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Intracellular pH reduction prevents excitotoxic and ischemic neuronal death by inhibiting NADPH oxidase

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Sustained activation of N-methyl-D-aspartate (NMDA) -type glutamate receptors leads to excitotoxic neuronal death in stroke, brain trauma, and neurodegenerative disorders. Superoxide production by NADPH oxidase is a requisite event in the process leading from NMDA receptor activation to excitotoxic death. NADPH oxidase generates intracellular H⁺ along with extracellular superoxide, and the intracellular H⁺ must be released or neutralized to permit continued NADPH oxidase function. In cultured neurons, NMDA-induced superoxide production and neuronal death were prevented by intracellular acidification by as little as 0.2 pH units, induced by either lowered medium pH or by inhibiting Na⁺/H⁺ exchange. In mouse brain, superoxide production induced by NMDA injections or ischemia–reperfusion was likewise prevented by inhibiting Na⁺/H⁺ exchange and by reduced expression of the Na⁺/H⁺ exchanger-1 (NHE1). Neuronal intracellular pH and neuronal Na⁺/H⁺ exchange are thus potent regulators of excitotoxic superoxide production. These findings identify a mechanism by which cell metabolism can influence coupling between NMDA receptor activation and superoxide production.

NOX2 | acidosis | Hv1 | cariporide

Many metabolic processes generate hydrogen ions, and hydrogen ions in turn influence cell metabolism and survival (1). Cerebral ischemia in particular produces acidosis of variable degree, depending upon blood glucose levels, degree of blood flow reduction, and other factors. Severe acidosis, below pH 6.4, exacerbates ischemic injury (2) by mechanisms involving protein denaturation, acid-sensing calcium channels, and release of ferrous iron (3–5). Conversely, lesser degrees of acidosis, in the range of 7.0–6.5, reduce both ischemic injury (6) and glutamate-induced neuronal death (7). These neuroprotective effects have been attributed to an inhibitory effect of hydrogen ions on NMDA receptor activation (8–10), but a causal link has not been demonstrated.

Excessive activation of N-methyl-D-aspartate (NMDA) type glutamate receptors leads to excitotoxic cell death in stroke and other neurological disorders (11, 12). Superoxide production by NADPH oxidase is a requisite event in the process leading from NMDA receptor activation to excitotoxic cell death (13–19). NADPH oxidase exists as several isoforms, of which NOX2 is the one most abundantly expressed in CNS neurons. NOX2 is also the isoform most abundantly expressed in phagocytes, microglia, and other immune cells, in which its regulation and function have been extensively characterized (20). NOX2 is composed of three cytosolic subunits, p47^{phox}, p67^{phox}, and p40^{phox}, which when phosphorylated bind with two membrane-bound subunits, p22^{phox} and gp91^{phox} (the catalytic unit), to form an active transmembrane enzyme complex. The transmembrane complex generates superoxide in the extracellular space and hydrogen ions in the intracellular space: $2O_2 + NADPH \rightarrow 2O_2^- + NADP^+ + H^+$. In immune cells, H⁺ concentration influences the phosphorylation status of the NOX2 p47^{phox} subunit, and the H⁺ generated by NOX2 must be transferred to the extracellular space to sustain NOX2 activity (21–24).

Together, the pH sensitivity of NOX2 and the role of NOX2 in NMDA receptor-mediated cell death suggest the possibility that reduced intracellular pH might limit neurotoxicity by dissociating NMDA receptor activation from superoxide production. Findings presented here confirm that both the superoxide production and cell death resulting from neuronal NMDA receptor activation are highly pH sensitive. We show that neurons use Na⁺/H⁺ exchange as a major route of proton efflux during NOX2 activation, and either genetic or pharmacologic inhibition of neuronal Na⁺/H⁺ exchange prevent both excitotoxic superoxide production and cell death.

Results

Mild Acidosis Blocks NMDA-Induced Superoxide Production and Cell Death. We first performed cell culture studies at a physiological medium pH to confirm NOX2 as the primary source of NMDA-induced neuronal superoxide production. Normal brain extracellular pH is 7.20–7.30 (25). Mouse cortical neurons exposed to 100 μM NMDA at medium pH 7.2 showed an immediate intracellular calcium elevation, followed by superoxide formation and subsequent cell death (Fig. S1). By contrast, neurons pretreated with a peptide inhibitor of NOX2 assembly (gp91ds-Tat) showed no increased superoxide formation or cell death after NMDA exposure, despite comparable calcium elevations (Fig. S1). These findings agree with prior reports identifying NOX2 as the primary source of NMDA-induced superoxide production (13, 14, 17–19), and they establish that both of the indicators

Significance

Activation of NMDA-type glutamate receptors produces neuronal excitotoxicity, a primary cause of cell death in stroke and other neurological disorders. This cell death process requires superoxide release by neuronal NADPH oxidase. Results presented here show that small reductions in intracellular pH uncouple neuronal NADPH oxidase from NMDA receptor activation, and thereby prevent neuronal death. The findings establish a link between metabolic activity and excitotoxicity, and identify a mechanism by which mild acidosis improves outcome after excitotoxic and ischemic brain insults. The findings also suggest that variations in intracellular pH associated with physiological brain activity may likewise influence the cell-to-cell signaling normally mediated by neuronal superoxide release.

