Acid-induced Death in Neurons and Glia

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Lactic acidosis has been proposed to be one factor promoting cell death following cerebral ischemia. We have previously demonstrated that cultured neurons and glia are killed by relatively brief (10 min) exposure to acidic solutions of pH <5 (Goldman et al., 1989). In the present series of experiments, we investigated the relationship between changes in intracellular pH (pH_i) and cellular viability. pH_i was measured using fluorescent pH probes and was manipulated by changing extracellular pH (pH_o). Homeostatic mechanisms regulating pH_i in neurons and glia were quickly overwhelmed: neither neurons nor glial cells were able to maintain baseline pH_i when incubated at pH_o below 6.8. Neuronal and glial death was a function of both the degree and the duration of intracellular acidification, such that the LD₅₀ following timed exposure to HCl increased from pH_o 3.5 for 10-min acid incubations to pH_o 5.9 for 2-hr exposures and pH_o 6.5 for 6-hr exposures.

Replacement of HCl with lactic acid raised the LD₅₀ to pH_o 4.5 for 10-min acid exposures, but did not change the LD₅₀ for longer exposures: pH_i measurements concurrent with extracellular acidification suggested that the greater cytotoxicity of lactic acid relative to that of HCl was caused by the more rapid intracellular acidification associated with lactic acid. The onset of death after exposure to moderately acidic solutions was delayed in some cells, such that death of the entire cell population became evident only 48 hr after acid exposure. During this latency period, cellular viability indices and ATP levels fell in parallel. The course of death could be experimentally manipulated: hypothermia initiated after the acid exposure attenuated the degree of cell death, indicating that processes occurring during this postinjury period were responsible for demise. Thus, a delayed process of cell death can be initiated *in vitro* by an initial insult other than glutamate exposure, and this process can occur in glia as well as in neurons.

Cerebral hypoxia-ischemia induces lactic acid formation through the accentuation of anaerobic glycolysis. The magnitude of this lactic acid accumulation depends largely upon the preischemic

glucose and glycogen concentrations of the affected tissue (Smith et al., 1986). Local accumulation of lactic acid to cytotoxic levels may play a causal role in the genesis of brain infarction following cerebral ischemia (Meyer and Yamaguchi, 1977; Siemkowitz and Hansen, 1978; Pulsinelli et al., 1982; Nedergaard, 1987).

Several authors have addressed directly the issue of acid-induced cell death. Cultured neurons and astrocytes die following relatively brief exposures to extracellular pH (pH_o) below 5.3 (Norenberg et al., 1987; Goldman et al., 1989). *In vivo*, exposure of rat parietal cortex to lactic acid below pH_o 5.3 for 20 min caused frank brain infarction (Kraig et al., 1987). However, such extremes of tissue pH may not be encountered during *in vivo* global or focal ischemia, unless the animals are exposed to marked degrees of preischemic hyperglycemia (Kraig and Chesler, 1990). Rather, tissue pH may be moderately lowered for many hours in those areas of focal ischemia that ultimately necrose (Kobatake et al., 1984; Nedergaard et al., 1990). This article examines the possibility that the prolongation of acid exposure does in fact lower the threshold for acid-induced cell death, thereby providing a mechanism by which brain tissue might infarct at pH levels within the range achieved *in vivo* during ischemic acidosis.

To test this hypothesis, neurons and glial cells were loaded with pH-sensitive dyes and exposed to extracellular acid for periods ranging from 10 min to several hours. Neuronal and glial intracellular pH (pH_i) was determined by microspectrofluorometry and was correlated with cell viability, as assessed by trypan blue exclusion. The results indicate that the cellular LD₅₀ for H⁺ ions is an inverse function of the duration of acid exposure.

Materials and Methods

Tissue dissociation and culture. Mixed forebrain cultures were derived from 16-d-gestation rat embryos and prepared as previously described (Goldman et al., 1989); 10⁶ cells were plated in each of 24 wells in poly-L-lysine treated plates (Falcon). For pH_i measurements, 2 × 10⁶ cells were plated on poly-L-lysine coated 25-mm round coverslips maintained in 35-mm Corning dishes. The cultures were kept at 37°C in 5% CO₂ humidified air. Cultures were exposed to acid after 8–10 d *in vitro* (DIV), unless otherwise stated.

