Role of transporters and ion channels in neuronal injury under hypoxia

Jin Xue,1 Dan Zhou,1 Hang Yao,1 and Gabriel G. Haddad1,2,3

Departments of 1Pediatrics and 2Neuroscience, University of California San Diego, La Jolla; and 3The Rady Children’s Hospital, San Diego, California

Submitted 23 July 2007; accepted in final form 25 October 2007

Xue J, Zhou D, Yao H, Haddad GG. Role of transporters and ion channels in neuronal injury under hypoxia. Am J Physiol Regul Integr Comp Physiol 294: R451–R457, 2008. First published October 31, 2007; doi:10.1152/ajpregu.00528.2007.—The aims of the current study were to 1) examine the effects of hypoxia and acidosis on cultured cortical neurons and 2) explore the role of transporters and ion channels in hypoxic injury. Cell injury was measured in cultured neurons or hippocampal slices following hypoxia (1% O2) or acidosis (medium pH 6.8) treatment. Inhibitors of transporters and ion channels were employed to investigate their roles in hypoxic injury. Our results showed that 1) neuronal damage was apparent at 5–7 days of hypoxia exposure, i.e., 36–41% of total lactate dehydrogenase was released to medium and 2) acidosis alone did not lead to significant injury compared with nonacidic, normoxic controls. Pharmacological studies revealed 1) no significant difference in neuronal injury between controls (no inhibitor) and inhibition of Na+-K+-ATP pump, voltage-gated Na+ channel, ATP-sensitive K+ channel, or reverse mode of Na+/Ca2+ exchanger; 2) inhibition of transporters and ion channels in neuronal death under hypoxia or acidosis; (2) roles of certain ionic exchangers, channels and pumps in hypoxic neuronal injury between controls (no inhibitor) and inhibition of NBCs with 500 μM DIDS did not cause hypoxic death in either cultured cortical neurons or hippocampal slices; 3) in contrast, inhibition of Na+/H+ exchanger isoform 1 (NHE1) with either 10 μM HOE-642 or 2 μM T-162559 resulted in dramatic hypoxic injury (+95% for HOE-642 and +100% for T-162559 relative to normoxic control, P < 0.001) on treatment day 3, when no death occurred for hypoxic controls (no inhibitor). No further damage was observed by NHE1 inhibition on treatment day 5. We conclude that inhibition of NHE1 accelerates hypoxia-induced neuronal damage. In contrast, DIDS rescues neuronal death under hypoxia. Hence, DIDS-sensitive mechanism may be a potential therapeutic target.

sodium/hydrogen exchanger 1; HCO3− transporters; disodium 4,4′-diisothiocyanatostilbene-2,2′-disulfonate; neuronal protection

CEREBRAL ISCHEMIA is the third most prevalent cause of death in the United States. Despite years of research efforts, the effective treatment and prevention of hypoxic/ischemic brain injury remains a major medical challenge. Because brain function is critically dependent on a proper O2 supply, mammalian neurons are exquisitely sensitive to oxygen shortage. Cerebral hypoxia can, in 1–2 min, slow electroencephalographic activity with subsequent depression of cortical-evoked potentials and loss of consciousness. Vulnerability of central neurons to O2 deprivation results from failure in a coordinated decrease in metabolism and ion pumping, leading to ATP loss, membrane depolarization, abnormal release of excitatory neurotransmitters, and injurious elevation in intracellular Ca2+ concentration (16, 22, 25). Ionic disturbance is an early event in the cascade leading to hypoxic injury in the mammalian brain (16, 22, 25). Some of the important ionic changes, as demonstrated by many studies, pertain to Ca2+, Na+, and H+. In the past decade, for example, extensive research has suggested that Na+/H+ exchanger isoform 1 (NHE1) activity is stimulated and contributes substantially to cardiac injury during ischemia and reperfusion. Inhibition of NHE1 was proved therefore to ameliorate detrimental consequences (2, 18). The principal mechanism underlying this cardioprotective action of NHE1 inhibition is the reduction of intracellular Na+ accumulation, thus prevention of intracellular Ca2+ overload via a reverse mode of Na+/Ca2+ exchanger (NCX) and avoidance of elevated Ca2+–triggered death cascade. However, limited knowledge is available about the effects of NHE1 inhibition on cerebral ischemic damage. Some data from ischemia-reperfusion experiments in brain appear to support the above-mentioned mechanism (17, 24). However, recent studies have implicated that NHE1 activity is required for cell survival (21, 33, 41, 42). Thus the exact role of NHE1 in cerebral ischemia is still an open question. Besides NHE1 and NCX, HCO3− transporters (NBCs), ATP-sensitive K+ channel (KATP channel), voltage-gated Na+ channel, and the Na+/K+-ATP pump are also important players in the regulation of ionic homeostasis and intracellular pH and contribute to ischemia-induced injury in the central nervous system (3, 7, 13, 22). In the present study, we examine (1) the response of cultured neurons or slices to hypoxia or acidosis; (2) roles of certain ionic exchangers, channels and pumps in hypoxic neuronal damage with aid of pharmacological inhibitors.

