Extracellular Alkalinity Exacerbates Injury of Cultured Cortical Neurons

Rona G. Giffard, MD, PhD; John H. Weiss, MD, PhD; and Dennis W. Choi, MD, PhD

Background and Purpose: We have previously shown that extracellular acidity protects cultured fetal murine neocortical neurons from glutamate toxicity and combined oxygen-glucose deprivation injury, an action at least in part mediated by reduction in N-methyl-D-aspartate receptor activation. We now investigate the effect of extracellular alkalinity on both glutamate neurotoxicity and injury due to combined oxygen-glucose deprivation.

Methods: The effects of extracellular alkalinity during injury induced by exposure of murine neocortical cultures to glutamate (0.5 mM for 5 minutes) or oxygen-glucose deprivation are characterized morphologically and quantitated by efflux of lactate dehydrogenase from both neurons and glia to the bathing medium. Calcium accumulation is measured with calcium-45.

Results: Moderate extracellular alkalinity is well tolerated by cortical cells but significantly potentiates both glutamate neuronal toxicity and oxygen-glucose deprivation neuronal injury. In contrast, glial viability in the face of combined oxygen-glucose deprivation is little affected by extracellular alkalinity. Increased accumulation of calcium-45 during oxygen-glucose deprivation in alkalotic medium and blockade of this increase by MK-801 is demonstrated.

Conclusions: These observations suggest that alkaline pH can exacerbate excitotoxic neuronal injury, most likely because of increased N-methyl-D-aspartate receptor activation. Metabolic alkalosis of any etiology may sensitize neurons to ischemic injury and potentiate reperfusion injury. (Stroke 1992;23:1817-1821)

KEY WORDS • cerebral ischemia • calcium • mice • glutamates

Lactic acidosis accompanies cerebral ischemia, and the effects of extracellular acidity on ischemic brain cells have been studied since the 1950s.1-3 In contrast, little information is presently available about the effects of extracellular alkalinity on ischemic injury. Reperfusion after ischemia causes a sudden pH shift in the alkaline direction,1 leading to transient intracellular alkalinity.4 Areas of chronic infarction have also been shown to be alkaline relative to normal brain.5 An ischemic event may occur during a period of systemic alkalosis due to metabolic or respiratory derangement. In particular, patients with chronic hypertension are at increased risk of stroke, and such patients are often treated with diuretics, which are well known to cause metabolic alkalosis. Recently, a correlation between severity of ischemia and a period of tissue alkalosis after the initial insult has also been suggested.6

The relation between interstitial and intracellular pH is complex. We chose to begin studying this problem by varying the extracellular pH. Here, the effect of extracellular alkalinity on cortical cell injury was determined for mixed neuronal and glial cultures from fetal mouse neocortex and essentially pure (>95% glial fibrillary acidic protein positive) astrocyte cultures from newborn mouse neocortex. We sought first to characterize cellular tolerance for extracellular alkalinity and then to determine if extracellular alkalinity affects the vulnerability of cortical cells to injury due to either glutamate exposure or combined deprivation of oxygen and glucose. We assessed cellular calcium accumulation during injury by adding calcium-45 to the bathing medium during oxygen-glucose deprivation at pH 7.4 and 8.0.

Materials and Methods

Culture medium was obtained from GIBCO, Grand Island, NY, as ×10 concentrated stocks lacking bicarbonate and glutamine; serum was from Hyclone Laboratories Inc., Logan, Utah; all other chemicals were reagent grade or better from Sigma Chemical Co., St. Louis, Mo. MK-801 was a gift from Merck Sharp & Dohme, West Point, Pa. "Ca"2+ was purchased from Amersham Corp., Arlington Heights, Ill. Swiss Webster mice from Simonsen Laboratories, Gilroy, Calif., were handled in accordance with a protocol approved by our institutional animal care committee.