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used to measure superoxide production are responsive to NOX2 inhibition.

Neurons were then exposed to NMDA in acidified culture medium to evaluate the effect of H^+ on superoxide production. Surprisingly, reducing medium pH by just 0.2 pH units, from 7.2 to 7.0, nearly eliminated both NMDA-induced superoxide formation and cell death (Fig. 1). A direct effect of acidosis on the responsiveness of the superoxide indicators was excluded by the lack of a pH effect on signal induced by exogenous oxidant (Fig. S2). The effect of acidosis on superoxide production was evident in both bicarbonate-containing and bicarbonate-free media (Fig. S3), and all subsequent studies were performed in bicarbonate-free medium.

The Effect of Mild Acidosis Is Not Attributable to NMDA Receptor Inhibition. Calcium influx through the NMDA receptor is required for NMDA-induced NOX2 activation (13). This influx is sensitive to H^+ binding to an extracellular site on NMDA receptors (8–10), suggesting a possible mechanism for the effect of medium acidification on NMDA-induced superoxide pro-

duction. However, the effect of H^+ on NMDA-induced current influx is relatively small between pH 7.2 and pH 6.8. Here, using the low-affinity dye Fura-4F, we confirmed a small but measurable attenuation of NMDA-induced calcium elevations over this pH range (Fig. 1F). To test the possibility that even small attenuations in calcium rise might prevent NOX2 activation, we increased the medium calcium concentration from the standard 1.26 mM to 2.0 mM, a value at which the Fura-4F signal induced by NMDA at pH 7.0 was at least equal to that induced by NMDA at pH 7.2 in 1.26 mM calcium. Under these conditions, NMDA-induced superoxide production was again prevented at the lower medium pH, despite comparable intracellular calcium elevations (Fig. S4).

Intracellular Acidification Is Sufficient to Block NMDA-Induced Superoxide Formation and Cell Death. NOX2 is sensitive to small changes in intracellular pH (pH_i) (24), and pH_i in cultured cells varies with the medium pH. Using the pH-sensitive dye 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF), we found neuronal pH_i to be at baseline about 0.2 pH units lower

