Excessive Na\(^+/\)H\(^+\) exchange in disruption of dendritic Na\(^+\) and Ca\(^{2+}\) homeostasis and mitochondrial dysfunction following \textit{in vitro} ischemia

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Neuronal dendrites are vulnerable to injury under diverse pathological conditions. However, the underlying mechanisms for dendritic Na\(^+\) overload and the selective dendritic injury remain poorly understood. Our current study demonstrates that activation of Na\(^+/\)H\(^+\) exchanger isoform 1 (NHE-1) in dendrites presents a major pathway for Na\(^+\) overload. Neuronal dendrites exhibited higher pHi regulation rates than soma as a result of a larger surface area to volume ratio. Following 2 h oxygen glucose deprivation (OGD) and 1 h reoxygenation (REOX), NHE-1 activity was increased by \sim 70-200\% in dendrites. This elevation depended on activation of p90 ribosomal S6 kinase. Moreover, stimulation of NHE-1 caused dendritic Na\(^+\) accumulation, swelling, and a concurrent loss of Ca\(^{2+}\) homeostasis. The Ca\(^{2+}\) overload in dendrites preceded the changes in soma. Inhibition of NHE-1 or the reverse mode of Na\(^+/\)Ca\(^{2+}\) exchange (NCX\(_{rev}\)) prevented these changes. Mitochondrial membrane potential (\(\Psi_m\)) in dendrites depolarized 40 min earlier than soma following OGD/REOX. Blocking NHE-1 activity not only attenuated loss of dendritic \(\Psi_m\) and mitochondrial Ca\(^{2+}\) homeostasis but also preserved dendritic membrane integrity. Taken together, our study demonstrates that NHE-1-mediated Na\(^+\) entry and subsequent NCX\(_{rev}\) activation contribute to the selective dendritic vulnerability to \textit{in vitro} ischemia.

Neuronal dendrites are vulnerable to injury under diverse pathological conditions including cerebral ischemia, epilepsy, and Alzheimer’s disease (1;2). The hallmark of dendritic injury is the formation of focal swelling or beads along the length of the dendritic arbor (3). However, the underlying mechanisms for this selective dendritic injury remain poorly understood. The initial NMDA or kainite-mediated swelling in dendrites of cultured neurons depends on intracellular accumulation of Na\(^+\) and Cl\(^-\), but not Ca\(^{2+}\) (4). On the other hand, excessive Ca\(^{2+}\) entry plays a role in the long lasting structural damage and delayed recovery in hippocampal slices in response to NMDA (4;5). A correlation between dendritic bead formation and ATP reduction/mitochondrial dysfunction has been demonstrated in cultured hippocampal neurons following glutamate exposure (6). However, the relationship between selective dendritic damage, loss of Na\(^+\) and Ca\(^{2+}\) homeostasis, and mitochondrial dysfunction following ischemia remains to be defined.

The Na\(^+/\)H\(^+\) exchanger isoform 1 (NHE-1) is a plasma membrane protein present in virtually all mammalian cells and plays a central role in intracellular pH (pHi) and cell volume regulation (7). NHE-1 activity is directly activated by intracellular acidification and/or by protein phosphorylation mediated by ERK-p90 ribosomal S6 kinase (p90\(^{RSK}\)) in ischemic neurons (8). Excessive NHE-1 activation results in intracellular Na\(^+\) accumulation which subsequently promotes...
Ca\(^{2+}\) entry via reversal of Na\(^{+}/Ca\(^{2+}\) exchange (NCX\(_{\text{rev}}\)) and plays an important role in myocardium ischemia/reperfusion injury (9). We recently reported that NHE-1 activity in the soma of neurons and astrocytes is stimulated following ischemia and inhibition of NHE-1 activity is neuroprotective (8;10). In addition, inhibition of NHE1 either pharmacologically or by genetic knockdown reduces infarction at 24 hr following \textit{in vivo} focal ischemia (11). However, it remains unexplored whether concurrent activation of NHE-1 and NCX\(_{\text{rev}}\) contributes to the selective vulnerability of postsynaptic neuronal dendrites to ischemic damage.

In the current study, we demonstrated that neurons exhibited robust NHE-1-dependent pH\(_i\) regulation in their dendrites as a result of their large surface area to volume ratio. Further, \textit{in vitro} ischemia (oxygen glucose deprivation and reoxygenation, OGD/REOX) stimulated NHE-1 activity in large (Lg)-Dendrites. NHE-1-mediated Na\(^{+}\) entry and subsequent stimulation of NCX\(_{\text{rev}}\) activity contributed to selective ischemic damage of dendrites. The underlying mechanisms involved the loss of mitochondrial Ca\(^{2+}\) homeostasis and mitochondrial membrane dysfunction.

**EXPERIMENTAL PROCEDURES**

\textit{Materials}- Hanks balanced salt solution (HBSS) was from Mediatech Cellgro (Manassas, VA). Neurobasal medium, B-27 supplement, fura-2-AM, SBFI-AM, BCECF-AM, rhod-2-AM, MitoTracker green, TMRE, calcein-AM, JC-1, Vybrant® DiO, SYTO 60, and 4-bromo A-23187 were from Invitrogen (Carlsbad, CA). Saponin, tetraphenylboron, gramicidin, and monensin were purchased from Sigma (St. Louis, MO). RU360 was from EMB Chemicals (Gibbstown, NJ). Pluronic F-127 was from BASF Corp (Parsippany, NJ). HOE 642 was a kind gift from Aventis Pharma (Frankfurt, Germany). SEA0400 was a kind gift from Taisho Pharmaceutical CO. Ltd. (Omiya, Saitama, Japan). BI-D1870 was purchased from the School of Life Science, University of Dundee, Scotland, UK.

Pure cortical neuron cultures- Pure cortical neurons from embryonic day 14-16 mouse fetuses (SV129/Black Swiss) were prepared as described previously (8). The cortices were removed from E14-16 fetuses and treated with 0.5 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 %) containing 0.5 mM L-glutamine and penicillin/streptomycin (100 units/ml and 0.1 mg/ml, respectively). The cells were seeded at a density of 1 x 10^5 cells/ cm\(^2\) on glass coverslips in 6-well plastic plates coated with poly-D-lysine. The cultures were maintained in an incubator (model 3130, Thermo Forma, Waltham, MA) with 5% CO\(_2\) and atmospheric air at 37°C. Half of the medium was replaced twice a week. DIV 10-15 cultures (days in culture) were used in the study.

\textit{Oxygen and glucose deprivation (OGD) treatment}- DIV 10-15 neuronal cultures grown on coverslips in 6-well plates were rinsed with an isotonic OGD solution (pH 7.4) containing (in mM): 0 glucose, 21NaHCO\(_3\), 120 NaCl, 5.36 KCl, 0.33 Na\(_2\)HPO\(_4\), 0.44 KH\(_2\)PO\(_4\), 1.27 CaCl\(_2\), and 0.81 MgSO\(_4\). This solution has a K\(^+\) concentration (~ 5.8 mM) which is similar to the neurobasal medium (5.6 mM) used for cell cultures. The cells were incubated in 1 ml of OGD solution for 2 h in a hypoxic incubator (model 3130, Thermo Forma) containing 94% N\(_2\), 1% O\(_2\), and 5% CO\(_2\). Normoxic control cells were incubated for 2 h in 5% CO\(_2\) and atmospheric air in a buffer identical to the OGD solution except for the addition of 5.5 mM glucose. REOX was achieved by addition of glucose (5.5 mM) and incubation at 37°C in 5% CO\(_2\) and atmospheric air. Alternately, REOX was performed on the microscope stage by superfusion with HCO\(_3\)--MEM at 37°C equilibrated with 5% CO\(_2\) and ~ 18% O\(_2\) (monitored by an in-line oxygen electrode, Model 16-730; Microelectrodes, Bedford, NH).

