BBB disruption (59, 70). To examine the effect of hypertension on ischemic stroke-induced infarct damage, we evaluated neurological deficits, infarction profile, and total infarct volume in the brain following MCAO in hypertensive SHR and age-matched WKY controls (7). Following permanent 4-h MCAO, both SHR and WKY show significant neurological impairment and cortical infarctions with early- and later-stage hypertensive rats displaying the most severe tissue damage compared with age-matched normotensive WKY rats. This increase in infarct volume is consistent with previous reports of increased cerebral infarct damage in SHR (15, 17, 25). Interestingly, neurological deficits were observed in 5-wk-old SHR following MCAO but the deficits did not necessarily correlate to increased infarct size. Brain tissue in the youngest group is likely the most resistant to ischemic tissue damage; thus, neurological deficits might not correlate with the extent of tissue necrosis, but rather with the extent of ischemia at the time of behavioral testing.

Endothelial cells of the BBB play a central role in sensing and responding to vascular stress (33). Prolonged exposure to hypertension may lead to dysregulation of vasoactive substances and altered BBB function. At the BBB, paracellular diffusion is limited by TJ’s, while transcellular movement is regulated by ion transporters and channels. These mechanisms
are altered during conditions associated with cerebral ischemia and linked to compromised BBB integrity (12, 20, 50, 53, 54, 68). Although this study demonstrated that no change in the expression of the TJ-associated proteins claudin-5, occludin, actin, and ZO-1 in SHR compared with age-matched WKY similar to previously reported findings in the stroke-prone SHR (45), a lack of expression modulation does not necessarily mean there is no change in function. Previously Hawkins et al. (27) showed that nicotine increased BBB permeability and altered cellular distribution of ZO-1 and claudin-3, but found no change in TJ protein expression. Hypertension may similarly modulate these TJ proteins via localization leading to a functional change in the BBB.

We found a decrease in ZO-2 expression in 15-wk-old SHR compared with control. Whether this change contributes to ischemic infarct volume remains unclear. However, our findings suggest an interaction between ZO-2 expression and prolonged hypertension in brain microvessels. ZO-2, a 160 kDa phosphoprotein and member of the membrane-associated guanylate kinase-like homolog family, is associated with cytoplasmic constituents of the TJ. Similar to ZO-1, ZO-2 acts as a signaling molecule to communicate the state of cell-cell contact of the TJ (28). ZO-2 contains nuclear localization and exportation signals and localizes to the cell nucleus in sparse or mechanically injured monolayers, suggesting that its subcellular localization is sensitive to the state of cell-cell contact (32). Furthermore, ZO-2 has also been associated with the transcription factors c-Jun, c-Fos, and C/EBP, suggesting that ZO-2 could modulate expression of proteins in response to changes in cell-cell contact at the TJ (8). Decreased expression of ZO-2 at the BBB may reflect an adaptation to chronic elevations in blood pressure where intraluminal shear stress diminishes endothelial cell-cell contact.

Another aspect of brain infarction involves the mechanisms by which ion transport pathways are modulated during ischemic stroke. The net uptake of ions and water from the blood into the brain across an intact BBB has been implicated in early edema formation following the onset of stroke (38, 52, 53). While NKCC in our model did not demonstrate a change in expression, there was a significant increase in brain microvessel NHE-1 expression in 15-wk-old SHR. The SHR has been widely reported to possess increased activity of both the ubiquitous and renal cell-specific isoforms of NHE and NKCC, respectively (56). Additionally, there is growing evidence that supports a relationship between primary hypertension and increased NHE activity in several cell types and disease states including blood cells (62), various organ tissues (37, 43), hypoxia (16) and ischemia-reperfusion models (2, 39). While we did not measure NHE-1 activity, we did observe a nearly twofold increase in NHE-1 expression during later-stage hypertension (15-wk-old SHR), which may account for the previously observed increase in activity. However, NHE-1 activity may also be modulated by growth factors, hormones, and neurotransmitters, as well as by hypertonic shrinking and mechanical stimuli (35).

