ERK1/2-p90RSK-mediated Phosphorylation of Na⁺/H⁺ Exchanger Isoform 1
A ROLE IN ISCHEMIC NEURAL DEATH*

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The function and regulation of Na⁺/H⁺ exchanger isoform 1 (NHE1) following cerebral ischemia are not well understood. In this study, we demonstrate that extracellular signal-related kinases (ERK1/2) play a role in stimulation of neuronal NHE1 following in vitro ischemia. NHE1 activity was significantly increased during 10–60 min reoxygenation (REOX) after 2-h oxygen and glucose deprivation (OGD). OGD/REOX not only increased the V_{max} for NHE1 but also shifted the K_m toward decreased [H^+]_i. These changes in NHE1 kinetics were absent when MAPK/ERK kinase (MEK) was inhibited by the MEK inhibitor U0126. There were no changes in the levels of phosphorylated ERK1/2 (p-ERK1/2) after 2 h OGD. The p-ERK1/2 level was significantly increased during 10–60 min REOX, which was accompanied by nuclear translocation. U0126 abolished REOX-induced elevation and translocation of p-ERK1/2. We further examined the ERK/90-kDa ribosomal S6 kinase (p90RSK) signaling pathways. At 10 min OGD, phosphorylated NHE1 was increased with a concurrent elevation of phosphorylation of p90RSK, a known NHE1 kinase. Inhibition of MEK activity with U0126 abolished phosphorylation of both NHE1 and p90RSK. Moreover, neuroprotection was observed with U0126 or genetic ablation or pharmacological inhibition of NHE1 following OGD/REOX. Taken together, these results suggest that activation of ERK1/2-p90RSK pathways following in vitro ischemia phosphorylates NHE1 and increases its activity, which subsequently contributes to neuronal damage.

The Na⁺/H⁺ exchanger (NHE)² family is a group of membrane transport proteins that catalyzes the secondary active electroneutral exchange of one Na⁺ for one H⁺. To date, nine NHE isoforms (NHE1–9) have been cloned in mammalian tissues (1). Na⁺/H⁺ exchanger isoform 1 (NHE1) is the most abundant NHE isoform in the rat central nervous system (2) and crucial in regulation of neuronal pH (3, 4). We have recently reported that NHE1 activity plays an important role in neuronal damage in both in vitro and in vivo ischemic models (4). Inhibition of NHE1 activity during OGD/REOX prevents intracellular Na⁺ and Ca²⁺ overload and thus reduces the Ca²⁺-mediated cascade of deleterious events (4). In acutely isolated CA1 neurons, an anoxia-triggered intracellular alkalization depends on activation of NHE1 (5). We also found that NHE1 activity is stimulated in cortical astrocytes following in vitro ischemia (6). However, whether NHE1 activity is over-stimulated in cortical neurons following in vitro ischemia remains unknown.

NHE1 has two large functional domains. The N-terminal transmembrane domain (~500 amino acids) is responsible for cation translocation, and the cytoplasmic C-terminal domain (~315 amino acids) is the main regulatory site for the NHE1 activity (7). The distal C-terminal tail of NHE1 contains a number of serine and threonine residues that are phosphorylated by several protein kinases, including extracellular signal-related kinases (ERK1/2) and 90-kDa ribosomal S6 kinase (p90RSK) (8). Activation of the ERK/p90RSK pathway is necessary for the increase in sarcoplasmic NHE activity induced by myocardial ischemia and reperfusion (8) and sustained intracellular acidosis (9). However, it is unknown whether the ERK signaling cascade plays a role in regulation of NHE1 activity in neurons following in vitro ischemia. Many factors released after ischemia may stimulate members of mitogen-associated protein kinases (MAPK) pathway, such as ERK1/2, c-Jun N-terminal kinase (c-JNK), and p38 MAPK. Among these, the ERK signaling cascade is emerging as an important regulator of neuronal responses to the pathologic stimuli (10). After activation, ERK1/2 phosphorylates several downstream elements, including Elk-1 or p90RSK. It functions as an important intermediate in signal transduction pathways to transmit extracellular signals into key regulatory membrane, cytoplasmic, and/or nuclear targets. However, the role of the activation of the ERK pathway in cerebral ischemia remains equivocal. Whereas activation of the ERK1/2 signaling pathway is involved in cell survival in programmed cell death (11) and deprivation; REOX, reoxygenation; GFAP, glial fibrillary acidic protein; PI, propidium iodide; PBS, phosphate-buffered saline; DAPI, 4',6-diamidino-2-phenylindole.
ischemia (12), there is growing evidence indicating a role for ERK1/2 in neuronal cell death during cerebral ischemia (13). Such multiple functions of ERK1/2 pathways may result from variation in intensity of stimuli, their subcellular localization, and the interplay with other pathways or cellular energy state.

In the current study, we investigated the ERK1/2-mediated regulation of NHE1 activity in cortical neurons following in vitro ischemia. Our results demonstrate that the stimulation of NHE1 in neurons following in vitro ischemia results from activation of ERK1/2-p90<sub>RSK</sub> signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Hanks’ balanced salt solution were from Mediatech Cellgro (Herndon, VA). Neurobasal Medium, B-27 supplement, acetyoxymethyl esters of 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein, were obtained from Invitrogen. Nigericin was purchased from Sigma. U0126 (1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene) was from Tocris (Ellisville, MO). The antibody for β tubulin type III was from Promega (Madison, WI). The antibody for glial fibrillary acidic protein (GFAP) was from Dako North America, Inc. (Fort Collins, CO). The following antibodies were from Cell Signaling Technologies (Beverly, MA): anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-p90<sub>RSK</sub>, anti-RSK, and anti-phospho-Ser 14-3-3 protein binding motif. HOE 642 was a kind gift from Aventis Pharma (Frankfurt, Germany). G116 antibody was a kind gift from Dr. Leong L. Ng (Leicester, UK).