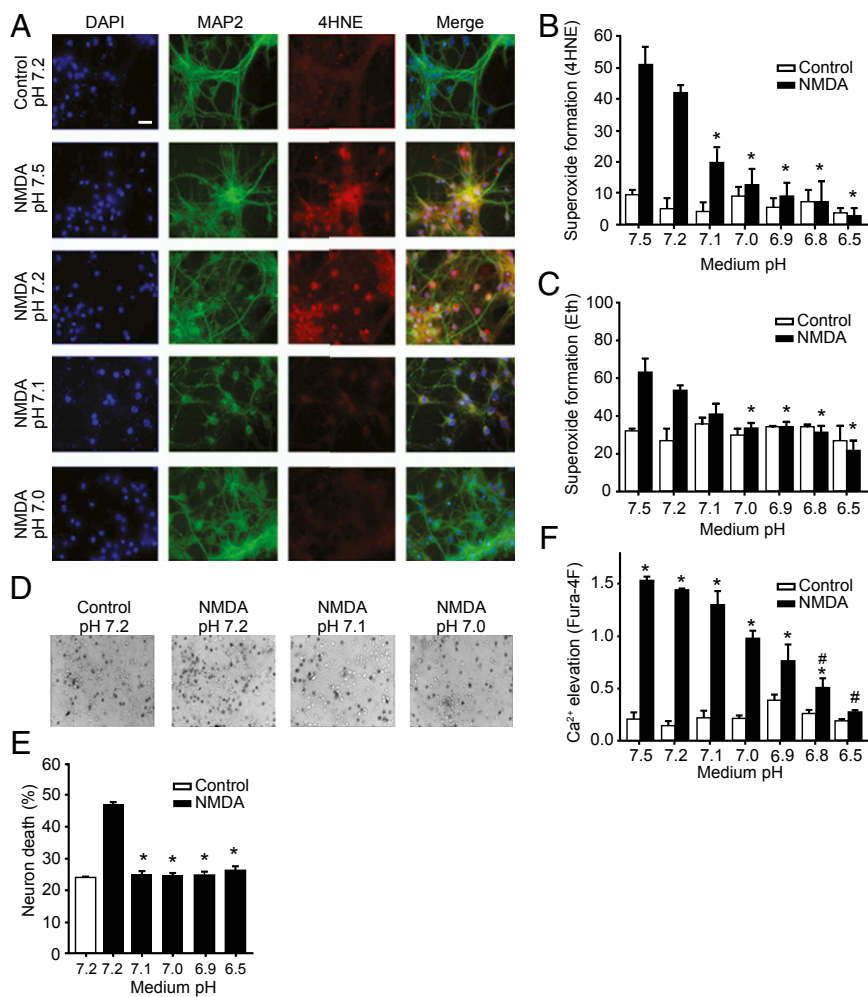


Fig. 1. NMDA-induced superoxide formation and cell death are blocked by small reductions in medium pH. (A) Immunostaining for 4-hydroxynonenal (4HNE; red) shows superoxide formation produced by 20-min incubations in 100 μ M NMDA at the designated medium pH. Staining for microtubule-associated protein 2 (MAP2; green) identifies neuronal processes, and DAPI (blue) identifies cell nuclei. (Scale bar: 25 μ m.) (B) Quantified 4HNE immunofluorescence. Control wells received medium exchanges only. ($n = 4$; $*P < 0.05$ vs. NMDA, pH 7.2). (C) Superoxide formation assessed by formation of fluorescent ethidium species (Eth) from dihydroethidium, conditions as in A. ($n = 4$; $*P < 0.05$ vs. NMDA, pH 7.2). (D) Neuronal death evaluated by trypan blue staining in phase-contrast photographs of cultures 24 h after treatment as in A. (E) Quantified neuronal death ($n = 3$; $*P < 0.05$ vs. NMDA, pH 7.2). (F) Intracellular calcium elevations assessed by Fura-4F, conditions as in A. ($n = 4$; $*P < 0.05$ vs. corresponding pH control; $\#P < 0.05$ vs. NMDA, pH 7.2).