MATERIALS AND METHODS

Primary neuronal culture. Animal use was approved by the Institutional Animal Care and Use Committee of the University of California, San Diego. Primary cortical neuronal cultures were prepared from embryonic day 14–17 mice (Charles River CD-1 strain; see Ref. 49). In brief, mice were decapitated, and cortical tissue was collected, minced, and dissociated with trypsin (0.25 mg/ml in Hanks’ balanced salt solution without Ca2+ and Mg2+) at 37°C for 15 min. Trypsin activity was stopped by a trypsin inhibitor (volume ratio 1:1). Next, the cell mixture was passed through 80 nm mesh to remove cell clumps and centrifuged at 1,400 revolutions/min for 5 min at 4°C. The cell pellet was resuspended in serum-free Neurobasal Medium (GIBCO-BRL, Grand Island, NY), supplemented with 1× B-27 Minus A0, 0.5 mM glutamine, 25 μM glutamate, 50 μg/ml penicillin, and 50 μg/ml streptomycin. Neurobasal/B27 culture medium was chosen instead of DMEM-F-12 medium because the former provides optimal growth and long-term viability of hippocampal and other central nervous system (CNS) neurons. In addition, glial cell growth is reduced to <0.5% for a nearly pure neuronal population (10). Cells were plated at 1 × 105 cells/ml on poly-d-lysine (0.1 mg/ml; Sigma, St. Louis, MO)-coated six-well plates. The cultures were maintained in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Half of the medium was replaced with fresh medium without glutamate every...
3 days. Two-week in vitro culture allows neurons grow back their processes and make cell contact. Afterward, hypoxia (1% O₂) or acidois (medium pH 6.8) was given for a period of 7 days. Culture media and cell lysates were taken and analyzed for lactate dehydrogenase (LDH) activity on treatment days 1, 3, 5, and 7. Hypoxia and acidois were studied separately because both could contribute to cell damage independently.

Treatments with pharmacological agents. Several pharmacological inhibitors of transporters and ion channels were chosen to study their roles during hypoxia (summarized on Table 1). Ouabain octahydrate, disodium 4,4′-disothiocyanatostilbene-2,2′-disulfonate (DIDS), benzamil hydrochloride, glybenclamide, and tetrodotoxin (TTX) were purchased from Sigma. 1,2-Bis(o-aminophenoxy)ethane-N,N,N′,N″-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA-AM) was obtained from Calbiochem (San Diego, CA). KB-R7943 mesylate was purchased from Tocris Bioscience (Ellisville, MO). Cariporide mesilate (HOE-642) was provided by Aventis Pharma Deutschland (Frankfurt am Main, Germany), and T-162559 was provided by Takeda Chemical Industries (Osaka, Japan). Pharmacological inhibitors were given during the treatments (normoxia as sham control vs. hypoxia or acidois). The culture medium was harvested on day 3 or 5 for LDH activity assay.

