Increased tolerance to oxygen and glucose deprivation in astrocytes from Na\(^{+}\)/H\(^{+}\) exchanger isofrom 1 null mice

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Increased tolerance to oxygen and glucose deprivation in astrocytes from Na\(^{+}\)/H\(^{+}\) exchanger isofrom 1 null mice. Am J Physiol Cell Physiol 287: C12–C21, 2004. First published March 10, 2004; 10.1152/ajpcell.00560.2003.—The ubiquitously expressed Na\(^{+}\)/H\(^{+}\) exchanger isofrom 1 (NHE1) functions as a major intracellular pH (pHi) regulatory mechanism in many cell types, and in some tissues its activity may contribute to ischemic injury. In the present study, cortical astrocyte cultures from wild-type (NHE1\(^{+/+}\)) and NHE1-deficient (NHE1\(^{-/-}\)) mice were used to investigate the role of NHE1 in pHi recovery and ischemic injury in astrocytes. In the absence of CO\(_{2}\), the mean resting pHi levels were 6.85 ± 0.03 in NHE1\(^{+/+}\) astrocytes and 6.53 ± 0.04 in NHE1\(^{-/-}\) astrocytes. Removal of extracellular Na\(^{+}\) or blocking of NHE1 activity by the potent NHE1 inhibitor HOE-642 significantly reduced the resting level of pHi in NHE1\(^{+/+}\) astrocytes. NHE1\(^{-/-}\) astrocytes exhibited a rapid pHi recovery (0.33 ± 0.08 pH unit/min) after NH\(_{4}\)Cl prepulse acid load. The pHi recovery in NHE1\(^{+/+}\) astrocytes was reversibly inhibited by HOE-642 or removal of extracellular Na\(^{+}\). In NHE1\(^{-/-}\) astrocytes, the pHi recovery after acidification was impaired and not affected by either Na\(^{+}\)-free conditions or HOE-642. Furthermore, 2 h of oxygen and glucose deprivation (OGD) led to an ~80% increase in pHi recovery rate in NHE1\(^{+/+}\) astrocytes. HOE-642 induced a 5-fold rise in intracellular [Na\(^{+}\)] and 26% swelling in NHE1\(^{+/+}\) astrocytes. HOE-642 or genetic ablation of NHE1 significantly reduced the Na\(^{+}\) rise and swelling after OGD. These results suggest that NHE1 is the major pHi regulatory mechanism in cortical astrocytes and that ablation of NHE1 in astrocytes attenuates ischemia-induced disruption of ionic regulation and swelling.

intracellular pHi; cortical astrocytes; sodium/calcium exchange; ischemia; intracellular sodium

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is, however, unknown whether these protective effects are attributable to inhibition of NHE1 activity in neurons and/or astrocytes. In addition, because these inhibitors are not isoform specific, it is not clear how dominant a role NHE1 plays compared with the other isoforms in the CNS.

In this study, we investigated the role of NHE1 in pH, recovery and cell damage in cultured cortical astrocytes after oxygen and glucose deprivation (OGD). We found that ablation of NHE1 activity decreased resting level pH, and impaired pH, recovery. OGD stimulated NHE1 activity in NHE1+/+ astrocytes; however, this increase in activity was absent in NHE1−/− astrocytes and was blocked by HOE-642 in NHE1+/+ astrocytes. Inhibition or genetic ablation of NHE1 reduced the OGD-mediated rise in intracellular Na+ concentration ([Na+]) and swelling, suggesting that the loss of NHE1 activity reduces ischemic injury in cortical astrocytes.

MATERIALS AND METHODS

Materials

Eagle’s modified essential medium (EMEM) and Hank’s balanced salt solution (HBSS) were obtained from Mediatech Cellgro (Henderson, CO). Fetal bovine serum (FBS) was obtained from HyClone Laboratories (Logan, UT). Collagen type I was purchased from Collaborative Biomedical Products (Bedford, MA). The acetoxy-methyl esters of 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF-AM) and sodium-binding benzofuran isothiopiate (SFBF-AM) were obtained from Molecular Probes (Eugene, OR). Pluronic acid was purchased from BASF (Ludwigshafen, Germany). Nigericin, dibutyryl cAMP (dbcAMP), monensin, and gramicidin were purchased from Sigma (St. Louis, MO). HOE-642 was a kind gift from Aventis Pharma (Frankfurt, Germany).

Animals and Genotype Analysis

The NHE1 null mutant (NHE1−/−) mice used were generated as previously described (3). We obtained NHE1 homozygous mutant and wild-type (NHE1+/+) mice by breeding gene-targeted NHE1 heterozygous mutant mice. Tail biopsies were obtained from 1-day-old mice. Genotypes were determined by polymerase chain reaction (PCR) analysis of DNA from tail biopsies. For PCR analysis, three primers were included in the reaction mixture. Forward (5'-GGT ATC CCC ACC ATC ATC AG-3') and reverse (5'-CGA AGA CAA GGA TGT GTA GC-3') primers were used to amplify a 450-base pair product from the wild-type gene. For the mutant gene, a reverse primer (5'-GCA TGG TCC AGA CGT CCT TG-3') and the same forward primer were used to amplify a 330-base pair product. PCR amplification was performed as follows: 94°C for 3 min, 40 cycles of 94°C for 30 s and 60°C for 30 s, and 72°C for 30 s. PCR products were run and revealed on a 2% agarose gel.

