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Gene inactivation of Na⁺/H⁺ exchanger isoform 1 attenuates apoptosis and mitochondrial damage following transient focal cerebral ischemia

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Abstract

We investigated mechanisms underlying the Na⁺/H⁺ exchanger isoform 1 (NHE1)-mediated neuronal damage in transient focal ischemia. Physiological parameters, body and tympanic temperatures, and regional cerebral blood flow during 30 min middle cerebral artery occlusion (MCAO) were similar in wild-type NHE1 (NHE1^{+/+}) and NHE1 heterozygous (NHE1^{+/-}) mice. NHE1^{+/+} mice developed infarct volume of 57.3 ± 8.8 mm³ at 24 h reperfusion (Rp), which progressed to 86.1 ± 10.0 mm³ at 72 h Rp. This delayed cell death was preceded by release of mitochondrial cytochrome c (Cyt. C), nuclear translocation of apoptosis-inducing factor (AIF), activation of caspase-3, and TUNEL-positive staining and chromatin condensation in the ipsilateral hemispheres of NHE1^{+/+} brains. In contrast, NHE1^{+/-} mice had a significantly smaller infarct volume and improved neurological function. A similar neuroprotection was obtained with NHE1 inhibitor HOE 642. The number of apoptotic cells, release of AIF and Cyt. C or levels of active caspase-3 was significantly reduced in NHE1^{+/-} brains. These data imply that NHE1 activity may contribute to ischemic apoptosis. Ischemic brains did not exhibit changes of NHE1 protein expression. In contrast, up-regulation of NHE1 expression was found in NHE1^{+/+} neurons after *in vitro* ischemia. These data suggest that NHE1 activation following cerebral ischemia contributes to mitochondrial damage and ischemic apoptosis.

Keywords

neuronal death; apoptosis-inducing factor; cytochrome c; HOE 642; infarction

INTRODUCTION

Cerebral ischemia triggers a complex series of pathophysiological events and eventually leads to neuronal death and subsequent neurological dysfunction (Schaller and Graf., 2004). Loss of ion homeostasis plays an important role in ischemia-induced cell damage (Hansen and Nedergaard., 1988; Siesjö., 1992). Na⁺/H⁺ exchanger isoform 1 (NHE1) is a plasma membrane

protein which is present in all eukaryotic cells, and plays a central role in regulation of intracellular pH and cell volume by exchanging intracellular H^+ with extracellular Na^+ . NHE1 activity is stimulated by ischemia-induced intracellular acidosis (Avkiran., 2001) and extracellular regulatory kinase-mediated phosphorylation (Malo et al., 2007; Luo et al., 2007). Inhibition of NHE1 function after ischemia/reperfusion injury has protective effects in cardiac and cerebral ischemia (Suzuki et al., 2002; Wang et al., 2003; Karmazyn et al., 2005; Luo et al., 2005). Our previous study demonstrated that inhibition of NHE1 with the pharmacological inhibitor HOE 642 or genetic ablation attenuates neuronal death following oxygen and glucose deprivation (OGD) or acute brain injury after transient focal ischemia (Luo et al., 2005). NHE1-mediated ischemic and reperfusion damage largely results from an increase in intracellular Na^+ which promotes Ca^{2+} influx by reverse mode operation of Na^+/Ca^{2+} exchanger (Lee et al., 2005; Kintner et al., 2007). However, it is unknown whether NHE1 activation is involved in mitochondrial dysfunction and apoptosis after focal ischemia.

Delayed neuronal cell death occurs days to weeks after reperfusion in ischemic stroke and is a primary target for neuroprotective strategies. Activation of caspase-mediated apoptosis has been documented in many cerebral ischemic studies (Chen et al., 1998; Graham and Chen., 2001). Mitochondria are central integrators and transducers for proapoptotic signals due to its storage of several critical proapoptotic activators, such as cytochrome c (Cyt. C) and apoptosis-inducing factor (AIF) (Christophe and Nicolas., 2006). Release of Cyt. C and translocation of AIF from mitochondria to nucleus will initiate caspase-dependent and -independent cell death pathways (Christophe and Nicolas., 2006), both of which could lead to the delayed neuronal death after transient cerebral ischemia (Chen et al., 1998; Cao et al., 2003).

In the present study, we investigated whether transgenic knockdown of NHE1 or pharmacological inhibition of NHE1 with its potent inhibitor HOE 642 reduces mitochondrial damage and apoptosis after middle cerebral artery occlusion (MCAO). We found that inhibition of NHE1 attenuated ischemic neuronal death. Furthermore, inhibition of NHE1 decreased release of Cyt. C from mitochondria, nuclear translocation of AIF, activation of caspase-3, and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining, and chromatin condensation in ischemic brains. The results suggest that NHE1 activation following cerebral ischemia contributes to ischemic apoptosis.

MATERIALS AND METHODS

Materials

FragEL™ DNA Fragmentation Detection Kit was from Calbiochem (La Jolla, CA, USA). Anti-Cyt C antibody was from BD Biosciences (556432, San Jose, CA, USA) and AIF antibody was from Santa Cruz Biotechnology (sc-13116, Santa Cruz, CA, USA). Neurobasal medium and B-27 were obtained from Invitrogen (Carlsbad, CA, USA). Anti-MnSOD antibody was from StressGene (02020602, Ann Arbor, MI, USA). Anti-Histone H1 antibody was from United States Biological (H5110-02A, Swampscott, MA, USA). Anti-HIF-1 α antibody was from Novus Biologicals (NB100-105, Littleton, CO, USA). Anti- β III-tubulin antibody was from Promega (G712A, Madison, WI, USA). Activate caspase-3 antibody was from Cell Signaling Technologies (Beverly, MA, USA).

Animal preparation

NHE1 null mice could not be used in this study because they develop epilepsy two weeks after birth as a result of altered expression of other membrane proteins including Na^+ channels (Bell et al., 1999; Zhou et al., 2004). There are no phenotype changes in NHE1 heterozygous (NHE1^{+/-}) mice (Luo et al., 2005). Therefore, NHE1^{+/-} mice and their NHE1^{+/+} littermates (SV129/Black Swiss; 25–30g) were used in the study. The genotype of each mouse was

determined by a polymerase chain reaction of DNA from tail biopsies as described before (Kintner et al., 2005). Animals were anesthetized with 5% halothane for induction and 0.8–1.0 % halothane plus N₂O and O₂ (3:2) for maintenance as described before (Chen et al., 2005). The left femoral artery was cannulated for blood pressure monitoring and sampling. Blood samples (80–100 µl) were taken before and during 15 min ischemia for analysis of P_aO₂, P_aCO₂, pH, Na⁺, and K⁺ with an i-STAT analyzer (i-STAT, East Windsor, NJ, USA). Any loss of plasma volume was replaced with saline. Rectal temperatures were monitored and maintained at 36.5 ± 0.5°C with a heating blanket and a heating lamp during MCAO and 30 min recovery. Tympanic membrane temperature was measured with an infrared thermometer (Thermoscan IRT 4020; Braun, Kronberg, Germany).