**Pure Cortical Neuron Cultures**—Preparation of NHE1<sup>+/+</sup> or NHE1<sup>−/−</sup> cortical neurons from E14–16 fetuses has been described previously with some modification (4). In brief, the cortices were removed from an E14–16 fetus 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 in B-27 supplemented Neurobasal medium (2%). The cells from individual fetal cortices were seeded separately at a density of 4 × 10<sup>4</sup> cells/cm<sup>2</sup> in 6-well plates or on glass coverslips coated with poly-D-lysin.

The cultures were maintained in an incubator of 5% CO<sub>2</sub> and atmospheric air at 37 °C. Half of the medium was replaced twice a week. 10–15 Days in vitro cultures were used in the study.

**OGD Treatment**—10–15 Days in vitro neuronal cultures were rinsed with an isotonic OGD solution (pH 7.4) containing (in mM): 0 glucose, 20 NaH<sub>2</sub>CO<sub>3</sub>, 120 NaCl, 5.36 KCl, 0.33 Na<sub>2</sub>HPO<sub>4</sub>, 0.44 KH<sub>2</sub>PO<sub>4</sub>, 1.27 CaCl<sub>2</sub>, 0.81 MgSO<sub>4</sub>. Cells were incubated in 0.5 ml of the OGD solution in a hypoxic incubator (model 3130 from Thermo Forma, Marietta, OH) containing 94% N<sub>2</sub>, 1% O<sub>2</sub>, and 5% CO<sub>2</sub>. Because similar cell death rates were found following 2 or 3 h OGD in our previous study (4), 2–3 h OGD was used in the current study. For reoxygenation, cells were incubated in 0.5 ml of B-27 supplemented Neurobasal medium at 37 °C in 5% CO<sub>2</sub> and atmospheric air. For pH<sub>i</sub> measurements, the OGD incubation was 2 h and REOX was performed on the microscope stage at 37 °C. Normoxic control cells were incubated in 5% CO<sub>2</sub> and atmospheric air in isotonic control buffer containing 5.5 mM glucose with the rest of the components identical to the isotonic OG solution.

**Measurement of Cell Death**—Cell viability was assessed by propidium iodide (PI) uptake and retention of calcein as described previously (4). Briefly, cultured neurons were rinsed and incubated with 1 µmol/liter calcein-AM and 10 µg/ml PI in HEPEs/minimal essential medium at 37 °C for 30 min. Cells were then rinsed and visualized using a Nikon TE 300 inverted epifluorescence (Tokyo, Japan) and ×20 objective lenses. Calcein and PI fluorescence signals were obtained using fluorescein isothiocyanate and Texas Red filters, respectively. Images were collected with a Princeton Instruments (Trenton, NJ) MicroMax CCD camera. In a blind manner, a total of 1000 cells/condition were counted using MetaMorph image-processing software (Universal Imaging Corp., Downingtown, PA). Cell mortality was expressed as the ratio of PI-positive cells to the sum of calcein-positive and PI-positive cells.

**Measurement of pH<sub>i</sub>**—pH<sub>i</sub> measurement and pre-pulse treatment were performed at 37 °C as described previously (4). Neurons grown on coverslips were incubated for 2 h with 2.5 µM 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein-AM under either normoxic control or OGD conditions at 37 °C. The coverslips were then washed and placed in the open bath imaging chamber. The chamber was mounted on the stage of the microscope and visualized by ×40 objective lens. The cells were excited every 10–30 s at 440 and 490 nm and the ratio of the fluorescence emission at 535 nm was recorded. Images were collected using the Princeton Instruments MicroMax CCD camera, and analyzed with MetaFluor image-processing software (Universal Imaging Corp.). The ratio of the fluorescence emissions (F<sub>Ex</sub>/F<sub>Em</sub>) was calibrated using the high K<sup>+</sup>/nigericin technique (14). NH<sub>4</sub><sup>+</sup>/NH<sub>3</sub> solutions (30 mM, 2 min) were used to induce an acid load as described before (4). The pH<sub>i</sub> recovery rate (ΔpH<sub>i</sub>/min) was determined from the slope of regression line fit to the 40 s following NH<sub>4</sub><sup>+</sup>/NH<sub>3</sub> pre-pulse (average pH, 6.6).

As described previously (15), buffer capacity (β<sub>i</sub>) was determined over a range of pH<sub>i</sub> by subjecting the cells to progressively decreasing concentrations of NH<sub>4</sub><sup>+</sup> in Na<sup>+</sup>-free HEPEs/minimal essential medium, based on β<sub>i</sub> = ΔNH<sub>4</sub><sup>+</sup>/ΔpH<sub>i</sub>. H<sup>+</sup> flux rates (I<sub>H<sup>+</sup></sub>; mm H<sup>+</sup>/min) were determined by multiplying β<sub>i</sub> by ΔpH<sub>i</sub>/Δt for each time interval during pH<sub>i</sub> recovery (1–2 min) following pre-pulse treatment.