\textit{pH\(_i\) measurement}- pH\(_i\) measurement and prepulse treatment were performed as described previously with some modifications (8). Briefly, pure neuronal cultures grown on coverslips were incubated with 2.5-5 μM BCECF-AM for 30 min during normoxia or during the last 30 min of REOX at 37°C. The coverslips were washed with HCO\(_3\)--free HEPES-MEM and placed in a temperature controlled (37°C) open-bath imaging chamber (Model RC24, Warner Instruments, Hamden, CT). The chamber was mounted on the
stage of the TE 300 inverted epifluorescence microscope and 1-3 neurons were visualized with a 100X oil-immersion objective. The cells were excited every 10-30 s at 440 and 490 nm, and the emission fluorescence at 535 nm recorded. Images were collected using a Princeton Instruments MicroMax CCD camera and analyzed with MetaFluor image-processing software. Fluorescence changes in regions of interest in soma, Lg- and small (Sm) dendrites were determined. Lg-Dendrites were defined as dendritic segments with a width of 5.3 ± 1.2 μm, while Sm-Dendrites were ones with 1.8 ± 0.4 μm. The ratio of the background-corrected fluorescence emissions (F490/F440) for each region was calibrated using the high K+/nigericin technique (8). pHi values were calculated for soma, Lg-Dendrites and Sm-Dendrites using the respective BCECF calibration values collected from each region.

For the pre-pulse treatment, cells were subjected to an acid load by a transient application (1.5 min) of a 30 mM NH₄⁺/NH₃ solution. NH₄⁺/NH₃ solutions were prepared by replacing 30 mM NaCl in the HEPES-buffered solution with an equimolar concentration of NH₄Cl. pHi recovery rates were determined from the slope of a fitted linear regression within the first min after NH₄⁺/NH₃ prepulse (8). To minimize differential allosteric effects of H⁺ on NHE-1 activity, pHi recovery rates were measured at pHi ~6.2 throughout the study. In the Na⁺-free experiments, NaCl in the HEPES-buffered solution was replaced with an equimolar concentration of NMDG. NMDG substituted Na⁺-free solutions (~5 min) do not cause cell swelling in acutely isolated CA1 neurons (12).

**Determination of intrinsic buffer power (β)** - βᵢ was determined in somata, Lg- and Sm-Dendrites over a range of pHᵢ by subjecting the cells to progressively decreasing concentrations of NH₄⁺ in Na⁺-free HEPES-MEM as previously described (13). The total H⁺ net efflux rate (JᵢH⁺, mM H⁺/min) was determined in three neuronal regions by multiplying βᵢ by ΔpHᵢ/Δt at pHᵢ ~6.2. In some experiments, JᵢH⁻ was also calculated in the presence of HCO₃⁻. The buffering by CO₂/HCO₃⁻ was determined as: βᵢHCO₃⁻ = 2.3 x [HCO₃⁻], where [HCO₃⁻]ᵢ = S x PCO₂ x 10^(pHi-pK) with S = 0.0314, PCO₂ = 40 mm Hg, and pK = 6.12. At pH 6.2, the contribution of βᵢHCO₃⁻ to total buffering in normoxic cells was ~ 6%.

**Intracellular Na⁺ measurement** - Intracellular Na⁺ concentration ([Na⁺]ᵢ) was measured with the fluorescent dye SBFI-AM as described previously with some modifications (14). Cultured neurons grown on coverslips were loaded with 30 μM SBFI-AM plus 0.02% pluronic acid during 45 min REOX following 2 h OGD. The coverslips were placed in the open-bath imaging chamber and superfused (1 ml/min) with HCO₃⁻-MEM at 37°C. Using the Nikon TE 300 inverted epifluorescence microscope and a 100X oil-immersion lens, neurons were excited at 345 nm and 385 nm and the emission fluorescence at 510 nm recorded. Regions of interest (1-3 cells/area) were drawn to determine SBFI fluorescence changes in soma, Lg-Dendrites, and Sm-Dendrites. The 345/385 ratios were analyzed with the MetaFluor image-processing software. Absolute [Na⁺]ᵢ was determined in normoxic controls or 45 min REOX-treated neurons.

**Intracellular Ca²⁺ measurement** - Neurons grown on coverslips were incubated with 5 μM fura-2 AM during 2 h OGD. Following OGD, the cells were placed in the open-bath imaging chamber and superfused (1ml/min) with HCO₃⁻-MEM at 37°C. Using the Nikon TE 300 inverted epifluorescence microscope and a 100X oil-immersion objective lens, neurons were excited every 5 min at 345 and 385 nm and the emission fluorescence at 510 nm recorded. Images were collected and analyzed with the MetaFluor image-processing software. At the end of each experiment, the cells were exposed to 1 mM MnCl₂ in Ca²⁺-free HCO₃⁻-MEM and 5 μM 4-bromo A-23187. The Ca²⁺-insensitive fluorescence was subtracted and the MnCl₂-corrected 345/385 emission ratios were converted to Ca²⁺ concentration ([Ca²⁺]) as described previously (14).

**Measurement of mitochondrial Ca²⁺** - Neurons on coverslips were incubated at 37°C for 60 min with
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200 nM MitoTracker green and 9 µM Rhod2-AM, which was reduced with a minimum of sodium borohydride in HCO3-MEM containing 3 mM sodium succinate and maintained for 2 h under either OGD or normoxic conditions. Coverslips were then placed in the perfusion chamber on the stage of the Leica DMIRE2 confocal microscope and superfused (1 ml/min) with HCO3-MEM at 37º C. Cells (1-3 in the field) were visualized with a 100x oil-immersion objective and scanned sequentially for MitoTracker green (ex. 488 nm argon laser line, em. 500-545 nm) and Rhod-2 (ex. 543 HeNe laser, em 544-677). The MitoTracker green signal was used to maintain focus prior to each sequential scan. Sequential scans were analyzed using the Leica confocal software. Average grayscale values were collected from regions of interest around mitochondrial clusters exhibiting colocalization of MitoTracker green and Rhod-2. Ca2+ levels were expressed as relative change of Rhod-2 signals from the baseline values and summarized data represent the average of the calculated values from 2-3 cells as described before (14).

Measurement of mitochondrial membrane potential ($\Psi_m$): The fluorescent probe JC-1 was used to monitor $\Psi_m$ as described previously (14). Neurons on coverslips were loaded with 9 µM JC-1 during 2 h OGD at 37º C. Following OGD, the cells were placed in the temperature controlled open-bath imaging chamber and superfused (1 ml/min) with HCO3-MEM at 37º C. Cells were visualized using the Nikon TE 300 inverted epifluorescence microscope and a 60x oil-immersion objective. Cells were excited at 480 nm and emission fluorescence images recorded at 535 nm (the monomer) and 640 nm (JC-1 aggregates). The ratio of the aggregate to monomer fluorescence was measured in regions of interest using the Leica DMIRE2 confocal microscope and software. Cells were excited at 480 nm using an open-path imaging chamber and superfluenced (1 ml/min) with HCO3-MEM at 37º C. Cells were visualized with a 100x oil-immersion objective and scanned sequentially for MitoTracker green (ex. 488 nm argon laser line, em. 500-545 nm) and Rhod-2 (ex. 543 HeNe laser, em 544-677). The MitoTracker green signal was used to maintain focus prior to each sequential scan. Sequential scans were analyzed using the Leica confocal software. Average grayscale values were collected from regions of interest around mitochondrial clusters exhibiting colocalization of MitoTracker green and Rhod-2. Ca2+ levels were expressed as relative change of Rhod-2 signals from the baseline values and summarized data represent the average of the calculated values from 2-3 cells as described before (14).

Determination of surface area to volume ratio in soma and dendrites: To determine differences in the ratio of surface area to volume in soma and dendrites, neurons grown on coverslips were loaded with 0.5 µM calcein-AM (cytosol dye) and 5 µM SYTO 60 (nucleus dye) for 30 min at 37º C. The coverslips were then placed in the perfusion chamber on the stage of the Leica DMIRE2 confocal microscope and visualized with a 100x oil-immersion objective. An 110 µm thick image stack (300 slices at 32x32 pixels) was collected. Each image slice was analyzed using Image J (Version 1.41, NIH). A cellular region (soma, nucleus, Lg- or Sm-Dendrites) was defined and the surface area measured. A cellular region (soma, nucleus, Lg- or Sm-Dendrites) was defined and the surface area measured.
was calculated by summing the product of the region perimeter by the distance between each image section (0.38 μm). The volume of the region was calculated with the region area and section distance. The soma volume was corrected by subtracting the calculated volume for the nucleus. No attempt was made to correct for the intracellular volumes of endoplasmic reticulum or mitochondria.