Focal ischemic model and drug administration

Focal cerebral ischemia in mice was induced by MCAO as described previously (Chen et al., 2005). Briefly, the left common carotid artery was exposed and the occipital artery branches of the external carotid artery (ECA) were isolated and coagulated. After coagulation of the superior thyroid artery, the ECA was dissected and coagulated. The internal carotid artery (ICA) was isolated and the extracranial branch of the ICA was then dissected and ligated. A polyamide resin glue-coated suture (6–0 monofilament nylon) was introduced into the ECA lumen and then advanced ~ 9–9.5 mm in the ICA lumen to block the middle cerebral artery (MCA) blood flow. The suture was withdrawn 30 min after the occlusion. After recovery, animals were returned to their cages with free access to food and water. At 24 or 72 h Rp, the animals were sacrificed for biochemical measurements. For NHE1 inhibitor HOE 642 treatment, 0.1–3 mg/kg HOE 642 in saline [a kind gift from Aventis Pharma (Frankfurt, Germany)] was administered through the left femoral vein immediately before the MCAO induction. Control groups were treated with saline. All animal procedures used in this study were conducted in strict compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the University of Wisconsin Center for Health Sciences Research Animal Care Committee.

Measurement of regional cerebral blood flow

Regional cerebral blood flow (rCBF) was measured with a laser Doppler probe as described previously (Chen et al., 2005). Changes in rCBF at the surface of the left cortex were recorded using a blood perfusion monitor (Laserflo BPM², Vasamedics, Eden Prairie, MN, USA) with a fiber optic probe (0.7 mm in diameter). The tip of the probe was fixed with glue on the skull over the core area supplied by MCA (2 mm posterior and 6 mm lateral from the bregma). Changes in rCBF after MCAO were expressed as a percentage of the baseline value. All of the mice underwent 30 min MCAO and subsequent 24 or 72 h Rp.

Investigation of intracranial vasculature

The experimental animals NHE1^{+/+} and NHE1^{+/-} mice (n = 3 for each genotype) were anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). After thoracotomy was performed, a cannula was introduced into the ascending aorta through the left ventricle. Transcardial perfusion fixation was performed with 2 ml saline and 2 ml of 3.7% formaldehyde. Carbon lampblack (C198–500, Fisher Scientific, NJ, USA) in an equal volume of 20% gelatin in ddH₂O (1 mL) was injected through the cannula. The brains were removed and fixed in 4% paraformaldehyde (PFA) overnight at 4° C. Posterior communicating artery (PComA) connect the carotid and vertebrobasilar arterial system and its development affects brain sensitivity to ischemia among different mouse strains (Barone et al., 1993). Development of PComAs in both hemispheres was examined and graded on a scale of 0 to 3, as reported previously (Murakami et al., 1997a; Murakami et al., 1997b; Majid et al., 2000). 0: no connection between

anterior and posterior circulation; 1: anastomosis in capillary phase (present but poorly developed); 2: small truncal PComA; 3: truncal PComA.

Infarction size measurement

After 24 or 72 h Rp, NHE1^{+/+} and NHE1^{+/-} mice were anesthetized with 5% halothane vaporized in N₂O and O₂ (3:2) and then decapitated. Brains were removed and frozen at -80°C for 5 min. Two-millimeter coronal slices were made with a rodent brain matrix (Ted Pella Inc., Redding, CA, USA). The sections were stained for 20 min at 37°C with 2% 2, 3, 5-triphenyltetrazolium chloride monohydrate (TTC, Sigma, St Louis, MO, USA). Infarction volume was calculated with the method reported by Swanson (Swanson et al., 1990) to compensate for brain swelling in the ischemic hemisphere. Briefly, the sections were scanned, and the infarction area in each section was calculated by subtracting the non-infarct area of the ipsilateral side from the area of the contralateral side with NIH image analysis software (Scion Image, Frederick, MD, USA). Infarction areas in each section were summed and multiplied by section thickness to give the total infarction volume.

Neurological deficit evaluation

Neurological deficit were evaluated in NHE1^{+/+} and NHE1^{+/-} mice after 30 min MCAO and 72 h Rp. The focal neurological status was scored (focal score) using the method of Clark (Clark et al., 1997). In brief, 7 different functional tests were conducted to evaluate focal neurological deficit (1. body symmetry; 2. gait; 3. climbing; 4. circling behavior; 5. front limb symmetry; 6. compulsory circling; 7. sensory response). Each test has a score from 0 (normal) to 4 (most severe deficit). The sum of 7 test scores was recorded as the final score for each animal.

Brain homogenate and subcellular fractionation preparations

Mice were anesthetized with 5% halothane vaporized in N₂O and O₂ (3:2) and decapitated. The ipsilateral and contralateral hemispheres were dissected and cut into small pieces in ice-cold brain isolation buffer (pH 7.4, in mmol/L: 225 mannitol, 75 sucrose, 1 EGTA), or in anti-phosphatase buffer (pH 7.4, mmol/L: 145 NaCl, 1.8 NaH₂PO₄, 8.6 Na₂HPO₄, 100 NaF, 10 Na₄P₂O₇, 2 Na₃VO₄, 2 EDTA containing protease inhibitors) as described previously (Luo et al., 2005). To obtain brain homogenate, tissues were homogenized by 7 strokes and centrifuged at 2,200g for 3 min at 4°C. The supernatant was saved and pellet was centrifuged for another 3 min at 2,200g at 4°C. The combined supernatant was centrifuged for 8 min at 12,100g at 4°C. Protein content was determined by BCA method (Pierce, Rockford, IL, USA).