Primary Culture of Mouse Cortical Astrocytes

Dissociated cortical astrocyte cultures were established as described previously (33). Cerebral cortices were removed from 1-day-old NHE1+/+ or NHE1−/− mice. The cortices were incubated in a trypsin solution (0.25 mg/ml in HBSS) for 25 min at 37°C. The tissue was then mechanically triturated and filtered through nylon meshes. The dissociated cells were rinsed and resuspended in EMEM containing 10% FBS. Viable cells (1 × 10<sup>5</sup> cells/well) were plated in 24-well plates coated with collagen type 1 or on collagen-coated glass coverslips in 6-well plates. Cultures were maintained in a 5% CO<sub>2</sub> atmosphere at 37°C and refed every 3 days throughout the study. To obtain morphologically differentiated astrocytes, we treated confluent cultures (10 days in culture, DIV10) with EMEM containing 0.25 mM DBcAMP to induce differentiation. DBcAMP has been widely used to mimic neuronal influences on astrocyte differentiation (14, 37). Experiments were routinely performed in DIV15–DIV28 astrocytes. This culture age was chosen because immature astrocyte cultures are resistant to damage under ischemic conditions (15).

Immunofluorescence Staining

Cultured cells grown on collagen type I-coated coverslips were rinsed with PBS (pH 7.4) and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After being rinsed, cells were incubated with blocking solution (10% normal goat serum, 0.4% Triton X-100, and 1% bovine serum albumin in PBS) for 1 h. Cells were then incubated with anti-glial fibrillary acidic protein (GFAP) monoclonal antibody (1:100) or anti-NHE1 rabbit polyclonal (G115) antibody (1:100) in blocking solution for 1 h. Cells were rinsed with PBS and incubated with goat anti-mouse Alexa 488- or anti-rabbit Alexa 595-conjugated antibodies (1:200) for 1 h. Cell images were captured using a Nikon TE 300 inverted epifluorescence microscope (×20) with a Princeton Instruments MicroMax charge-coupled device (CCD) camera and MetaMorph image-processing software (Universal Imaging, Downingtown, PA). Identical digital imaging acquisition parameters were used in both negative control and experimental images.

The G115 antibody was raised in rabbit against amino acids 627–820 of the rat NHE1 sequence in the laboratory of L. L. Ng. The antibody was purified according to the same method described previously for the G116 antibody preparation (17).

Measurement of pH

Solutions. The HEPES-buffered solution (pH 7.4) contained (in mM) 140 NaCl, 5.36 KCl, 0.81 MgSO<sub>4</sub>, 1.27 CaCl<sub>2</sub>, 0.44 K<sub>H</sub>PO<sub>4</sub>, 0.33 Na<sub>2</sub>HPO<sub>4</sub>, 5.55 glucose, and 20 HEPES. Calibration solutions for pH contained (in mM) 140 NaCl, 5.36 KCl, 0.81 MgSO<sub>4</sub>, 1.27 CaCl<sub>2</sub>, 0.44 K<sub>H</sub>PO<sub>4</sub>, 0.33 Na<sub>2</sub>HPO<sub>4</sub>, 5.55 glucose, and 20 HEPES plus 10 µM nigericin. In Na<sup>+</sup>-free solution, NaCl in the HEPES-buffered solution was replaced with an equimolar concentration of NMDG. In experi-
ments in which CO2/HCO3- buffered solutions were used, HEPES was replaced with 26 mM NaHCO3 and equilibrated with 5% CO2. NH4+/NH3 solution was prepared by replacing 10 mM NaCl in either the HEPES-buffered solution or the CO2/HCO3- buffered solution with an equimolar concentration of NH4Cl.

pHi measurements. Cells grown on coverslips were incubated with 2.5 μM BCECF-AM in HEPES-buffered solution for 10 min at ambient temperature (see Figs. 2–5). For experiments in Fig. 6, cells were incubated for 2 h with 2.5 μM BCECF-AM under either normoxic control or OGD conditions (37°C). The coverslips were then washed in HEPES-buffered solution and placed in an open-bath imaging chamber (volume = 40 μl, model RC24; Warner Instruments, Hamden, CT) containing HEPES-buffered solution. The chamber was mounted on the stage of the TE 300 inverted epifluorescence microscope, and the astrocytes were visualized with a ×40 Super Fluor oil-immersion objective lens (1.3 NA, 0.22 WD). The cells were excited every 10–30 s at 440 and 490 nm, and the ratio of emission fluorescence at 535 nm was recorded. Images were collected by using a Princeton Instruments MicroMax CCD camera and analyzed with MetaFluor image-processing software (Universal Imaging). The ratio of fluorescence emissions (F490/F440) was calibrated by using the high-K+/nicergicin technique (5). The background-corrected data were fit with a variant of the Michaelis-Menten equation (5). Typically, six cells were monitored in each coverslip. NHE1+/+ and NHE1−/− astrocytes (75 cells from 4 different cultures) were exposed to the pH calibration solutions with pH 5.6–8.0. The calculated pH was not significantly different between NHE1+/+ and NHE1−/− astrocytes (7.13 vs. 7.14).

Prepulse treatment. Cells were subjected to an acid load by the transient application (1–2 min) of a NH4+/NH3 solution, pH rose as NH4+ accumulated during the prepulse. Cells were subsequently returned to a HEPES-buffered solution. Acidification of the cytoplasm occurred when NH4+ quickly diffused out of the cell. Unless otherwise stated, pHi recovery was determined during the first minute after NH4+/NH3 prepulse from the slope of a fitted linear regression.

OGD Treatment

NHE1+/+ or NHE1−/− cultures grown on coverslips in six-well plates were rinsed twice 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, and 0.81 MgSO4. Cells were incubated in 1.0 ml of OGD solution in a hypoxic incubator at 37°C (Forma model 3130; Thermo Fisher Scientific, Marietta, OH) equilibrated with 94% N2–1% O2–5% CO2. The oxygen level in the medium of cultured cells was monitored with an oxygen probe (model M1-730). Microelectrodes, Bedford, NH) and decreased to ~2–3% after 60 min in the hypoxic incubator. The OGD incubation lasted 2 h. 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.