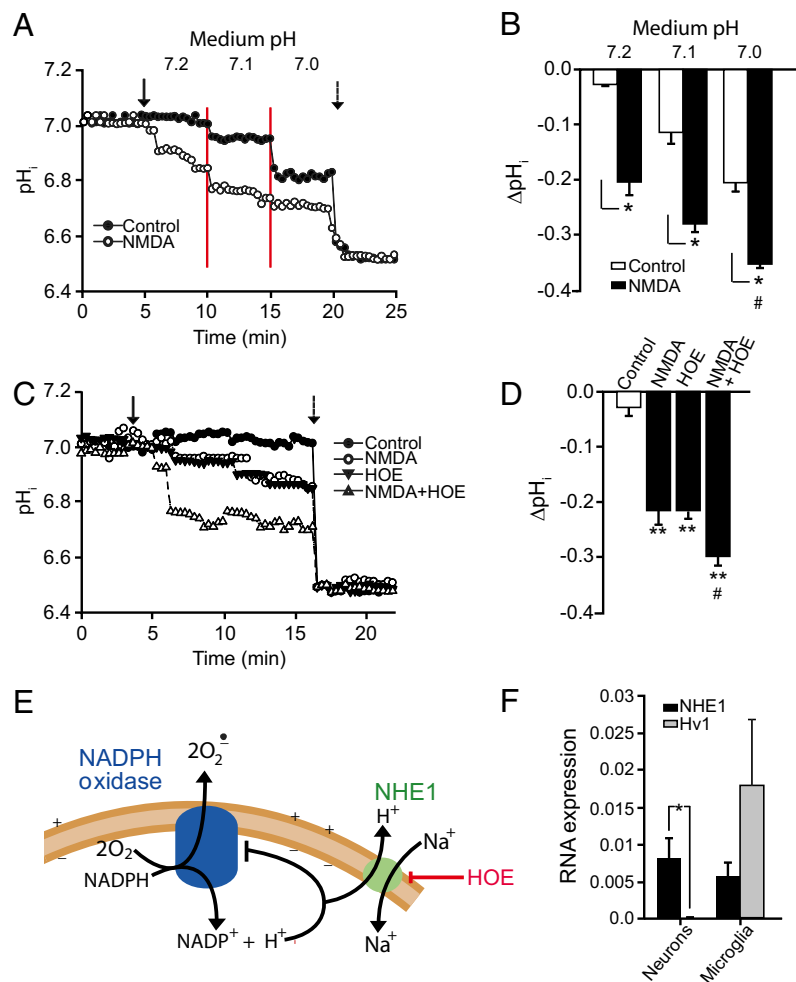


Fig. 2. Effects of NMDA, medium pH, and Na^+/H^+ exchange inhibition on intracellular pH. (A) Representative single-cell BCECF traces show that both NMDA and medium acidification reduce intracellular pH (pH_i), with additive effect. Arrow indicates NMDA addition, red lines delineate stepwise medium acidification, and dotted arrow shows pH 6.5 calibration. (B) Quantified aggregate results; $n = 4$, $*P < 0.05$, $\#P < 0.05$ vs. pH 7.2. (C) Representative single-cell BCECF traces show that both NMDA and the Na^+/H^+ exchange inhibitor HOE (1 μM) reduce intracellular pH and have additive effects. Solid arrow indicates time of NMDA and HOE addition, dotted arrow shows pH 6.5 calibration. (D) Quantified aggregate results; $n = 4$, $**P < 0.05$ vs. control, $\#P < 0.05$ vs. NMDA. (E) Intracellular H^+ inhibits NADPH oxidase. H^+ produced by NADPH oxidase and other sources may exit neurons via the Na^+/H^+ exchanger NHE1. HOE inhibits Na^+/H^+ exchange and thereby produces intracellular acidification. (F) RT-PCR assessment of cultured neurons and microglial shows that, in neurons, gene expression of NHE1 is high relative to the voltage-dependent H^+ channel Hv1. ($n = 4$, $P < 0.01$).

than the medium pH, and to drop further with reductions in medium pH (Fig. 2A and B). NMDA receptor stimulation also reduces pH_i (26), and acidification induced by NMDA was found to be additive with that induced by medium acidification (Fig. 2A and B). We next evaluated the effect of selective acidification of the intracellular space using the Na^+/H^+ exchange inhibitor, 4-isopropyl-3-methylsulfonyl-benzoyl-guanidine-methanesulfonate (HOE). HOE produced an intracellular acidification comparable to that produced by reducing medium pH to 7.0, and this acidification was likewise additive to that induced by NMDA (Fig. 2C and D). Importantly, neurons treated with 1 μM HOE showed a near-complete elimination of NMDA-induced superoxide formation and neuronal death, with no significant reduction in NMDA-induced calcium elevation (Fig. 3A–F).

H^+ ions produced by NOX2 must be transferred to the extracellular space to prevent intracellular acidification and resultant inhibition of NOX2 function (Fig. 2E). In immune cells, in which NOX2 function has been extensively characterized, H^+ translocation occurs through both the voltage-sensitive proton channel Hv1 and the Na^+/H^+ exchanger NHE1 (21–23). A com-

parison of Hv1 and 4-hydroxynonenal (4HNE) gene expression in cultured neurons and microglia showed relatively greater NHE1 gene expression in neurons (Fig. 2F), consistent with the robust effect of HOE.

HOE blocks Na^+ entry as well H^+ efflux, and consequently the observed effects of HOE on superoxide production could alternatively stem from reduced Na^+ entry. If HOE blocks superoxide production by reducing pH_i , then normalizing pH_i should reverse the effect of HOE. This prediction was confirmed: pH_i in neurons treated with NMDA plus HOE was normalized by raising medium pH to 7.5 and, under this condition, HOE failed to prevent NMDA-induced superoxide formation (Fig. S5 and Fig. 3E–H).

Reduced NHE1 Function Prevents NMDA- and Ischemia-Induced Superoxide Formation in Brain. We next used HOE to evaluate the effect of intracellular acidosis on superoxide formation and neuronal death induced by NMDA injection into mouse striatum. NMDA induced a neuronal superoxide signal (ethidium; Eth) in neurons around the injection site within 1 h, and produced neuronal death within 24 h (Fig. 4A–D). By contrast, mice