The subcellular fractionation was prepared as described before (Matsumori et al., 2005) with some modification. Brain tissues were dissected and gently homogenized with a tissue pestle grinder (Kontes, Vineland, NJ, USA) for 10 strokes in 5 volumes of buffer A [pH 7.9, in mmol/L: 20 HEPES-KOH, 250 sucrose, 10 KCl, 1.5 MgCl₂, 1 EDTA, 1 dithiothreitol (DTT)], and protease inhibitor cocktail (Kintner et al., 2005). Samples were centrifuged at 200g at 4°C for 5 min to remove unbroken cells, then centrifuged at 750g at 4°C for 15 min to separate into supernatant A (containing cytosolic and non-synaptosomal mitochondrial proteins, see below) and pellet A. Pellet A containing the nuclear fraction was resuspended in 90 µL of buffer B (pH 7.9, in mmol/L: 20 HEPES-KOH, 1.5 MgCl₂, 20 KCl, 0.2 EDTA, 0.5 DTT, and protease inhibitor cocktail), and mixed with 30 µL of buffer C (pH 7.9, in mmol/L: 20 HEPES-KOH, 1.2 KCL, 0.2 EDTA, 0.5 DTT, and protease inhibitor cocktail). The samples were placed on ice for 30 min during extraction and then centrifuged at 12,000 g for 10 min. Supernatants containing nuclear fraction were transferred and stored at -70°C. Supernatant A containing cytosolic and mitochondrial proteins was further centrifuged at 16,000 g for 30 min at 4°C to separate supernatant B from pellet B. Supernatant B was used as the cytosolic fraction, and

pellet B was used as the non-synaptosomal mitochondrial fraction after resuspension in buffer A. Protein concentrations were determined by the BCA method.

Gel Electrophoresis and Western Blotting

Protein samples and prestained molecular mass markers (Bio-Rad, Hercules, CA, USA) were denatured in sodium dodecyl sulfate (SDS) reducing buffer (1:1 by volume) and heated at 37°C or 100°C for 5 min before gel electrophoresis. The samples were then electrophoretically separated on 6 or 15 % SDS gels and the resolved proteins were electrophoretically transferred to a PVDF membrane (0.45µm, Millipore Corporation, Bedford, MA, USA). The blots were incubated in 7.5% nonfat dry milk in tris-buffered saline (TBS) for 2 h and then incubated with the following primary antibodies: anti-NHE1 (G115, 1:500, polyclonal, a kind gift from Dr. Leong L. Ng, University of Leicester, Leicester, UK), anti-βIII tubulin (1:3000), anti-AIF (1:200), and anti-HIF-1α (1:500) overnight at 4 °C. β tubulin III (1:3000), MnSOD (1:5000) and histone H1 (1:200) were used as protein loading controls and markers for cytosol, mitochondrial or nuclear fractions, respectively. The blots were rinsed with TBS and incubated with goat anti-rabbit (1:1000), goat anti-mouse (1:3000) and goat anti-sheep (1:1000) horseradish peroxidase-conjugated secondary IgG for 1 hour. Bound antibody was visualized using the enhanced chemiluminescence assay (Pierce, Rockford, IL, USA). Densitometric measurement of each protein band was performed with Un-Scan-It software (Silk Scientific, Orem, UT, USA). Average pixel intensity was recorded.

TUNEL staining

After 30 min ischemia and 72 h Rp, the mice were deeply anesthetized with ketamine (100 mg/kg, ip) and xylazine (10 mg/kg, ip) and transcardially perfused with 4% PFA in 0.1 M PBS (pH 7.4). Brains were removed and postfixed in 4% PFA overnight at 4 °C and cut into coronal sections (30 µm) on a freezing microtome (Leica SM 2000 R, Nussloch, Germany). Sections at the level of 2.06 mm posterior to the bregma were selected and processed for in situ labeling of DNA fragmentation using TUNEL staining (FragEL™ DNA Fragmentation Detection Kit). For negative controls, slices were treated similarly but in the absence of the TdT enzyme. TUNEL-positive cells were viewed using a Nikon TE300 inverted epifluorescence light microscope (Nikon, Japan) and data were analyzed with MetaMorph image-processing software (Universal Imaging Corp., Downingtown, PA, USA). The TUNEL-positively stained cells were counted in four evenly distributed areas (0.26 mm²/each area) of the ipsilateral hemispheres and averaged.

Immunofluorescence staining

After 30 min ischemia and 24 h Rp, the mice were deeply anesthetized and transcardially perfused with PFA as described above. Sections at the level of 2.06 mm posterior to the bregma were selected and were incubated with anti-Cyt C monoclonal antibody (1:100) or anti-AIF monoclonal (1:50) overnight at 4°C. After rinsing with TBS, sections were incubated with goat anti-mouse Alexa Fluor 488-conjugated IgG (1:200) for 1 h at 37°C. Images were captured on a Leica DMIRE 2 inverted confocal laser-scanning microscope (40×) using the Leica confocal software (Leica Microsystems Inc., Mannheim, Germany). DAPI staining was performed using mounting medium containing the DAPI (Vector Laboratories, Burlingame, CA, USA).

Quantification of Cyt. C release from mitochondria

Cyt. C release into the cytosol was assessed in subcellular fraction preparations as described above. Level of Cyt. C in cytosol and mitochondrial fractions were measured using the Quantikine M Rat/Mouse Cytochrome C Immunoassay kit (R&D Systems, Minneapolis, MN, USA). Data were expressed as ng/mg protein.

Primary cultures of mouse cortical neurons and astrocytes

Preparation of NHE1^{+/+} cortical neurons from E14–16 fetuses has been described previously (Luo et al., 2005). In brief, the cortices were removed from E14–16 fetuses and each mouse fetus was genotyped. The tissues were treated with 0.2 mg/ml trypsin at 37°C for 25 min. The cells were centrifuged at 300 g for 4 min. The cell pellet was diluted with Neurobasal medium supplemented with B-27 (2 %). The cells from individual fetal cortices were seeded separately (4×10^4 cells/cm²) in 6-well plates coated with poly-D-lysine. The cultures were maintained in an incubator of 5% CO₂ and atmospheric air at 37°C. Half of the medium was replaced twice a week. The culture contains > 99% neurons (Luo et al., 2007). DIV 12–13 cultures (days in culture) were used in this study.

Dissociated cortical astrocyte cultures were established as described (Kintner et al., 2005). Cerebral cortices were removed from 5–7-day-old mice. The cell suspension was prepared as described above and diluted in EMEM containing 100 units/ml of penicillin and 100 µg/ml streptomycin, 10 mM glutamine, and 10% fetal bovine serum. 1.0×10^4 cells/well were seeded on collagen-coated 6-well plates.