Intracellular Na+ Measurement

Cultured astrocytes grown on coverslips were loaded with 10 μM SBFI-AM in HCO3−-free HEPES-buffered solution containing 0.05% Pluronic for 45 min at room temperature (34). The coverslips were placed in the open-bath imaging chamber containing HCO3−-free HEPES-buffered solution at ambient temperature. With the use of the Nikon TE 300 inverted epifluorescence microscope and a ×40 Super Fluor oil-immersion objective lens, astrocytes were excited every 60 s at 345 and 385 nm, and the emission fluorescence at 510 nm was recorded. Images were collected and analyzed with the MetaFluor image-processing software as described previously (34). Absolute [Na+]i was determined in the cytoplasm of cells by calibrating the SBFI fluorescence ratio with solutions containing 0, 10, 20, 40, or 80 mM external Na+ (NaCl) plus monensin (10 μM) and gramicidin (3 μM), as described previously (33).

To minimize the dye loss and phototoxicity, we performed these experiments at ambient temperature. In one set of experiments, [Na+]i was measured and calibrated at 37°C (see Fig. 7B). In another set of experiments, [Na+]i was measured in CO2/HCO3- buffered solutions at 37°C (see Fig. 7C).

Measurement of Cell Swelling

NHE1+/+ or NHE1−/− cultures grown on coverslips were placed in a closed-bath imaging chamber (volume = 36 μl, model RC20H; Warner Instruments). The chamber was mounted on a heated platform (model TC-344B; Warner Instruments) to allow regulation of buffer temperature (37°C). The chamber was perfused by gravity (0.25 ml/min) via buffer reservoirs containing either standard HEPES-buffered solution or OGD solution continuously bubbled with N2. Flow from the reservoirs was directed through a manifold, a flow valve, and an in-line oxygen monitor and then to the chamber. When flow was switched between the control reservoir and the OGD reservoir, the O2 level fell to 2–3% within 1.5 min, as monitored by an oxygen probe (model M1-730).

As an estimate of relative cell volume changes, relative cross-sectional areas (CSA) were monitored before and during OGD with the use of video-enhanced differential interference contrast (DIC) microscopy, as reported previously (33, 34). A single astrocyte was visualized by using a Nikon ×60 Plan Fluor DIC objective lens, and an image was recorded. The mean CSA was calculated after the perimeter of the cell body was traced in triplicate with MetaMorph image-processing software as described in our previous study (34).

Statistics

In statistical analysis, n values refer to the number of coverslips in each experimental condition for Figs. 2–5 and 7. Significant differences were determined using ANOVA with the Bonferroni post hoc test for multiple comparisons. Data were considered significant if a 95% confidence level was reached.

RESULTS

NHE1 Expression in Cortical Astrocytes

The genotype of newborn mice was determined by PCR. As shown in Fig. 1A, a single DNA band (~330 bp) was amplified from tail biopsies of NHE1−/− mice. In contrast, a larger DNA band (~450 bp) was detected in NHE1+/+ tail biopsies. In a heterozygous sample, both PCR products were present. Immunoblotting was performed to analyze expression of the NHE1 protein in cortical astrocytes. An ~96-kDa protein was recognized by an anti-NHE1 antibody in NHE1+/+ astrocytes (Fig. 1B). No protein band was detected by the anti-NHE1 antibody in NHE1−/− astrocytes. A lack of NHE1 bands was not due to insufficient protein loading, because, as shown on the same blot, a similar amount of β-actin protein was observed in samples from both NHE1+/+ and NHE1−/− astrocytes (Fig. 1B).

NHE1+/+ and NHE1−/− astrocytes were stained with anti-GFAP antibody. As shown in Fig. 1, C and D, abundant levels of GFAP expression were found in both NHE1+/+ and NHE1−/− astrocytes. The GFAP immunosignal was absent when the primary antibody was omitted (Fig. 1C, inset). Immunosignals with the anti-NHE1 antibody were observed only in NHE1+/+ astrocytes, not in NHE1−/− astrocytes (Fig. 1, E and F). Taken together, these results indicate that NHE1 is abundant in mouse NHE1+/+ cortical astrocytes.
solution, they rapidly recovered to a normal basal pH (Fig. 3A, b and c). In response to Na⁺-free HEPES-buffered solution, the resting pH of NHE1⁻⁻ astrocytes further decreased by 0.13 ± 0.02 pH unit (Fig. 3A, inset). The possible mechanisms for this decrease are discussed later (see DISCUSSION).

Figure 3B shows that exposing NHE1⁺⁺ astrocytes to 1 μM HOE-642 caused pH to decrease progressively, resulting in a total reduction of 0.19 ± 0.06 pH unit (P < 0.01). The effects of HOE-642 on NHE1⁺⁺ astrocytes were reversible, because pH returned to basal levels when HOE-642 was removed (Fig. 3B, b and c). In contrast, HOE-642 had no effects on the resting pH of NHE1⁻⁻ astrocytes.