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Cultures were made essentially as previously described,3 with the modification that mixed cultures were prepared by plating the 15–17-day gestation fetal neocortical cell suspension on preexisting glial cultures. Cultures were maintained in humidified 5% CO₂ atmosphere incubators at 37°C. Neocortical glial cultures were prepared from 1–3-day-old mice, plated in medium supplemented with epidermal growth factor (10 ng/ml), and fed once weekly once confluent. Glial cultures were used 10–30 days after plating. Cells were exposed to extracellular alkalinity in a balanced salt solution containing (mM) NaCl (see below), CaCl₂ 1.8, MgSO₄ 0.8, KCl 5.4, NaH₂PO₄ 1.0, phenol red (10 mg/l), and glucose 5.5. N-(2-Hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES) (20 mM, pKₗ 7.5) free acid was titrated with NaOH and was the sole buffer for glutamate toxicity experiments or was used at 10 mM with the addition of that amount of bicarbonate that would result in the desired pH when equilibrated with 5% CO₂ at 37°C for other experiments. Final solution pH was determined after equilibration with 5% CO₂ for solutions containing bicarbonate. In all cases, NaCl was added to maintain constant osmolarity such that total Na⁺ was 143 mM.

For all experiments, the cells were washed into balanced salt solution at the desired pH. Cell damage was evaluated 1 day after exposure to glutamate or oxygen-glucose deprivation by light microscopy (phase-contrast and trypan blue dye exclusion) and by measurement of lactate dehydrogenase (LDH) lysed by subjecting the cultures to freezing at 37°C in an anoxic chamber (Forma Scientific, Marietta, Ohio) with the oxygen concentration maintained at <0.2% as previously described.12 All test medium was preequilibrated with anoxic gas mixture: 5% CO₂, 85% N₂, and 10% O₂. Cells were then washed into deoxygenated medium lacking glucose in the anoxic chamber. At the end of the period of combined oxygen-glucose deprivation, cultures were washed into Eagle’s minimal essential medium (Earle’s salts), containing freshly added glutamine (2 mM), oxygen, and 5.5 mM glucose, and were returned to the normoxic incubator to allow full expression of injury. Assessment of injury was performed the following day. For glial injury by combined oxygen-glucose deprivation, the period of deprivation was terminated by addition of 10 μl of 200 mM glucose per well to bring the concentration to 5.5 mM at the time of removal from the anoxic chamber. Medium overlying the cultures was sampled at the indicated times to quantitate injury.

Assessment of intracellular calcium accumulation was performed by adding 0.2 μCi of ⁴⁰Ca²⁺ to the medium in each well at the beginning of the period of combined oxygen-glucose deprivation or sham wash. After the anoxic incubation, the cultures were washed three times with 0.75 ml of buffer then solubilized in hot 0.2M sodium dodecyl sulfate; aliquots were counted in a

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**TABLE 1. Effect of Extracellular Alkalinity on Glial and Glial Injury by Oxygen-Glucose Deprivation**

<table>
<thead>
<tr>
<th>Condition</th>
<th>pH 7.4</th>
<th>pH 7.7</th>
<th>pH 8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>+O₂, +glucose</td>
<td>5.5±0.3</td>
<td>3.6±0.6</td>
<td>4.5±0.6</td>
</tr>
<tr>
<td>-O₂, -glucose</td>
<td>52.0±4.4</td>
<td>50.7±3.0</td>
<td>54.3±4.4</td>
</tr>
</tbody>
</table>

Values are mean±SEM (pooled from four experiments); n=6–8 cultures per condition. Values are expressed as percent of maximal lactate dehydrogenase (LDH) released by freeze-thaw insult. There was no statistically significant difference between different pH levels within any condition; all oxygen-glucose deprivation conditions were significantly different from all conditions with oxygen and glucose present; p<0.05 by analysis of variance and Student-Newman-Keuls test.