OGD treatment

DIV 12–13 cultures were rinsed with an isotonic OGD solution (pH 7.4) containing (in mM): 0 glucose, 20 NaHCO₃, 120 NaCl, 5.36 KCl, 0.33 Na₂HPO₄, 0.44 KH₂PO₄, 1.27 CaCl₂, 0.81 MgSO₄, as described before (Luo et al., 2005). Cells were incubated in 2 ml of OGD solution in a hypoxic incubator (model 3130 Thermo Forma, Marietta, OH) containing 94% N₂, 1% O₂ and 5% CO₂. Following 2 h OGD, the cells were incubated for 22 h in the normal medium in a normoxic incubator. Normoxic control cells were incubated in 5% CO₂ and atmospheric air in isotonic control buffer containing 5.5 mM glucose with the rest of the components in the buffer identical to the isotonic OGD solution. At the end of OGD and specific REOX time points, cell lysates were prepared as described before (Luo et al., 2005).

Statistical Analysis

Comparisons between groups were made by student t test, Mann-Whitney rank sum test, or ANOVA using the Bonferroni post test (SigmaStat, Systat Software, Point Richmond, CA, USA). $p < 0.05$ was considered as significant difference.

RESULTS

Physiological parameters, tympanic temperature, and rCBF in NHE1^{+/+} and NHE1^{+/-} mice

Mean arterial blood pressure (MABP), pH, P_aCO₂, P_aO₂, Na⁺, and K⁺ were not significantly different between NHE1^{+/+} and NHE1^{+/-} mice under control or ischemic conditions (Table 1). P_aO₂ tended to decrease at 15 min MCAO in both NHE1^{+/+} and NHE1^{+/-} mice, but was not statistically different from the pre-ischemic values ($p > 0.05$). Tympanic temperatures were at similar levels prior to or during ischemia in both NHE1^{+/+} and NHE1^{+/-} mice (Table 2). At 15 and 30 min MCAO, rCBF was less than 10% of the control in both NHE1^{+/+} and NHE1^{+/-} mice ($p < 0.05$, Figure 1 A). Upon reperfusion, rCBF recovered by ~46% in NHE1^{+/+} mice and ~55 % in NHE1^{+/-} mice. The small difference in rCBF between NHE1^{+/+} and NHE1^{+/-} mice was not statistically significant ($p > 0.05$).

We further examined potential gross anatomic differences in the cerebral circulation of NHE1^{+/+} and NHE1^{+/-} mice (Figure 1 B). The circle of Willis was observed and the distribution of MCA trunk and branch appeared to be anatomically identical in both genotypes (Figure 1 B). The pComA development was similar in NHE1^{+/+} and NHE1^{+/-} brains (Figure 1 B). The mean pComA development score in two hemispheres was 3.0 ± 0.0 in NHE1^{+/+} mice and 2.6 ± 0.6 in NHE1^{+/-} mice. There was no significant difference between the two genotypes ($p >$

0.05, $n = 3-4$). These scores are within the range of normal PComA development (Murakami et al., 1997).

Reduced infarct volume by inhibition of NHE1 after ischemia-reperfusion injury

As shown in Figure 2 A, infarct volume in NHE1^{+/+} mice was $57.3 \pm 8.8 \text{ mm}^3$ at 24 h Rp. It expanded to $86.1 \pm 10.0 \text{ mm}^3$ at 72 h Rp ($p < 0.05$), suggesting a delayed neuronal damage. In contrast, NHE1^{+/-} mice exhibit an infarct volume of $27.8 \pm 7.6 \text{ mm}^3$ at 24 h Rp, which was significantly smaller than NHE1^{+/+} brains ($p < 0.05$). The infarct volume in NHE1^{+/-} brains at 72 h Rp was only ~50% of the NHE1^{+/+} controls ($43.2 \pm 13.0 \text{ mm}^3$, $p < 0.01$, Figure 2 A, **lower panel**).

To further confirm that decreased neuronal damage in NHE1^{+/-} brains resulted from reduction of NHE1 activity, we investigated the effects of NHE1 inhibitor HOE 642 in NHE1^{+/+} mice. HOE 642 was administered by i.v. immediately prior to MCAO at a dose of 0.1, 1, and 3 mg/kg. Figure 2 B shows that HOE 642 at 3 mg/kg reduced infarct volume ~50% at 72 h Rp ($85.7 \pm 10.9 \text{ mm}^3$ in control vs. 34.1 mm^3 in HOE-treated group, $p < 0.05$, **top and lower panels**).

We then investigated whether NHE1^{+/-} mice exhibit improved neurological function compared to NHE1^{+/+} mice. As shown in Figure 2 C, the neurological deficit score of NHE1^{+/+} mice was 13.0 ± 2.9 , which is significantly higher compared to the score of NHE1^{+/-} mice (9.2 ± 0.1) at 72 h Rp ($p < 0.05$). These data indicate that NHE1^{+/-} mice not only have less ischemic infarct but also maintain better neurological function.

Reduced mitochondrial damage in NHE1^{+/-} brains after ischemia-reperfusion injury

We investigated the role of NHE1 in mitochondrial damage by detecting Cyt. C release from mitochondria and nuclear translocation of AIF. As shown in Figure 3 A, in the contralateral hemispheres of NHE1^{+/+} or NHE1^{+/-} brains, all cells were positively stained for Cyt. C with a punctate perinuclear expression pattern (**arrowhead**), which represents its mitochondrial localization in normoxic cells. In contrast, a few cells stained for Cyt. C in the ipsilateral side of NHE1^{+/+} brains at 24 h Rp (Figure 3 A). The Cyt. C immunosignal in these cells was diffuse and located in both cytoplasm and nuclei (**arrow, insets**). This implies that Cyt. C is released from mitochondria and may be subsequently degraded in the damaged cells. Overall, ~20% cells among total DAPI-positive cells showed Cyt. C release in NHE1^{+/+} brains (Figure 3 B). However, in the ipsilateral hemispheres of NHE1^{+/-} brains, although the Cyt. C immunosignal was reduced in some cells (**arrow**), many cells exhibit perinuclear Cyt. C expression similar to contralateral hemispheres (Figure 3 A, **arrowhead**). Release of Cyt. C in NHE1^{+/-} ipsilateral hemispheres was reduced by ~87% (Figure 3 B; $p < 0.05$). In parallel, Cyt. C release was also determined in cytosol and mitochondrial subcellular fractions. Cytosolic Cyt. C level in NHE1^{+/+} ipsilateral hemispheres was $63.3 \pm 12.0 \text{ ng/mg protein}$, which was ~50% higher than the contralateral hemispheres (Figure 3 C). In contrast, Cyt. C release was absent in ipsilateral NHE1^{+/-} brains (Figure 3 C). No significant changes in mitochondrial Cyt. C levels were detected (data not shown).