**Immunofluorescence Staining**—Cultured cells grown on coverslips were rinsed with PBS (pH 7.4) and fixed with 4% paraformaldehyde in PBS (4). After rinsing, 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 incubated with primary antibodies for 1 h at 37 °C. After rinsing in PBS, cells were incubated with Alexa Fluor<sup>TM</sup> 488 goat anti-mouse (1:200; Invitrogen) and Alexa Fluor<sup>TM</sup> 594 goat anti-rabbit IgG (1:200; Invitrogen) for 1 h at 37 °C. The coverslips were then covered with Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). Fluorescence images for GFAP and βIII tubulin staining were captured by the Nikon TE 300 inverted epifluorescence microscope (×40) using a Princeton Instruments MicroMax CCD camera and MetaMorph image-processing software. Fluorescence images for NHE1, β tubulin III, p-ERK1/2, and ERK1/2 were obtained using a Leica DMIRE2 inverted confocal laser-scanning microscope (×63) and Leica confocal software (Leica Microsystems Inc., Mann-
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heim, Germany). Identical digital imaging acquisition parameters were used in both negative control and experimental images.

Polyclonal antibody against ERK1/2 (1:100) and monoclonal antibody against p-ERK1/2 (1: 200) were used for detection of total ERK1/2 and p-ERK1/2, respectively. Polyclonal G116 antibody against NHE1 (1:50) (16), anti-GFAP polyclonal antibody (1:200), and anti-β tubulin monoclonal antibody (1:200) were used for detection of NHE1, GFAP, and βIII tubulin, respectively.

Subcellular Fractionation Preparation—For nuclear fractionation, cells were harvested in PBS (pH 7.4) containing (mM): 10 NaCl, 10 Na2PO4, 5 MgCl2, 10 β-glycerophosphate, 0.1% Nonidet P-40, 0.065, Na3VO4, and protease inhibitors as described previously (4). The cells were incubated for 10 min on ice and homogenized by 20 strokes with a tight-fitting homogenizer. After removing unbroken cells by centrifugation at 150 × g for 2 min, nuclei were collected via centrifugation at 900 × g for 10 min at 4 °C and prepared for Western blot analysis. The supernatant containing the cytoplasmic proteins was also collected for Western blotting.

Gel Electrophoresis and Western Blotting—Cells were washed twice with ice-cold PBS and harvested on ice in an antiphosphatase buffer (pH 7.4) containing (mM): 150 NaCl, 20 HEPES (pH 7.4), 100 NaF, 10 Na2HPO4, 2 Na3VO4, 5 EDTA, and 0.2 μm microcystin and protease inhibitors as described previously (4). After brief centrifugation (300 × g), cell pellet was collected and resuspended in the same buffer containing 1% Triton X-100. After sonication, samples were centrifuged at 10,000 × g at 4 °C for 10 min. Supernatants were collected for immunoblotting. Protein content was determined by the bicinchoninic acid method.

Protein samples (15 μg/lane) and pre-stained molecular mass markers (Bio-Rad) were denatured in SDS reducing buffer (1:2 by volume). The samples were then electrophoretically separated on 6 or 12% SDS gels and the resolved proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (4). The blots were incubated in 5% nonfat dry milk in Tris-buffered saline for 1 h at room temperature, and then incubated overnight at 4 °C with a primary antibody. The blots were rinsed with Tris-buffered saline and incubated with horse-radish peroxidase-conjugated secondary IgG for 1 h. Bound antibody was visualized using the enhanced chemiluminescence assay (Pierce).

Polyclonal antibody against ERK1/2 (1:1000) and monoclonal antibody against p-ERK1/2 (1:2000) were used for detection of total ERK1/2 and p-ERK1/2, respectively. Polyclonal antibodies against RSK (1:1000) and phosphorylated p90RSK (p-p90RSK, 1:1000) were used for detection of total RSK and p-p90RSK, respectively. Polyclonal G116 antibody against NHE1 (1:1000) and anti-β tubulin monoclonal antibody (1:3000) were used for detection of NHE1 and βIII tubulin, respectively.

Measurement of NHE1 Expression in Phosphoprotein and Nonphosphoprotein Fractions—The PhosphoCruz™ Protein Purification System from Santa Cruz (Santa Cruz, CA) was used to isolate phosphoproteins from neuronal lysates. Cellular lysates were prepared as described by Lehoux (17) with some modification. Cells were washed twice in PBS and lysed in ice-cold antiphosphatase buffer with 1% Triton X-100 as described above. Cell lysates were sonicated at 4 °C and centrifuged at 10,000 × g for 10 min. The supernatants were collected. According to the manufacturer’s instruction, 5 mg of total protein was incubated with the phosphoprotein affinity resin. Proteins that carry a phosphate group were specifically retained on the resin, whereas the nonphosphorylated proteins flow through the column and were collected. The phosphoproteins retained on the resin were subsequently eluted from the resin to obtain the phosphoenriched protein fraction of interest for analysis. Protein content in elution was concentrated by microconcentrators supplied in the kit. The levels of NHE1 in different fractions were measured using gel electrophoresis and Western blotting as described above. NHE1 recovery rate in normoxic control samples was 40 ± 9% by this technique. Phosphorylated NHE1 was 5% of the total NHE1 proteins and nonphosphorylated was about 95%, which is consistent with the report from other analyses.