Detection of dendritic beading formation (varicosities)- To monitor dendritic beading formation, neurons grown on coverslips were loaded with the plasma membrane dye Vybrant® DiO as per manufacturer’s instructions. Following OGD, the coverslips were placed in the open-bath imaging chamber and superfused (1ml/min) with HCO₃⁻-MEM at 37°C on the stage of a Leica DMIRE2 confocal microscope. A single neuron was visualized with a 100x oil-immersion objective and scanned (512 x 512, 200 Hz) with an argon laser (ex. 488 nm, em. 500-545 nm). The images were analyzed for dendrite beading with Image J analysis software. Beads with a diameter ~ 4 x larger than the width of the corresponding dendrite were counted in a 90 μm x 90 μm area.

Data represent the average of the calculated values from 3-4 experiments.

Immunoblotting- Cells were washed with ice-cold PBS and lysed with 30 sec sonication at 4°C in anti-phosphatase buffer (pH 7.4) containing (mM): 145 NaCl, 1.8 NaH₂PO₄, 8.6 Na₂HPO₄, 100 NaF, 10 Na₂P₂O₇, 2 Na₃VO₄, 2 EDTA and 0.2 μM microcystin and protease inhibitors as described previously (11). Protein content was determined by the bicinchoninic acid method. Protein samples (40 μg/lane) and pre-stained molecular mass markers (Bio-Rad, Hercules, CA) were denatured in SDS 2X sample buffer and then electrophoretically separated on 8 % SDS gels. The resolved proteins were electrophoretically transferred to a PVDF membrane (14). The blots were incubated in 7.5% nonfat dry milk in tris-buffered saline (TBS) overnight at 4°C and then incubated for 1 h with polyclonal anti-NHE-1 (1:500), polyclonal anti-NHE-2 (1:500) (16), polyclonal anti-NHE-3 (1:1000, Alpha Diagnostic International), polyclonal anti-NHE-5 (1:1000). The blots were rinsed with TBS and incubated with horseradish peroxidase-conjugated secondary IgG for 1 h. Bound antibody was visualized using an enhanced chemiluminescence assay (Amersham Corp, Piscataway, NJ).

Immunofluorescence staining- Cells grown on coverslips were fixed in 4% paraformaldehyde in PBS for 15 min. After rinsing, cells were incubated with a blocking solution for 20 min followed by application of a primary polyclonal antibody for NHE-1 (1:50, Abcam Inc.). After rinsing in PBS, cells were incubated with Alexa Fluor™ 488 goat anti-rabbit IgG (1:200; Invitrogen) for 1 h. The coverslips were then covered with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Fluorescence images were captured by the Nikon TE 300 inverted epifluorescence microscope (40X) using a Princeton Instruments MicroMax CCD camera and MetaMorph image-processing software.

Statistics- Statistical significance was determined by student’s t-test or an ANOVA (Bonferroni post-hoc test) in the case of multiple comparisons. A P-value smaller than 0.05 was considered statistically significant. N values represent the number of cultures in each experiment.

RESULTS

Surface area to volume (A/V) ratio in soma and dendrites. In order to accurately calculate ionic flux rates in soma and dendrites, we first estimated surface area to volume (A/V) ratios in these cellular regions. Figure 1 A shows a single slice 2-D image of cultured neurons from a confocal stack image (300 slices, 110 μm thick). Arrows in Figure 1 A illustrate the areas in the somata, Lg- and Sm-Dendrite where the A/V ratios and ionic changes were determined. Figure 1 B is a 3-D reconstruction of the stack of images with Metamorph software, highlighting neuronal morphology with the distinctly higher A/V ratios in Lg- and Sm-Dendrites. The A/V ratio in Lg-Dendrites was 3.8 times larger than somata (Figure 1 C). An ~ 7 times larger A/V ratio was estimated for Sm-Dendrites. Interestingly, 2 h of OGD and 1 h REOX did not significantly change the A/V ratio either in soma or in dendrites.

We then determined buffer capacity (β), in each
region was plotted against pH and fit with a linear regression. The slopes of the lines in the three regions were not significantly different under normoxic control or OGD/REOX conditions. These findings imply that the changes of pH regulation may result from altered function of H+ transporters such as NHEs.

Changes of pH in soma and dendrites following OGD/REOX. Lg-or Sm-Dendrites exhibited more alkaline resting pH values than soma under normoxic conditions (Figure 2 A). Inhibition of NHE-1 with its potent inhibitor HOE 642 (1 µM) or the newly developed NHE-1 kinase p90sk inhibitor fluoromethylketone [FMK, IC$_{50}$ of 15 nM, (17)] acidified pH and significantly decreased pH recovery rates (Figure 2 A, B). OGD/REOX caused an alkalization of pH in soma (a shift from 6.96 ± 0.03 to 7.19 ± 0.05, p < 0.05). Inhibition of NHE-1 reversed the OGD/REOX-mediated increase in pH (Figure 2 C). Moreover, inhibition of the NHE-1 kinase p90sk with FMK prevented the post-OGD alkalization. OGD/REOX did not trigger additional changes in pH in dendrites. But, either HOE 642 or FMK significantly acidified dendrites following OGD/REOX. These data suggest that NHE-1 activation plays a role in resting pH maintenance and contributes to the intracellular post-OGD alkalization.

Increased H$^+$ efflux in soma and dendrites following OGD/REOX. We further determined NHE-1 activity in soma and dendrites by measuring pH recovery rate following the NH$_3$/NH$_4^+$ prepulse-induced acidification. As shown in Figure 3 A, when neurons were exposed to 30 mM NH$_3$/NH$_4^+$, pH in Lg-Dendrites rose rapidly as NH$_3$ diffused into the cell and combined with H$^+$ to form NH$_4^+$ (a-b) and then declined slowly (b-c). Returning cells to the standard HCO$_3^-$-free HEPES-MEM solution caused pH to decrease due to the rapid diffusion of NH$_3$ which was dissociated from the newly formed NH$_4^+$, and trapping H$^+$ inside the cells (c-d). Both normoxic control and OGD/REOX-treated cells were able to restore pH$_i$ to their basal levels (Figure 3 A). However, pH$_i$ recovery rate increased by ~2-fold in Lg-Dendrites following OGD/REOX (1.23 ± 0.24 unit/min vs. 0.57 ± 0.05 unit/min in normoxic neurons, p < 0.05).

pH$_i$ recovery rates were significantly higher in the Lg-Dendrites (90%) and in the Sm-Dendrites (330%) than the soma under normoxic conditions (Figure 3 B). The apparent higher pH$_i$ recovery rates in the dendrites could result from the larger A/V ratios in the dendrites. Thus, we corrected the pH$_i$ recovery rate for A/V ratio in the three different regions. After the correction, the rates were similar in all three regions under normoxic control conditions (Figure 3 C). 2 h OGD/1 h REOX triggered a further increase in the H$^+$ efflux in the soma (264%), the Lg-Dendrites (218%), and the Sm-Dendrites (69%, Figure 3 B). After the correction for the A/V ratio, the OGD/REOX-induced elevation in pH$_i$ recovery rates remained significant in soma, and Lg-Dendrites (Figure 3 C).

This finding was further validated by calculating $J_{H^+}$ (Figure 3 D). Dendrites exhibited smaller $J_{H^+}$ than soma under normoxia and OGD/REOX conditions. The OGD/REOX-mediated selective stimulation of $J_{H^+}$ persisted in soma and Lg-Dendrites, but not in Sm-Dendrites. Similar changes of $J_{H^+}$ were observed in the presence of HCO$_3^-$ (21 mM, Figure 3 D). The lack of changes in the A/V ratios and $\beta_i$ following OGD/REOX suggest that the OGD/REOX-induced stimulation of pH$_i$ recovery rates mainly reflect $J_{H^+}$.