LDH activity assay. Cellular injury was evaluated in the neuronal cultures by measuring LDH activity released in the media using the CytoTox96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI) and quantitated using wavelength absorbance at 490 nm. Briefly, the cultured neuronal cells were treated under different conditions (21% O₂ vs. 1% O₂ vs. acidois, with or without various pharmacological inhibitors) in six-well plates. Nine separate samples were examined from nine individual cultures, with each culture obtained from one litter of pups (about 10 pups/litter). At the end of each treatment, the culture medium from each well was collected separately and stored at −80°C. To normalize to the total amount of LDH, cultured cells in the corresponding wells were lysed with 0.1% Triton X-100 in PBS for 45 min at 37°C in a 5% CO₂ incubator. The plates were then centrifuged at 250 g for 4 min, and the supernatant was harvested individually for intracellular LDH determinations. To measure LDH activity, 50 µl substrate mixture (provided in the kit) and either 50 µl of culture medium or cell lysate were added together in a 96-well plate, incubated for 30 min at room temperature, and protected from light. The reaction was stopped by adding 50 µl of 1 M acetic acid. Absorbance (A) in samples was measured at 490 nm with the aid of an Ultramark Microplate Imaging System (BioRad Laboratory, Hercules, CA). LDH release (%A) was determined using the ratio (A<sub>490</sub> in medium)/(A<sub>490</sub> in medium + A<sub>90g</sub> in cell lysate).

Organotypic hippocampal slice cultures. Hippocampal slice cultures were prepared and maintained as described previously (36). Briefly, CD1 mice (Charles River Laboratories, Raleigh, NC), 6–8 days old, were anesthetized with halothane. The brains were removed and transferred quickly to an ice-cold dissection medium (Gey’s balanced salt solution supplemented with 6.5 mg/ml D-glucose). Hippocampi were dissected out and cut transversely into slices 300 µm thick using a Vibratome 800-McIlwain Tissue Chopper (Vibratome, St. Louis, MO). Slices were carefully transferred in the 30-mm Millicell-CM tissue culture inserts (Millipore, Bedford, MA) and placed in 35-mm culture dishes. The stock of culture medium contained basal medium Eagle medium (50%), Earle’s balanced salt solution (25%), horse serum (25%), and l-glutamine (1 mM). Before each use, the above culture medium was supplemented with 50 U/ml penicillin/streptomycin and 36 mM glucose. Cultures were maintained in a 5% CO₂, 37°C incubator, and culture medium was half-replaced the 2nd day after plating and twice a week thereafter. In vitro culture day 14, normoxia (21% O₂) or hypoxia (1% O₂) was given with or without DIDS (400 µM) for 5 days. Chemicals used in the above media were obtained from Invitrogen (Grand Island, NY) or Sigma.

Quantification of cell death. Cell damage was assessed in the cultured slices with propidium iodide (PI), which enters the cell and intercalates into the DNA with enhanced fluorescence. PI (5 µg/ml) was added into the culture medium 24 h before image acquisition and kept at the same concentration throughout the experiment. PI fluorescence was observed on an inverted microscope equipped with a rhodamine filter set that has a 540- to 552-nm band-pass filter for excitation and a 590-nm long-pass filter for emission (Zeiss Axiovert 200M microscope; Zeiss, Yena, Germany). A F-Fluar ×5 objective (Zeiss) and the attached 12-bit CCD camera (C4742; Hamamatsu, Herrsching, Germany) were routinely used for image acquisition. The light source, microscope, and the camera were controlled by a computer (Universal Imaging, Downington, PA). For data collection, the parameters of the microscope such as light intensity for excitation, exposure time, camera gain etc., were kept constant. Images were acquired and analyzed with MetaFlour imaging-processing software (Universal Imaging). The fluorescence intensity was measured offline in the hippocampal CA1 region, and the mean fluorescence intensity was expressed as MFI (47).