Analysis of NHE1 Phosphorylation by Immunoprecipitation—In parallel, the phosphorylation level of NHE1 was determined using a method established by Snabaitis et al. (18). Cells were washed with ice-cold antiphosphatase buffer and lysed in ice-cold immunoprecipitation lysis buffer (pH 7.5) containing (mM): 20 Tris-HCl, 150 NaCl, 1 EDTA, 1 EGTA, 2.5 sodium pyrophosphate, 1 β-glycerophosphate, 1 Na4P2O7, 100 NaF, 1% Triton X-100, 0.1% SDS and protease inhibitors as described previously (4). The samples were centrifuged at 14,000 × g for 60 min at 4 °C. Supernatant containing 0.4 mg of solubilized membrane proteins was removed and incubated overnight at 4 °C with mouse monoclonal antibody against phospho-Ser 14-3-3 protein binding motif (1:15). Immunocomplexes were mixed with protein G (Sigma) for 2 h at 4 °C and washed three times with ice-cold modified immunoprecipitation lysis buffer without containing SDS. The immunocomplexes were dissociated from beads with the Laemmli sample buffer and heated for 5 min at 70 °C. Protein samples from crude lysate or the immunocomplex were resolved on 6% SDS-PAGE and analyzed by immunoblotting using rabbit polyclonal NHE1 antibody as described above.

Statistics—Throughout the study, n values represent numbers of cultures used in each experiment. Routinely, parameters for live cell imaging were measured in ~20 cells on a coverslip from each culture. Statistical significance was determined by the Mann-Whitney nonparametric test at a confidence of 95% (p < 0.05) or by Student’s t test (p < 0.05). Error bars in figures represent either standard deviation (S.D.) or standard error of means (S.E.).

RESULTS

Expression of NHE1 in Pure Neuron Cultures—The pure neuronal cultures were characterized by triple staining with astrocyte-specific marker GFAP (red, Fig. 1A), neuron-specific marker β tubulin III (green, Fig. 1B), and nucleus marker DAPI (blue, Fig. 1, A and C). As shown in Fig. 1A, no GFAP-staining signal was found in the neuronal cultures. Positive GFAP-staining signal was shown in the mixed neuronal cultures (inset, Fig. 1A). Fig. 1C demonstrates characteristic neuronal morphology in the cultures. Fig. 1, D–F, shows expression of NHE1 in neu-
neurons by immunofluorescence staining. Colocalization of NHE1 (red, Fig. 1D) and βIII tubulin (green, Fig. 1E) was found in all neurons (Fig. 1F).

**Stimulation of NHE1 Activity following OGD/REOX Depends on Activation of MEK-mediated Pathways**—Fig. 2A illustrates pH$_i$ regulation in response to NH$_4$Cl-mediated pre-pulse in neurons under normoxic or OGD/REOX conditions. In the standard HCO$_3$-free HEPES-buffered solution, basal pH$_i$ in cortical neurons was 7.05 ± 0.05 (Fig. 2, A and B). Following the NH$_4$Cl-mediated pre-pulse, pH$_i$ was recovered within 5 min in normoxic control cells, which largely reflects NHE1 activity (4). In neurons exposed to 2 h OGD and 10 min REOX, basal pH$_i$ was reduced moderately (pH$_i$ of 6.91 ± 0.08, p < 0.05, Fig. 2, A and B). By 1 h REOX, pH$_i$ increased to 7.38 ± 0.18 (p < 0.05). Moreover, there was a significant increase in pH$_i$ recovery rate in neurons following 2 h OGD and 10 min REOX (0.49 ± 0.08 unit/min, p < 0.05, Fig. 2C), compared to a control of 0.19 ± 0.02 unit/min. This suggests an increase in NHE1 activity. This H$^+$ extrusion rate remained elevated at 1 h REOX (0.61 ± 0.15 unit/min, p < 0.05, Fig. 2C). Inhibition of NHE1 activity with its potent inhibitor HOE 642 (1 μM) abolished the pH$_i$ recovery following 2 h OGD and 10 min REOX (p < 0.05, Fig. 2C). Moreover, the resting pH$_i$ was reduced to 6.87 in the presence of HOE 642 (p < 0.05, Fig. 2B).

Interestingly, in the presence of the MEK inhibitor U0126 (10 μM), the OGD/REOX-mediated increase in the pH$_i$ recovery rate was abolished (Fig. 2). The alkaline rebound during REOX was absent in the presence of U0126 (pH$_i$ of 6.89 ± 0.11). This suggests that the ERK kinase (MEK1/2) and ERK1/2 pathways are involved in activation of NHE1 activity in neurons following OGD/REOX.

**OGD/REOX Increases the Maximal Velocity (V$_{max}$) and Proton Affinity of NHE1, Which Depends on ERK1/2 Activation**—To further investigate NHE1 kinetics in neurons following OGD/REOX, we first examined whether the intrinsic buffer
capacity $\beta_i$ of neurons was altered by OGD/REOX. Changes in $\text{pH}$ were induced by applying progressively decreasing concentrations of $\text{NH}_3/\text{NH}_4^+$. In normoxic neurons, $\beta_i$ increased linearly from a value of about 12.5 mM/pH at a pH of 7.0 to about 23.5 mM/pH at a pH of 6.2. The fitted regression was $\beta_i = 109 + (-13.8 \times \text{pH})$. $\beta_i$ was also determined in neurons following 2 h OGD and 1 h REOX. OGD/REOX did not cause a significant change in the fitted line of $\beta_i$ versus pH ($\beta_i = 98 + (-12.2 \times \text{pH}), p > 0.05$).