Differential NHE-1 activity in soma and dendrites. We directly evaluated NHE-1-dependent pH$_i$ regulation activity in the soma and the dendrites using the NHE-1 inhibitor HOE 642 (1 µM, IC$_{50}$ of 0.08 µM), at a concentration that inhibits only the NHE-1 isoform (18). As shown in Figure 4 A, the OGD/REOX-mediated elevation of H$^+$ extrusion rate in the somata was nearly abolished in the presence of HOE 642. However, HOE 642 only partially blocked the elevated pH$_i$, recovery rate in the dendrites (~50-60%). To determine the possible role of other isoforms of NHE in neuronal processes, we examined the effects of removing extracellular Na$^+$, which inhibits the function of all NHE isoforms by abolishing the inward Na$^+$ driving force. In the absence of extracellular Na$^+$, H$^+$ extrusion was absent in soma, similar to the NHE-1 inhibition via HOE 642 (Figure 4 A). In the dendrites, pH$_i$, recovery rate was eliminated by ~76-86%. Inhibiting all NHE isoforms with a general NHE inhibitor EIPA (100 µM) had a
similar effect as removing extracellular Na⁺. Moreover, the residual Na⁺-independent H⁺ extrusion in the Sm-Dendrites could be mediated by vacuolar H⁺-ATPase. Inhibition of vacuolar H⁺-ATPase with a specific inhibitor bafilomycin (1 µM) abolished the residual H⁺ extrusion in both the Lg- and the Sm-Dendrites (Figure 4 A). These data imply that NHE-1 is the dominant isoform in soma. However, in the dendrites, pHᵢ regulation is governed by NHE-1 as well as other NHE isoforms and H⁺-ATPases.

The HOE-642 sensitive portion of H⁺ extrusion rate was obtained under normoxic control and OGD/REOX conditions (Figure 4 B). Consistently, NHE-1 activity (HOE-642 sensitive portion) in the soma and the Lg-Dendrites was significantly elevated following OGD/REOX. Sm-Dendrites exhibited a significantly higher basal activity of NHE-1 and OGD/REOX did not cause additional activation. Figure 4 C demonstrates that NHE-1 is the dominant form in cortical neurons, while NHE-3 isoform is restricted to cerebellum (19). Localization of NHE-1 protein in soma, Lg- and Sm-Dendrites was also shown in Figure 4 D.

NHE-1-mediated Na⁺ entry in soma and dendrites following OGD/REOX. The robust NHE-1 activation in the soma and the dendrites following REOX led us to speculate that NHE-1 plays a role in the dendritic Na⁺ᵢ dysregulation following in vitro ischemia. [Na⁺ᵢ] in the soma and the dendrites were monitored under normoxic controls and at 45 min REOX. There were no significant differences in the baseline [Na⁺ᵢ] between the soma and the dendrites (Figure 5 A, arrowhead). 45 min REOX following 2 h OGD led to an increase in [Na⁺ᵢ] throughout the neuron. Localized increases in [Na⁺ᵢ] were detected in the dendrites (Figure 5 A, arrow). Summary data show that [Na⁺ᵢ], increased from a resting level of 12.5 ± 0.3 to 44.4 ± 2.6 mM in the soma (p < 0.05, Figure 5 B). The Sm-Dendrites exhibited the largest Na⁺ᵢ accumulation (56.5 ± 4.1 mM, p < 0.05). Inhibition of NHE-1 with HOE 642 during REOX abolished the OGD/REOX-induced Na⁺ᵢ overload in both the soma and the dendrites. The rise in HOE 642-sensitive changes in [Na⁺ᵢ], was shown in Figure 5 C. These data implies that OGD/REOX-mediated accumulation of [Na⁺ᵢ] is largely mediated via NHE-1 activation.

We recently reported that stimulation of NHE-1 depends on activation of the ERK-p90RSK signal transduction pathways and phosphorylation of NHE-1(8). In the current study, we examined whether direct inhibition of the NHE-1 kinase p90RSK with its potent inhibitor BI-D1870 [BI-D, IC₅₀ of 10-30 nM, (20)] could reduce OGD/REOX-mediated NHE-1 activation. The OGD/REOX-induced Na⁺ᵢ loading in Lg- and Sm-Dendrites was abolished by BI-D1870 (Figure 5 B). This effect is similar to the one mediated by NHE-1 inhibitor HOE 642. Thus, p90RSK function is largely responsible for NHE-1 activation and Na⁺ᵢ entry. These data also imply that the initial increased intracellular acidification associated with OGD (8) is not sufficient to drive NHE-1 activity, but requires altered phosphorylation of the transporter.

We further compared the effects of BI-D along with FMK on inhibition of NHE-1 activation. Both BI-D and FMK abolished the OGD/REOX-mediated stimulation of pHᵢ recovery in the soma and the dendrites (Figure 5 D). Especially in the Sm-Dendrites, BI-D or FMK profoundly suppressed the pHᵢ recovery rate, which was only ~ 25% of the normoxic basal levels (p < 0.05, Figure 5 D). These data suggest that p90RSK pathways play a more dominant role in pHᵢ regulation in the dendrites than in the soma.

Lastly, the pHᵢ recovery rates in all three regions were determined in the presence of 21 mM bicarbonate under normoxic and OGD/REOX conditions (Figure 5 E). The results are similar to ones in the absence of physiological bicarbonate. These data suggest that the role for bicarbonate-dependent ion transporters in regulation of pHᵢ is negligible under these conditions.

To further test NHE-1-mediated Na⁺ overload, we examined whether there was a differential Na⁺ᵢ overload in soma and Sm-Dendrites when Na⁺/K⁺-ATPase was blocked by ouabain (0.1 mM) during REOX. As shown in Suppl. Figure 1 A, Na⁺ᵢ clearly loaded faster in Sm-Dendrites than in soma under these conditions. Moreover, at 30 min REOX, blocking NHE-1 activity decreased the Na⁺ᵢ influx in Sm-Dendrites (Suppl. Figure 1 B). A similar trend was also observed in Lg-Dendrites (Suppl. Table-1).
Delayed dysregulation of Ca\(^{2+}\) depends on a concurrent activation of NHE-1 and NCX\(_{\text{rev}}\). One consequence of Na\(^+\) overload is to trigger NCX\(_{\text{rev}}\) and Ca\(^{2+}\) entry. To investigate the possible concerted activation of NHE-1 and NCX\(_{\text{rev}}\), we first monitored changes of local [Ca\(^{2+}\)]\(_{\text{i}}\), following REOX. The somata [Ca\(^{2+}\)]\(_{\text{i}}\) was 68 ± 9 nM under normoxic control conditions and increased modestly to 104 ± 8 nM after 2 h OGD (p < 0.05), which remained unchanged over the initial 35 min REOX (Figure 6 A, B). In contrast, 2 h OGD triggered a secondary rise in the dendritic [Ca\(^{2+}\)]\(_{\text{i}}\), (157 ± 21 nM, p < 0.05). Twenty min of REOX triggered a secondary rise in the dendritic [Ca\(^{2+}\)], that initiated from local “hot spots” and then spread toward the soma over time (Figure 6 A, C). The amplitude of the dendritic Ca\(^{2+}\) dysregulation was significantly higher than the soma. By 45 min REOX, dendritic [Ca\(^{2+}\)]\(_{\text{i}}\) rose dramatically to 1206 ± 440 nM and spread to the soma which exhibited slightly lower levels (766 ± 300 nM). Sustained elevation in [Ca\(^{2+}\)]\(_{\text{i}}\) during REOX (60-100 min) resulted in cell death as reflected by a sudden loss of the dye (data not shown).

We speculated that NCX\(_{\text{rev}}\) contributes to this Ca\(^{2+}\) dysregulation as a result of the robust NHE-1 activation and Na\(^+\) overload. First, we investigated whether inhibition of NHE-1 activity with HOE 642 could block the delayed rise in [Ca\(^{2+}\)]. As shown in Figure 6 (B, C), when HOE 642 was present only during 60 min REOX, no secondary rise in [Ca\(^{2+}\)]\(_{\text{i}}\) occurred in the soma and the dendrites following REOX. These data imply that NHE-1 activation is involved in the secondary loss of Ca\(^{2+}\) homeostasis during REOX. To establish whether this Ca\(^{2+}\) rise results from activation of NCX\(_{\text{rev}}\), we conducted the experiments in the presence of SEA0400 (1 µM), a potent inhibitor of NCX\(_{\text{rev}}\). As shown in Figure 6 B and C, REOX failed to elicit the secondary elevation in [Ca\(^{2+}\)]\(_{\text{i}}\) in both the soma and the dendrites. The results were similar to those of the HOE 642-treated cells. This led us to conclude that concerted activation of NHE-1 and NCX\(_{\text{rev}}\) contributed to the delayed Ca\(^{2+}\) dysregulation in soma and dendrites following in vitro ischemia. However, these inhibitors did not affect basal levels of [Na\(^+\)]\(_{\text{i}}\) and [Ca\(^{2+}\)]\(_{\text{i}}\) under normoxic control conditions (Suppl. Table-2).