We then investigated whether translocation of AIF from mitochondria to nucleus occurred in ischemic brains at 24 h Rp. The contralateral sides of NHE1^{+/+} brains showed strong immunostaining for mitochondrial AIF (perinuclear pattern) (Figure 4 A, **arrowhead**). In contrast, only a few cells were positively stained for AIF in the ipsilateral hemispheres of NHE1^{+/+} brains (Figure 4 A, **arrow**). Several cells showed nuclear translocation of AIF with condensed and shrunken DNA morphology (Figure 4 A, **arrow**). In contrast, immunostaining pattern of AIF was not significantly altered in the ipsilateral hemispheres of NHE1^{+/-} brains (Figure 4 A, **arrowhead**) with only ~2% AIF translocation (Figure 4 B).

Furthermore, nuclear translocation of AIF was also examined in subcellular fractions. Figure 4 C illustrates that no changes in mitochondrial AIF levels occurred at 24 h Rp in either NHE1^{+/+} brains or NHE1^{+/-} brains. However, the amount of AIF in the nuclear fraction was significantly increased in ipsilateral hemispheres of NHE1^{+/+} brains but not in the NHE1^{+/-} brains (There was some contamination of synaptosomal mitochondria in the nuclear fraction). The ratio of AIF/histone H1 was analyzed and shown in Figure 4 D. Taken together, we consistently observed that NHE1^{+/-} brains exhibit less release of mitochondrial Cyt. C and AIF. These data imply that NHE1 activity may contribute to mitochondrial damage after transient cerebral ischemia.

TUNEL staining in NHE1^{+/+} and NHE1^{+/-} brains following transient focal ischemia

TUNEL-positive cells were analyzed in NHE1^{+/+} and NHE1^{+/-} brains at 72 h Rp. No TUNEL-positive cells were detected in the contralateral sides of either NHE1^{+/+} or NHE1^{+/-} brains (Figure 5 A, **top panel, insets**). In ipsilateral hemispheres of NHE1^{+/+} brains, many cells were TUNEL-positive (Figure 5 A, **top panel, arrows**). The mean value of TUNEL-positive cells was 437 ± 74 cells / mm² in NHE1^{+/+} ipsilateral hemispheres. In contrast, only 139 ± 71 TUNEL-positive cells/ mm² were found in ipsilateral hemispheres of NHE1^{+/-} brains ($p < 0.05$, Figure 5 A, **lower panel**). These data suggest that NHE1 plays a role in ischemic apoptosis.

To further strengthen this finding, we also examined chromatin condensation in ipsilateral brains at 24 h Rp. Apoptotic cells showed condensed chromatin stained with DAPI (Figure 5 B, top panel, arrows). There were more cells with condensed DNA in NHE1^{+/+} ipsilateral brains (36 ± 15 cells/mm²) than in NHE1^{+/-} ipsilateral brains (10 ± 3 cells/mm², $p < 0.05$, Figure 5 B, low panel). Taken together, these data imply that there is less apoptotic cell death in NHE1^{+/-} ipsilateral brains.

Gene inactivation of NHE1 reduces stimulation of caspase 3 following ischemia

In addition to nuclear condensation and TUNEL staining, we examined changes of active caspase-3 protein level, another characteristic feature of apoptosis, in NHE1^{+/+} and NHE1^{+/-} brains. As demonstrated in Figure 6 A, active caspase-3 levels increased in ipsilateral NHE1^{+/+} brains at 6 h Rp, reached statistically significant levels at 24 h Rp, and remained elevated at 72 h Rp. In contrast, cleaved caspase-3 levels in ipsilateral NHE1^{+/-} brains were similar to sham control or contralateral controls (Figure 6 B).

In an *in vitro* ischemia model, active caspase-3 protein level in NHE1^{+/+} neurons rose at 2 h OGD/2 h REOX and reached significance at 4 h REOX (Figure 6 C). Similar to the *in vivo* model, this rise was sustained during 22 h REOX (Figure 6 C). However, inhibition of NHE1 activity in NHE1^{+/+} neurons with HOE 642 abolished caspase-3 cleavage at 22 h REOX (Figure 6 C). Taken together, these data clearly demonstrate that inhibition of NHE1 activity pharmacologically or by transgenic knockdown reduces caspase-3 activation following ischemia/reperfusion.

NHE1 expression in NHE1^{+/+} and NHE1^{+/-} brains after ischemia-reperfusion injury

NHE1 protein is reported to be degraded by caspase 3 in apoptotic renal tubular epithelial cells induced by staurosporine (Wu et al., 2003). In the current study, we monitored changes of NHE1 protein in NHE1^{+/+} and NHE1^{+/-} brains at 0 h, 6 h, 24 h or 72 h Rp. As shown in Figure 7 A, no obvious decrease in NHE1 protein level (~110 kDa) was found in ipsilateral hemispheres of NHE1^{+/+} brains during 0–72 h Rp. β III-tubulin was probed in all samples on the same blot. In fact, NHE1 level of ipsilateral hemispheres at 6 h Rp tends to increase. Densitometric analysis of the ratio of NHE1/ β III-tubulin revealed a moderate but statistically significant increase in NHE1 expression (Figure 7 A; $p < 0.05$).

To further confirm that no degradation of NHE1 occurs following ischemia/reperfusion, changes of NHE1 protein in neuronal or astrocyte cultures were monitored following 2 h OGD and 1–22 h REOX. As shown in Figure 7 B, no significant change in NHE1 protein level occurred at 2 h OGD in NHE1^{+/+} neurons. NHE1 protein expression was elevated at 1 h REOX and reached a peak value at 2 h REOX (~1.8 fold increase). This rise was sustained during 4 h REOX. NHE1 protein in NHE1^{+/+} neurons returned to the basal level by 22 h REOX (Figure 7 B). No NHE1 degradation was found either at 2 h OGD or 1–22 h REOX. Expression of NHE1 protein in astrocytes at 2 h OGD or 1–24 h REOX was not significantly altered (Figure 7 C).