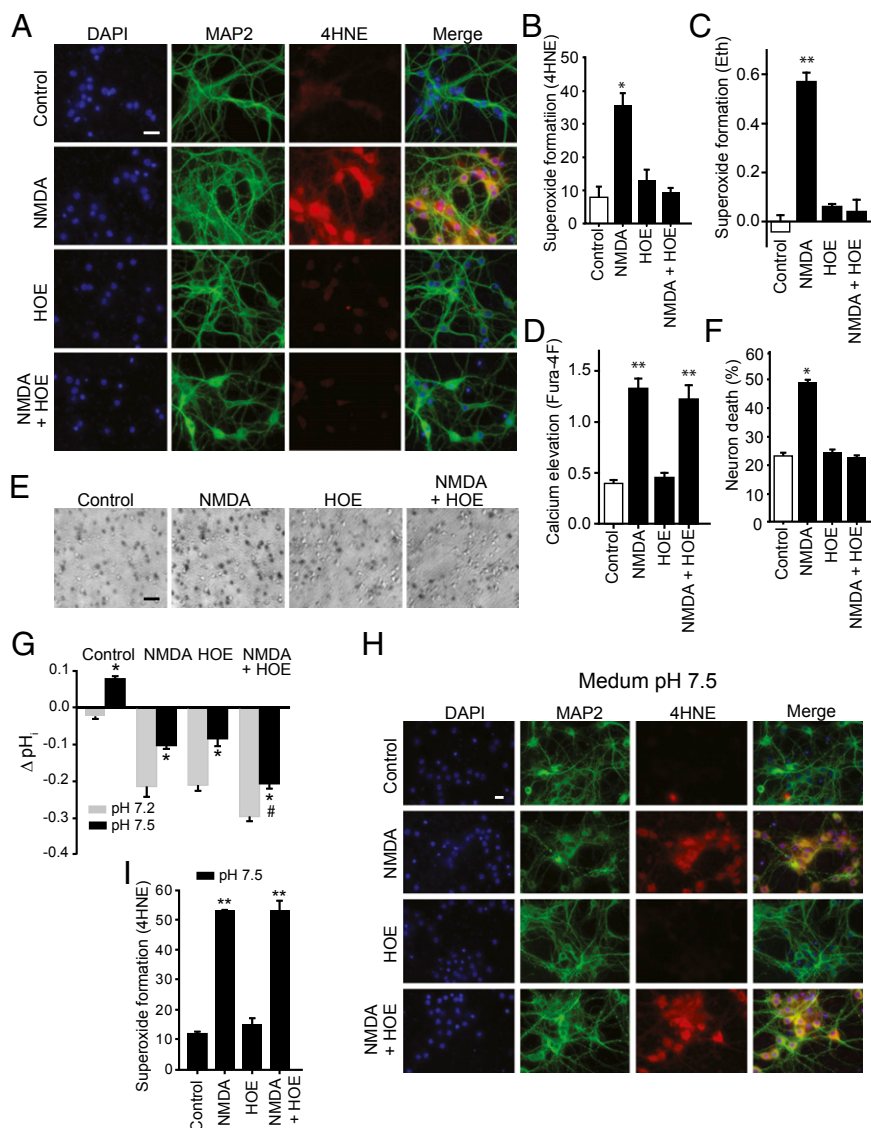


Fig. 3. Na⁺/H⁺ exchange inhibition blocks NMDA-induced superoxide formation and neuronal cell death. (A) Superoxide formation assessed by immunostaining for 4HNE (red). MAP2 (green) identifies neuronal processes, and DAPI (blue) identifies cell nuclei. Neurons were exposed to 100 μM NMDA for 20 min with or without the Na⁺/H⁺ exchange inhibitor HOE (1 μM). (Scale bar: 25 μm.) (B) Quantified 4HNE immunofluorescence (*n* = 4; **P* < 0.05 vs. all other groups). (C) Superoxide formation assessed by Eth formation, conditions as in A; *n* = 4; ***P* < 0.01 vs. all other groups. (D) Calcium elevation assessed by Fura-4F, conditions as in A; *n* = 4; ***P* < 0.01 vs. control. (E) Neuronal death evaluated by trypan blue staining in phase-contrast photographs of cultures 24 h after treatment as in A. (Scale bar: 60 μm.) (F) Quantified neuronal death (*n* = 3; **P* < 0.05 vs. all other groups). (G) Change in intracellular pH induced by 20-min exposure to 100 μM NMDA and 1 μM HOE, with medium pH set to either pH 7.2 or 7.5. (*n* = 4; **P* < 0.05 vs. control, #*P* < 0.05 vs. NMDA). (H) Alkalinization reverses the effect of HOE on NMDA-induced superoxide formation. Formation of 4HNE (red) is not suppressed by HOE at medium pH 7.5. MAP2 (green) identifies neuronal processes. (Scale bar: 25 μm.) (I) Quantification of 4HNE formation (*n* = 4, ***P* < 0.01 vs. control).

coinjecting with NMDA and HOE showed no increase in superoxide formation or cell death over that produced by vehicle injection alone.

NHE1 is the major Na⁺/H⁺ exchanger isoform expressed in brain (27, 28), and HOE is a relatively specific inhibitor of this isoform (29). To confirm the role of NHE1 in this process, we performed NMDA injections in NHE1^{+/-} mice, which have a 70% reduction in brain expression of NHE1 relative to wild-type, NHE1^{+/+} mice (29). NHE1^{+/-} mice, like HOE-treated wild-type mice, showed no increased superoxide formation or neuronal death following striatal NMDA injections (Fig. 4 E–H). Immunostaining for 4HNE formation was limited by technical issues in the NMDA-injected brains.

The release and impaired reuptake of endogenous glutamate during brain ischemia–reperfusion activates NMDA receptors,

and the resulting production of superoxide by neuronal NOX2 is a primary cause of ischemic neuronal death (30–34). Therefore, we also compared superoxide formation after ischemia–reperfusion in wild-type and NHE1^{+/-} mouse hippocampus. Wild-type mice showed a robust and rapid increase in superoxide production in CA1 pyramidal neurons, consistent with prior results (31, 32). By contrast, NHE1^{+/-} mice showed no significant increase in neuronal superoxide production, as measured by either Eth or 4HNE formation (Fig. 5). NHE1^{+/-} mice have previously been shown to have reduced neuronal death after ischemia–reperfusion (29, 35).