We also calculated $J_{\text{H}^+}$ during the pre-pulse-induced pH recovery and plotted it against the corresponding $[\text{H}^+]$, (Fig. 3, A and B). In normoxic control neurons, the velocity of $\text{H}^+$ efflux from cells increased as $[\text{H}^+]$ increased and was best described by a sigmoid fit ($V_{\text{max}} = 8.2 \pm 0.8 \text{ mM/min}, K_m = 0.29 \pm 0.01 \mu M$; Fig. 3A) with a Hill coefficient of 2.03 ± 0.07. The latter is consistent with other reports and suggests that there might be more than one binding site for $\text{H}^+$ on the intracellular face of the protein (19). When neurons were exposed to 2 h OGD and 10 min REOX, the kinetics of NHE1 were dramatically changed (Fig. 3A). $V_{\text{max}}$ was increased about 3 times ($23.5 \pm 1.9 \text{ mM/min}, p < 0.05$), whereas the $K_m$ shifted to a lower concentration of protons ($0.21 \pm 0.04 \mu M, p < 0.05$). The sigmoid curve fit of the OGD/REOX data were significantly different from the curve fit to the normoxic control data in neurons ($p < 0.05$). The elevation in $V_{\text{max}}$ and proton affinity was maintained during 60 min REOX following 2 h OGD (Fig. 3B). The Hill coefficients for the OGD/REOX data were similar to the control (2.26 ± 0.09; 2.58 ± 0.15, respectively). In contrast, inhibition of MEK1/2 activity with 10 $\mu M$ U0126 significantly attenuated OGD/REOX-mediated changes in NHE1 kinetics (Fig. 3, A and B). $V_{\text{max}}$ was reduced by ~50% ($p < 0.05$ versus OGD/REOX) and $K_m$ shifted back to a higher concentration of protons (0.30 ± 0.07 $\mu M, p > 0.05$ versus normoxic control) in the presence of 10 $\mu M$ U0126 (Fig. 3, A and B). This suggests that the MEK1/2 and ERK1/2 pathways are involved in stimulation of NHE1 activity following OGD/REOX.

**Activation of ERK1/2 Pathway in Cortical Neurons following OGD/REOX**—We further investigated activation of ERK1/2 in response to OGD/REOX by measuring the phosphorylation status of ERK1/2. p-ERK1/2 levels in NHE1+/-+ neurons were not increased at the end of 2 h OGD (Fig. 4, A and B), which is consistent with data in ischemic heart (20). However, 10 min REOX triggered a rapid increase in levels of ERK1/2 phosphorylation (~3.4-fold, $p < 0.001$), which remained elevated during REOX (~2.7-fold at 30 min REOX, $p < 0.001$; ~2.5-fold at 60 min REOX, $p < 0.001$). Total ERK1/2 protein levels were not significantly altered following OGD/REOX (Fig. 4A). Exposing neurons to U0126 during OGD/REOX abolished ERK phosphorylation (Fig. 4, A and B).

It was recently observed in vascular smooth muscle cells that NHE1 activity regulates ERK phosphorylation (21). We performed additional experiments in NHE1+/-+ neurons to investigate whether ERK phosphorylation was altered in the absence of NHE1 function. Interestingly, a similar rapid increase in phosphorylation of ERK1/2 was found in NHE1+/-+ neurons following OGD/REOX (~3-fold, $p < 0.001$, Fig. 4, C and D). The NHE1 inhibitor HOE 642 had no effects on ERK1/2 phosphorylation either (data not shown). This suggests that ERK phosphorylation does not depend on NHE1 activity in neurons under OGD/REOX conditions.

**Translocation of Activated ERK1/2 Kinases into Nuclei after OGD/REOX in Cortical Neuron**—The OGD/REOX-induced ERK1/2 activation was further examined in cortical neurons by immunofluorescence staining. Constitutive expression of ERK1/2 was found within the cytoplasm and processes with a weak immunoreactive signal in nucleus under normoxic control conditions (red, Fig. 5A). No immunoreactive signals of p-ERK1/2 were observed in these neurons (green, Fig. 5B, the signals in the inset were manually amplified 4 times). Either 10 or 60 min REOX had no significant effects on distribution of ERK1/2 immunostaining (red, Fig. 5, C and E). In contrast, immunoreactive signals of p-ERK1/2 were significantly increased at 10 and 60 min REOX (green, Fig. 5, D and F). Additionally, some p-ERK1/2 was also localized in the nucleus (Fig. 5, D and F, arrows). It has been suggested that persistent nuclear retention of activated ERK-1/2 may be a critical factor in eliciting pro-apoptotic effects in neurons.
subjected to glutamate-induced oxidative stress (22). Moreover, inhibition of MEK with U0126 prevented nuclear accumulation of activated ERK1/2 after OGD/REOX (Fig. 5H). The change of ERK1/2 phosphorylation after OGD/REOX was further examined by immunoblotting analysis (Fig. 5, I and J). Because four times more nuclear proteins were loaded in Fig. 5, I and J, the level of nuclear p-ERK signals in control conditions in Fig. 5, I and J, appear to be higher than that in Fig. 5B. After 10 min REOX following 2 h OGD, neurons displayed increased levels of p-ERK in the nuclei (~2.3-fold, p < 0.05) as well as in the cytosol (~3-fold, p < 0.05, Fig. 5, I and J). Consistently, ERK phosphorylation was abolished by U0126 (Fig. 5, I and J).