Culture medium. The culture medium consisted of 20% fetal calf serum and 80% Dulbecco's modified Eagle's medium, supplemented with 4 mM L-glutamine, 100 μM nonessential amino acids, 8 mg/ml D-glucose, 5 μg/ml insulin, 20 U/ml penicillin-G, 20 μg/ml streptomycin, and 50 ng/ml amphotericin. Half-volume media changes were performed every third day.

Acid exposure. Following an 8–10-d incubation period, test cultures were exposed to HEPES-buffered Ringer's solution (HBS), titrated with HCl to a pH within the range of 2.2–7.3. Each culture was washed three times in the appropriate acidified HBS, then exposed to the test solutions from 10 to 360 min in a CO₂-free incubator at 37°C. The test solutions were changed every 2 hr during those acid exposures exceeding 120

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min. Following acid exposure, each culture was washed once in fresh medium and left for 24 hr in the incubator before viability counting, unless otherwise indicated. The HEPES-buffered solution was composed of 150 mM NaCl, 1 mM MgSO₄, 1 mM K₂HPO₄, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid), 10 mM glucose, and 1.5 mM CaCl₂. In some experiments, 20 mM sodium L-lactate was substituted for 20 mM NaCl. Solutions containing CO₂ were bubbled with 5% CO₂/95% air for 30 min before titration with HCl. Extracellular pH was determined with an electronic pH meter (Beckman Instruments, model 55), in combination with a Microelectrodes MI-410 pH probe. Both were reliable to 0.01 pH unit. All pH measurements were made at 37°C.

Hypothermia. The effect of lowered incubation temperatures upon acid-induced injury was examined by lowering the ambient incubation chamber temperature to 32°C after acid exposure at 37°C. After the exposure, one set of cultures was kept in this incubator for 4 hr and then transferred to an incubator at 37°C.

Viability criteria and index. Loss of membrane integrity, as determined by trypan blue inclusion, was used as a marker of cell death (Phillips, 1969; Choi et al., 1987; Goldman et al., 1989). A total of 496 cultures were exposed to acidified solutions, spanning a wide range of pH_i and exposure duration, as noted above. After appropriate postexposure survival times, the cells, both adherent and detached, were counted by hemocytometer, following 15 min incubation in trypan blue as previously described (Goldman et al., 1989). Neurons and glia were pooled for this determination, as no effort was made to assess their differential sensitivities to acidosis.

In a separate experiment, lactate dehydrogenase (LDH) release into the culture medium was correlated to trypan blue-stained cells in the same cultures. LDH activity was measured spectrophotometrically (Schnaar et al., 1987). The percentage cell death was defined as the amount of LDH released into the culture medium, divided by LDH activity in lysed sister cultures. The lysis buffer used for the latter cultures was composed of 1 ml of 0.1 M phosphate buffer containing 1% Triton X-100.

Metabolic measurements. ATP concentrations were measured by the enzyme-fluorometric method described by Lowry and Passonneau (1972) and expressed as μmol/gm protein. Briefly, 0.1 ml of ice-cold 3 M perchloric acid was added to each well for 15 min, then diluted with 20 μl of 8 M EDTA. The mixtures were removed from the dish and centrifuged at 5000 × *g* for 10 min, and the supernate was neutralized to pH 6.8–7.0 with a mixture of 2 M KOH, 0.4 M imidazole, and 0.4 M KCl. The mixtures were centrifuged at 5000 × *g* for 10 min, and the supernates were stored at –80°C until analyzed. Protein content was measured in sister cultures using the Bio-Rad Coomassie-based protein assay, with a bovine serum albumin standard. Protein was collected by applying 0.4 ml of boiling distilled water to each cell well; the contents were then suctioned off and boiled for 5 min before frozen storage and measurement.

Immunocytochemistry. Immunocytochemical stains for glial fibrillar acidic protein (GFAP; Sigma clone GA-5) and neurofilament protein (Sigma clone NN-18) or microtubule-associated protein (MAP-2; Sigma clone HM-2) were used to identify astrocytes and neurons, respectively. Procedures were as previously described (Goldman, 1989). In selected experiments, phase-contrast photomicrographs were taken immediately after pH measurement and were used to reidentify the measured cells following immunohistochemistry.