**Impaired pH⁻⁻ Recovery in NHE1⁻⁻ Astrocytes**

We then studied how NHE1⁻⁻ astrocytes regulate pH after intracellular acidosis using the NH₄⁺ prepulse technique. As shown in Fig. 4A, when either NHE1⁺⁺ or NHE1⁻⁻ astrocytes were exposed to 10 mM NH₃/NH₄⁺, pH rapidly rose as NH₃ diffused into the cell and combined with H⁺ to form NH₄⁺ (Fig. 4A, a and b) and then declined slowly (Fig. 4A, b and c). When cells (either NHE1⁺⁺ or NHE1⁻⁻ astrocytes) were returned to the standard HCO₃⁻-free HEPES-buffered solution, pH decreased sharply as NH₄⁺ rapidly left cells as NH₃, trapping H⁺ inside (Fig. 4A, c and d). After the acid load, pH recovered within 2 min in NHE1⁺⁺ astrocytes (Fig. 4A, d and e). However, in NHE1⁻⁻ astrocytes, pH recovery was delayed for 1–2 min and then slowly returned to near the basal pH level over the following 10 min.

The pH⁻⁻ recovery rate was summarized by calculating the slope of pH change during the first minute after NH₄⁺ prepulse acid load in NHE1⁺⁺ and NHE1⁻⁻ astrocytes (Fig. 4A, inset). The NH₄⁺ prepulse acidified NHE1⁺⁺ and NHE1⁻⁻ astrocytes (pH of 6.43 ± 0.20 and 6.29 ± 0.09, respectively, at the first minute after the prepulse). This difference in the mean pH in NHE1⁺⁺ and NHE1⁻⁻ astrocytes did not reach statistical significance. During the first minute after NH₄⁺ prepulse acid

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**Decrease in Resting pH of NHE1⁻⁻ Astrocytes**

The pH⁻⁻ frequency distribution in NHE1⁺⁺ astrocytes ranged from 6.5 to 7.2 (Fig. 2). The mean steady-state pH in NHE1⁺⁺ astrocytes was 6.86 ± 0.03 in the presence of a HEPES-buffered solution (pH 7.4, CO₂/HCO₃⁻-free). This is in agreement with previous reports by others that astrocytes have a resting level pH below neutral under HCO₃⁻-free conditions (4, 5, 21, 26, 28). However, the pH in NHE1⁻⁻ cells had a larger range (6.04 to 6.84) and was not normally distributed (Kolmogorov-Smirnov normality test, P < 0.05). The mean steady-state pH in NHE1⁻⁻ astrocytes was significantly lower (6.53 ± 0.04, P < 0.01). In the presence of CO₂/HCO₃⁻, the mean steady-state pH in NHE1⁻⁻ astrocytes was increased relative to that observed in HEPES-buffered solution but remained significantly lower than in NHE1⁺⁺ astrocytes (6.80 ± 0.02 vs. 7.00 ± 0.02, n = 6, 2 cultures, P < 0.001).

To further examine the role of NHE1 in the maintenance of resting pH in astrocytes, we monitored pH in NHE1⁺⁺ and NHE1⁻⁻ astrocytes in a Na⁺-free HEPES-buffered solution (Fig. 3A). In the absence of Na⁺, the pH of NHE1⁺⁺ astrocytes progressively decreased (Fig. 3A, a and b). The average decrease in pH was 0.28 ± 0.05 pH unit (Fig. 3A, inset). When the NHE1⁺⁺ astrocytes were returned to the HEPES-buffered solution, they rapidly recovered to a normal basal pH (Fig. 3A, b and c). In response to Na⁺-free HEPES-buffered solution, the resting pH of NHE1⁻⁻ astrocytes further decreased by 0.13 ± 0.02 pH unit (Fig. 3A, inset). The possible mechanisms for this decrease are discussed later (see DISCUSSION).

Figure 3B shows that exposing NHE1⁺⁺ astrocytes to 1 μM HOE-642 caused pH to decrease progressively, resulting in a total reduction of 0.19 ± 0.06 pH unit (P < 0.01). The effects of HOE-642 on NHE1⁺⁺ astrocytes were reversible, because pH returned to basal levels when HOE-642 was removed (Fig. 3B, b and c). In contrast, HOE-642 had no effects on the resting pH of NHE1⁻⁻ astrocytes.

We then studied how NHE1⁻⁻ astrocytes regulate pH after intracellular acidosis using the NH₄⁺ prepulse technique. As shown in Fig. 4A, when either NHE1⁺⁺ or NHE1⁻⁻ astrocytes were exposed to 10 mM NH₃/NH₄⁺, pH rapidly rose as NH₃ diffused into the cell and combined with H⁺ to form NH₄⁺ (Fig. 4A, a and b) and then declined slowly (Fig. 4A, b and c). When cells (either NHE1⁺⁺ or NHE1⁻⁻ astrocytes) were returned to the standard HCO₃⁻-free HEPES-buffered solution, pH decreased sharply as NH₄⁺ rapidly left cells as NH₃, trapping H⁺ inside (Fig. 4A, c and d). After the acid load, pH recovered within 2 min in NHE1⁺⁺ astrocytes (Fig. 4A, d and e). However, in NHE1⁻⁻ astrocytes, pH recovery was delayed for 1–2 min and then slowly returned to near the basal pH level over the following 10 min.