Changes of Ca\(^{2+}\)\(_{\text{m}}\) and \(\Psi_{\text{m}}\) in soma and dendrites following OGD/REOX. Excessive dendritic Na\(^+\)\(_{\text{i}}\) and Ca\(^{2+}\)\(_{\text{i}}\) overload will affect mitochondrial Ca\(^{2+}\) homeostasis and mitochondrial function. To investigate this, we first monitored changes of Ca\(^{2+}\)\(_{\text{m}}\) in the soma and the dendrites during 0-60 min REOX. There was a slow and progressive elevation in the somata Ca\(^{2+}\)\(_{\text{m}}\) starting by 10 min REOX and reaching a plateau value (~ 2.5-fold of control) by 40 min REOX (Figure 7 A). Interestingly, the somata \(\Psi_{\text{m}}\) did not decrease significantly while Ca\(^{2+}\)\(_{\text{m}}\) was increasing during early REOX. But, \(\Psi_{\text{m}}\) depolarized significantly after Ca\(^{2+}\)\(_{\text{m}}\) reached its plateau levels and it was reduced to 47 ± 6 % of control at 60 min REOX (Figure 7 A).

By 10 min of REOX, Ca\(^{2+}\)\(_{\text{m}}\) levels had increased significantly in the Sm-Dendrites but not in the Lg-dendrites (Figure 7 B, C). The rate of Ca\(^{2+}\)\(_{\text{m}}\) increase in the Sm-Dendrites during early REOX was significantly faster than in the soma (0.018 vs. 0.003 relative change/min, p < 0.05). At 60 min REOX, Ca\(^{2+}\)\(_{\text{m}}\) was increased by ~4.5-fold in the Sm-Dendrites and ~3.2-fold in the Lg-Dendrites. Ca\(^{2+}\) accumulation in the mitochondria remained elevated throughout the neuron until ~100 min, when a sudden loss in the rhod-2 dye signal occurred, likely due to a collapse of mitochondrial function (data not shown).

The earlier rise in Ca\(^{2+}\)\(_{\text{m}}\) in the dendrites was accompanied with a faster decrease of \(\Psi_{\text{m}}\) (Figure 7 B and C). \(\Psi_{\text{m}}\) in the dendrites (particularly in the Sm-Dendrites) depolarized at a rate twice that of the somata (1.2 vs. 0.7 %/min). \(\Psi_{\text{m}}\) in the Sm-Dendrites dropped to the lowest level (17 ± 4 % of control) by 40 min REOX. The kinetics of the \(\Psi_{\text{m}}\) collapse in the dendrites exhibited a significant negative correlation with the dendritic Ca\(^{2+}\)\(_{\text{m}}\) accumulation (Pearson product moment correlation coefficient = -0.964, p < 0.001). Thus, compared to the soma, the dendrites show two characteristics: earlier onset time and larger magnitude in the loss of mitochondrial Ca\(^{2+}\) homeostasis and \(\Psi_{\text{m}}\). This demonstrates that the dendritic mitochondria are more sensitive to OGD/REOX damage than the soma. These changes are consistent with the earlier loss of Na\(^+\) and Ca\(^{2+}\) homeostasis in the dendrites.
Interestingly, inhibition of NHE-1 activity with 1 μM HOE 642 prevented the REOX-mediated changes of Ca^{2+} and Ψ_m in soma. In the presence of 1 μM HOE 642, there were no significant increases in Ca^{2+} in the Lg-Dendrites and the somata. A slow accumulation in Ca^{2+} was detected in the Sm-Dendrites, which was not statistically significant from 0 min REOX. Moreover, the delayed depolarization of the somata Ψ_m during 50-60 min REOX was absent with the HOE 642 treatment (Figure 7 A). Strong protective effects of HOE 642 on Ψ_m were also found in the Lg- and the Sm-Dendrites. The Sm-Dendritic Ψ_m depolarized to ~ 44% of control (instead of 17% of control) at 60 min REOX when NHE-1 activity was inhibited (p < 0.05). Thus, preservation of Ψ_m by NHE-1 inhibition may result from decreased mitochondrial Ca^{2+} loading.

To determine the role of NHE-1 in mitochondrial dysfunction following OGD/REOX, we examined whether FMK (3 μM) would prevent mitochondrial damage in the soma and the dendrites. As shown in Figure 8 A, B, inhibition of the NHE kinase p90RSK during REOX attenuated loss of Ψ_m following OGD/REOX in the soma and the Lg-Dendrites. In the Sm-Dendrites, FMK effectively prevented depolarization of Ψ_m as early as 10 min of REOX (Figure 8 C). Interestingly, these effects were similar to the direct inhibition of NHE-1 mediated by HOE 642. Taken together, we can firmly conclude that either blocking NHE-1 or p90RSK significantly preserves mitochondrial function in ischemic neurons.

In a parallel study, in order to confirm reliability of the Ψ_m determination with JC-1, we also used the cationic dye TMRE to monitor changes of Ψ_m at 0 min and 60 min REOX (Suppl. Figure 2 A, B). Both JC-1 and TMRE measurements indicated that the REOX-induced decrease in Ψ_m was more profound in the Lg- and the Sm-Dendrites. Additionally, inhibition of NHE-1 with HOE 642 during REOX significantly attenuated the OGD/REOX-induced decrease in Ψ_m in all areas of the cells (p < 0.05, Suppl. Figure 2 B).

**Role of mitochondrial uniporter in mitochondrial Ca^{2+} accumulation.** To investigate the role of the uniporter in mitochondrial Ca^{2+} loading following OGD/REOX, we determined changes of Ca^{2+} when the mitochondrial uniporter was inhibited with 10 μM RU360 during 60 min REOX. Inhibition of the uniporter prevented accumulation of Ca^{2+} in the mitochondria in all regions of the neuron (Figure 9).

**Dendritic damage is reduced by inhibition of NHE-1 following OGD/REOX.** We further investigated dendritic damage by monitoring the dendritic beading (varicosities) and membrane integrity changes following OGD/REOX. As shown in Figure 10 A and B, 2 h OGD caused some swelling in the dendrites without formations of varicosities (arrow). Varicosities developed in the dendrites over 30-60 min REOX (arrowhead, Figure 10 C). At 60 min REOX, the bead density increased by > 13 times compared to the normoxic neurons (Figure 10 C, E). In contrast, in the presence of 1 μM HOE 642 during REOX, the bead formation increased only by ~ 3 times (arrow, Figure 10 D, E, p < 0.05). These data suggest that inhibition of NHE-1 activity with HOE 642 not only reduces the dendritic ionic dysregulation but also decreases dendritic swelling following OGD/REOX.

**DISCUSSION**

**Robust NHE-1 activity in dendrites.** In the current study, we characterized NHE-1 activity in the soma, the Lg- and the Sm-Dendrites under normoxic and OGD/REOX conditions. We observed that pH_i regulation in the Lg- and the Sm-Dendrites was ~ 90% - 300% faster than in the soma. The differential pH_i regulation rates between the dendrites and the soma were abolished when they were corrected by the differences in the A/V ratios. Therefore, the data illustrate that the dendrites can change pH_i more rapidly than the soma due to the small cytosolic volume compared to its surface area. Robust NHE activity has previously been detected in the hippocampal nerve terminals following intracellular acidification under normoxic conditions (21). In our study, following OGD/REOX, the Na^+-dependent H^+ extrusion activity was further elevated in the soma (264%), and the Lg-Dendrites (218%) while the A/V ratios remained unchanged.
The H⁺ extrusion mechanisms in the soma and the dendrites have not been well defined. In this study, we concluded that the somata pHᵢ regulation under HCO₃⁻ free conditions is exclusively mediated by NHE-1 activity, which is consistent with our previous findings using both HOE 642-mediated pharmacological inhibition and NHE-1 genetic knockout approaches (11). Moreover, the significance of NHE-1 in neuronal ionic regulation is further highlighted by the abundant expression of NHE-1 compared to NHE-2, NHE-3, or NHE-5 in the neurons. This is consistent with the earlier reports on the preeminence of both NHE-1 mRNA and protein expression in brains over the isoforms NHE 2-4 and the abundance of NHE-3 in the cerebellum (19;22).