Intracellular pH measurements. Cytosolic pH was determined in a total of 79 cultures, by microspectrofluorometry of cells loaded with pH-sensitive fluorescent dyes. These dyes are esterified compounds, which are all membrane permeable. Once gaining cellular entry, the dye esters are hydrolyzed by cytosolic esterases to membrane-impermeant ions (Rink et al., 1982). Three dyes were used in this study (Molecular Probes), each employed over its effective pH range, as determined by its p*K*_a: BCECF (2',7'-bis-[2-carboxyethyl]-5-[and-6] carboxyfluorescein), DCF (5-[and-6]-carboxyfluorescein), and CDCF (5-[and-6]-carboxy-2',7'-dichlorofluorescein). BCECF has a p*K*_a of 7.0 (Rink et al., 1982); DCF, a p*K*_a of 6.4 (Thomas, 1986); and CDCF, a p*K*_a of 4.2 (Nedergaard et al., 1990). The cells were dye loaded by incubation in either 5 μM BCECF acetoxymethyl ester (AM) for 15 min, 1 μM of DCF AM for 10 min, or 1 μM CDCF diacetate for 10 min, all at 37°C. Following dye incubation, the culture-bearing coverslips were washed in HBS and mounted in a Leiden chamber maintained at 37°C by a TC-102 temperature controller (Medical System Corp.). All changes of solutions bathing the cultures were done by injection through a syringe,

and excess medium was aspirated; complete solution exchange was accomplished in less than 5 sec. All measurements of pH_i were made in HBS. Intracellular pH calibration standards for individual cells were established by exposing the cultures, after measurement, to a potassium Ringer's solution containing 10 μM of the ionophore nigericin. Nigericin in combination with high potassium equalizes extracellular and intracellular pH (Chaillet and Boron, 1985). The potassium Ringer's solution employed consisted of 70 mM KCl, 10 mM glucose, 1 mM CaCl₂, 2.5 mM K₂HPO₄, 10 mM HEPES, 1 mM MgSO₄, and 90 mM sucrose.

Fluorescence from individual neurons and glia, as well as from small cellular aggregations, was monitored by a microspectrofluorometer. Measurements were obtained from a 10-μm-diameter field with an adjustable field aperture placed in front of the photomultiplier tube. In all measurements of neurons, we focused upon the cell body. As a result of poor dye uptake among glia, relative to their neuronal counterparts (Connor et al., 1987), as well as to the thin, well-spread topography of cultured astrocytes, the contamination of neuronal signal by glial counts was low. Also, we attempted to measure neurons positioned in areas free of underlying glia. Monochromatic excitation was accomplished using a Photon Technology Delta Scan, in which a xenon arc beam is split by a rotating chopper into two beams, each of which passes through a narrow-range monochromator (4-nm band width), which permits precise wavelength selection. In this study, dual excitation at 495 nm (pH sensitive) and 440 nm (pH insensitive) was used for both BCECF and CDCF, while DCF was excited at 487 nm (pH sensitive) and 450 nm (pH insensitive). The Delta Scan was attached to a Leitz Diavert microscope equipped with a Nikon 40× 1.3-NA Fluor objective. Emissions of >510 nm were measured for each excitation wavelength, for 4.5 msec of every 10-msec cycle. The ratio of emission intensities at the 495- and 440-nm excitation wavelength was then calculated (the 495:440 ratio); these measurements were integrated over 1 sec and stored in an NEC 386 Powermate computer. Intracellular pH was then calculated by the formula

$$\text{pH} = \text{p}K_a + \log((R - R_{\min})/(R_{\max} - R)),$$

where *R* is the ratio measured at the given pH, and *R*_{min} and *R*_{max} are the limiting values of this ratio at the extremes of acid and alkaline pH, respectively. For CDCF, *R*_{max} = 8.4 and *R*_{min} = 2.7. CDCF was used in the pH range 3.5–5.3. DCF was used in the pH range 5.4–6.4, and BCECF was used in the pH range 6.5–7.3; within these respective ranges, the relation between *R* and pH is linear, thus allowing a simple transformation of the signal-intensity ratio into pH.

Statistical analysis. All results are expressed as the mean ± SD. One-way analysis of variance (ANOVA) was used to compare mean values of ATP content and viability index among groups (see Fig. 5), while Tukey's multiple range test was used to determine which groups significantly differed from control values. Two-tailed Student's *t* test was used to analyze differences among paired groups in Tables 2 and 3. LD₅₀ was determined using probit analysis, in which the percentage of cells killed was transformed according to a probit transformation. Probit was regressed on pH, and LD₅₀ was estimated by inverting the regression equation at probit = 0. Observations with percent killed = 0 or 100 were assigned probits of –5 and +5, respectively.

