Increased tolerance to oxygen and glucose deprivation in astrocytes from Na+/H+ exchanger isoform 1 null mice

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Kintner, Douglas B., Gui Su, Brett Lenart, Andy J. Ballard, Jamie W. Meyer, Leong L. Ng, Gary E. Shull, and Dandan Sun. Increased tolerance to oxygen and glucose deprivation in astrocytes from Na+/H+ exchanger isoform 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 isoform 1 (NHE1) functions as a major intracellular pH (pH) 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 pH recovery and ischemic injury in astrocytes. In the absence of HCO3−, 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 NH4Cl 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 a ~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 pH regulatory mechanism in cortical astrocytes and that ablation of NHE1 in astrocytes attenuates ischemia-induced disruption of ionic regulation and swelling.

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

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THE PLASMA MEMBRANE PROTEIN Na+/H+ exchanger isoform 1 (NHE1) is ubiquitously expressed in mammalian tissues (8, 25). Under physiological conditions, NHE1 plays an important role in intracellular pH (pHi) and volume regulation in most cells (29). To date, eight NHE isoforms (NHE1–NHE8) have been identified (8, 12, 4, 25). In contrast to the plasma membrane localization of NHE1–NHE5, NHE6 and NHE7 are expressed in endosomes and trans-Golgi network, respectively (6, 24). Although NHE8 is more closely related to the organelar NHEs, it has been localized to brush border membranes of the renal proximal tubule (12). In situ hybridization analysis revealed that NHE1 is by far the most abundant of all plasma membrane NHEs in the rat central nervous system (CNS) (19).

Pharmacological approaches have been used to elucidate the role of NHEs in astrocyte pHi regulation. In HCO3−-free buffer, the NHE blocker amiloride decreased the resting pHi by 0.10–0.42 pH units in rat cortical astrocytes (23, 31). Similar decreases in the resting pHi by amiloride were found in rat cerebellar and hippocampal astrocytes (7, 26, 28). Amiloride abolished pHi recovery in astrocytes after NH4Cl prepulse acid loads (4, 21, 28, 31). However, the relative role of NHE1 in pHi regulation in cortical astrocytes is not clear because of the poor NHE1 isoform selectivity of amiloride. Thus definitive studies are warranted to assess the role of NHE1 in pHi recovery in cortical astrocytes.

To date, a genetic ablation approach has revealed an attenuated pHi recovery in freshly isolated CA1 neurons from NHE1 gene defect slow-wave epilepsy mutant mice (9, 41). However, it is not known how genetic ablation of NHE1 affects pHi recovery in cortical astrocytes. In the current study, both the more potent NHE1 inhibitor HOE-642 and genetic ablation approaches were taken to elucidate the function of NHE1 in mouse cortical astrocytes.

In the past decade, the results of a large body of research suggest that stimulation of NHE1 activity contributes to cardiovascular pathology after ischemia (1, 16). Inhibition of NHE1 has been shown to attenuate the detrimental consequences of myocardial ischemia and reperfusion (1). The principal mechanism underlying the cardioprotective actions of NHE1 inhibition is the attenuation of the intracellular Na+ accumulation during ischemia. However, limited information is available about the effects of NHE inhibition on cerebral ischemic damage. Pretreatment of gerbils with the amiloride derivative ethylisopropanylamiloride (EIPA), a NHE inhibitor, significantly reduced the extent of CA1 pyramidal neuron loss after global ischemia (27). The NHE inhibitor SM-20220 (N-aminomiminomethyl-1-methyl-1-indole-2-carboxamide methanesulfonate) attenuated the increase in brain Na+ and water contents after 2 h of transient focal ischemia and 4 h of reperfusion in rats (18). It
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 isof orm specific, it is not clear how dominant a role NHE1 plays compared with the other isof orms in the CNS.