**Results**

The ability of cortical cells to tolerate exposure to extracellular alkalinity was studied in glucose containing salt solutions buffered either with HEPES and bicarbonate or with only bicarbonate. Little effect of extracellular alkalinity was noted on glial cell survival under normoxic conditions after 24 hours (Table 1). Glial injury due to oxygen-glucose deprivation did not depend on whether the extracellular pH was 7.4 or 8.0 (Table 1). Similar results were obtained whether buffer containing only bicarbonate or bicarbonate and HEPES was used. For mixed cultures, 24-hour exposure to pH 8.0 was also generally well tolerated (Figure 2A), although slight injury by lactate dehydrogenase release was occasionally noted (three of six experiments) after this exposure or after 48-hour exposure to pH 7.7 (Figure 1). Blocking

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**FIGURE 1.** Bar graph shows effect of prolonged extracellular alkalinity on mixed cultures. Cultures were exposed to indicated pH levels in balanced salt solution containing both 10 mM HEPES and bicarbonate for 24 or 48 hours before medium was sampled for lactate dehydrogenase (LDH) efflux. Mean±SEM (n=4 cultures per condition) expressed relative to LDH released by 24-hour exposure to 500 μM N-methyl-d-aspartate (=100). *Significantly different from pH 7.4 (p<0.05) at same time point by analysis of variance and Student-Newman-Keuls test.
FIGURE 2. Photomicrographs show alkalinity increases glutamate neurotoxicity. Three sets of sister cultures were washed into balanced salt solution at either pH 8.0 (panels A and C) or 7.4 (panel B). Cultures in panels B and C were then exposed to 500 μM glutamate for 5 minutes, after which all three were maintained in minimum essential medium for 24 hours at pH 7.4. At the end of this time, cultures were stained with trypan blue to identify nonviable cells. Bar represents 50 μm and applies to all three panels.

NMDA receptor activation with MK-801 reduced the injury noted after 24-hour exposure to pH 8.0 to a level not statistically different from pH 7.4.

The effect of extracellular alkalinity on neuronal injury due either to direct application of glutamate or to combined deprivation of oxygen and glucose was then studied. Mixed cultures were exposed to 0.5 mM glutamate for 5 minutes. If this exposure was carried out at pH 7.4, widespread neuronal injury resulted by the next day; exposure at pH 8.0 led to essentially complete neuronal loss (Figure 2). The effect of alkaline pH can also be seen as a shift in the glutamate toxicity concentration-response curve (Figure 3). At glutamate concentrations that cause intermediate levels of injury, significantly greater injury is seen at pH 8.0, but the maximum extent of injury (neuronal death without glial death) caused by high concentrations of glutamate was not altered.

Cultures subjected to combined oxygen-glucose deprivation for 40 minutes sustained only modest injury at pH 7.4 but sustained significantly increased injury at pH 8.0 (Figure 4). No lactate dehydrogenase release was found at the time the insult was terminated (40 minutes), so lactate dehydrogenase was routinely measured the following day after cells had been transferred back into medium containing oxygen and glucose at pH 7.4 to allow for the full expression of delayed injury. As both voltage-gated calcium channels and sodium-calcium exchange could allow increased calcium influx at alkaline pH, we also determined the effect of adding a noncompetitive NMDA antagonist to see if the enhanced vulnerability seen at pH 8.0 was mediated by NMDA receptors. MK-801 was essentially able to prevent the injury observed at either pH (Figure 4).

Since pathological NMDA receptor activation is thought to produce injury in large part by allowing excessive calcium influx, accumulation of calcium was assessed directly by addition of ⁴⁵Ca²⁺ to the extracellular medium for the period of oxygen-glucose deprivation. Cells deprived of oxygen and glucose at higher pH were found to accumulate significantly more calcium (Figure 5); MK-801 blocked ⁴⁵Ca²⁺ accumulation at either pH 7.4 or 8.0. Similar studies carried out on glial cultures revealed only a small total accumulation of ⁴⁵Ca²⁺, which was unchanged by alkaline pH.