Results

Comparison among viability determination methods. LDH release to the culture medium 24 hr following acid exposure (pH 4.1–7.3) was compared to the viability index determined by trypan blue uptake in 56 cultures in a separate experiment (Fig. 1). A high correlation (*r* = 0.92) was found between these independent modalities of viability assessment.

Cellular viability following 10-min HCl exposure. Twenty-four hours following acid exposure, cultures exposed to HCl at pH_i 3.5 and above retained normal morphology and excluded trypan blue (Fig. 2A). In contrast, cells exposed to pH_i below 3.1 underwent process retraction and somal rounding, with detachment from their substrate and trypan blue inclusion. Probit analysis of cellular viability yielded an LD₅₀ for HCl of pH_i 3.3.

Cellular viability following 10-min lactic acid exposure. HBS solutions containing 20 mM lactic acid were titrated over a pH range of 4.0–7.3. The cells were exposed for 10 min to the test

Figure 1. Validation of viability index. Cellular death as used in this article is indicated at the *x*-axis and is defined as the percentage of cells stained with trypan blue 24 hr after the exposure. LDH release to the culture medium is indicated at the *y*-axis. % LDH release was defined as LDH activity in culture medium divided by LDH activity from lysed sister cultures. The regression line of LDH release as a function of trypan blue-stained cells described $y = 3.96 + 1.02x$; $r = 0.92$.

acid solution. Virtually all cells exposed for 10 min to pH_e 4.4 or below incorporated trypan blue when stained 24 hr following exposure (Fig. 2B). In contrast, most cells survived such exposure to pH_e over 4.8 (Fig. 3). Probit analysis yielded an LD_{50} for lactic acid of pH_e 4.6.

Cellular viability following 10-min exposure to acidified culture media. Exposure to HCl-acidified culture media, rather than acidified HBS, raised the LD_{50} from pH_e 3.3 to 4.1 ($n = 32$). Addition of 20 mM lactate to the culture medium increased the threshold from pH_e 4.6 to 4.9 ($n = 27$).¹

¹ These data confirm an earlier report (Goldman et al., 1989) in which culture medium was used as the vehicle for acid exposure, rather than the HBS used in the present study. Unlike HBS, the culture medium is buffered with $\text{HCO}_3^-/\text{CO}_2$. CO_2 is a highly membrane-permeable weak acid, capable of mediating rapid intracellular acidification (Roos and Boron, 1981). Thus, the cellular LD_{50} s for acid injury were found to be at higher pH levels (lower acid concentrations) in bicarbonate-buffered media than in HBS.

Figure 2. Determination of threshold for acid-induced cell death. The cultures were exposed for 10 min to HBS titrated to the pH levels noted on the *abscissa*. Cellular death is indicated on the *y*-axis and is defined as the percentage of cells stained with trypan blue 24 hr after exposure. Neurons and glial cells were pooled for this determination. *A*, HBS titrated with HCl: $\text{LD}_{50} = \text{pH}$ 3.3. *B*, Lactate (20 mM) added to the HBS titrated with HCl: $\text{LD}_{50} = \text{pH}$ 4.6.

Intracellular acidification by extracellular HCl. Using CDCF, pH_i was measured in single neurons and glial cells. Exposure to HCl in HBS at the LD_{50} of pH_e 3.3 induced a slow, steady decline of pH_i , which reached 3.52 ± 0.18 ($n = 5$) in neurons and 3.53 ± 0.33 ($n = 4$) in glia by the end of the 10-min acid exposure (Fig. 4A, Table 1). pH_i rapidly normalized when the acidified HBS solution was replaced by one with a normal pH (7.3).

Intracellular acidification by extracellular lactic acid. Exposure of cells to HBS containing lactate at its threshold (LD_{50})

Table 1. pH_i during acid exposure

Exposure time	pH _e	pH _i	
		Neurons	Glia
10 min			
Without lactate	3.3	3.52 ± 0.18 (5)	3.53 ± 0.33 (4)
With lactate	4.6	4.48 ± 0.12 (10)	4.48 ± 0.10 (4)
2 hr			
Without lactate	5.4	5.58 ± 0.04 (9)	5.64 ± 0.05 (8)
Without lactate	5.6	5.87 ± 0.05 (9)	5.90 ± 0.02 (12)
With lactate	5.6	5.87 ± 0.08 (12)	5.93 ± 0.09 (12)
Without lactate	6.0	6.12 ± 0.08 (11)	6.02 ± 0.06 (9)
4 hr, without lactate	6.1	6.01 ± 0.06 (11)	6.10 ± 0.15 (17)
6 hr, without lactate	6.4	6.46 ± 0.09 (15)	6.40 ± 0.11 (14)

Data are presented as mean $\pm$ SD; numbers in parentheses are number of observations. The measurements of pH_i were performed at the end of the exposure periods. With lactate: 20 mM sodium lactate; without lactate: 0 mM sodium lactate.