In this study, we investigated the role of NHE1 in pH<sub>1</sub> 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<sub>1</sub> and impaired pH<sub>1</sub> recovery. OGD stimulated NHE1 activity in NHE1<sup>+/+</sup> astrocytes; however, this increase in activity was absent in NHE1<sup>−/−</sup> astrocytes and was blocked by HOE-642 in NHE1<sup>+/+</sup> astrocytes. Inhibition or genetic ablation of NHE1 reduced the OGD-mediated rise in intracellular Na<sup+</sup> concentration ([Na<sup+</sup>]) 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 (Hernando, VA). Fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT). Collagen type I was purchased from BD Biosciences (San Jose, CA). FBS was purchased from Hyclone (Logan, UT). Cultured astrocytes were prepared as described previously (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-covered 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 by using a Nikon TE 300 inverted epi-fluorescence microscope (×20) with a Princeton Instruments MicroMax charge-coupled device (CCD) camera and MetaMorph image-processing software. Identical digital imaging acquisition parameters were used in both negative control and experimental images.

**Measurement of pH<sub>1</sub>**

**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 KH<sub>2</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) 30 KCl, 20 N-methyl-d-glucamine (NMDG), 0.81 MgSO<sub>4</sub>, 1.27 CaCl<sub>2</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.

**pH₁ 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 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 (F₅₉₀/F₄₈₀) was calibrated by using the high-K⁺/citric acid 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 pK 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 NH₄Cl/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 NH₃ quickly diffused out of the cell. Unless otherwise stated, pH recovery was determined during the first minute after NH₃/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 Forma, Marietta, OH) equilibrated with 94% N₂/1−5% 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 acid 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⁺ (NaCl₂) 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⁺], was measured and calibrated at 37°C (see Fig. 7B). In another set of experiments, [Na⁺], 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 pHᵢ 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 pHᵢ returned to basal levels when HOE-642 was removed (Fig. 3B, b and c). In contrast, HOE-642 had no effects on the resting pHᵢ of NHE1⁻⁻ astrocytes.

**Impaired pHᵢ Recovery in NHE1⁻⁻ Astrocytes**

We then studied how NHE1⁻⁻ astrocytes regulate pHᵢ after intracellular acidosis using the NH₄⁺ prepulse technique. As shown in Fig. 4A, when either NHE1⁻/+ or NHE1⁻⁻ astrocytes were exposed to 10 mM NH₄Cl/HCO₃⁻, pHᵢ 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, pHᵢ decreased sharply as NH₄⁺ rapidly left cells as NH₃, trapping H⁺ inside (Fig. 4A, c and d). After the acid load, pHᵢ recovered within 2 min in NHE1⁻/+ astrocytes (Fig. 4A, d and e). However, in NHE1⁻⁻ astrocytes, pHᵢ recovery was delayed for 1–2 min and then slowly returned to near the basal pHᵢ level over the following 10 min.

pHᵢ recovery rate was summarized by calculating the slope of pHᵢ 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 (pHᵢ of 6.43 ± 0.20 and 6.29 ± 0.09, respectively, at the first minute after the prepulse). This difference in the mean pHᵢ 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 pHᵢ of NHE1⁻⁻ Astrocytes**

The pHᵢ frequency distribution in NHE1⁻/+ astrocytes ranged from 6.5 to 7.2 (Fig. 2). The mean steady-state pHᵢ 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 pHᵢ below neutral under HCO₃⁻-free conditions (4, 5, 21, 26, 28). However, the pHᵢ 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 pHᵢ in NHE1⁻⁻ astrocytes was significantly lower (6.53 ± 0.04, P < 0.01). In the presence of CO₂/HCO₃⁻, the mean steady-state pHᵢ 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 pHᵢ in astrocytes, we monitored pHᵢ in NHE1⁻/+ and NHE1⁻⁻ astrocytes in a Na⁺-free HEPES-buffered solution (Fig. 3A). In the absence of Na⁺, the pHᵢ of NHE1⁻/+ astrocytes progressively decreased (Fig. 3A, a and b). The average decrease in pHᵢ 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 pHᵢ 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 pHᵢ 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 pHᵢ returned to basal levels when HOE-642 was removed (Fig. 3B, b and c). In contrast, HOE-642 had no effects on the resting pHᵢ of NHE1⁻⁻ astrocytes.