We report here that NHE-1 plays a dominant role in the regulation of dendritic pHᵢ (60%). The remaining pHᵢ regulation in the dendrites depended on the functions of the less abundant NHE isoforms (NHE-2, NHE-5) and vacuolar H⁺-ATPases. The H⁺ pumps are highly expressed in the vesicles of synaptic terminals and responsible for acid loading and accumulation of neurotransmitters (23). Although the H⁺ pumps are typically expressed in membranes of organelles, they have been detected in the plasma membrane of hippocampal astrocytes and are active in pHᵢ regulation under Na⁺- and HCO₃⁻-free conditions (24;25). Our findings of H⁺ pump activity in the dendritic plasma membrane suggest that dendrites are equipped with multiple H⁺ extrusion mechanisms to counteract the robust Ca²⁺-dependent intracellular acidification during synchronous neural activity (26).

Recently, spatial nonuniformity in pHᵢ has been reported in the proximal and distal dendrites of oligodendrocytes (27). The alkaline microdomains in the perikaryon and proximal dendrites of the oligodendrocytes were attributed to localized increases in NHE activity, while the acidic pHᵢ in the distal dendrites may be the result of Na⁺/HCO₃⁻ cotransporter-mediated HCO₃⁻ extrusion (27). Thus, the pHᵢ microdomain and regulatory mechanisms in the oligodendrocyte distal dendrites appear to be different from those in the Sm-Dendrites of neurons. These findings suggest that different cell types express different pHᵢ regulating mechanisms in regulating microdomain pHᵢ.

**Lack of NHE-1 activation in Sm-Dendrites following OGD/REOX.** The basal level of NHE-1-mediated H⁺ extrusion was high in the Sm-Dendrites compared to soma. OGD/REOX did not further stimulate it. This suggests that, given their large A/V ratio and their higher basal JᵢH⁺, Sm-Dendrites are able to maintain pHᵢ without significant further elevation of NHE activity. This may also be a reflection of the sophisticated pHᵢ regulatory mechanisms expressed in Sm-Dendrites, preventing over-stimulation of H⁺ extrusion. On the other hand, this Sm-Dendritic JᵢH⁺ phenomenon may be unique under the circumstances of culture models in plastic plates, but not characteristic of dendrites under in vivo conditions. Indeed, we have observed much higher A/V ratios and faster pHᵢ recovery rates in neurites grown in microfluidic devices, a model that mimics the slow diffusion and convection of the in vivo microenvironment (28). It remains to be investigated whether OGD/REOX affects NHE-1 function differently in the Sm-Dendrites using the microfluidic device model.

**p90RSK-mediated stimulation of NHE-1 activity following OGD/REOX.** In the current study, OGD/REOX-mediated stimulation of NHE-1 activity was abolished in both the soma and the dendrites when NHE-1 kinase p90RSK was inhibited by its potent inhibitors BI-D1870 and FMK. Activation of p90RSK and NHE-1 phosphorylation is downstream of the ERK1/2 signaling pathways (8). FMK is a novel, specific inhibitor for p90RSK isoform 1 and 2 (17). FMK blocks the α₁-adrenoceptor-mediated NHE-1 phosphorylation and stimulation in rat ventricular myocytes (29). On the other hand, BI-D1870 has been shown to be a potent ATP-competitive inhibitor of all p90RSK isoforms (20). In this study, we found that FMK and BI-D1870 were equally effective in inhibiting OGD/REOX-mediated NHE-1 activation, implying a role for p90RSK isoforms 1 and 2. Moreover, the p90RSK inhibitors reduced the NHE-1 activity to below the baseline levels. This suggests that p90RSK is also involved in the regulation of basal NHE-1 activity in all three regions.
**NHE-1-mediated Na⁺ entry following OGD/REOX.** Disruption of dendritic ionic homeostasis occurs during early cerebral ischemia and may play a role in irreversible dendritic damage. Excessive Na⁺ influx via ionotropic glutamate receptors or TTX-sensitive Na⁺ channels leads to neuronal death under excitotoxic or hypoxic conditions (4;30;31). However, subsequent studies have suggested that hypoxia-induced Na⁺ influx could be through pathways other than ionotropic glutamate receptors or TTX-sensitive channels (32). In cultured hippocampal neurons, Na⁺ entry immediately after anoxia results from activation of NHE and a Gd³⁺-sensitive pathway (33). Recent reports demonstrate that dendritic damage following brief *in vivo* ischemia (34) or axonal morphological changes following *in vitro* hypoxia (35) are independent of ionotropic glutamate receptor activation. In the present study, we observed that OGD/REOX triggered ~3-fold increase in [Na⁺]i (~50 mM). The Na⁺ accumulation was eliminated when NHE-1 activity was inhibited by its inhibitor HOE 642 or the p90RSK kinase inhibitor BI-D1870. Thus, the elevated NHE activity in the dendrites not only accelerate pHi recovery after OGD/REOX but also intensify disruption of Na⁺ ionic homeostasis and cause dendritic vulnerability to ischemic damage. These findings also suggest that Na⁺/K⁺-ATPase function is not sufficient to maintain Na⁺ homeostasis following OGD/REOX when there is an increase in Na⁺ influx. Blocking NHE-1 activity would decrease the need for Na⁺ extrusion via Na⁺/K⁺-ATPase and preserve cellular ATP levels (Figure 11). This imbalance between Na⁺ extrusion via Na⁺/K⁺-ATPase and NHE-1-mediated Na⁺ influx can also have a significant impact on [Na⁺]i in Sm-Dendrites, particularly because of their large A/V ratio and high basal J₀i, even without further elevation of NHE-1 activity following OGD/REOX. Taken together, we conclude that the OGD/REOX-mediated stimulation of NHE-1 plays a dominant role in dendritic Na⁺ overload.

We failed to detect elevation of NHE-1-mediated H⁺ extrusion in the Sm-Dendrites after OGD/REOX. The causes for the discrepancy between the NHE-1-mediated H⁺ extrusion and Na⁺ overload in the Sm-Dendrites are not apparent. One possible explanation is that a subtle increase in NHE-1 activity may not be detected with the instantaneous measurement of H⁺ extrusion (dpH/dt), while its impact on Na⁺ overload over time (at a steady-state level) can be revealed. This speculation is also supported by HOE 642-sensitive effects on mitochondrial dysfunction and Ca²⁺ dysregulation. Future study is needed to further address this issue.

**NHE-1-dependent changes in dendritic Ca²⁺ following OGD/REOX.** Activation of NHE activity in hippocampal nerve terminals following intracellular acidification is accompanied with an elevation in [Na⁺]i and [Ca²⁺]i, as well as increased postsynaptic currents (21). The authors attribute these changes to the concurrent activation of NHE and NCXrev in the nerve terminals (21). These findings suggest NHE and NCXrev could play a coordinating role in the regulation of dendritic Na⁺ and Ca²⁺ homeostasis and affect Ca²⁺-dependent release of neurotransmitters. In dendrites and dendritic spines close to postsynaptic localities, all three isoforms of NCX (NCX1-3) are preferentially expressed, suggesting a role for NCX in Ca²⁺ signaling at the excitatory postsynaptic sites (36).

In this study, 2 h OGD triggered a moderate elevation in dendritic Ca²⁺ but, during 60 min REOX, a delayed accelerated Ca²⁺ rise occurred. The OGD/REOX-induced Ca²⁺ deregulation was initiated in the dendrites and then propagated to the soma. Interestingly, the secondary Ca²⁺ deregulation can be prevented when either NHE-1 activity was inhibited by HOE 642 or NCXrev was blocked by SEA0400. These findings imply that a coupled NHE-1 and NCXrev function is a major contributor to Ca²⁺ deregulation in the dendrites of cultured cortical neurons. Moreover, we believe that NCX-1 is the dominant NCX isoform in this study because SEA0400 has a high affinity against the reverse mode function of NCX-1 (IC50 ~ 50 nM) as compared to NCX-2 (IC50 ~ 1 μM) and is ineffective against either NCX-3 or NCKX-2 (37).