Figure 4. pH_i during acid exposure. *A*, Ten minutes exposure to pH 3.3 HCl in HBS in an astrocyte. pH_i declines slowly to 3.5 and returns quickly to baseline pH_i upon normalizing pH_e to 7.3. *B*, Neuron exposed to pH 4.6 in an HBS containing 20 mM lactate. The cell rapidly acidifies to pH 4.45. CDCF, with a pK_a of 4.2, was used as the pH probe in these experiments.

pH_e of 4.6 induced a rapid intracellular acidification. pH_i stabilized at 4.6 ± 0.21 after 2 min in both neurons (Fig. 4*B*) and glia. Over the subsequent 8 min, pH_i slowly declined to 4.48 ± 0.12 in neurons ($n = 10$) and 4.48 ± 0.10 in glia ($n = 4$). pH_i quickly returned to baseline upon normalizing pH_e . Thus, pH_i declined to levels of roughly 4.5 and 3.5 following cellular exposure to HBS solutions titrated to pH_e 4.6 and 3.3. These points represent the LD_{50} pH_e values for 10-min exposure to lactic acid and HCl, respectively.

The apparent difference between the LD_{50} for HCl and lactic acid solutions may reflect the more rapid intracellular acidification achieved by lactic acid, and therefore the longer duration of toxic intracellular acidosis.

Cellular viability following 2–6 hr acid exposure. In order to test the possibility that acid-induced death might be a function of both the degree and the duration of intracellular acidification, viability was assessed among cells exposed to acidified solutions, at various pH levels and for varying durations of time including

Figure 5. Determination of thresholds for acid-induced cell death. Cellular death is indicated on the y -axis and is defined as the percentage of cells stained with trypan blue. The cultures were exposed to HBS titrated to the pH levels noted on the $abscissa$. *A*, $LD_{50} = pH$ 5.6; 2-hr exposure. *B*, $LD_{50} = pH$ 6.1; 4-hr exposure. *C*, $LD_{50} = pH$ 6.4; 6-hr exposure.

2, 4, and 6 hr, and examined for cell survival 24 hr later. Figure 5*A–C* illustrates cellular viability among cultures assessed 24 hr after acid exposure: LD_{50} varied as a function of the time and degree of acid exposure, such that 50% of cells died following each of several paradigms, including incubation at pH_e 5.6 for 2 hr, pH_e 6.1 for 4 hr, and pH_e 6.4 for 6 hr. Using DCF, it was found that a minor proton gradient was maintained at threshold pH_e (LD_{50}) in both cell types (Table 1).

No difference was observed in viability between solutions with or without lactate at extended exposure times (Table 1). Buffering of the acid solution with bicarbonate instead of HEPES did not affect cellular viability with long (2 hr) exposure times (Table 2).

Figure 3. Long-term course of 10-min exposure to an HBS containing 20 mM sodium lactate titrated to various levels of pH . *A*, Control exposed to pH 7.3. *B*, Culture exposed to pH 4.9. Both neurons and glial cells are morphologically indistinguishable from those in the control culture. *C*, Astrocytic process retraction, with membrane ruffling and substrate denudement after exposure to pH 4.5. Some neurons show normal morphology, indicating a slightly higher tolerance toward acidity. *D*, Culture exposed to pH 3.9. Very few cells, if any, remain viable. These phase-contrast photomicrographs were taken 24 hr after acid exposure. Scale bar, 25 μm .