The pH⁻⁻ recovery rate was summarized by calculating the slope of pH change during the first minute after NH₄⁺ prepulse acid load in NHE1⁺⁺ and NHE1⁻⁻ astrocytes (Fig. 4A, inset). The NH₄⁺ prepulse acidified NHE1⁺⁺ and NHE1⁻⁻ astrocytes (pH of 6.43 ± 0.20 and 6.29 ± 0.09, respectively, at the first minute after the prepulse). This difference in the mean pH in NHE1⁺⁺ and NHE1⁻⁻ astrocytes did not reach statistical significance. During the first minute after NH₄⁺ prepulse acid
load, the average pH recovery in NHE1\(^{+/+}\) astrocytes was 0.33 ± 0.08 pH units (at a pH of 6.43 ± 0.20). In contrast, no pH recovery was found in NHE1\(^{+/−}\) astrocytes (0.04 ± 0.08 pH unit at a pH of 6.29 ± 0.09). Figure 4B shows the pH summary data for NHE1\(^{+/+}\) and NHE1\(^{+/−}\) astrocytes at resting level, 2 min, and 10 min after NH\(_4\)\(^+\) prepulse. pH in NHE1\(^{+/+}\) astrocytes returned to the steady resting level by 2 min after acid loading. However, pH in NHE1\(^{+/−}\) astrocytes dropped to ~6.2 after the NH\(_4\)\(^+\) prepulse. It took 10 min for NHE1\(^{+/−}\) astrocytes to recover pH to the resting level. This indicates that NHE1 is critical for initial pH recovery after an acid load in CO\(_2\)/HCO\(_3\)^−-free solution.

**pH\(_i\) Recovery in Astrocytes Is Mediated by NHE1 Activity**

The data in Fig. 4 indicate that initial pH\(_i\) recovery after an acid load in astrocytes is exclusively mediated by NHE1 in the absence of HCO\(_3\)^−. To further establish this point, we investigated the Na\(^+\) dependence of pH\(_i\) recovery after an acid load in NHE1\(^{+/+}\) and NHE1\(^{+/−}\) astrocytes in the absence of HCO\(_3\)^− (Fig. 5, A and B). When NHE1\(^{+/+}\) or NHE1\(^{+/−}\) astrocytes were exposed to 10 mM NH\(_4\)\(^+\)/NH\(_3\)^−, pH\(_i\) increased (Fig. 5, A and B) and then declined gradually (Fig. 5, A and B, b and c). Both NHE1\(^{+/+}\) and NHE1\(^{+/−}\) astrocytes exhibited acidification after the NH\(_4\)\(^+\) acid load (Fig. 5, A and B, c and d). In contrast to the results shown in Fig. 4, in the absence of extracellular Na\(^+\), NHE1\(^{+/+}\) astrocytes failed to regulate pH\(_i\) (Fig. 5A, d and e) after acidosis. pH\(_i\) in NHE1\(^{+/+}\) astrocytes decreased by 0.19 ± 0.04 pH unit/min at a pH of 6.36 ± 0.012 (Fig. 5A, inset) during the first minute of recovery. NHE1\(^{−/−}\) astrocytes exhibited no pH\(_i\) recovery in the absence of Na\(^+\) and no further decrease in pH\(_i\) (0.02 ± 0.02 pH unit/min at a pH\(_i\) of 6.09 ± 0.14) (Fig. 5A). NH\(_4\)/NH\(_3\)\(^−\) pulse led to a significantly more acidification in NHE1\(^{−/−}\) astrocytes than in NHE1\(^{+/+}\) astrocytes in the absence of extracellular Na\(^+\) (P < 0.05). pH\(_i\) recovered when NHE1\(^{+/+}\) cells were returned to the control conditions (Fig. 5A, e and f). However, there was only a slow recovery of pH\(_i\) in NHE1\(^{−/−}\) astrocytes (Fig. 5A, e and f).

As shown in Fig. 5B, inhibition of NHE1 activity with the potent NHE1 blocker HOE-642 had a similar effect on pH\(_i\) to that of removal of extracellular Na\(^+\). In the presence of 1 μM HOE-642, NH\(_4\)/NH\(_3\)\(^−\) triggered acid loading in NHE1\(^{+/+}\) and NHE1\(^{+/−}\) astrocytes (Fig. 5B, b–d). However, after acidification, there was little increase in pH\(_i\) in NHE1\(^{+/+}\) cells (Fig. 5B, d and e). The rate of pH\(_i\) recovery in NHE1\(^{+/+}\) astrocytes in the presence of HOE-642 was 0.03 ± 0.1 pH units/min at a pH\(_i\) of 6.35 ± 0.19 (Fig. 5B, inset). When HOE-642 was removed from the buffer, pH\(_i\) rapidly returned to basal levels in

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**Fig. 3. Effects of Na\(^+\)-free buffer and HOE-642 on resting pH\(_i\) levels. A:** representative trace of pH\(_i\) from either a NHE1\(^{+/+}\) or NHE1\(^{+/−}\) astrocyte when exposed to Na\(^+\)-free HEPES-buffered solution for 10 min (a–b) and then returned to standard HEPES-buffered solution (b–c). Inset: mean decrease in pH\(_i\) (ΔpH\(_i\)) under Na\(^+\)-free conditions for either NHE1\(^{+/+}\) (n = 3) or NHE1\(^{+/−}\) astrocytes (n = 3). B: representative trace of pH\(_i\) from either a NHE1\(^{+/+}\) or NHE1\(^{+/−}\) astrocyte when exposed to 1 μM HOE-642 for 10 min (a–b) and then returned to standard HEPES-buffered solution (b–c). Inset: ΔpH\(_i\) with HOE-642 in either NHE1\(^{+/+}\) (n = 4) or NHE1\(^{+/−}\) astrocytes (n = 6). Arrow indicates the time when ΔpH\(_i\) was measured. Data are expressed as means ± SE. *P < 0.01 vs. NHE1\(^{+/+}\) astrocytes.