Statistical analyses. All values were represented as means ± SE. Statistical significance was calculated by the one-way ANOVA with Bonferroni post test. Differences in means were considered significant if P < 0.05.

RESULTS

Neuronal injury under hypoxia (1% O₂) or acidois (medium pH 6.8). Primary cultured neurons were exposed to either 1% O₂ or pH 6.8 medium for 7 days starting with in vitro culture day 14. The culture medium was collected on treatment days 1, 3, 5, and 7, and medium LDH activity (in %total LDH) was used as an index of cell death. Our data (Fig. 1) revealed that 3 days of 1% O₂ did not increase medium LDH activity, indicating that cultured neurons maintained their integrity within the first 3 days of hypoxia. However, neuronal damage occurred thereafter and was evident around days 5–7, i.e., 36–41% of total LDH was released into medium (P < 0.001,

Table 1. Pharmacological inhibitors of transporters and ion channels

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration, µM</th>
<th>Description</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIDS</td>
<td>500</td>
<td>Anion transporter inhibitor, mainly block HCO⁻ transporters</td>
<td>5</td>
</tr>
<tr>
<td>Ouabain</td>
<td>100</td>
<td>A classical Na⁺-K⁺-ATP pump inhibitor</td>
<td>1</td>
</tr>
<tr>
<td>Glybenclamide</td>
<td>100</td>
<td>Selectively block ATP-sensitive K⁺ channels</td>
<td>28</td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>50</td>
<td>Membrane permeable and hydrolyzed by cytosolic esterase and trapped intracellularly as the active Ca²⁺ chelator</td>
<td>6</td>
</tr>
<tr>
<td>KB-R7943</td>
<td>20</td>
<td>Potent, selective inhibitor of the reverse mode of Na⁺/Ca²⁺ exchanger</td>
<td>7</td>
</tr>
<tr>
<td>TTX</td>
<td>1</td>
<td>Reversible, selective blocker of Na⁺ channels</td>
<td>13</td>
</tr>
<tr>
<td>HOE-642</td>
<td>10</td>
<td>Specific inhibitor of Na⁺/H⁺ exchanger isoform 1</td>
<td>40</td>
</tr>
<tr>
<td>T-162559</td>
<td>2</td>
<td>Potent, selective inhibitor of Na⁺/H⁺ exchanger isoform 1</td>
<td>20</td>
</tr>
</tbody>
</table>

TTX, tetrodotoxin.
Fig. 1. Effects of hypoxia and acidosis on cultured cortical neurons. The primary cultured cortical neurons were treated with either hypoxia (1% O2) or acidosis (medium pH 6.8) starting in vitro on culture day 14 for 7 days. The control was cultured neurons under normoxic, normoxia (21% O2, pH 7.2) condition. Lactate dehydrogenase (LDH) activity in culture medium and cell lysate was measured, and the ratio of medium LDH to (medium + lysate) LDH was used as an index of cell injury. Note that hypoxic injury was not observed on 3-day 1% O2. However, significant neuronal damage occurred on day 5 to day 7 under 1% O2 (P < 0.05, n = 9 experiments). There was no difference in cell death between normoxic control and acidosis at pH 6.8 (P > 0.05, n = 9). *Statistical significance.

n = 9), suggesting that the neurons were dying by days 5–7. In contrast, no significant difference in medium LDH activity was observed when acidosis (pH 6.8, 21% O2) was compared with nonacidic, normoxic control (pH 7.2, 21% O2) during a 7-day treatment period. There was a slight and progressive elevation of medium LDH activity (day 7 relative to that of day 1, 47% increase for normoxia, P < 0.01, n = 9 and 24% increase for acidosis, P > 0.05, n = 9), suggesting that neurons die slowly during the in vitro culture period.