Figure 6. ATP content and death after 10-min exposure to pH 7.3, 4.9, 4.5, and 3.9 (A–D, respectively). ATP content and viability remained normal after exposure to either pH 7.3 or pH 4.9. Exposure to pH 4.5 triggered a process of delayed death, with ATP content and viability normal until 2 hr after the exposure. First, after 6 hr, death increased to significant levels as compared to control, whereas ATP content showed an insignificant decline at the same time point. Loss of viability progressed until the next day, when 100% of the cells were dead and ATP was unmeasurable. In contrast, loss of viability and ATP content occurred instantaneously after exposure to pH 3.9. ANOVA was used to compare the groups, and Tukey's multiple comparison test was used to determine which groups were significantly different from control. Data points with asterisks were significantly different from control (culture at same time point exposed to pH 7.3 for 10 min), with $p < 0.05$, $n \geq 4$ for all values. ATP content is given in percent of control values obtained from sister cultures.

Temporal profile of viability and ATP content. Figure 6A–D illustrates those changes in cellular viability and ATP content occurring after 10-min exposures to varying degrees of lactic acidosis. ATP concentrations in normal cultures averaged 27.5

Table 2. Influence of 2-hr exposure of lactate and CO_2 on cell viability

Treatment	pH_e	Percent dead	
		With	Without
Lactate	5.7	47 ± 8 (7)	52 ± 7 (9)
	7.3	3 ± 4 (4)	5 ± 5 (4)
$\text{CO}_2/\text{HCO}_3^-$	5.5	61 ± 6 (6)	64 ± 4 (6)
	7.3	5 ± 3 (4)	4 ± 2 (4)

Data are presented as mean ± SD; numbers in parentheses are number of observations.

* $p < 0.01$, paired t test.

± 4.9 $\mu\text{mol/gm}$ protein ($n = 65$; range, 16.8–38.1 $\mu\text{mol/gm}$ protein). Baseline ATP content and viability were unchanged for 48 hr following exposure to both pH_e 7.3 and pH_e 4.9 (Fig. 6A,B). In contrast, ATP and cellular viability remained normal only initially following exposure to pH 4.5. At 6 hr, an insignificant decrease of ATP was first evident, and viability was significantly ($p < 0.05$) diminished, with $31 \pm 4\%$ ($n = 6$) of the cells incorporating trypan blue (Fig. 6C). By 12 hr following exposure, significant drops were evident in both ATP levels and viability index; by 24 hr, $80 \pm 10\%$ ($n = 11$) of the cells were dead, and ATP levels had fallen to $7 \pm 9\%$ ($n = 4$) of control values. By 48 hr, 100% of the cells were dead, and ATP was unmeasurable (Fig. 6C). Viability and ATP content were both lost when assessed 30 min after exposure to pH_e 3.9 (Fig. 6D).

Regulation of pH_i in an acid environment. When pH_e was lowered from 7.3 to 6.8 (Table 3, Fig. 7A–C), all neurons regained normal pH_i , while no glia did. Five of nine neurons initially acidified, but then slowly normalized for pH_i over the next 30–40 min, despite sustained extracellular acidification ($n = 9$; Fig. 7B). Four of nine neurons maintained normal pH_i during the lowering of pH_e to 6.8 (Table 3, Fig. 7A). In contrast, all glia ($n = 7$) acidified and showed little tendency towards normalization of pH_i during the 25-min observation period (Fig. 7C). All neurons and glia alkalized in a rebound fashion to levels higher than the initial pH_i , once the pH_e was returned to 7.3 (Table 3).

The pH_i in both neurons and glia after prolonged exposures (2, 4, and 6 hr) to toxic levels of acid, with or without lactate, was invariably slightly higher than pH_e (Table 1). Thus, the threshold for acid-induced death does not represent a discrete acid level at which pH_i homeostasis is suddenly lost in stepwise fashion. Acid-induced cell death is rather a function of both the time and the degree of intracellular acidification. Addition of 20 mM lactate to HBS did not cause a significant change in the degree of intracellular acidification at 2 hr (Table 1).

Effects of incubation temperature. Matched series of lactic acid exposures were performed at 37°C, including both 10- and 120-min exposures to LD_{50} pH_e . After acid exposure, one set of cultures from each series was maintained at 32°C for 4 hr and at 37°C for the remaining 20 hr. The other set was kept at 37°C for the entire 24 hr. The 4-hr period of postacidification hypothermia improved cell viability after both short and long acid exposures (Table 4).

Sensitivity to acid as a function of cellular maturation. Cultures exposed to acid at various ages, ranging from 2 to 30 DIV, were equally vulnerable to acid exposure (Fig. 8).