**Fig. 4. Delayed pH\(_i\) recovery in NHE1\(^{−/−}\) astrocytes after an acid load. A:** representative trace of pH\(_i\) from a prepulse experiment with either NHE1\(^{+/+}\) or NHE1\(^{+/−}\) astrocytes. Cells were exposed to 10 mM NH\(_4\)\(^+\)/NH\(_3\)^− for 1 min (b–c) and then returned to standard HEPES-buffered solution (c–e), and pH\(_i\) was recovery monitored (d–e). pH\(_i\) recovery rate was determined by calculating the slope of ΔpH\(_i\) during the first minute after NH\(_4\)\(^+\) prepulse acid load. Inset: mean pH\(_i\) recovery rate in either NHE1\(^{+/+}\) (n = 6) or NHE1\(^{−/−}\) astrocytes (n = 6). Data are expressed as means ± SE. *P < 0.01 vs. NHE1\(^{+/+}\) astrocytes. **B:** summary pH\(_i\) data during baseline, 2 min after prepulse, and 10 min after prepulse for NHE1\(^{+/+}\) and NHE1\(^{−/−}\) astrocytes. Data are expressed as means ± SE. *P < 0.001 vs. NHE1\(^{+/+}\) control. #P < 0.001 vs. NHE1\(^{−/−}\) at each time point.
NHE1 activity was elevated in astrocytes after OGD

We hypothesize that NHE1 activity is stimulated in astrocytes and promotes the rapid extrusion of cytosolic H+ that is accumulated during OGD. First, we investigated whether pH0 was reduced after 2 h of OGD. Figure 6A shows that at 2 min after 2 h of OGD in the presence of extracellular pH (pH0) of 7.4, pH1 in NHE1+/+ astrocytes was significantly decreased from 7.09 ± 0.07 to 6.80 ± 0.02 (P < 0.05). Inhibition of NHE1 activity during OGD with HOE-642 (1 μM) further acidified NHE1+/+ astrocytes (6.66 ± 0.03 vs. 6.44 ± 0.01, P < 0.05). pH1 was similarly reduced in NHE1−/− astrocytes during OGD (6.65 ± 0.06 vs. 6.42 ± 0.13). These data suggest that OGD caused intracellular acidosis in astrocytes. This low pH1 may subsequently stimulate NHE1 activity to extrude excessive H+ from astrocytes.

Under in vivo ischemic conditions, pH0 decreases. Low pH0 has been reported to inhibit NHE activity in astrocytes (22). Therefore, we investigated pH0 changes in NHE1+/+ astrocytes after 2 h of OGD at pH0 6.6 and 2 min of reoxygenation at pH0 7.4. Figure 6A, inset, shows that pH0 in NHE1+/+ astrocytes decreased to 6.67 ± 0.02 after 2 h of OGD at pH0 6.6, which is significantly less than pH0 after 2 h of OGD at pH0 7.4 (6.80 ± 0.02, P < 0.05). Moreover, in NHE1+/+ astrocytes treated with 1 μM HOE-642, pH0 decreased further to 6.48 ± 0.04 (P < 0.05). These findings imply that NHE1 in NHE1+/+ astrocytes is still active under OGD conditions at pH0 6.6.

We then measured NHE1 activity in NHE1+/+ and NHE1−/− astrocytes under both normoxic and OGD conditions. As shown in Fig. 6B, in normoxia-treated NHE1+/+ astrocytes, the rate of pH0 recovery in response to the acid loading was 0.30 ± 0.01 pH unit/min at a pH0 of 6.12 ± 0.07 (Fig. 5B, inset). Returning NHE1−/− astrocytes to the control solution had no effect on the rate of pH0 recovery. The difference in the mean pH0 during the initial pH0 recovery between NHE1+/+ and NHE1−/− astrocytes was not statistically significant.

pH0 recovery was also measured in NHE1+/+ astrocytes from separate cultures in the presence of CO2/HCO3 26 mM. In two experiments, the pH0 recovery rates in NHE1+/+ astrocytes averaged 0.13 (1 coverslip, 6 cells) and 0.17 pH0/min (1 coverslip, 6 cells), respectively. When NHE1 activity was blocked by HOE-642 (1 μM), the average pH0 recovery rate was reduced to 0.11 ± 0.04 pH unit in NHE1+/+ astrocytes (n = 3, P < 0.001). These data suggest that NHE1 in astrocytes plays a dominant role in pH0 recovery after acidosis, regardless of the presence of HCO3−-dependent transport systems. In two experiments, the pH0 recovery rates in NHE1−/− astrocytes averaged 0.42 (1 coverslip, 20 cells) and 0.22 pH0 unit/min (1 coverslip, 20 cells), respectively. This implies that HCO3−-dependent transport systems might be upregulated in NHE1−/− astrocytes.

NHE1+/+ and NHE1−/− astrocytes were exposed to 2 h of OGD followed by 1 h of reoxygenation, and [Na+]i was determined at ambient temperature or 37°C. NHE1+/+ and NHE1−/− astrocytes from sister cultures were incubated in
A pool of reoxygenation, the NH$_3$/NH$_4^+$ levels measured at 2 min of reoxygenation were significantly lower than that in NHE1$^{+/+}$ astrocytes (P < 0.001). Inhibition of NHE1 with 1 μM HOE-642 during OGD and reoxygenation also significantly attenuated the rise in [Na$^+$], (30.0 ± 8.3 mM; Fig. 7A) in NHE1$^{+/+}$ astrocytes.