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pHᵢ recovery rate was summarized by calculating the slope of pHᵢ 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 (pHᵢ of 6.43 ± 0.20 and 6.29 ± 0.09, respectively, at the first minute after the prepulse). This difference in the mean pHᵢ 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
33 ± 0.08 pH units (at a pH of 6.43 ± 0.20). In contrast, no
pH recovery was found in NHE1−/− astrocytes (6.04 ± 0.08
pH unit at a pH of 6.29 ± 0.09). Figure 4B shows the pHi
summary data for NHE1+/+ and NHE1−/− astrocytes at rest-
ing level, 2 min, and 10 min after NH4+ prepulse. pHi in
NHE1+/+ astrocytes returned to the steady resting level by 2
min after acid loading. However, pHi in NHE1−/− astrocytes
dropped to ~6.2 after the NH4+ prepulse. It took 10 min for
NHE1−/− astrocytes to recover pHi to the resting level. This
indicates that NHE1 critical for initial pHi recovery after an
acid load in CO2/HCO3− free solution.

pHi Recovery in Astrocytes Is Mediated by NHE1 Activity

The data in Fig. 4 indicate that initial pHi recovery after an
acid load in astrocytes is exclusively mediated by NHE1 in
the absence of HCO3−. To further establish this point, we
investigated the Na+ dependence of pHi recovery after an acid load
in NHE1+/+ and NHE1−/− astrocytes in the absence of HCO3−
(Fig. 5, A and B). When NHE1+/+ or NHE1−/− astrocytes
were exposed to 10 mM NH3/NH4+, pHi increased (Fig. 5, A
and B, a and b) and then declined gradually (Fig. 5, A and B,
b and c). Both NHE1+/+ and NHE1−/− astrocytes exhibited
acidification after the NH3 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 pHi
(Fig. 5A, d and e) after acidosis. pHi 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 pHi recovery in the absence of Na+ and
no further decrease in pHi (0.02 ± 0.02 pH unit/min at a pHi
of 6.09 ± 0.14) (Fig. 5A). NH3/NH4+ pulse led to a significa-
tantly more acidification in NHE1−/− astrocytes than in
NHE1+/+ astrocytes in the absence of extracellular Na+ (P <
0.05). pHi recovered when NHE1+/+ cells were returned to the
control conditions (Fig. 5A, e and f). However, there was only a
slow recovery of pHi 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 pHi to
that of removal of extracellular Na+. In the presence of 1 μM
HOE-642, NH3/NH4+ triggered acid loading in NHE1+/+ and
NHE1−/− astrocytes (Fig. 5B, b–d). However, after acidifica-
tion, there was little increase in pHi in NHE1+/+ cells (Fig. 5B,
d and e). The rate of pHi recovery in NHE1+/+ astrocytes in
the presence of HOE-642 was 0.03 ± 0.1 pH units/min at a pHi
of 6.35 ± 0.19 (Fig. 5B, inset). When HOE-642 was removed
from the buffer, pHi rapidly returned to basal levels in

![Fig. 3. Effects of Na+-free buffer and HOE-642 on resting pH levels. A: representative trace of pHi 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 pHi (ΔpHi) under Na+-free conditions for either NHE1+/+ (n = 3) or NHE1−/− astrocytes (n = 3). B: representative trace of pHi 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: ΔpHi, with HOE-642 in either NHE1+/+ (n = 4) or NHE1−/− astrocytes (n = 6). Arrow indicates the time when ΔpHi was measured. Data are expressed as means ± SE. *P < 0.01 vs. NHE1+/+ astrocytes.](http://ajpcell.physiology.org/)
NHE1\(^{+/+}\) astrocytes (Fig. 5B, e and f). In some NHE1\(^{-/-}\) astrocytes, pH\(_i\) slowly increased despite NHE1 blockade (Fig. 5B, d and e). However, there was no change in the mean pH\(_i\) recovery rate (0.00 ± 0.01 pH unit/min at a pH\(_o\) of 6.12 ± 0.07) (Fig. 5B, inset). Returning NHE1\(^{-/-}\) astrocytes to the control solution had no effect on the rate of pH\(_i\) recovery. The difference in the mean pH\(_i\) during the initial pH\(_i\) recovery between NHE1\(^{+/+}\) and NHE1\(^{-/-}\) astrocytes was not statistically significant.