Discussion

Both direct glutamate neurotoxicity and combined oxygen-glucose deprivation neuronal injury were exac-
erbated by extracellular alkalinity. Such enhancement of neuronal vulnerability to injury can be explained by increased NMDA receptor-mediated currents, as demonstrated for extracellular alkalinity in previous electrophysiological studies. Consistent with this explanation, we observed that increasing extracellular pH to 8.0 increased the $\text{Ca}^{2+}$ accumulation by cultures deprived of oxygen and glucose and that at pH 8.0 both neuronal injury and $\text{Ca}^{2+}$ accumulation could still be blocked by MK-801. Our previous studies showed a similar pH dependence for NMDA neurotoxicity. Furthermore, injury to glia, which lack NMDA receptors, was not influenced by extracellular alkalinity. These results in cortical neurons are consistent with the findings of Elmerl and Schramm that alkalinity increases glutamate toxicity in cerebellar granule cells.

Extracellular alkalinity might also have injury-potentiating actions unrelated to the NMDA receptor. The effect of extracellular alkalinity during hypoxia has been studied in other cell types, including cardiac myocytes, renal cortical cells, and Ehrlich ascites tumor cells, and was found to be deleterious to all of them. Glycolysis shows marked stimulation as the pH is raised, attributed largely to the pH sensitivity of phosphofructokinase. If glycolysis were accelerated, cells might more rapidly exhaust their energy stores and sustain increased damage. In addition, alkalinity might increase calcium influx via voltage-gated calcium channels or the sodium-calcium exchanger. However, as MK-801 can still fully protect neurons at pH 8.0, any additional injury-potentiating actions of extracellular alkalinity were not sufficient to cause neuronal death in the absence of NMDA receptor activation.

Previous study has indicated that extracellular acidity can reduce the vulnerability of cultured cortical and hippocampal neurons to glutamate neurotoxicity and oxygen-glucose deprivation injury. The protective effect of acidity correlated with a decrease in NMDA receptor-mediated current and decreased $\text{Ca}^{2+}$ accumulation. Thus, the extracellular pH dependence of excitotoxic neuronal injury observed for changes in either direction near normal pH closely parallels the pH dependence of NMDA receptor activation.

While moderate extracellular alkalosis accompanies such clinical situations as metabolic alkalosis, seen commonly with diuretic therapy and nasogastric suctioning, severe metabolic alkalosis with markedly elevated bicarbonate levels also occurs after resuscitation from cardiac arrest, with pH levels as high as 7.9 reported with the use of bicarbonate. This is also a time of potential cerebral ischemia, and the degree of alkalosis in these circumstances could increase the susceptibility of some neurons to ischemic damage.

Furthermore, intracellular alkalosis has been demonstrated in relation to ischemic brain injury both immediately after recirculation and in areas of chronic infarction. The pH of the extracellular microenvironment under these circumstances is not known. The abrupt tissue alkalinization seen on reperfusion after ischemia in vivo has been proposed to be central to reperfusion injury in the liver. This may also be true in
the brain, although further study will be needed to substantiate this possibility. Although cells in culture may not always faithfully reproduce the behavior of their counterparts in vivo, present observations indicate that a possible contribution of alkalosis to ischemic brain injury in vivo merits further study.

Acknowledgment

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Editorial Comment

Using a well-established murine cortical neuronal culture, Giffard et al reported that extracellular alkalinity (i.e., pH 8.0) significantly exacerbates glutamate toxicity and neuronal death induced by combined oxygen-glucose deprivation. MK-801, a noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist reduces alkalosis-induced injury as well as Ca2+ uptake in oxygen-glucose deprived neurons. These authors have also reported earlier that extracellular acidosis, as opposed to alkalosis, reduces NMDA receptor activation, glutamate neurotoxicity, and combined oxygen-glucose deprivation neuronal injury in murine cortical neurons. Although these in vitro findings cannot be equated with those of the complicated events that occur in cerebral ischemia in vivo, they nevertheless indicate the functional dynamics of the neuronal NMDA receptors that can be modulated by extracellular pH. It is known that the connection between pH changes in the brain extracellular compartment, cerebral spinal fluid, and neuro-logical diseases and stroke is a rather complicated and dynamic process. The extracellular pH changes may also be different from those that occur intracellularly after stroke. Thus, further study on the mechanisms underlying the effects of extracellular pH on neuronal injury after stroke is deemed necessary so that strategies for therapeutic interventions can be developed.

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