The role of NCXrev in dendritic Ca²⁺ deregulation has also been examined during sustained NMDA exposure. Delayed Ca²⁺ deregulation in CA1 neurons of acute hippocampal slices depended on Na⁺ loading, but was not prevented by the non-specific NCX inhibitor KB-R7943 (38). However, when Na⁺ loading was potentiated with low levels of ouabain (30 μM),
NMDA can trigger secondary Ca\(^{2+}\) deregulation in dendrites which is completely blocked by KB-R7943 (39). These findings further suggest that activation of NCX\(_{\text{rev}}\) requires excessive Na\(^+\) loading. Our previous thermodynamic analysis predicts that NCX\(_{\text{rev}}\) occurs when [Na\(^+\)]\(_i\) is elevated to ~ 20 mM in cortical neurons at a resting membrane potential of -60 mV (40).

The vulnerability of neuronal dendrites is characterized by the initial membrane depolarization, mitochondrial structure collapse, and dendritic beading in the dendrites and the subsequent propagation towards the soma during hypoxia and activation of NMDA receptors (1, 6). Our current study illustrates that blocking of NHE-1 activity attenuated many similar changes in the dendrites following OGD/REOX. Therefore, concerted activation of NHE-1 and NCX\(_{\text{rev}}\) may also play a role in dendritic injury in conditions such as glutamate-mediated neurotoxicity, epilepsy, etc.

Changes of dendritic $\Psi_m$ and Ca\(^{2+}\)\(_m\) following OGD/REOX. In the current study, depolarization of $\Psi_m$ in the Sm-Dendrites occurred 40 min earlier than the soma following OGD/REOX. The loss of $\Psi_m$ in the dendrites closely correlated with the Ca\(^{2+}\)\(_m\) accumulation. Mitochondria are capable of sequestering large amounts of Ca\(^{2+}\) under various pathological conditions (41). Increases in free mitochondrial Ca\(^{2+}\) would occur when Ca\(^{2+}\) entry into the mitochondria exceeds the capacity of mitochondrial Ca\(^{2+}\) extrusion and the mitochondrial robust phosphate buffering system (42). The Ca\(^{2+}\)\(_m\) accumulation reported in this study likely reflects Ca\(^{2+}\) entry via a voltage-dependent Ca\(^{2+}\) uniporter before the collapse of $\Psi_m$ (41). Small increases in Ca\(^{2+}\)\(_m\) stimulate Ca\(^{2+}\)-dependent dehydrogenases and mitochondrial metabolism, but massive Ca\(^{2+}\) loading of mitochondria leads to depolarization of $\Psi_m$ (6;42). Sustained loss of $\Psi_m$ will eventually trigger the opening of the permeability transition pore (MPTP) and release of Ca\(^{2+}\)\(_m\) and collapse of mitochondria bioenergetics (6). In the current study, most of the Ca\(^{2+}\)\(_m\) accumulation occurred before $\Psi_m$ decreased below 50% and remained at a sustained level when $\Psi_m$ was ~45% of control in the soma and ~ 20% of control in the Sm-Dendrites. This implies that a residual level of $\Psi_m$ (20%) for a short period (~ 40 min) is sufficient to maintain high mitochondrial Ca\(^{2+}\) levels. But, when low $\Psi_m$ and high Ca\(^{2+}\)\(_m\) were extended past 60 min, there was a sudden loss of residual $\Psi_m$ that coincided with the loss of the rhod-2 signal, suggesting a release of Ca\(^{2+}\)\(_m\) from the permeability transition pore under these conditions.

Interestingly, when either NHE-1, p90\(_{\text{RSK}}\), or NCX\(_{\text{rev}}\) was inhibited during 60 min REOX, loss of $\Psi_m$ and Ca\(^{2+}\)\(_m\) accumulation in dendrites was significantly reduced. This finding is consistent with the earlier reports on NHE-1 inhibition-induced attenuation of the mitochondrial Ca\(^{2+}\) overload and MPTP opening in cardiomyocytes and in ischemic/reperfused rat hearts (43-45). Taken together, these studies demonstrate a conserved role of the NHE-1 signaling mechanism in ischemic reperfusion injury among multiple cell types. Moreover, it has been suggested that NHE-1 inhibitors including HOE 642 have a direct effect on ROS production and MPTP formation (46). In the current study, it is unknown whether any protective effects mediated by HOE 642 results from its direct actions on mitochondria. We speculate that such a possibility is low in light of the similar protective effects offered by inhibition of p90\(_{\text{RSK}}\) or NCX\(_{\text{rev}}\) in this study as well as the protection observed in NHE-1 transgenic knockout neurons (11).

Moreover, in general, mild acidosis can inhibit neurotransmission while alkaline pH\(_i\) stimulates excitability. Thus, we cannot rule out that HOE 642 may protect neurons in part via directly correcting NHE-1 mediated alkalinization.

In summary (Figure 11), the current study reports that NHE-1-mediated Na\(^+\) entry and subsequent stimulation of NCX\(_{\text{rev}}\) activity contribute to the selective vulnerability of dendrites following in vitro ischemia. A newly emerging hypothesis speculates that dendritic Na\(^+\) overload, and the subsequent activation of Na\(^+\)/K\(^+\)-ATPase would consume more ATP and further collapse mitochondrial biogenesis (6). However, to date, the mechanisms underlying the excessive Na\(^+\) influx and mitochondrial dysfunction are not well defined. Our current study demonstrates that activation of NHE-1 in dendrites presents a major pathway for Na\(^+\) overload. NHE-1 inhibition prevents Na\(^+\) accumulation which is required for dendritic beading. Blocking NHE-1 function also
attenuates loss of the dendritic $\Psi_m$ and $\text{Ca}^{2+}_m$ homeostasis and preserves mitochondrial bioenergetics and dendritic membrane integrity.

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**FOOTNOTES**

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**FIGURE LEGENDS**

**Figure 1.** Surface area to volume ratios in soma and dendrites. **A**. A single 2-D confocal image taken from a 300-slice image stack (110 μm thick, 512 by 512 pixels) of a neuron loaded with calcein-AM. Soma, large (Lg-), and small (Sm)-Dendrites were indicated (arrows). **B**. The 300-slice image stack was rendered into a 3-D image using Metamorph software to illustrate surface area to volume (A/V) ratios determined in soma, Lg- and Sm-Dendrites (arrowheads). **C**. A/V ratios were calculated in 3 regions of normoxic control neurons or neurons subjected to 2 h OGD and 1 h REOX. Data are mean ± SEM. n = 4. * p < 0.05 vs. soma. **D**. Intrinsic buffer capacity (β) was determined in three regions under normoxia and following 2 h OGD/1 h REOX. Data are mean ± SEM. n = 4. * p < 0.05 vs. normoxic (p = 0.97) or OGD/REOX (p = 0.474) conditions.
Figure 2. A. Resting pH in the soma, Lg- and Sm-Dendrites under normoxic conditions. In the drug treatment experiments, neurons were exposed to either HOE 642 (1 µM) or FMK (3 µM) for 30 min prior to the pH determination. B. pH regulation in the soma, Lg- and Sm-Dendrites under normoxic conditions. Data are mean ± SEM, n = 3-4. * p < 0.05 vs. corresponding normoxia; # p < 0.05 vs. corresponding soma. C. Changes of pH at 60 min REOX following 2 h OGD. In the drug treatment experiments, either HOE 642 (1 µM) or FMK (3 µM) was present only during the 60-min REOX. Data are mean ± SEM, n = 3-4. * p < 0.05 vs. corresponding normoxia; # p < 0.05 vs. OGD/REOX.

Figure 3. Increased H^+ efflux rate in somata and dendrites following OGD/REOX. A. Representative pH changes in Lg-Dendrites subjected to NH4^+/NH3 prepulse-mediated acid-loading. pH_i recovery rate was determined by fitting a slope to the pH_i values within the first minute following the prepulse in either normoxic or 2 h OGD/1 h REOX-treated neurons. pH_i recovery rates were determined at ~ 6.2 to normalize for the allosteric regulation of H^+ on NHE-1 activity. B. Summary data of pH_i recovery rates under normoxic and OGD/REOX conditions. Data are mean ± SEM. n = 3-4. * p < 0.05 vs. corresponding normoxia. # p < 0.05 vs. corresponding soma. C. pH_i recovery rates were corrected for the relative differences in surface area to volume ratios in three regions. Data are mean ± SEM. n = 3-4. * p < 0.05 vs. corresponding normoxia. # p < 0.05 vs. corresponding soma. D. Proton flux (J^+H) was calculated at ~pH 6.2 during pH recovery following NH4^+/NH3 prepulse. Data are mean ± SEM. n = 3-4. * p < 0.05 vs. corresponding normoxia. # p < 0.05 vs. corresponding soma.