pH\(_o\) recovery was also measured in NHE1\(^{+/+}\) astrocytes from separate cultures in the presence of CO\(_2\)/HCO\(_3^-\) (26 mM). In two experiments, the pH\(_o\) recovery rates in NHE1\(^{+/+}\) astrocytes averaged 0.38 (1 coverslip, 6 cells) and 0.37 pH/min (1 coverslip, 6 cells), respectively. When NHE1 activity was blocked by HOE-642 (1 \(\mu\)M), the average pH\(_o\) 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 pH\(_o\) recovery after acidosis, regardless of the presence of HCO\(_3^-\)-dependent transport systems. In two experiments, the pH\(_i\) recovery rates in NHE1\(^{-/-}\) astrocytes averaged 0.42 (1 coverslip, 20 cells) and 0.22 pH unit/min (1 coverslip, 20 cells), respectively. This implies that HCO\(_3^-\)-dependent transport systems might be upregulated in NHE1\(^{-/-}\) astrocytes.

**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 pH\(_o\) was reduced after 2 h of OGD. Figure 6A shows that to 2 min after 2 h of OGD in the presence of extracellular pH (pH\(_o\)) of 7.4, pH\(_i\) 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 \(\mu\)M) further acidified NHE1\(^{+/+}\) astrocytes (6.66 ± 0.03 vs. 6.44 ± 0.01, P < 0.05). pH\(_i\) 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 pH\(_i\) may subsequently stimulate NHE1 activity to extrude excessive H\(^+\) from astrocytes.

Under in vivo ischemic conditions, pH\(_o\) decreases. Low pH\(_o\) has been reported to inhibit NHE activity in astrocytes (22). Therefore, we investigated pH\(_o\) changes in NHE1\(^{+/+}\) astrocytes after 2 h of OGD at pH\(_o\) 6.6 and 2 min of reoxygenation at pH\(_o\) 7.4. Figure 6A, inset, shows that pH\(_i\) in NHE1\(^{+/+}\) astrocytes decreased to 6.67 ± 0.02 after 2 h of OGD at pH\(_o\) 6.6, which is significantly less than pH\(_i\) after 2 h of OGD at pH\(_o\) 7.4 (6.80 ± 0.02, P < 0.05). Moreover, in NHE1\(^{-/-}\) astrocytes treated with 1 \(\mu\)M HOE-642, pH\(_i\) 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 pH\(_o\) 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 pH\(_i\) recovery in response to the acid loading was 0.30 ± 0.01 pH unit/min at a pH\(_o\) of 6.40 ± 0.11. After 2 h of OGD at pH\(_o\) 7.4 and 15 min of reoxygenation, pH\(_i\) recovery rate was increased to 0.53 ± 0.03 pH unit/min at a pH\(_o\) of 6.42 ± 0.12 (P < 0.05). In contrast, pH\(_i\) recovery rate after 2 h of OGD at pH\(_o\) 7.4 and 15 min of reoxygenation was nearly absent in NHE1\(^{-/-}\) astrocytes or in HOE-642-treated NHE1\(^{+/+}\) astrocytes (0.03 ± 0.013 pH unit/min at a pH\(_o\) of 6.13 ± 0.07 and 0.01 ± 0.01 pH unit/min at a pH\(_o\) of 14 ± 0.05, respectively). These findings indicate that OGD treatment stimulates NHE1 activity and that NHE1 plays an important role in pH\(_i\) recovery after OGD.