Figure 7B shows the resting [Na$^+$], levels measured at 37°C. Basal [Na$^+$], levels were 12.4 ± 0.1 mM in NHE1$^{+/+}$ astrocytes and 12.1 ± 0.2 mM in NHE1$^{-/-}$ astrocytes. After 2 h of OGD and 1 h of reoxygenation, [Na$^+$], levels increased to 40.9 ± 2.5 mM in NHE1$^{+/+}$ astrocytes. In contrast, 2 h of OGD and 1 h of reoxygenation resulted in [Na$^+$], of only 20.9 ± 2.2 mM in NHE1$^{+/+}$ astrocytes treated with 1 μM HOE-642 and [Na$^+$], of 22.0 ± 0.7 mM in NHE1$^{-/-}$ astrocytes. Taken together, these data show that inhibition of NHE1

Normoxic control buffer in a normoxic incubator for 3 h. As shown in Fig. 7A, under control conditions, average [Na$^+$], measured at ambient temperature was 11.3 ± 2.3 mM in NHE1$^{+/+}$ astrocytes and 10.9 ± 1.6 mM in NHE1$^{-/-}$ astrocytes. After 2 h of OGD and 1 h of reoxygenation, [Na$^+$], increased to 61.2 ± 9.8 mM in NHE1$^{+/+}$ astrocytes. However, in NHE1$^{-/-}$ astrocytes, [Na$^+$], was 23.4 ± 5.0 mM, significantly lower than that in NHE1$^{+/+}$ astrocytes (P < 0.001).
activity significantly attenuated intracellular Na⁺ accumulation after OGD.

The cells in Fig. 7, A and B, were exposed to 1 h of reoxygenation in the HCO₃⁻-free/HEPES-buffered solution. Additional experiments were performed in the presence of HCO₃⁻ (26 mM) throughout 2 h of OGD and 1 h of reoxygenation. As shown in Fig. 7C, Na⁺ accumulation was significantly lowered in NHE1⁻/⁻ astrocytes or in NHE1⁺/⁺ astrocytes treated with HOE-642. Thus these results are in agreement with the Na⁺ accumulation data determined in the absence of HCO₃⁻ (Fig. 7, A and B). This finding implies that Na⁺-HCO₃ cotransport systems contribute little to intracellular Na⁺ loading after OGD.

**NHE1⁻/⁻ Astrocytes Exhibited Resistance to OGD-Mediated Swelling**

Accumulation of intracellular Na⁺ leads to astrocyte swelling. CSA was measured as an estimate of cell swelling during OGD in NHE1⁺/⁺ and NHE1⁻/⁻ astrocytes. In control experiments, 100 min of perfusion with iso-HEPES did not cause any significant changes in CSA (data not shown). However, OGD induced a gradual increase in CSA over time in NHE1⁺/⁺ astrocytes. After 60 min, CSA increased by 26 ± 2% in NHE1⁺/⁺ astrocytes (Fig. 8, A and B). In contrast, treatment with 1 μM HOE-642 significantly attenuated the OGD-induced rise in CSA in NHE1⁺/⁺ astrocytes (9 ± 2%; Fig. 8B). In a similar manner, NHE1⁻/⁻ astrocytes exhibited only an 8 ± 2% increase in CSA after 60 min of OGD (Fig. 8, A and B).

**DISCUSSION**

**Dominant Role of NHE1 in pHᵢ Regulation in Cortical Astrocytes**

Resting pHᵢ in NHE1⁻/⁻ astrocytes. In this study, we found that the resting pHᵢ in NHE1⁺/⁺ astrocytes was 6.86 and was decreased after removal of Na⁺ or application of the potent NHE1 inhibitor HOE-642. Genetic ablation of NHE1 in NHE1⁻/⁻ astrocytes resulted in a new “set point” for resting level pHᵢ (6.53), which was significantly lower than in NHE1⁺/⁺ astrocytes in the absence of CO₂/HCO₃⁻. In the presence of CO₂/HCO₃⁻, despite an ~0.24-pH unit increase, presumably by HCO₃⁻ transporters, the resting pHᵢ in NHE1⁻/⁻ astrocytes remained significantly lower than in NHE1⁺/⁺ astrocytes. Thus both pharmacological inhibition and genetic ablation of NHE1 decrease the resting pHᵢ in astrocytes. This suggests that NHE1 is active in maintaining the steady-state level of pHᵢ in mouse cortical astrocytes. In contrast, NHE1 activity in many cell types is quiescent at resting pHᵢ (8).

In NHE1⁻/⁻ astrocytes, removal of extracellular Na⁺ caused a small but significant decrease in pHᵢ. This could be attributable to functions of other NHE isoforms, although this would require considerable upregulation of these isoforms in NHE1⁻/⁻ astrocytes. NHE1 is normally expressed in the CNS but not abol-
ished. pH recovery was quickest in NHE1−/− astrocytes during the first 2 min after an acid load, but the recovery rate gradually increased, and the original resting pH level was reached within 10 min. The ability of the NHE1−/− cells to slowly restore pH may be due to upregulation of other NHE isoforms in NHE1−/− astrocytes. This view is based on the following information: (1) the slow pH recovery rate was Na+-dependent, because removal of extracellular Na+ abolished this regulation. This would argue against a role for the V-type ATPase that has been described in hippocampal astrocytes and does not require Na+ (26); (2) in HEPES buffer, HCO3−-dependent transport activity (i.e., electrogenic Na+-HCO3− cotransporters) should be minimal, because the apparent KCr for external HCO3− is 6–10 mM (13); and (3) a residual pH recovery in NHE1−/− astrocytes is resistant to 1 μM HOE-642. The apparent KCr for HOE-642 in transected fibroblast cell lines is 0.05 μM for NHE1, 3 μM for NHE2, and 1 μM for NHE3 (30). Thus, in NHE1−/− astrocytes, other NHEs are likely to be upregulated to compensate for the loss of NHE1 activity.