Figure 7. pH_i during exposure to pH_e 6.8 in three representative cells. *A*, pH_i remained normal during the exposure, but increased to pH 7.4 upon terminating the exposure (*neuron*). *B*, pH_i acidified to 6.7 during exposure to an HBS with pH_e 6.8, but pH_i normalized within 1500 sec. An abrupt alkalinization was evident when the acid exposure was terminated (*neuron*). *C*, This glial cell showed little tendency for pH_i normalization during the observation period. The cell alkalinized upon returning to pH_e .

Discussion

Traditionally, the homeostatic mechanisms subserving cellular pH_i have been thought of as tightly regulating pH_i . The buffer capacity of brain cells is estimated at approximately 20 mM

Figure 8. Determination of threshold for acid-induced cell death. Cultures at various DIV were exposed for 10 min each to an HBS titrated to the pH levels noted on the *abscissa*. Cellular death is indicated on the *y-axis* and is defined as the percentage of cells stained with trypan blue 24 hr after exposure. There was no significant influence of DIV on the threshold for acid-induced death.

(Moody, 1984), with the result that approximately 20,000 protons are buffered for each free hydrogen ion appearing in the cytoplasm. In addition, such pH regulators as the proton/hydrogen exchanger are effective proton extruders; proton transport rates increase exponentially with the degree of intracellular acidification (Moolenaar, 1986). Nevertheless, when the proton influx exceeds the cellular capacity for buffering and proton extrusion, net intracellular acidification occurs. The present study indicates that the pH_i homeostatic mechanisms are readily overwhelmed in neurons, and even more so in astrocytes during exposure to extracellular acidosis. pH_i always fell significantly at pH_e lower than 6.8. Furthermore, this study demonstrates that the threshold for acid-induced death in both cell types does not represent a fixed level of acidosis at which pH_i homeostasis is lost in stepwise fashion; cell death is rather a combinatorial function of both the time and the degree of intracellular acidification, such that LD_{50} increases from pH_i 3.5 for 10-min exposures to pH_i 6.5 after 6-hr acid exposures.

Local accumulation of lactic acid has been implicated in the development of ischemic tissue damage (Plum, 1983). This study demonstrated that a degree of intracellular acidity, spanning the range of pH_i 5.5–6.5, which is achieved during *in vivo* normoglycemic-ischemia (Smith et al., 1986), kills both neurons and glia after 2–6 hr incubation *in vitro*. Intracellular acidosis may therefore be deleterious to cells during focal ischemia, in which the intracellular hydrogen ion concentration is elevated for hours (Nedergaard et al., 1990). We have no evidence suggesting that lactate in itself is harmful. Control cultures exposed to 20 mM lactate at normal pH_e for up to 6 hr were undamaged. Rather, the rapid intracellular acidification obtained with lactate-containing solutions, as compared to HCl-titrated HEPES buffer,

Table 3. Changes in pH_i during exposure to pH_e 6.8

	pH_i Baseline	Decline	Recovery	Final	Time (sec)
Neuron (n = 9)	6.95 ± 0.12	6.80 ± 0.15	6.94 ± 0.12	7.26 ± 0.14	1625 ± 663
Neuron ^a (n = 4)	6.89 ± 0.10	—	—	7.16 ± 0.08	—
Glial cell (n = 7)	7.09 ± 0.09	6.78 ± 0.08	6.82 ± 0.09	7.15 ± 0.18	1551 ± 203

^a Neurons that did not change pH_i during exposure to media acidified to pH_e 6.8.

Table 4. Effect of incubation temperature after acid exposure

Exposure time	pH _e	Percent dead	
		32°C	37°C
10 min	4.4	46 ± 11 (10)	99 ± 3* (7)
	4.6	23 ± 5 (5)	57 ± 7* (5)
	7.3	6 ± 4 (8)	4 ± 3 (8)
2 hr	5.6	9 ± 4 (4)	46 ± 6* (4)
	5.8	7 ± 5 (6)	31 ± 10* (6)
	7.3	1 ± 2 (3)	4 ± 3 (4)

Data are presented as mean ± SD; numbers in parentheses are number of observations. All solutions contained 20 mM sodium lactate.