Discussion

Superoxide contributes to neuronal injury in stroke and other conditions that lead to NMDA excitotoxicity. The present findings show that NMDA-induced superoxide production is highly

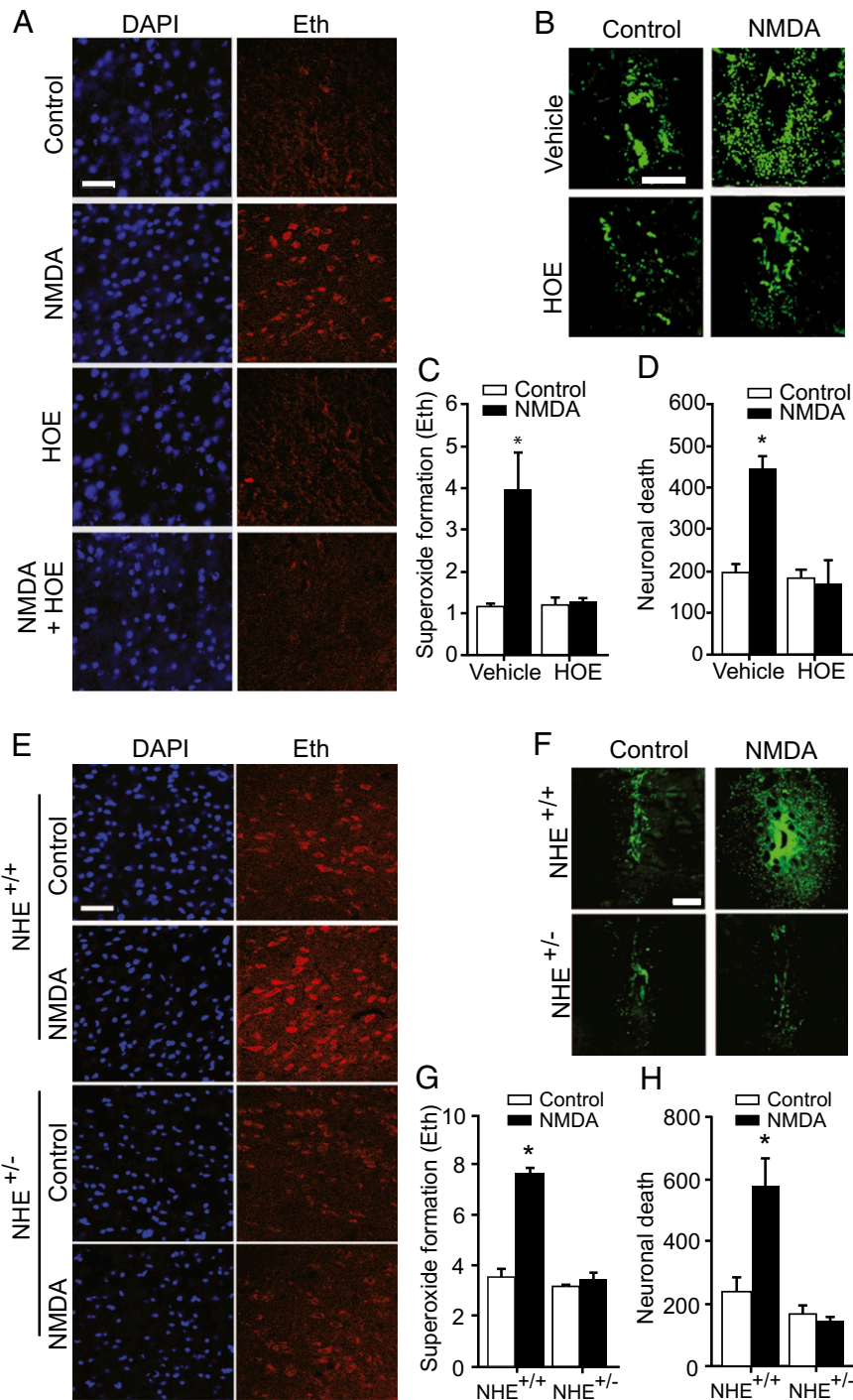


Fig. 4. Reduced Na^+/H^+ exchange prevents neuronal superoxide production after NMDA injections into brain. (*A*) Eth fluorescence (red) in representative striatal sections harvested 30 min after 6 nmol NMDA injections shows reduced superoxide formation in mice coinjected 6 nmol HOE. DAPI provides nuclear counterstaining. (*B*) Degenerating neurons identified by Fluoro-Jade B (green) in striatal sections harvested 1 d after NMDA injections. There is scattered neuronal death adjacent to the needle track in vehicle-injected brains. The more extensive neuronal death in NMDA-injected brains was reduced by HOE. (*C* and *D*) Quantified data; $n = 4$, $*P < 0.05$ vs. all other groups. (*E*) Eth fluorescence (red) in striatal sections harvested 30 min after 6 nmol NMDA injections shows reduced superoxide formation in mice with reduced expression of the NHE1 Na^+/H^+ exchanger (NHE1^{+/-} mice). (*F*) Degenerating neurons identified by Fluoro-Jade B (green) in striatal sections harvested 1 d after NMDA injections. (*G* and *H*) Quantified data; $n = 4$, $*P < 0.05$ vs. all other groups. (Scale bars: 30 μm for *A* and *E*, 100 μm for *B* and *F*.)