We also investigated changes of NHE1 protein in NHE1^{+/-} brains following focal ischemia. The NHE1 protein level in NHE1^{+/-} brains was only ~50% of NHE1^{+/+} brains. Interestingly, no significant changes in NHE1 protein level were observed in ipsilateral hemispheres of NHE1^{+/-} mice at 6 h Rp when NHE1^{+/+} brains showed a tendency of NHE1 protein elevation. Moreover, OGD/REOX-induced up-regulation of NHE1 was absent in NHE1^{+/-} neurons (Figure 8 A, B).

The underlying mechanisms causing a lack of NHE1 up-regulation in NHE1^{+/-} mice after ischemia are unknown. HIF-1 α has been reported to play a role in hypoxia-induced up-regulation of NHE1 expression in pulmonary arterial myocytes (Shimoda et al., 2006). We examined HIF-1 α protein level following *in vitro* and *in vivo* ischemia. As shown in Figure 9 A, 2 h OGD triggered a significant stabilization of HIF-1 α in NHE1^{+/+} neurons that was sustained at 1 h REOX. Similarly, the level of HIF-1 α in ipsilateral hemispheres of NHE1^{+/+} brains was significantly higher than the contralateral hemispheres after 30 min MCAO (Figure 9 C). But, it returned to the basal level by 3 h Rp (Figure 9 C). Interestingly, no increases of HIF-1 α protein level were detected in either NHE1^{+/-} neurons or NHE1^{+/-} brains after ischemia (Figure 9 B, D).

DISCUSSION

Gene inactivation of NHE1 is neuroprotective in ischemic damage

The role of NHE1 in cardiac ischemic and reperfusion damage has been well established (Avkiran., 2001). Accumulation of intracellular H⁺ during ischemia stimulates NHE1 activity by protein phosphorylation and up-regulation of myocardial NHE1 expression (Avkiran., 2001). Genetic ablation of NHE1 or inhibition of NHE1 by HOE 642 produces protection in hearts subjected to ischemia-reperfusion injury (Stromer et al., 2000; Wang et al., 2003).

Our previous study demonstrated that inhibition of NHE1 is neuroprotective against acute cell death at 24 h Rp (Luo et al., 2005). To examine whether this merely just delays the cell damage, in this study, we induced mild infarction in NHE1^{+/+} mice that developed at 24 h Rp after 30 min MCAO and progressed further by 72 h Rp. In contrast, infarction volume in NHE1^{+/-} brains was significantly less at 24 h Rp with a smaller progression by 72 h Rp. A similar neuroprotection was obtained with the potent NHE1 inhibitor HOE 642. More importantly, NHE1^{+/-} mice also exhibit improved neurological function compared to NHE1^{+/+} mice. Taken together, these data suggest that NHE1 is indeed involved ischemic damage.

NHE1^{+/-} brains exhibit reduced apoptotic ischemic damage

In the current study, we investigated whether NHE1 activation contributes to ischemic apoptotic cell death. Release of mitochondrial Cyt. C and AIF, and nuclear chromatin condensation were found in NHE1^{+/+} brains at 24 h Rp, which is consistent with findings on caspase-dependent or -independent apoptosis activation following cerebral ischemia (Fujimura et al., 1998; Zhao et al., 2005). We subsequently observed an increase in TUNEL-

positively stained cells in NHE1^{+/+} brains at 72 h Rp. Moreover, we detected time-dependent activation of caspase-3 in both *in vitro* and *in vivo* models of ischemia. Intriguingly, we observed less TUNEL-positively stained cells and chromatin condensation, less activity of caspase 3, less release of mitochondrial Cyt. C or less AIF translocation in ipsilateral hemispheres of NHE1^{+/-} brains. Inhibition of NHE1 activity with its potent inhibitor HOE 642 abolished activation of caspase-3 in neurons after *in vitro* ischemia. These data lead us to conclude that NHE1 activity plays a role in cerebral ischemic apoptosis.

Activation of NHE1 has been reported to contribute to apoptosis in ischemic myocardium. Sustained activation of NHE1 increases Na⁺ influx and intracellular Na⁺ accumulation, which triggers reversal mode operation of NCX1 and intracellular Ca²⁺ overload (Karmazyn et al., 1999; Pedersen, 2006). Mitochondria can take up the excessive Ca²⁺ through its uniporter, which may activate the permeability transition pore (PTP), depolarize mitochondrial membrane potential, and stimulate ROS generation and release pro-apoptosis proteins such as Cyt. C and AIF (Brookes et al., 2004). It has been reported that inhibition of NHE1 activity by HOE 642 attenuates the mitochondria-mediated death in myocytes (Teshima et al., 2003; Toda et al., 2007). HOE 642 also significantly reduces the number of TUNEL-positive cells in cardiomyocytes during hypoxia/reoxygenation (Chakrabarti et al., 1997; Otani et al., 2000; Sun et al., 2004). We recently reported that either pharmacological inhibition or genetic ablation of NHE1 significantly reduces mitochondrial Ca²⁺ overload following OGD/REOX in astrocyte and neuron (Kintner et al., 2005; Luo et al., 2005). These data further support the view that less neuronal death in ischemic NHE1^{+/-} brains may result from less mitochondrial dysfunction and apoptosis.

We notice that a more optimal protocol for selective study of delayed cell death or apoptosis will be needed to further investigate role of NHE1 in apoptosis (such as a global ischemia model or milder ischemic insult, and trophic factor withdraw models).

Changes of NHE1 protein level after ischemia-reperfusion injury

Little is known about changes of NHE1 expression in brains following cerebral ischemia-reperfusion injury. In the current study, we observed a transient up-regulation of NHE1 protein following both *in vitro* and *in vivo* ischemia. However, this ischemia/reperfusion-mediated change in NHE1 protein expression was absent in NHE1^{+/-} brains or ischemic NHE1^{+/-} neurons. The mechanisms underlying this difference are unknown.

In an attempt to understand this issue, we determined changes of HIF-1 α protein level in both *in vivo* and *in vitro* ischemic models. The promoter region of NHE1 gene contains a candidate-binding site for HIF-1 α (Miller et al., 1991; Dyck et al., 1995; Facanha et al., 2000). HIF-1 α plays a role in hypoxia-induced up-regulation of NHE1 mRNA and protein expression and subsequent alkalization of pulmonary arterial myocytes (Shimoda et al., 2006). In the current study, elevation of HIF-1 α protein occurred after *in vitro* or *in vivo* ischemia and this change preceded the up-regulation of NHE1 protein during post-ischemia recovery. However, in our focal ischemic model, HIF-1 α level returned to a basal level prior to 6 h Rp. On the other hand, no changes of HIF-1 α were detected in either ipsilateral NHE1^{+/-} brains or ischemic NHE1^{+/-} neurons, which is consistent with the lack of NHE1 protein up-regulation. These data show some correlative changes of HIF-1 α and NHE1 in *in vivo* and *in vitro* ischemic models. However, we cannot conclude whether there is a causative relationship between changes in HIF-1 α and NHE1 proteins following ischemia. Understanding how NHE1 protein expression is regulated in ischemic NHE1^{+/+} and NHE1^{+/-} brains requires further study.