NHE1 Kinase \(p90^{RSK}\) Is Downstream Effector of ERK1/2—

\(p90^{RSK}\), which is activated by ERK1/2, has been shown to phosphorylate the regulatory domain of NHE1 and may mediate serum- or endothelin-induced stimulation of NHE activity in cultured fibroblasts (23) and neonatal rat ventricular myocytes (24). We investigated whether \(p90^{RSK}\) is responsible for activation of NHE1 in neurons following OGD/REOX. \(p90^{RSK}\) activation was determined by measuring the phosphorylation status of \(p90^{RSK}\). As shown in Fig. 6, A and B, a low level of \(p-p90^{RSK}\) was found in neurons under normoxic control conditions. There were no changes in \(p-p90^{RSK}\) at 2 h OGD, which is consistent with ERK1/2 phosphorylation data (Fig. 4). Moreover, 2 h OGD/10 min REOX caused a ~3-fold increase in \(p90^{RSK}\) phosphorylation. Furthermore, the OGD/REOX-induced \(p90^{RSK}\) phosphorylation was abolished by U0126 (Fig. 6, A and B), suggesting that activation of \(p90^{RSK}\) depends on the ERK pathway. A role for \(p90^{RSK}\) in direct phosphorylation of NHE1 has been found in the myocardium in response to \(\alpha_{1A}-AR\) stimulation (25).

Phosphorylation of NHE1 in Neurons via ERK Pathway—

We then investigated whether OGD/REOX leads to phosphorylation of NHE1 in neurons. Nonphosphorylated and phosphorylated fractions of total proteins were separated and probed for NHE1 protein as described under “Experimental Procedures.” As shown in Fig. 7A, a basal level of NHE1 phosphorylation was detected in normoxic control conditions. 2 h OGD/10 min REOX caused an increase in expression of NHE1 in phosphorylated fractions and a decrease in non-phosphorylated fractions. Moreover, total NHE1 proteins were not altered by OGD/REOX. Interestingly, U0126 treatment selectively abolished the increase in NHE1 phosphorylation and had no effect on total NHE1 protein content (Fig. 7, A and C). On the same blot, expression of \(\beta III\) tubulin in each fraction was determined to confirm equal protein loading (Fig. 7A). Furthermore, the efficiency of phosphoprotein isolation from the total proteins was confirmed by the inability to detect phosphorylated ERK1/2 in the nonphosphoprotein fractions (data not shown). This result suggests that phosphorylation of NHE1 is stimulated after OGD/REOX at least in part through the ERK pathway.

We then used the phosho-Ser 14-3-3 binding motif antibody to detect the phosphorylation of NHE1 in neurons. This method was established by Snabaitis et al. (18) and confirmed by parallel radioactive kinase assays. As illustrated in Fig. 7E, the intensity of a protein band migrating at ~100 kDa, which represents the phosphorylation level of NHE1, was increased in neurons exposed to 2 h OGD and 10 min REOX (p < 0.05, Fig. 7, E and F). In the presence of U0126, the increase in NHE1 phosphorylation was abolished (p < 0.05, Fig. 7, E and F). These data further confirmed that phosphorylation of NHE1 is indeed elevated after OGD/REOX and depends on the ERK pathway.

Prolonged Acidosis Is Not Responsible for NHE1 Activation following OGD/REOX—

It has been reported that prolonged acidosis (\(pH_i\) of 6.55) for 3 min in rat ventricular cardiomyocytes leads to ERK1/2-dependent stimulation of NHE1 activity (26): 2 h OGD reduced basal \(pH_i\) from 7.09 to 6.80 in astrocytes (6). To study the possible influence of OGD-mediated acidosis on NHE1 activity, we investigated whether prolonged acidosis
triggered by the NH₄Cl pre-pulse can activate NHE1 activity. Fig. 8A shows that neurons were first exposed to 30 mM NH₃/NH₄⁺/H₁₁₀₀₁ for 2 min and returned to the control HEPES buffer containing Na⁺/H₁₁₀₀₁. The prolonged acidosis was induced by exposing cells to a second NH₃/NH₄⁺/H₁₁₀₀₁ treatment and returned them to a Na⁺-free HEPES buffer for 5 min (Fig. 8A). pHᵢ was reduced from 7.05 ± 0.05 to 6.33 ± 0.07 during the second exposure. However, when the cells were returned to HEPES buffer with Na⁺, the pHᵢ recovery rate was not significantly different from the first brief acidosis (p > 0.05). Moreover, the ERK1/2 phosphorylation level was not significantly altered following 5 min acidosis (p > 0.05, Fig. 8A, inset of A and C). This suggests that OGD/REOX-mediated stimulation of NHE1 activity is unlikely due to prolonged acidosis during OGD.

Inhibition of Phosphorylation of ERK1/2 Significantly Reduces OGD/REOX-induced Neuronal Death—A basal level of cell death occurred in NHE1⁻/⁻/⁻ neurons under normoxic conditions (Fig. 9, A and B). 3 h OGD and 21 h REOX led to a significant cell death in NHE1⁻/⁻/⁻ neurons (78 ± 11%, p < 0.001, Fig. 9, C, D, and G). Inhibition of phosphorylation of ERK1/2 with 10 μM U0126 was neuroprotective in NHE1⁻/⁻/⁻ neurons (40 ± 20%, p < 0.001, Fig. 9, E–G). Consistent with our

inhibitory phosphorylation of ERK1/2, inhibition of ERK1/2 phosphorylation by U0126 significantly reduced OGD/REOX-induced neuronal death.