sensitive to intracellular pH. Intracellular acidification in cultured neurons by as little as 0.2 pH units, induced by either lowered medium pH or with an NHE1 inhibitor (HOE), prevented NMDA-induced superoxide production and neuronal death. In mouse brain, both HOE and reduced NHE1 expression blocked

superoxide production induced by NMDA or ischemia–reperfusion. Intracellular pH is thus a potent regulator of excitotoxic superoxide production.

Although superoxide can originate from multiple sources including mitochondria, suppression of NOX2 activity by phar-

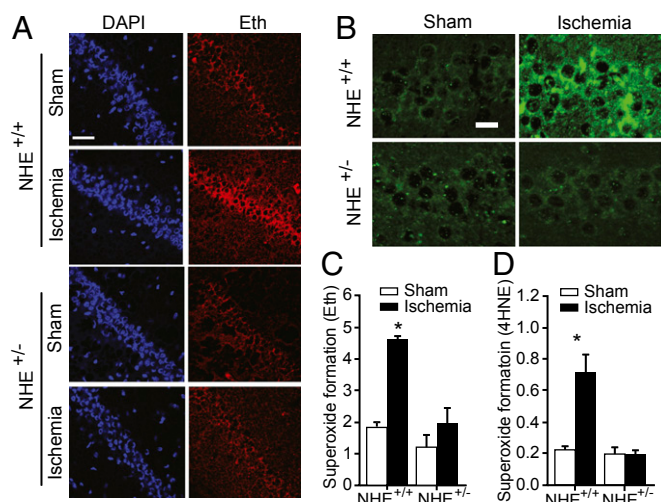


Fig. 5. Reduced Na^+/H^+ exchange prevents neuronal superoxide production after ischemia-reperfusion. (A and B) Eth fluorescence (red) and 4HNE (green) in representative hippocampal sections harvested 30 min after ischemia-reperfusion show reduced superoxide formation in $\text{NHE1}^{-/-}$ mice. (Scale bar: 60 μm in A, 15 μm in B.) (C and D) show quantified data; $n = 4$, * $P < 0.05$ vs. all other groups.

macologic agents, peptide inhibitors, or genetic deletion eliminates both the superoxide production and neuronal death caused by either NMDA receptor activation (Fig. S1; refs. 13–19) and reduce that caused by ischemia-reperfusion (30–32, 34, 36). Eth and 4HNE formation can be induced by factors other than superoxide (37), but the results presented here (Fig. S1) and elsewhere (13, 14, 19) confirm that the Eth and 4HNE formation induced by neuronal NMDA receptor activation are blocked by NOX2 inhibition, and thereby establish their specificity for superoxide under the conditions of these studies. However, both Eth and 4HNE formation are likely insensitive to superoxide production below levels that are normally scavenged by neurons, and superoxide-induced cell death likewise requires a minimum threshold of superoxide production. These threshold effects may partly explain why the relationships between pH and Eth formation, 4HNE formation, and cell death are not identical (Fig. 1).

A neuroprotective effect of mild acidosis in excitotoxicity and ischemia-reperfusion is well established (6, 7). This effect has been postulated to result from the inhibitory effect of extracellular H^+ on NMDA-gated calcium influx (8, 9), but a causal relationship has not been demonstrated. Here we show instead that, in neuronal cultures, both NMDA-induced cell death and NOX2 activation are eliminated by small reductions in medium pH that have minimal effect on NMDA-induced Ca^{2+} influx. Moreover, selective acidification of the intracellular space by the NHE1 inhibitor HOE likewise prevented NMDA-induced cell death and NOX2 inactivation, despite no reduction in NMDA-induced Ca^{2+} influx. These findings argue that the neuroprotective effect of mild acidosis is not mediated by extracellular H^+ on the NMDA receptor, but instead by intracellular acidification. NOX2 activity in nonneuronal cell types has previously been shown to be sensitive to modest intracellular pH reductions (24). The mechanism of this effect is unlikely to be simple mass action, given its steep pH dependence; instead, evidence suggests that H^+ concentration influences the phosphorylation status of p47^{phox} , a cytosolic “organizer” subunit of the NOX2 complex (20, 23).