Lack of effect of Na+/K+-ATP pump, voltage-gated Na+ channel, KATP channel, and NCX inhibition in cultured neurons under hypoxia. Previous studies have reported that Na+/K+ ATP pump, voltage-gated Na+ channel, KATP channel, and NHE play pathophysiological roles in hypoxia under certain circumstances (3, 7, 13, 22). In the current study, specific inhibitors were chosen to investigate their impacts on cell fate in primary cultured neurons under hypoxia. Drugs alone under normoxia were examined and used as sham controls. No significant drug toxicity was observed, except for intracellular Ca2+ chelator BAPTA-AM (50 μM). BAPTA elicited a twofold increase in medium LDH activity compared with control (no inhibitor) under normoxia (P < 0.001, n = 9; data not shown), indicating that a certain level of intracellular Ca2+ is required by neurons to survive under normoxia. After 5 days of 1% O2 exposure, the percentage of cell death relative to its corresponding normoxic sham control was determined. Our results showed that, in the absence of any inhibitor, 1% O2 increased injury by 75% (P < 0.01, n = 9) compared with normoxic control. Similar degrees of hypoxic damage were found when the Na-K ATP pump (100 μM ouabain; +47%), the voltage-gated Na+ channel (1 μM TTX; +57%), the KATP channel (100 μM glybenclamide; +56%), and the reverse mode of NCX (20 μM KB-R7943; +46%) were blocked (data not shown). One-way ANOVA did not demonstrate statistical significance (P > 0.05, n = 9). A decrease on neuronal injury was observed in BAPTA-treated neurons under hypoxia compared with BAPTA-treated normoxic controls (−26%, P < 0.01, n = 9), suggesting that prevention of excessive hypoxia-induced intracellular Ca2+ elevation is protective.

Aggravation of neuronal damage by NHE isoform 1 inhibition under hypoxia. NHE1 is a ubiquitously expressed integral membrane protein. It is involved in numerous pathophysiological processes, including intracellular pH regulation, cell volume control, cytoskeletal organization, cell proliferation and differentiation, as well as apoptosis (4, 26). Furthermore, extensive research has also implicated a role of NHE1 in ischemia-reperfusion injury of the heart. In the present study, two specific NHE1 inhibitors (10 μM HOE-642 and 2 μM T-162559) were used to examine their effects on primary cultured neurons. In contrast to previously published data in heart (2, 18), our results from the cultured neurons have shown that NHE1 inhibition resulted in dramatic neuronal damage at an early stage (day 3) of hypoxia treatment (+95% for HOE-642 and +100% for T-162559 relative to their normoxic sham controls, P < 0.001, n = 9), whereas no obvious injury was detected in the hypoxic control group (no inhibitor; P > 0.05, samples n = 9; Fig. 2A). On treatment day 5, control neurons (no inhibitor) showed significant cell death under 1% O2 (+75% relative to its normoxic control, P < 0.001, n = 9). However, no further damage was observed in the NHE1 inhibition group on day 5 (+114% for HOE-642 and +90% for T-162559 relative to their normoxic sham controls) compared with those on day 3 (P > 0.05, n = 9; Fig. 2B).

Protection from neuronal damage by DIDS under hypoxia. The BT superfamily is composed of the Na+ -coupled NBCs and the AE family of anion exchangers (i.e., Cl−/HCO3− exchangers). BTs play key roles in pHi and ionic homeostasis (8, 30). It has been reported that BTs contribute to cell injury in a cardiac model of ischemia (19, 29, 31). DIDS (500 μM), a inhibitor of BTs, was tested in cultured neurons. Our data revealed that there was no difference in neuronal damage between blockade of BTs and control after 3 days of 1% O2 exposure (P > 0.05, n = 9; Fig. 3A). However, on day 5 of 1% O2, in contrast to evident injury in the control group (+75% of normoxia, P < 0.001, n = 9), DIDS almost completely blocked low oxygen-induced neuronal death (+14% of its normoxic sham control, P > 0.05, n = 9; Fig. 3B).