* $p < 0.01$, paired t test.

might explain why the pH_e LD₅₀ rose from 3.3 to 4.6 for 10-min exposures in the presence of lactate. The combination of hypoxia and acidosis has recently been shown to lower further the pH threshold for irreversible injury (Tombaugh and Sapolsky, 1990). Thus, acidosis might also play a role in injury associated with relatively brief episodes of transient global ischemia. Interestingly, moderate acidosis decreases glutamate neurotoxicity in cultures, presumably through a reduction in the NMDA-associated channel conductance (Tang et al., 1988; Giffard et al., 1990).

Cellular vulnerability to intracellular acidification presumably is a product of the many cellular processes sensitive to changes in pH_i. Glycolysis, protein synthesis, Ca²⁺ buffering, adenylate cyclase activity, and mitosis have all been shown to be exquisitely sensitive to pH (Busa and Nuccitelli, 1984). In addition, severe acidosis causes protein denaturation: the excess of intracellular protons binds to available negative groups on proteins, especially the imidazole moiety of histidine (Tanford, 1968). The resulting structural instability of the proteins and concurrent alteration of covalent bonds cause irreversible denaturation (Tanford, 1968). Available data indicate that cellular proteins vary considerably in their stability at low pH. Some proteins, such as lysozyme, retain their native conformation below pH 2, while others such as glyceraldehyde-3-phosphate unfold below pH 4, irreversibly losing their enzymatic activity (Tanford, 1968).

We observed two patterns of cellular death. Exposure to moderate levels of acidity induced a process of cell injury with long latency. The dying cells excluded trypan blue for several hours after the exposure, and their ATP level remained normal during this period. Nevertheless, this population of cells began to lose viability after 6–24 hr, such that within 48 hr following acid exposure, all cells incorporated trypan blue and lost their intracellular ATP stores. During this period, viability loss and ATP decline occurred in parallel. This delayed process of cell injury contrasted with that in cells exposed to highly acidic solutions, in which all cells died within 30 min after the exposure. This rapid loss of viability and ATP content following exposure to low pH suggests direct disruption of the cell membrane. Lipid components of the plasma membrane might be expected to be very resistant to low pH: models of planar lipid bilayers, such as the lecithin-cholesterol membrane and phospholipid bilayers, resist exposure below pH 2 without conformational changes (Ohki and Goldup, 1969; Gutknecht, 1987). Most likely, the denaturation of structural and integral proteins under these highly acidic conditions might cause secondary disruption of and leakage through the plasma membrane.

The mechanism behind the slow process of cell death is unknown. Membrane disruption, energy depletion, and metabolic derangement are all likely participants. In addition, irreversible denaturation of a cohort of key regulatory proteins, in combination with a failure in protein synthesis, might even prematurely trigger a genetically predetermined process of programmed cell death. The present study demonstrates that slow cell death can be initiated *in vitro*, and that this process can occur in glial cells as well as neurons. Both the time course and the outcome of cell injury could be experimentally manipulated: postinjury incubation hypothermia improved cellular viability to a significant degree. This hypothermic rescue of acid-exposed cells suggests that a temperature-dependent metabolic slowing may allow the cells to survive an initial critical period, following which recovery becomes possible.

Cultures only a few days *in vitro* were composed of more than 95% neuronal cells as determined by double immunocytochemistry for MAP-2 and GFAP, whereas older cultures contained successively greater proportions of glial cells. We did not maintain enriched neuronal cultures by addition of mitotic inhibitors, because such treatment might have conceivably changed the threshold for acid-induced death. We made no direct effort to assess the differential sensitivities of neurons and glia to acidosis in these experiments. Nevertheless, during microscopic examinations of the acid-exposed cultures, it was often noted that neurons appeared to be more tolerant than glial cells to threshold acid exposures (Fig. 1). However, three lines of evidence indicate that such differential sensitivities are minimal. First, acid exposure performed on cultures from 2 to 21 DIV revealed the same LD₅₀ at all maturational ages *in vitro* (Fig. 8). Second, the regression line for acid-induced death was steep and linear; the absence of plateau values suggests uniform susceptibility. Third, pH_i declined to the same level during extracellular acidification in neurons and glial cells. Thus, though the precise threshold for acid-induced death appears to differ somewhat between neurons and glial cells (Goldman et al., 1989), it is likely that intracellular acidosis disrupts basic mechanisms common to all brain cells in an analogous fashion.

In summary, we have shown that acid-induced neurotoxicity is a product of both the degree and the duration of intracellular acidity, that exposure to moderate acidity triggers death in some cells with a latency of as long as 24–48 hr, and that delayed lethal injury may be attenuated by postinjury hypothermia.

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