NOX2 produces extracellular superoxide by transferring electrons from NADPH to O_2 , and in the process generates intracellular H^+ (Fig. 2E). In microglia and other immune cells, the H^+ generated by NOX2 activity is released to the extracel-

lular space through both the voltage-sensitive proton channel Hv1 and the Na^+/H^+ exchanger NHE1, and inhibition of either process causes intracellular acidification and resultant cessation of NOX2 activity (21–23). A distinction between Hv1 and NHE1 is that protons released through Hv1 relieve the plasma membrane depolarization caused by intracellular H^+ accumulation, whereas Na^+/H^+ exchange is electroneutral. The present results identified a much higher ratio of NHE1:Hv1 gene expression in neurons than microglia. The reason for this difference is not evident, but it may be that NMDA-induced neuronal acidification results largely from processes other than NOX2 activation, such as Ca^{2+} -ATPase activity, glycolytic activity, and mitochondrial depolarization (26, 28), that require electroneutral H^+ disposition to avoid a net charge transfer across the cell membrane.

Neurons express bicarbonate-coupled mechanisms of pH regulation in addition to NHE1 (38). However, studies comparing bicarbonate-containing and bicarbonate-free media showed no difference in the effect of medium pH on superoxide formation (Fig. S3), and studies performed *in vivo* support a dominant role for NHE1 (Fig. 4). These findings are consistent with reports that NHE1 is crucial for both bicarbonate-independent and bicarbonate-independent regulation of intracellular pH under acidosis conditions (39).

HOE (also known as cariporide) is a relatively selective inhibitor of NHE1, with an IC_{50} of 0.08 μM (29). The structure of HOE predicts that it does not cross an intact blood-brain barrier (40), necessitating delivery by stereotactic injection in the studies of its effect on acute excitotoxicity *in vivo*. Homozygous $\text{NHE1}^{-/-}$ mice cannot be used because they have seizures, spontaneous mortality, and compensatory changes in gene expression (29); however, $\text{NHE1}^{+/-}$ mice provide a useful complement to the HOE studies because these mice have reduced NHE1 activity. $\text{NHE1}^{+/-}$ mice have previously been shown to have reduced neuronal death after brain ischemia (29). It should be noted, however, that NHE1 deficiency has additional effects that can influence outcome from brain ischemia. Reduced sodium accumulation in $\text{NHE1}^{+/-}$ neurons may promote neuronal survival (29), and reduced sodium accumulation in nonneuronal cells limits brain edema (40). Moreover, suppression of NOX2 activation by NHE1 or Hv1 inhibition in microglia inhibits the innate immune response, and may thereby promote neuronal survival in the postischemic interval (21–23).

It is possible that intracellular acidosis could function in a teleologically advantageous way to limit superoxide production during ischemia. Neurons acidify during ischemia as a result of anaerobic lactic acidosis, in addition to processes induced by NMDA receptor activation. Impaired blood flow prevents removal of accumulated H^+ , and reperfusion clears accumulated H^+ . The present findings suggest that this pH-normalizing effect of reperfusion may explain, in part, why superoxide production is greater in reperused than nonreperused ischemic brain, and why both NOX2 inhibitors and NMDA receptor antagonists are better neuroprotective agents in reperused brain than nonreperused brain (41–43). The degree of intracellular acidification shown here to influence neuronal superoxide production is within the range induced by physiological brain activity (28, 44). Intracellular pH changes may thereby also influence the normal, physiological intercellular signaling mediated by neuronal superoxide production (45).

Materials and Methods

Studies were performed in accordance with protocols approved by the San Francisco Veterans Affairs Medical Center animal studies subcommittee. $\text{NHE1}^{+/-}$ and $\text{NHE1}^{+/+}$ littermate controls on SV129/Black Swiss background were bred as described (29). Both males and females were used, equally proportioned between control and experimental groups. All other studies used wild-type Black Swiss mice (Simonsen). *N*-(diaminomethylidene)-3-methanesulfonyl-4-(propan-2-yl)benzamide (HOE642; abbreviated as “HOE”) was obtained from Sanofi-Aventis (Frankfurt). Cell culture reagents were obtained from Mediatech, and all other reagents were obtained from Sigma-

Aldrich except where noted. Data analyses for all studies were performed by observers blinded to the experimental conditions.

Neuronal Cultures. Primary neuron cultures were prepared as described (46) and used at 10–14 d in vitro, at which time greater than 95% of the cells are neurons (46). Primary microglia were isolated from primary mouse astrocyte/microglial cocultures as described (46). Except where otherwise noted, experiments were initiated by placing the cultures in balanced salt solution (BSS) containing 1.2 mM CaCl₂, 0.8 mM MgSO₄, 5.3 mM KCl, 0.4 mM KH₂PO₄, 137 mM NaCl₂, 0.3 mM NaHPO₄, 5 mM glucose, and 10 mM 1,4 piperazinediethanesulfonate (PIPES), adjusted to the designated pH. Exposures to 100 μM NMDA were performed in BSS with reduced Mg²⁺ (0.4 mM MgSO₄). Where used, drugs were added from concentrated stocks in BSS 10 min before the addition of NMDA. HOE was used at 1 μM, a concentration at which it has high selectivity for the NHE1 isoform of Na⁺/H⁺ exchanger (29).

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