Figure 6C shows that 2 h of OGD at pH\(_o\) 6.6 did not block the increase in pH\(_i\) recovery rate in NHE1\(^{+/+}\) astrocytes (0.56 ± 0.05 pH unit/min at a pH\(_o\) of 6.33 ± 0.014, P < 0.05). To further establish that NHE1 was responsible for the accelerated pH\(_i\) recovery during reoxygenation, we then examined whether HOE-642 would block pH\(_i\) recovery in NHE1\(^{+/+}\) astrocytes. Astrocytes underwent 2 h of OGD at pH\(_o\) 6.6 in the presence of 1 \(\mu\)M HOE-642. pH\(_i\) recovery was abolished by HOE-642, with a pH\(_i\) recovery rate of 0.04 ± 0.01 pH unit/min at a pH\(_o\) of 6.22 ± 0.11 (Fig. 6C). This indicates that NHE1 activity remains stimulated by low pH\(_o\), even under conditions that would be expected to cause low pH\(_o\)-mediated inhibition.

An Increase in [Na\(^+\)] Was Significantly Attenuated in NHE1\(^{-/-}\) Astrocytes After OGD

NHE1\(^{+/+}\) and NHE1\(^{-/-}\) astrocytes were exposed to 2 h of OGD followed by 1 h of reoxygenation, and [Na\(^+\)], was determined at ambient temperature or 37°C. NHE1\(^{+/+}\) and NHE1\(^{-/-}\) astrocytes from sister cultures were incubated in
Fig. 6. pH recovery rate is increased in NHE1
astrocytes after 2 h of oxygen and glucose deprivation (OGD). A: NHE1+/− or NHE1−/− astrocytes were exposed to 2 h of OGD in the presence of HCO3− buffer. Mean pHi in these cells was then determined at 2 min of reoxygenation at pH 7.4. Note that 2 min may not be long enough for some cells to reach complete equilibration between the HCO3−/H2CO3− buffered OGD buffer and the HEPES buffer. Inset: OGD incubation was carried out at extracellular pH (pH e) 6.6 by increasing CO2 in the incubator to 20% in NHE1+/− astrocytes; mean pHi, at 2 min of reoxygenation at pH 7.4 was then determined. In HOE studies, 1 μM HOE-642 was present during OGD and reoxyenation. Data are expressed as means ± SE; n = 3–7, *P < 0.05 vs. NHE1+/− control (Con). #P < 0.001 vs. NHE1+/− OGD. ΨP < 0.005 vs. NHE1+/− OGD at pH 6.6. B: pH recovery rate was determined in NHE1+/− or NHE1−/− astrocytes after 2 h of OGD at pH 7.4. After 15 min of reoxyenation, the NH4/HCO3− prepulse was applied and pHi recovery rate was measured (see MATERIALS AND METHODS and Fig. 4). In HOE studies, 1 μM HOE-642 was present during OGD and reoxyenation. Data are expressed as means ± SE; n = 4–5. *P < 0.05 vs. NHE1+/− control. #P < 0.001 vs. NHE1+/− OGD. C: OGD incubation was carried out at pH 6.6 by increasing CO2 in the incubator to 20%. These studies were done in either the presence or absence of 1 μM HOE-642. Data are expressed as means ± SE; n = 3. *P < 0.05 vs. control. #P < 0.001 vs. OGD at pH 6.6.

Fig. 7. NHE1−/− astrocytes exhibit a smaller rise in intracellular Na+ concentration ([Na+]i) after OGD. [Na+]i was determined at either 22°C (A) or 37°C (B) in NHE1+/− and NHE1−/− astrocytes after 2 h of OGD in the presence of 26 mM HCO3− and 1 h of reoxyenation (HCO3−/free/HEPES). In a control study, sister cultures were incubated under normoxic conditions for 3 h. C: [Na+]i was determined at 37°C in NHE1+/− and NHE1−/− astrocytes after 2 h OGD and 1 h of reoxyenation in the presence of 26 mM HCO3−. Data are expressed as means ± SE; n = 3–6. *P < 0.001 vs. control. #P < 0.001 vs. NHE1+/− OGD.
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 HCO\(_3\)\(^-\)-free/HEPES-buffered solution. Additional experiments were performed in the presence of HCO\(_3\)\(^-\) (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 HCO\(_3\)\(^-\) (Fig. 7, A and B). This finding implies that Na\(^-\)-HCO\(_3\) 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 \(\mu\)M 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 pH\(_i\) Regulation in Cortical Astrocytes**