To further understand the role of NHE-1 in mediating ischemic tissue injury, we investigated the effect of NHE-1 inhibition on ischemia-induced infarct volume following permanent 4-h MCAO in 15-wk-old SHR and WKY rats via intracerebroventricular administration of DMA, a selective inhibitor. Selective inhibition of NHE-1 significantly atten-
ated the increase in infarct volume in SHR (Figs. 6 and 7). TTC uptake in tissues (red in color) is used to measure mitochondrial viability in cells; hence, necrotic tissue does not absorb TTC and remains blanched in appearance. We observed that with NHE-1 inhibition in SHR, 40–50% of the rats in this group demonstrated one of two patterns of staining: 1) infarcted regions were either small, focused lesions; or 2) mixed lesions, which were stained pink in color as opposed to the blanched, necrotic tissue observed in all other groups. The staining pattern of these lesions suggests that NHE-1 inhibition may offer some neuroprotection resulting in the preservation of some viable cells. Moreover, it appears that the ischemic penumbral regions of SHR are most sensitive to DMA treatment rather than the ischemic core as evidenced by the degree of infarct damage observed in the most extreme anterior and posterior coronal slices of the forebrain. Finally, the fact that DMA had no effect on infarct volume in the age-matched WKY rats (Fig. 7C) suggests that abnormally high expression and/or activity of NHE-1 in SHR may account for the increased infarct volume observed in this strain.

This finding is supported by previous studies using other selective inhibitors of NHE which report varying degrees of neuroprotection in both in vitro and in vivo models of ischemic brain injury (30, 40, 48, 49, 51). A unique aspect of our study was the intracerebroventricular injection of DMA into the lateral ventricle of the rat brain, allowing for direct action of the DMA in the MCA territory and reducing systemic metabolism of the drug. This is the first study to demonstrate decreased infarct volume in hypertensive animals by central administration of DMA, implicating NHE-1 in exacerbation of ischemic tissue injury by hypertension.

The results of our current investigation suggest that activation of NHE-1 may be involved in the development of neuronal damage during focal cerebral ischemia in vivo. Vornov et al. (67) reported that in cerebral ischemia, DMA has been shown to protect neurons from the effects of acidosis by either suppressing pH-sensitive mechanisms from injury or by blocking sodium entry due to Na+/H+ exchange. Activation of NHE-1 may also play a major role in the development of ischemic brain injury by limiting Na+ overload. Ennis et al. (18) used DMA in transport studies to investigate the mechanism of unidirectional transport of sodium from blood to brain using in situ brain perfusion. They found that 50% of transcellular transport of sodium from blood to brain occurs through NHE and a sodium channel in the luminal membrane of the BBB, which suggests that the NHE may be involved in increased sodium transport during the early hours of edema formation associated with cerebral ischemia (18). Regardless of the mechanism(s) by which it works, inhibition of NHE-1 may be a novel approach to protect cerebral tissue against ischemic insult, particularly in patients with hypertension.

We have shown that the development of hypertension increased ischemia-induced infarct volume, decreased expression of ZO-2, and increased protein expression of NHE-1 in hypertensive SHR. Previous reports suggest that ZO-2 might be involved in the endothelial response to increased intraluminal pressure. Upon selective inhibition of NHE-1, we observed a significant reduction of cerebral infarct volume in hypertensive animals. These data suggest a novel role for NHE-1 at the neurovascular unit in the regulation of ischemia-induced infarct volume in hypertensive SHR. An implication of these findings is that NHE-1 in cerebral microvessels may be a potential target for therapeutic modulation of the neurovascular unit following acute ischemia or traumatic brain injury. This work represents a novel contribution to the understanding of how the BBB/neurovascular unit may be regulated in hypertension, and may present targets for potential therapeutics to improve the clinical outcome of stroke.

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REFERENCES

16. Cutaiia MV, Parks N, Centracchio J, Rounds S, Yip KP, Sun AM. Effect of hypoxic exposure on Na+/H+ antiport activity, isoform expres-