To further substantiate the protective role of DIDS, we examined the effect of DIDS in cultured hippocampal slices. Brain slices generally maintain the cellular organization and preserve glial and neuronal communications. Hippocampal slices were investigated for the following two reasons: 1) the hippocampus is generally well studied, and comparisons between this study and others could be made; and 2) if DIDS acts also on the hippocampus, this would allow us to conclude potentially that the effect of DIDS is not limited to cortical neurons. PI uptake was measured in hippocampal CA1 region following 5 days of normoxia or hypoxia (1% O2) incubation with or without DIDS (400 μM). Mean PI uptake was significantly higher in hypoxia than in normoxia in the absence of DIDS (Fig. 4, bar on left in each panel). DIDS alleviated PI uptake in both normoxia- and hypoxia-treated slices (Fig. 4, bar on right in each panel), and the reduction of PI uptake was statistically significant in the hypoxia group (Fig. 4, right).
were damaged by 1% O2 (study on pH i regulation in neurons cultured from mouse). It is well known that neuronal injury may be related to pHi and its regulation. A previous study that inhibition of NHE1 and NBCs affect hypoxia-induced neuronal injury (12, 32, 44). Because of its high metabolic rate, the CNS produces a large amount of metabolic acid, particularly under stressful conditions, such as hypoxia/ischemia, excessive activity, and hyperglycemia. This feature renders neurons more susceptible to injury if subjected to wide swings in acid or alkaline loads. Thus the mechanisms for acid extrusion or acid loading in neurons would be important for pHi homeostasis and proper brain function. A previous study on pHi regulation in neurons cultured from mouse cerebral cortex (27) has demonstrated that a 5’-(N-ethyl-N-isopropyl)amiloride (EIPA, a blocker of NHE)-sensitive NHE as well as a DIDS-sensitive Na+ and HCO3−-dependent mechanism, possibly a Na+HCO3− cotransporter (NBC), are functioning at physiological pHi to maintain steady-state pHi. pHi recovery after acid load is mediated mainly by an EIPA-sensitive NHE and a DIDS-sensitive Na+ and HCO3−-dependent Cl−/HCO3− exchanger. Therefore, the discovery in the present study that inhibition of NHE1 and NBCs affect hypoxia-induced neuronal injury may be related to pHi and its regulation.

NHE1, ubiquitously expressed NHE, acts as an acid extruder by mediating an electroneutral 1:1 exchange of intracellular H+ for extracellular Na+. The decreased pHi, known to occur in hypoxia, activate acid-base transporters, such as NHES, Na+−dependent NBCs, and/or H+ ATPase, to maintain pHi. Inhibition of a major acid extruder, NHE1, may exacerbate intracellular acidification during hypoxia. Indeed, our previous data have shown that O2 deprivation induced a small intracellular acidification in CA1 neurons. This pHi drop was mark-

---

**DISCUSSION**

The major finding of the current study is that DIDS, a known inhibitor of NBCs and Cl− channels (11), rescues hypoxia-induced injury on both cultured cortical neurons and hippocampal slices. However, HOE-642 (40) or T-162559 (20), specific inhibitors of NHE1, aggravate neuronal damage under hypoxia.

Both NBCs and NHE1 belong to acid-base regulatory systems and have been shown to be present in the central nervous system at both mRNA and protein levels (12, 32, 44). Because of its high metabolic rate, the CNS produces a large amount of metabolic acid, particularly under stressful conditions, such as hypoxia/ischemia, excessive activity, and hyperglycemia. This feature renders neurons more susceptible to injury if subjected to wide swings in acid or alkaline loads. Thus the mechanisms for acid extrusion or acid loading in neurons would be important for pHi homeostasis and proper brain function. A previous study on pHi regulation in neurons cultured from mouse cerebral cortex (27) has demonstrated that a 5’-(N-ethyl-N-isopropyl)amiloride (EIPA, a blocker of NHE)-sensitive NHE as well as a DIDS-sensitive Na+ and HCO3−-dependent mechanism, possibly a Na+−HCO3− cotransporter (NBC), are functioning at physiological pHi to maintain steady-state pHi. pHi recovery after acid load is mediated mainly by an EIPA-sensitive NHE and a DIDS-sensitive Na+−dependent Cl−/HCO3− exchanger. Therefore, the discovery in the present study that inhibition of NHE1 and NBCs affect hypoxia-induced neuronal injury may be related to pHi and its regulation.