FIGURE 5. Activation and translocation of ERK1/2. A–H, immunostaining for ERK1/2 and p-ERK1/2 in NHE1⁻/⁻/⁻ neurons. Representative image of three experiments is presented. A, C, E, and G, ERK1/2 (red), B, D, F, and H, p-ERK1/2 (green). A and B, normoxic control. C and D, 2 h OGD/10 min REOX. E and F, 2 h OGD/1 h REOX. G and H, 2 h OGD/1 h REOX plus 10 μM U0126. Identical digital imaging acquisition parameters were used in experimental images. Arrows, nuclear translocation of p-ERK1/2. Inset, p-ERK1/2 signals in nuclei under normoxic conditions in B were enhanced 4 times. Scale bar, 15 μm. I and J, level of p-ERK1/2 was determined by immunoblotting. Samples were prepared as control (Con), 2 h OGD/10 min REOX (O/R), or 2 h OGD/10 min REOX plus U0126 (O/R + U). 10 μg of cytosolic proteins and 40 μg of nuclear proteins were loaded. Data are mean ± S.D. (n = 4). *, p < 0.001 versus Con; #, p < 0.001 versus OGD/REOX.
pH<sub>i</sub> recovery rate after NH<sub>4</sub>Cl pre-pulse was determined in normoxic control and OGD/REOX-treated neurons. We found that after 10 min REOX following 2 h OGD, pH<sub>i</sub> recovery rate was increased by 2.4-fold and the increase was statistically significant. This finding suggests that in our study, the ERK1/2 pathway contributes to neuronal death by stimulation of NHE1 activity following OGD/REOX.

**DISCUSSION**

**NHE1 Activity Is Stimulated in Cortical Neurons after OGD/REOX**—We recently reported that NHE1 activation contributes to Na<sup>+</sup> and Ca<sup>2+</sup> overload in conjunction with reverse mode operation of Na<sup>+</sup>/Ca<sup>2+</sup> exchanger in neuronal damage following OGD (4). This implies that NHE1 activity is elevated after ischemia. However, little is known about regulation of NHE1 activity in ischemic neurons.

In this study, pH<sub>H</sub>, recovery rate after NH<sub>4</sub>Cl pre-pulse was determined in normoxic control and OGD/REOX-treated neurons. We found that after 10 min REOX following 2 h OGD, pH<sub>H</sub> recovery rate was increased by ~2.4-fold and the increase was
Our analysis of the kinetics of NHE1 in neurons revealed that the $V_{\text{max}}$ of Na$^+$/H$^+$ exchange was increased by 3-fold following OGD/REOX. There are at least three possible factors that could increase $V_{\text{max}}$: 1) OGD-mediated increase in NHE1 synthesis; 2) increased expression of NHE1 in the surface of neurons via protein trafficking following OGD; and 3) phosphorylation of NHE1 at the distal C terminus of NHE1. At 10 min REOX following 2 h OGD, there were no significant changes in total NHE1 protein expression in cortical neurons in the immunoblots assay (Fig. 7). Similar results were observed at 30 min REOX following 2 h OGD (data not shown). This implies that the elevation in $V_{\text{max}}$ of NHE during 10–30 min REOX is unlikely the result of an increase in NHE1 synthesis. But, we cannot rule out an increase in NHE1 expression on the plasma membrane via protein trafficking during early REOX following OGD.

Activation of ERK1/2-p90RSK Signaling Pathways Is Responsible for OGD/REOX-mediated Phosphorylation and Stimulation of NHE1 in Neurons—The other possible regulatory mechanism for NHE1 activation is via protein phosphorylation. The C-terminal cytoplasmic regulatory domain of NHE1 may modulate transport activity by altering the affinity of a H$^+$ transport site in the transmembrane domain (28). In our study, OGD/REOX results in an alkaline shift in the pH dependence ($K_m$), which may be attributed to phosphorylation of NHE1. It has been suggested that ERK and p90RSK can directly phosphorylate NHE1 at the distal C terminus of NHE1 and activate NHE1 in ischemic myocardium (8). Mutation of Ser$^{770}$ and Ser$^{771}$ in NHE1 abolishes ERK1/2-p90RSK-mediated phosphorylation and activation of NHE1 following sustained intracellular acidosis (9). Serum stimulation of fibroblasts leads to phosphorylation of NHE1 and this phosphorylation is greatly diminished in the presence of the MEK1 inhibitor PD98059 (23). A serine/threonine kinase p90RSK, a downstream substrate of ERK1/2, has been reported to directly phosphorylate NHE1 at Ser$^{703}$ in the regulatory C-terminal domain and activate the exchanger activity in response to growth factors (23). Phosphorylation of Ser$^{703}$ on NHE1 by p90RSK creates a binding motif for protein 14-3-3 $\beta$ (17) and stabilizes NHE1 in an active state in ischemic cardiomyocytes (29).

In the present study, 10 min REOX following 2 h OGD led to activation of the ERK1/2-p90RSK cascade. Marked increase in the phosphorylation levels of ERK1/2 and p90RSK reflected the increased activity in post-ischemic neurons. A concurrent increase in NHE1 phosphorylation and NHE1 activity was found in neurons at 10 min REOX following OGD. Importantly, upstream inhibition of the ERK-p90RSK cascade by the specific MEK1 inhibitor, U0126, abolished both the stimulation of NHE1 activity and phosphorylation of NHE1 following OGD/REOX. This firmly establishes a role for the MAPK-ERK-p90RSK signaling pathway in stimulation of NHE1 activity in neurons following OGD. The OGD/REOX-mediated alkaline shift of $K_m$ of NHE1 was blocked by U0126. This implies that phosphorylation of the exchanger following activation of ERK1/2-p90RSK alters the “pH set point” of NHE1.