In summary, we investigated the role for NHE1 in mitochondrial damage and apoptosis after focal ischemia. We found that both transgenic knockdown and pharmacological inhibition of NHE1 reduced ischemic neuronal death over time and concurrently improved neuronal

function. NHE1^{+/-} brains exhibit less Cyt. C release, nuclear translocation of AIF, activation of caspase-3, and positive TUNEL-staining, as well as chromatin condensation. Similar results were also found in *in vitro* model of ischemia. No degradation of NHE1 protein was detected in either model. Taken together, our results suggest that NHE1 activation following ischemia is involved in mitochondrial damage and ischemic apoptosis.

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ABBREVIATIONS

AIF	apoptosis inhibitor factor
Cyt. C	cytochrome c
HIF-1 α	hypoxia- inducible factor-1 α
ICA	internal carotid artery
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
NHE1	Na ⁺ /H ⁺ exchanger isoform 1
NHE1 ^{+/+}	NHE1 wild type mice
NHE1 ^{+/-}	NHE1 heterozygous mice
OGD	oxygen and glucose deprivation
pComA	posterior communicating artery
Rp	reperfusion
rCBF	Regional cerebral blood flow
TBS	tris-buffered saline
TUNEL	terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling

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Figure 1. Cerebral vasculature and regional cerebral blood flow in NHE1^{+/+} and NHE1^{+/-} mice
A. Changes of regional cerebral blood flow (rCBF) during MCAO and reperfusion. rCBF was determined before, during 30 min MCAO, and 15 min reperfusion (Rp). Data are expressed as percentage of the baseline, $n = 3-4$. * $p < 0.001$ vs. pre-MACO; # $p < 0.001$ vs. 30 min MCAO.
B. Cerebral vasculature anatomy of NHE1^{+/+} and NHE1^{+/-} mice. **Left panel:** Representative image of bilateral posterior communicating arteries (pComA, arrows) in NHE1^{+/+} and NHE1^{+/-} mice. Scale bar = 5 mm. **Lower panel:** amplified images of pComA from the top panel. Scale bar = 1 mm. **Right panel:** Mean pComAs scores in NHE1^{+/+} and NHE1^{+/-} mice. Data are expressed as mean $\pm$ SD, $p > 0.05$, $n = 3-4$.

Figure 2. Neuroprotection in NHE1^{+/-} mice or NHE1^{+/+} mice treated with HOE 642

A. Top panel: Representative image of infarct in NHE1^{+/+} and NHE1^{+/-} brains after 30 min MCAO and 72 h Rp. Scale bar = 5 mm. **Lower panel:** Summary data of infarct volume in NHE1^{+/+} and NHE1^{+/-} brains after 30 min MCAO and 72 h Rp. **B. HOE 642-mediated reduction in infarct volume in NHE1^{+/+} mice. Top panel:** Representative image of infarct volume in NHE1^{+/+} brains. Scale bar = 5 mm. HOE 642 was administered i.v. immediately prior to MCAO. **Lower panel:** Summary data of infarction. **C. Neurological function.** The neurological deficit scores in NHE1^{+/+} and NHE1^{+/-} mice were determined at 72 h Rp. Data are expressed as mean $\pm$ SD, n = 3–5. * p < 0.05 vs. NHE1^{+/+}; # p < 0.05 vs. 24 h in NHE1^{+/+}.

Figure 3. Gene inactivation of NHE1 reduced Cyt. C release after MCAO

A. Immunofluorescence staining of Cyt. C in the ipsilateral (IL) and contralateral (CL) hemispheres of NHE1^{+/+} and NHE1^{+/-} brains at 24 h Rp following 30 min MCAO. *: Nuclei. **Arrowhead:** Cyt. C retained cells. **Arrow:** cells with released Cyt. C. **Insets:** Double staining with Cyt. C and DAPI. Scale bar = 15 μ m. **B.** Summary data. Percentage of cells with Cyt. C release among total DAPI-positive cells was calculated in IL and CL hemispheres. Data are expressed as mean $\pm$ SD, n = 3–4. * p < 0.05 vs. NHE1^{+/+}. **C.** Cytosolic Cyt. C release measured by the immunoassay kit. Data are expressed as mean $\pm$ SD, n = 3–4. * p < 0.05 vs. NHE1^{+/+} CL.

Figure 4. Decreased translocation of AIF into nucleus in NHE1^{+/-} brains following MCAO

A. Immunofluorescence staining for AIF in the ipsilateral (IL) and contralateral (CL) hemispheres of NHE1^{+/+} and NHE1^{+/-} brains at 24 h Rp. **Arrowhead:** AIF staining in normal cells. *****, nuclei. **Arrow:** translocation of AIF. **Insets:** Double staining with AIF and DAPI. **Scale bar, 14 μ m.** **B.** Summary data. Data were expressed as mean $\pm$ SD, $n = 3-4$. * $p < 0.05$ vs. NHE1^{+/+}. **C.** Immunoblotting data for AIF expression. Mitochondrial and nucleus fractions were probed for AIF. On the same blot, MnSOD and histone H1 were examined for sample loading controls. **D.** The ratio of nuclear AIF/ histone H1 was analyzed. Data were expressed as mean $\pm$ SD, $n = 3-4$. * $p < 0.05$ vs. NHE1^{+/+} CL.