NHE1, ubiquitously expressed NHE, acts as an acid extruder by mediating an electroneutral 1:1 exchange of intracellular H+ for extracellular Na+. The decreased pHi, known to occur in hypoxia, activate acid-base transporters, such as NHES, Na+−dependent NBCs, and/or H+ ATPase, to maintain pHi. Inhibition of a major acid extruder, NHE1, may exacerbate intracellular acidification during hypoxia. Indeed, our previous data have shown that O2 deprivation induced a small intracellular acidification in CA1 neurons. This pHi drop was mark-

---

**Fig. 2.** Inhibition of Na+/H+ exchanger isoform 1 (NHE1) aggravated hypoxic injury in cultured cortical neurons. The primary cultured cortical neurons were treated with NHE1 specific inhibitors (10 μM HOE-642 or 2 μM T-162559) under nonacidic, normoxia (21% O2, pH 7.2, sham control), or hypoxia (1% O2) or acidosis (medium pH 6.8) starting in vitro on culture day 14 for 3 days (A) and 5 days (B). Medium LDH activity was measured and shown as the percentage of its corresponding sham control. Note that on treatment day 3 (A), NHE1 inhibition leads to significant hypoxic injury (+95% for HOE-642 and +100% for T-165229 relative to sham controls, \( P < 0.001 \)), whereas no hypoxic damage was observed in controls (no inhibitor). On treatment day 5 (B), control neurons were damaged by 1% O2 (+75%, \( P < 0.001, n = 9 \)). However, NHE1 inhibition did not cause further neuronal death (+114% for HOE-642 and +90% for T-165229) compared with those on day 3 (A). No difference was detected between acidosis at pH 6.8 and normoxic control, with or without inhibitors. *Statistical significance (one-way ANOVA with Bonferroni post test, \( P < 0.05, n = 9 \)) relative to corresponding sham control.

**Fig. 3.** Disodium 4,4’-diisothiocyanatostilbene-2,2’-disulfonate (DIDS) attenuated hypoxic injury in cultured cortical neurons. The primary cultured cortical neurons were treated with DIDS (500 μM), an inhibitor for HCO3− transporter, under nonacidic, normoxia (21% O2, pH 7.2, sham control), or hypoxia (1% O2) or acidosis (medium pH 6.8) starting in vitro on culture day 14 for 3 days (A) and 5 days (B). Medium LDH activity was measured and shown as the percentage of its corresponding sham control. Note that on treatment day 3 (A), no hypoxia injury occurred in the presence or absence of DIDS. On treatment day 5 (B), control neurons were damaged by 1% O2 (+75%, \( P < 0.001, n = 9 \)). However, DIDS protected cultured neurons from hypoxic injury (+14% relative to sham control, \( P > 0.05, n = 9 \)). No difference was detected between acidosis at pH 6.8 and normoxic control, with or without DIDS. *Statistical significance (one-way ANOVA with Bonferroni post test, \( P < 0.05, n = 9 \)) relative to corresponding sham control.
investigations are required to delineate the precise contribution of each ionic change (Na\(^{+}\), K\(^{+}\), Cl\(^{-}\), HCO\(_3\)^{-}, H\(^{+}\)) to ischemic injury, the cellular processes/signaling pathways involved (apoptosis, neurotoxicity, ionic and pH disturbance).