Resting pH\(_i\) in NHE1\(^{-/-}\) astrocytes. In this study, we found that the resting pH\(_i\) 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 pH\(_i\) (6.53), which was significantly lower than in NHE1\(^{+/+}\) astrocytes in the absence of CO\(_2\)/HCO\(_3\). In the presence of CO\(_2\)/HCO\(_3\), despite an ~0.24-pH unit increase, presumably by HCO\(_3\) transporters, the resting pH\(_i\) in NHE1\(^{-/-}\) astrocytes remained significantly lower than in NHE1\(^{+/+}\) astrocytes. Thus both pharmacological inhibition and genetic ablation of NHE1 decrease the resting pH\(_i\) in astrocytes. This suggests that NHE1 is active in maintaining the steady-state level of pH\(_i\) in mouse cortical astrocytes. In contrast, NHE1 activity in many cell types is quiescent at resting pH\(_i\) (8).

In NHE1\(^{-/-}\) astrocytes, removal of extracellular Na\(^+\) caused a small but significant decrease in pH\(_i\). 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 and at a level 10 times that of the other isoforms (19). Alternatively, in the absence of extracellular HCO\(_3\), removal of external Na\(^+\) could acidify the cell by triggering reversal of the electrogenic Na\(^-\)-HCO\(_3\) cotransporter (31).

Moreover, the resting pH\(_i\) exhibited by NHE1\(^{-/-}\) astrocytes was significantly lower than that of NHE1\(^{+/+}\) astrocytes when NHE1 activity was inhibited by Na\(^+\)-free buffer or the NHE1 inhibitor HOE-642. This implies that other pH\(_i\) regulatory pathways are altered in NHE1\(^{-/-}\) astrocytes in addition to an absence of NHE1 activity. A steady-state pH\(_i\) is affected by many factors, including cell growth and metabolism or mitochondrial function, in addition to membrane transport systems. NHE1-mediated H\(^+\) extrusion is only one of the factors in determination of the steady-state pH\(_i\) in cells. To our knowledge, no studies have been done to compare rates of glucose consumption, lactate production, or other factors such as glycerogen levels in NHE1\(^{+/+}\) to NHE1\(^{-/-}\) astrocytes during either normoxic or OGD conditions. Thus we do not know whether the proton production rate differs between NHE1\(^{+/+}\) and NHE1\(^{-/-}\) astrocytes. Future studies are needed to investigate whether acid loading systems are stimulated in NHE1\(^{-/-}\) astrocytes.

**pH\(_i\) recovery in NHE1 astrocytes after acid loading.** In response to NH\(_4\)Cl-mediated acidosis, NHE1\(^{+/+}\) astrocytes restored pH\(_i\) to the resting level within 1–2 min. This robust recovery was abolished when NHE1 activity was inhibited by either HOE-642 or removal of external Na\(^+\) in the absence of extracellular HCO\(_3\). The pH\(_i\) recovery rate was decreased by ~70% with HOE-642 in the presence of extracellular HCO\(_3\). This finding suggests that NHE1 is the dominant mechanism for alkalinizing astrocytes after acidosis.

**pH\(_i\) recovery in NHE1\(^{-/-}\) astrocytes after an acid load was delayed in the absence of extracellular HCO\(_3\) 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+/H+ cotransporters (26); 2) in HEPS buffer, HCO3−-dependent transport activity (i.e., electrogenic Na+/HCO3− cotransporters) should be minimal, because the apparent Km 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 Km for HOE-642 in transiented fibroblast cell lines is 0.05 μM for NHE1, 3 μM for NHE2, and 1 mM 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 pH (ΔFBCF/ΔpH) decreases at low pH. In a pilot study, we found that in NHE1+/− astrocytes at pH 6.15, ΔFBCF/ΔpH was ~25% of the maximum response we observed at pH 7.25. In the current study, pH 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 pH.

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 pH levels (pH 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 ~21 times normal. A similar attenuation of the rise in [Na+]i after OGD and repurification 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, Shull 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 dimethylbenzamil (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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GRANT

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