Figure 4. Differential NHE-1 activity in soma and dendrites. A. 2 h OGD/1 h REOX-treated neurons exhibited different pH_i recovery rates following the NH4^+/NH3 prepulse. To inhibit NHE-1 activity, 1 µM HOE 642 was present during 60 min REOX. In some pre-pulse studies, Na^+-dependent H^+ extrusion was blocked by replacing NaCl in the HEPES-buffered solution with an equimolar concentration of NMDG. Na^+-free HEPES-MEM, either with or without 1 µM bafilomycin, was used to evaluate function of other NHE isoforms and vacuolar H^+-ATPases. Inhibition of all NHE isoforms was examined with the general NHE inhibitor EIPA (100 µM). Data are mean ± SEM. n = 3-5. * p < 0.05 vs. soma under OGD/REOX. # p < 0.05 vs. corresponding OGD/REOX. B. HOE 642-sensitive pH_i regulation. OGD/REOX data are from the experiments in Figure 3 B. Data are mean ± SEM. n = 3-5. * p < 0.05 vs. soma under normoxia. # p < 0.05 vs. corresponding normoxia. C. NHE-1, NHE-2, NHE-3, or NHE-5 protein expression in cultured cortical neurons. Cerebellar tissue was used as positive control for NHE-3 and the same blot probed for β-tubulin as a loading control. D. Expression of NHE-1 protein in soma (arrow), Lg-Dendrite (arrowhead), and Sm-Dendrite (open arrowhead). Negative control: primary antibody was omitted. Scale bar: 10 µm.

Figure 5. NHE-1-mediated Na^+ entry in soma and dendrites following OGD/REOX. A. Representative SBFI pseudocolored images of changes in [Na^+]i in normoxic and OGD/REOX-treated neurons. arrowhead: low levels of [Na^+]i; arrow: localized increases in [Na^+]i. B. Summary data of [Na^+]i in soma, Lg- and Sm-Dendrites of neurons under normoxic conditions and 2 h OGD/45 min REOX. Data are mean ± SEM. n = 3-5, * p < 0.05 vs. normoxia; # p < 0.05 vs. OGD/REOX; α p < 0.05 vs soma OGD/REOX. C. HOE 642-sensitive change in [Na^+]i. Data are mean ± SEM. n = 3-5. * p < 0.05 vs. corresponding normoxia. # p < 0.05 vs. corresponding normoxia. D. pH_i recovery rates were determined. Normoxia and OGD/REOX data are from the experiments in Figure 3B. BI-D1870 (1 µM), FMK (3 µM) or HOE 642 (1 µM) was present only during REOX. Data are mean ± SEM. n = 3, * p < 0.05 vs. normoxia; # p < 0.05 vs. OGD/REOX; α p < 0.05 vs soma OGD/REOX. E. pH_i recovery rates were determined in the presence of 21 mM HCO3^-. HOE 642 (1 µM) was present only during REOX. Data are mean ± SD, n = 4-8 cells. * p < 0.05 vs. normoxia; # p < 0.05 vs. OGD/REOX.
Figure 6. Changes in $[\text{Ca}^{2+}]_i$ in soma and dendrites following OGD/REOX. A. Representative fura-2 pseudocolored images of changes in $[\text{Ca}^{2+}]_i$ in neurons at 0, 20, 30, 40, and 45 min REOX. *arrowhead*: low levels of $[\text{Ca}^{2+}]_i$. *Arrow*: localized increases in $[\text{Ca}^{2+}]_i$. B/C. Summarized changes of $[\text{Ca}^{2+}]_i$ in soma (B) or Sm-Dendrites (C). In the HOE 642 or SEA0400 studies, the drugs (1 μM) were present only during 1 h REOX. Data are mean ± SEM. n = 4. * p < 0.05 vs. 0 min REOX. # p < 0.05 HOE vs. non-treated OGD/REOX.

Figure 7. Changes in $\text{Ca}^{2+}$m and $\Psi_m$ in soma and dendrites following OGD/REOX. Changes in $\text{Ca}^{2+}$m and $\Psi_m$ were monitored in soma (A), Lg-Dendrites (B), and Sm-Dendrites (C). *Blue lines*: $\Psi_m$ data determined with JC-1 and expressed as the percentage of the maximal FCCP-induced change in the JC-1 ratio of normoxic controls. *Red lines*: $\text{Ca}^{2+}$m determined by the relative change in rhod-2 fluorescence. In the HOE study, 1 μM HOE 642 was present only during 0-1 h REOX. Data are mean ± SEM. n = 3-4. * p < 0.05 vs. 0 min REOX; # p < 0.05 vs. OGD/REOX.

Figure 8. Effects of FMK on changes of $\Psi_m$ in soma and dendrites following OGD/REOX. Changes in $\Psi_m$ were determined with JC-1 and expressed as the percentage of the maximal FCCP-induced change in the JC-1 ratio of normoxic controls. In the FMK study, 3 μM FMK was present during 0-1 h REOX. Data are mean ± SEM. n = 4. * p < 0.05 vs. 0 min REOX; # p < 0.05 vs. OGD/REOX.

Figure 9. Effects of mitochondrial uniporter inhibitor RU360 on $\text{Ca}^{2+}$m in soma and dendrites following OGD/REOX. $\text{Ca}^{2+}$m was assessed by the relative change in rhod-2 fluorescence when the mitochondrial uniporter inhibitor, RU360 (10 μM), was present during 60 min REOX. Data were plotted against $\text{Ca}^{2+}$m data from Figure 7 for OGD/REOX and OGD/REOX + HOE. Data are mean ± SEM. n = 3-4. * p < 0.05 vs. 0 min REOX; # p < 0.05 vs. OGD/REOX.

Figure 10. Dendritic beading in neurons following OGD/REOX. A-D. Dendritic beading formation in DiO-loaded cells was detected at 0, 30, 45, and 60 min REOX. In the HOE studies, 1 μM HOE 642 was present only during 0-1 h REOX. *Arrow*: swelling in dendrites without formations of varicosities; *Arrowhead*: varicosities. E. Summary data of dendritic beading. The data of normoxia control and normoxia plus HOE 642 (red) groups overlap. Data are mean ± SEM. n = 3-4. * p < 0.05 vs. 0 min REOX. # p < 0.05 vs. OGD/REOX.

Figure 11. Illustration of dendritic ionic disruption and mitochondrial dysregulation in ischemic neurons. Following ischemia, activation of NHE-1 causes an increase in dendritic $[\text{Na}^+]$, which triggers NCXrev, and leads to increases in $[\text{Ca}^{2+}]$. The $[\text{Na}^+]$, overload also causes increased consumption of ATP by Na⁺/K⁺-ATPase to maintain dendritic ionic homeostasis. On the other hand, the $[\text{Ca}^{2+}]$, overload stimulates $\text{Ca}^{2+}$m uptake by the uniporter (UP) and formation of the mitochondrial permeability transition pore (PTP). Blocking NHE-1 and NCXrev would reduce disruption of Na⁺ and Ca²⁺ homeostasis and preserve mitochondrial bioenergetics and dendritic membrane integrity.
Figure 1

A. 2-D

B. 3-D

C. Bar graph showing surface area/volume ratios for Soma, Lg-Dendrites, and Sm-Dendrites under Normoxia and OGD/REOX conditions. Bars marked with an asterisk (*) indicate significant differences.

D. Graphs showing $\beta_i$ (in mM/pH) against $pHi$ for Normoxia and OGD/REOX conditions. Symbols represent Soma (black circle), Lg-Dendrite (red circle), and Sm-Dendrite (blue triangle).
Figure 2
Figure 3
Figure 4

A. pH recovery rate in different experimental conditions.

B. pH regulation in Normoxia and OGD/REOX conditions.

C. Western blot analysis of NHE-1, NHE-2, NHE-3, NHE-5, and β-tubulin in cerebellum lysates.

D. Imaging of Negative Control and NHE1 expression in neuronal cells.
Figure 5

A

B

C

D

E

Figure 5
Figure 6
Figure 7
Figure 8
Figure 9
Figure 10
Excessive Na+/H+ exchange in disruption of dendritic Na+ and Ca2+ homeostasis and mitochondrial dysfunction following in vitro ischemia
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