An intriguing finding in this work is that DIDS prevents hypoxia-induced neuronal injury. DIDS is a known inhibitor of NBCs and Cl\(^{-}\)-channels (11). The NBC superfamily comprises Cl\(^{-}\)-HCO\(_3\)^{-} exchangers (AEs), Na\(^{+}\)-driven Cl\(^{-}\)-HCO\(_3\)^{-} exchangers (NDCBE), and electroneutral (1:1) and electrogenic (1:2 or 1:3) NBCs (8). Among these, NDCBE and NBCs with 1:1 and 1:2 stoichiometry are believably acid extruders, whereas AEs and NBCs with 1:3 stoichiometry are acid loaders. Functional studies in hippocampal CA1 neurons have proven that NBCs, AEs, and NDCBE all contribute to pH\(_{i}\) regulation (9, 45). If pH\(_{i}\) homeostasis is critical for neuronal survival under hypoxia, as proposed above, the opposite effects of inhibition of NHE1 vs. NBCs revealed by the current study suggest that an acid-loading type of NBC (AEs and/or NBCs with 1:3 stoichiometry) may play a role in neuronal death during O\(_2\) deprivation. This notion is supported by the findings from our group that anoxia activates a DIDS-sensitive, Na\(^{+}\)-dependent acid loader (45). Because of lack of specific inhibitor for each type, the identity of this DIDS-sensitive transporter has remained unclear. Nevertheless, evidence has accumulated that blockade of DIDS-sensitive, NBCs can have a protective action in ischemia-reoxygenation injury (19, 29, 31).

Besides its impact on pH\(_{i}\) regulation via inhibition of NBCs, DIDS has recently been shown to have multiple roles in cell death, including apoptosis. For instance, DIDS can act as a weak N-methyl-D-glucamine (NMDA) receptor antagonist to reduce NMDA receptor-mediated current and Ca\(^{2+}\) influx via this receptor, thereby attenuating neurotoxicity (38); 2) DIDS protects the ischemic myocardium by inhibition of fatty acid uptake, improved glucose metabolism, and enhanced functional recovery on reperfusion (29); 3) DIDS rescues cardiomyocytes from apoptosis by blocking Cl\(^{-}\) channel (37); 4) DIDS attenuates reactive oxygen species released from mitochondria during hypoxia, thus protecting the myocardium (39); 5) DIDS inhibits cardiac voltage-gated Na\(^{+}\) channels, potentially preventing Na\(^{+}\) influx (23); and 6) DIDS blocks K\(_{ATP}\) channels in guinea pig ventricular myocytes (14). The last two possibilities can be excluded based upon data from this study, i.e., TTX, a blocker of voltage-gated Na\(^{+}\) channels, and glybenclamide, an inhibitor of K\(_{ATP}\) channels, fail to protect neurons from hypoxia-induced death. Which exact mechanism(s) is important in hypoxic rescue by DIDS in our cultured neurons and slices is not certain at this stage. The present findings of NHE1 inhibition and DIDS on hypoxic injury favor a pH\(_{i}\) regulatory mechanism. Furthermore, our preliminary data from an ischemic infarct rim model (48) have suggested that lowered Cl\(^{-}\) in ischemic solution is detrimental. Thus we believe that the protective action of DIDS can be at least partially explained by blocking Cl\(^{-}\} efflux.

**Perspectives and significance.** One significant finding of the current study is that DIDS almost completely abolishes the hypoxia-induced neuronal damage. DIDS has various beneficial actions in distinct cellular processes as discussed above. Although the neuroprotection by DIDS makes this agent an attractive therapeutic target against ischemia injury, DIDS may cause adverse effects because of its multiple effects on different pathways. Ongoing studies should investigate the exact mechanism underlying DIDS protection, such as the relative contribution of each ionic change (Na\(^{+}\), K\(^{+}\), Cl\(^{-}\), HCO\(_3\)^{-}, H\(^{+}\}) to ischemic injury, the cellular processes/signaling pathways involved (apoptosis, neurotoxicity, ionic and pH\(_{i}\) disturbance, etc.).
metabolism, etc.), and the effects of DIDS on the in vivo ischemia model.

ACKNOWLEDGMENTS

We thank Orit Gavrialov for technical assistance.

GRANTS

This work was supported by National Institutes of Health Grants PO1HD-32573 and RO1NS-037756 to G. G. Haddad and a Parker B. Francis Fellowship grant to J. Xue.

REFERENCES