Both ERK1/2 and p90RSK could directly phosphorylate NHE1, it remains to be determined which one and/or both are the direct regulators of NHE1 phosphorylation in neurons fol-

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**FIGURE 9. Inhibition of ERK1/2 activity attenuates OGD/REOX-mediated neuronal death.** Cell mortality was assessed in neuronal cultures after 3 h OGD and 21 h REOX. For drug treatment, neurons were incubated in Earle’s minimal essential medium in the presence of 10 $\mu$M U0126 or 1 $\mu$M HOE 642 at 37 °C during OGD and REOX. Sister NHE1+/− or NHE1−/− cultures were incubated for 24 h in normoxic control buffers (Con). At the end of the experiment, cells were stained with PI and calcein-AM and cell images were acquired. A, C, and E, calcein (green). B, D, and F, PI (red). A and B, control. C and D, OGD/REOX; E and F, OGD/REOX + U0126. Scale bar, 100 μm. G, summary data. Data are mean ± S.E. (n = 4–8). *, p < 0.001 versus control. #, p < 0.01 versus NHE1+/− OGD/REOX.
lowing OGD/REOX. Additional studies with specific inhibition of p90RSK are needed to resolve this issue.

In addition to oxidative stress, the ERK pathway can be activated by many other signals, such as sustained intracellular acidosis (30) and NHE1 activation (21). In our study, the pH recovery rate was not altered by intracellular acidosis in NHE1+/+ neurons. Neither did intracellular acidosis induce activation of the ERK pathway. Thus, stimulation of NHE1 following OGD is unlikely the result of a sustained intracellular acidosis in our in vitro ischemic model. But, a role for intracellular acidosis in stimulation of NHE1 following in vivo cerebral ischemia cannot be ruled out. On the other hand, it has been reported that acidosis-mediated stimulation of NHE1 is not effective unless cultures are serum deprived previously (9). Our pure neuron cultures were maintained in the presence of B27 supplement.

Our data demonstrated that NHE1 is a substrate of ERK-p90RSK because ERK phosphorylation (~3-fold) during REOX occurred in NHE1−/− neurons or in NHE1+/+ neurons treated with HOE 642. This is different from a previous report that NHE1 plays a regulatory role in the stimulation of ERK1/2 pathways in vascular smooth muscle cells (21). It remains to be determined whether REXO-induced rapid ERK1/2 activation is mediated through the classic Ras/Raf/MEK/ERK pathway in neurons.

The Role of ERK1/2-p90RSK Pathway in Cerebral Ischemic Injury—Robust activation of ERK occurs upon immediate reperfusion following transient focal cerebral ischemia (31) or at 4 h reperfusion after spinal cord ischemia (32). Inhibition of ERK1/2 with the MEK inhibitors SL327, PD98058, or U0126 significantly reduces ischemic infarct volume (31, 33, 34). Although the precise mechanisms of the MEK/ERK pathway in ischemic injury remain to be determined, activation of ERK1/2 following ischemia may have deleterious effects via enhancing glutamate release (33), activation of caspase 3 and apoptosis (31), and stimulation of cytokines (31).

In the current study, we found that inhibition of the ERK-p90RSK cascade in neurons not only suppressed stimulation and phosphorylation of neuronal NHE1 following OGD/REOX, but also was neuroprotective. U0126 had similar neuroprotection as inhibition of NHE1 activity by HOE 642 or genetic ablation. It has been reported that inhibition of p90RSK by transgenic expression of a p90RSK mutant enhances the tolerance of mouse myocardium to ischemia and reperfusion-induced injury, probably through reduced sarcosommal NHE1 activation (29). This is consistent with our result that activation of the ERK-p90RSK pathway is detrimental and responsible for the activation of NHE1 in ischemic neurons. Our study also suggests that although the ERK-p90RSK pathway has multiple cellular substrates, it appears that NHE1 is the critical one for the protective effect in our ischemic model. Overstimulation of NHE1 activity following ischemia may disrupt intracellular H+ homeostasis. An alkaline brain pH may be detrimental to cell survival by influencing pro-apoptotic mechanisms (35), and/or stimulating N-methyl-D-aspartate receptor, thus exacerbating excitotoxic neuronal injury (36). Therefore, mild acidosis after ischemia is neuroprotective (37). In our study, a significant part of OGD/REOX-induced neuronal death was independent of ERK1/2 activation (Fig. 9). This implies that other signaling transduction mechanisms also play a role in neuronal death. For example, N-methyl-D-aspartate receptor-triggered necrosis and ischemic apoptosis have been established in neuronal death following OGD/REOX (38).

Specific subcellular location of ERK1/2 is critical for its protective or toxic effects. Restriction in the cytoplasm of activated ERK1/2 is important for hepatocyte survival following H2O2 treatment (39). On the other hand, persistent nuclear retention of activated ERK1/2 is reported in eliciting pro-apoptosis in glutamate-treated neurons (22). Blockade of active ERK1/2 nuclear translocation is neuroprotective (40). These studies suggest that subcellular localization of active ERK1/2 plays an important role in determining its responses to stress stimuli. In our current study, nuclear accumulation of ERK1/2 was detected, at least during early REOX after OGD, which may contribute to neuronal ischemic damage independent of affecting NHE1 stimulation.

In conclusion, we report here that phosphorylation and activity of neuronal NHE1 were stimulated by ERK1/2-p90RSK signaling pathways during REOX following in vitro ischemia. Inhibition of either ERK1/2 or NHE1 not only abolished alka-linization but also reduced ischemic neuronal death following in vitro ischemia.

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