Figure 5. TUNEL-positive cells and DNA condensation in NHE1^{+/-} brains after MCAO
A. Representative staining of TUNEL-positive cells was from the ipsilateral hemispheres (2.06 mm posterior from bregma) at 72 h Rp following 30 min MCAO (**Top panel**). **Inset:** contralateral hemispheres. **Arrow:** TUNEL-positive cells. **Scale bar = 75 μ m.** **Lower panel:** Summary data. Data were expressed as mean $\pm$ SEM of TUNEL-positive nuclei per field (0.26 mm²) in the ipsilateral hemispheres. n = 4–5. * p < 0.05 vs. NHE1^{+/+}. **B.** Chromatin condensation with DAPI staining. **Top panel:** Representative staining of cells in the ipsilateral hemispheres of NHE1^{+/+} and NHE1^{+/-} brains at 24 h Rp following 30 min MCAO. **Arrows:** cells with condensed nucleus; **Arrowheads:** normal cell. **Scale bar = 25 μ m.** **Low panel:**

summary data. Data were expressed as mean $\pm$ SD of DAPI-positive nuclei per field (mm^2) in the ipsilateral hemispheres. $n = 3$. * $p < 0.05$ vs. NHE1^{+/+}.

Figure 6. Gene inactivation of NHE1 reduces stimulation of caspase 3 following ischemia

A. Time-dependent changes of active caspase-3 protein levels in NHE1^{+/+} ischemic brains. Brain lysate was prepared from contralateral (CL) and ipsilateral (IL) NHE1^{+/+} brains at 6, 24 or 72 h Rp after 30 min MCAO. The blot was probed with anti-active caspase-3 polyclonal antibody or anti- β III tubulin monoclonal antibody. Summary data are presented as a ratio of active caspase-3/ β III tubulin, which was normalized to either sham or normoxic control. Data are expressed as mean $\pm$ SD, n = 3. * p < 0.05 vs. CL. **B.** Active caspase-3 protein levels in NHE1^{+/-} brains at 72 h Rp. n = 3. **C.** Time-dependent changes of active caspase-3 protein levels in NHE1^{+/+} neuronal cultures. Cell lysates were prepared from neuronal cultures at 0 h, 1 h, 2 h, 4 h, 22 h REOX following 2 h OGD. For the HOE 642 treatment, 1 μ M HOE 642 was

presented during both OGD and 22 h REOX. Normoxic control (Con) study was used in sister cultures incubated in normoxic buffers for 24 h. Data are expressed as mean $\pm$ SD, n = 4. * p < 0.05 vs. Con. # p < 0.05 vs. 2 h OGD/22 h REOX.

Figure 7. Increased expression of NHE1 following ischemia

A. Brain crude membrane fractions were prepared from contralateral (CL) and ipsilateral (IL) NHE1^{+/+} brains at 0, 6, 24 or 72 h Rp after 30 min MCAO. The blot was probed with anti-NHE1 polyclonal antibody (G115 1:500) or anti-βIII tubulin monoclonal antibody (1:3000). **Lower panel**, Summary data. **B.** Time-dependent changes of NHE1 proteins in NHE1^{+/+} neuronal cultures. Cell lysates were prepared from neuronal cultures at 0 h, 1 h, 2 h, 4 h or 22 h REOX following 2 h OGD. Control (Con) study was used in sister cultures incubated in normoxic buffers for 24 h. The blots were probed with anti-NHE1 or re-probed with anti-β III-tubulin antibodies. Densitometric analysis of immunoblots is presented as a ratio of NHE1/β-tubulin III band intensity, normalized to control. **C.** Expression of NHE1 in astrocytes

following 2 h OGD and 0 h, 1 h, or 24 h REOX. Summary data were the ratio of NHE1/GFAP in astrocytes. Data are expressed as mean $\pm$ SD, n = 3–4. * p < 0.05 vs. Con or CL.

Figure 8. No change of NHE1 expression in NHE1^{+/-} brains following ischemia

A. Changes of NHE1 expression in NHE1^{+/-} brains following 30 min MCAO and 6 h Rp. **B.** Summary data. Data are mean $\pm$ SD, n = 3. **C.** Changes of NHE1 expression in NHE1^{+/-} neurons following 2 h OGD and 2 h REOX. **D.** Summary data. Data are mean $\pm$ SD, n = 3.

Figure 9. Changes of HIF-1α expression following ischemia

A. Changes of HIF-1α proteins in NHE1^{+/+} neurons following OGD and REOX. The blots were probed with anti-HIF-1α antibody. The same blot was re-probed with anti-βIII tubulin antibodies. **B.** Levels of HIF-1α protein in NHE1^{+/-} neurons. **C.** HIF-1α proteins in the ipsilateral (IL) and contralateral (CL) hemispheres of NHE1^{+/+} brains at 0 h and 3 h Rp following 30 min MCAO. **D.** HIF-1α protein in NHE1^{+/-} brains. Summary data are the ratio of HIF-1α /β-tubulin III band intensity. Data are expressed as mean ± SD, n = 3. * p < 0.05 vs. control or CL.

Table 1Physiological parameters in NHE1^{+/+} and NHE1^{+/-} mice

	NHE1 ^{+/+}		NHE1 ^{+/-}	
	Pre-ischemia	Ischemia	Pre-ischemia	Ischemia
pH	7.33 ± 0.06	7.28 ± 0.03	7.31 ± 0.11	7.23 ± 0.07
MABP (mm Hg)	79.8 ± 7.4	80.7 ± 4.0	83.0 ± 5.7	84.5 ± 2.1
P _a CO ₂ (mm Hg)	45.6 ± 11.3	44.2 ± 6.2	46.0 ± 11.9	47.3 ± 14.7
P _a O ₂ (mmHg)	133.8 ± 44.8	105.3 ± 9.7	133.7 ± 51.8	100.8 ± 22.8
Na ⁺ (mmol/L)	145.0 ± 1.2	141.3 ± 3.5	140.7 ± 3.0	148.0 ± 14.1
K ⁺ (mmol/L)	4.2 ± 0.6	3.7 ± 1.4	4.4 ± 0.7	4.6 ± 0.8

Values are expressed as mean ± SD, n = 3–6. The parameters were measured 15 min before MCAO and at 15 min during MCAO.

Table 2Tympanic membrane temperature in NHE1^{+/+} and NHE1^{+/-} mice

Temperature (°C)	NHE1 ^{+/+}		NHE1 ^{+/-}	
	Contralateral	Ipsilateral	Contralateral	Ipsilateral
Before MCAO (10 min)	36.2 ± 1.0	36.3 ± 0.8	36.0 ± 0.1	36.1 ± 0.1
During MCAO (15 min)	36.4 ± 1.0	36.0 ± 0.6	36.2 ± 0.2	36.3 ± 0.3
During MCAO (30 min)	37.1 ± 0.4	36.3 ± 0.4	36.5 ± 0.2	36.3 ± 0.8

Values are expressed as mean ± SD, n = 3–5.