NHE1 activity is known to be stimulated in a dose-dependent manner below pH 6.7 in astrocytes (21). In the current study, we found that pH recovery was persistently impaired in NHE1−/− astrocytes under pH 6.10, compared with pH 6.36 in NHE1+/+ astrocytes (Fig. 5). However, the sensitivity of BCECF, as determined by the ratio of the change in BCECF fluorescence with changes in pHr (ΔF/BCECF/ΔpH) decreases at low pHr. In a pilot study, we found that in NHE1−/− astrocytes at pH 6.15, ΔF/BCECF/ΔpH was ~25% of the maximum response we observed at pH 7.25. In the current study, pHr in NHE1−/− astrocytes decreased to 6.10 after NH4Cl pulse (Fig. 5). Thus the lack of pH recovery in NHE1−/− astrocytes could be due, in part, to an insensitivity of BCECF at low pHr.

Protective Effects of NHE1 Inhibition in Astrocytes

Little is known about the role of NHEs in ischemic damage in astrocytes. In this study, we examined whether NHE1 is involved in astrocyte damage after OGD. We first demonstrated that OGD induces acidosis in cortical astrocytes. OGD-induced intracellular acidosis may lead to stimulation of NHE1 activity allosterically by cytosolic H+ (25). In addition, prolonged intracellular acidosis can elevate NHE1 activity by ERK- and p90RSK-mediated phosphorylation (13a). We found that, measured at the same pHr levels (pHr 6.4 vs. 6.42), NHE1 activity in OGD-treated NHE1+/+ astrocytes was increased by ~80% compared with that in normoxic NHE1+/+ astrocytes (Fig. 6B). This finding suggests that OGD-mediated stimulation of NHE1 is sustained.

After 2 h of OGD and 1 h of reoxygenation, [Na+]i in NHE1+/+ astrocytes rose to ~5.4 times normal. In contrast, in NHE1−/− astrocytes subjected to OGD and reoxygenation, [Na+]i was only ~2.1 times normal. A similar attenuation of the rise in [Na+]i after OGD and reperfusion was observed in NHE1+/+ astrocytes (2.7 times normal) when NHE1 activity was inhibited by HOE-642. The data suggest that NHE1 activity in astrocytes plays an important role in Na+ loading during reoxygenation. Furthermore, OGD-mediated swelling was significantly reduced when NHE1 activity was eliminated by genetic ablation or inhibited by HOE-642.

However, blockade of NHE1 activity by HOE-642 only attenuated the Na+ loading by ~60%. The mechanisms responsible for the remaining Na+ influx in astrocytes are unknown. Several other Na+ transport proteins could play a role in this Na+ loading, such as the Na+ channels, Na+-HCO3− cotransporters, or the Na-K-2Cl cotransporter. Na-K-2Cl cotransporter activity is stimulated in neurons (2) and astrocytes (Lenart B, Kintner DB, Shall GE, and Sun D, unpublished data) under OGD conditions. In addition, inhibition of Na+-K+-ATPase activity may also contribute to the Na+ accumulation.

Our data provide strong evidence that inhibition of NHE1 is partially protective against OGD-mediated damage in astrocytes. The cellular mechanisms underlying the role of NHE1 in ischemic astrocyte damage are not well understood. We demonstrated that NHE1 activity is stimulated during OGD and early reoxygenation. NHE1 triggers an acute accumulation of intracellular Na+ and swelling after OGD. A rise in [Na+]i, and a reduction in the transmembrane Na+-gradient can worsen the energy state and exacerbate cell damage during ischemia. This view is supported by the report that 10 μM HOE-642 prevented a significant reduction of PCr/Cr in C6 glioma cells after hypoxia (11). It has been suggested that astrocyte death is related to the extent of Na+ influx under conditions in which Na+-K+-ATPase activity is not maintained (38). Thus the Na+ overload mechanism is likely the primary means whereby NHE1 contributes to ischemic astrocyte damage.

In addition, a rise in [Na+]i could reduce Ca2+ extrusion and/or reverse Na+/Ca2+ exchange and lead to accumulation of intracellular Ca2+ (32). This hypothesis is supported by several previous studies. Activation of NHE activity in hippocampal neurons by acidosis triggered Na+ influx and subsequent elevation in [Ca2+]i, (40). The rise in both Na+ and Ca2+ was blocked by the NHE inhibitor EIPA and the Na+/Ca2+ exchange blocker dimethylbenzamid (40). Thus these findings suggest that stimulation of NHE activity can lead to accumulation of intracellular Na+ and Ca2+. In addition, reversal of Na+/Ca2+ exchange activity and subsequent Ca2+ influx have been observed in depolarized and glucose-deprived cerebellar granule cells (10). Abundant expression of Na+/Ca2+ exchange proteins NCX1 and NCX2 has been found in cultured hippocampal astrocytes (39). When the Na+/Ca2+ exchange inhibitor SEA0400 was applied to cultured astrocytes subjected to paradoxical Ca2+ challenge-induced injury, there was a reduction in Ca2+ influx, production of reactive oxygen species, and cell damage (20). Taken together, the results of these studies suggest that an initial intracellular Na+ overload and subsequent stimulation of Ca2+ influx may contribute to ischemic astrocyte damage. However, further studies are needed to determine whether stimulation of NHE1 activity would alter Na+/Ca2+ exchange and promote Ca2+ influx in astrocytes after OGD.

In summary, we have shown that both pharmacological inhibition and genetic ablation of NHE1 reduced the steady-state levels of pH in cultured cortical astrocyte. Inhibition of NHE1 activity impaired pH recovery after intracellular acidosis. NHE1 activity was elevated after OGD at either neutral or low pH. In addition, NHE1−/− astrocytes exhibited significantly less damage after OGD. These findings suggest that stimulation of NHE1 activity in astrocytes may contribute to disruption of ion homeostasis and ischemic damage.
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REFERENCES