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Effects of Lactacidosis on Glial Cell Volume and Viability

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Summary: Effects of severe lactacidosis were analyzed in vitro by employment of C6 glioma cells and astrocytes from primary culture. The cells were suspended in a physiological medium, which was rendered acidotic by addition of lactic acid in rising concentrations. A pH range of 7.4–4.2 was studied under maintenance of isotonicity and a normal electrolyte concentration of the medium. Cell swelling was quantified by flow cytometry using an advanced Coulter system with hydrodynamic focusing. The method was also utilized for assessment of cell viability by exclusion of the fluorescent dye propidium iodide. The volume of C6 glioma cells was found to increase if the pH was titrated to pH 6.8 or below. From this level downward, the extent of cell swelling depended on the degree of acidosis and the duration of exposure. For example, lactacidosis of pH 6.2 for 60 min led to an increase in cell size to 124.5% of normal, while pH 5.0 or 4.2 led to a cell size of 151.1 or 190.9%, respectively. A comparative analysis of the acidosis-induced cell swelling was made by using sulfuric acid. Swelling of C6 glioma at a given pH was only half of what was found when using lactic acid. This indicates specific swelling-inducing properties of lactic acid, while cell viability was not differently affected by both acids. Of the C6 glioma cells, 89.1% were viable under control conditions at pH 7.4. The viability remained unchanged down to pH 6.2. At pH 5.6, viability remained normal for 30 min, but it decreased to 73.4% after 60 min. Further lowering of pH to 5.0 or 4.6 respectively, decreased the number of viable cells to 47.8 or 40.3%. At pH 4.2 only 21.1% of the cells were surviving 1 h of lactacidosis. Cell swelling from lactacidosis could be largely inhibited by replacement of Na^+ and bicarbonate ions in the medium by choline chloride and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer, suggesting an involvement of the Na^+/H^+ and Cl^-/HCO_3^- antiporters in the swelling process. Omission of Na^+ and bicarbonate was, however, associated with reduced viability of the glial cells in acidosis. The swelling response of astrocytes obtained from primary culture was similar to that of C6 glioma. Lactic acid was also more effective in inducing cell swelling than sulfuric acid at the same level of acidosis. In astrocytes, viability at, e.g., pH 5.6 appeared to be more affected by lactic than by sulfuric acid. Taken together, the current results demonstrate different thresholds of acidosis, which are associated with either cell swelling or cell death. Cell swelling was induced from pH 6.8 downward, whereas cell viability started to decline at pH 5.6. Since increasing levels of acidosis were associated with an increasing degree of glial swelling, which resulted, at least in part, from an exchange of intracellular H^+ ions against extracellular Na^+ ions, the swelling response may be interpreted as a protective mechanism, which was activated to maintain or reestablish a normal intracellular pH, thereby mitigating cell damage from acidosis. **Key Words:** Astrocytes—C6 glioma—Cell swelling—Cell viability—Lactacidosis—Na^+/H^+ antiporter.

Cerebral ischemia, seizures, and severe head injury are associated with an enhancement of anaerobic metabolism resulting in intra- and extracellular lactacidosis (Siesjö, 1981, 1988). Lactacidosis may be involved in the formation of cytotoxic brain edema and irreversible damage of nerve and glial cells (Kraig et al., 1987; Kempski et al., 1988*b*; Goldman et al., 1989). Besides, lactic acid formed in necrobiotic tissue of an ischemic or traumatic focus can be expected to leak through interstitial routes into perifocal brain areas and to cause acidosis in the penumbra zone. Its severity may determine whether or not the penumbra zone is doomed. The penumbra zone, characterized as an area with blood flow too low to support a normal neuronal function, however not low enough to cause infarction (Astrup et al., 1981), would constitute a promising target for salvage, if specific methods of treatment were available. Such treatment should interfere with the

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Abbreviations used: FCS, fetal calf serum; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

mechanisms of secondary brain damage and thereby prevent an increase of irreversibly injured tissue.

Methods of treatment that are specifically aimed at underlying pathophysiological processes are in short supply, however. An impediment to a deeper understanding is the complexity of pathomechanisms occurring at all levels, including the cellular and molecular basis. Approaches are required that make possible investigations of relevant phenomena, such as cell swelling or permanent cellular injury in isolation without interference by other processes. Many mechanisms evolving in the ischemic core and the penumbra zone can be subjected to in vitro studies. Respective models have been developed and utilized (Ames and Nesbett, 1983; Kempski et al., 1983, 1987*a;* Choi, 1987; Rothman et al., 1987). Quantification of a single phenomenon, for example, of cell swellling, can be carried out more reliably in vitro than in vivo, since the experiment can be better controlled.

Respective investigations have utilized established cell lines such as C6 glioma, astrocytes from primary culture, and mixed neuronal and glial cultures (Kempski et al., 1988*b;* Goldman et al., 1989; Jakubovicz and Klip, 1989). The role of acidosis in cell swelling and irreversible damage has been studied in glial cells suspended in a medium containing increasing concentrations of sulfuric acid (Kempski et al., 1988*a*). Acidosis was found to induce cell swelling once the pH fell to 6.8. At this pH threshold and below, the glial volume increased immediately to 110% of normal. Acidosis of pH 6.2 or longer periods of exposure did not augment cell swelling from sulfuric acid. Experiments with administration of inhibitors of ion-exchange mechanisms or replacement of Na^+ and bicarbonate ions in the medium with choline and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer provided support for a role of the Na^+/H^+ and Cl^-/HCO_3^- antiporters in the acidosis-induced cell swelling (Grinstein and Rothstein, 1986; Kempski et al., 1988*a,b;* Jakubovicz and Klip, 1989). The present study was conducted in continuation of this research. Its purpose was to investigate the specificity of lactic acid in both cell swelling and cell death, since lactacidosis is a major determinant of damage in ischemic brain tissue (Siesjö, 1988). Levels of acidosis were studied between pH 7.4 and 4.2 for analysis of a dose response of glial swelling and permanent cell damage. Thereby, two important aspects of ischemic brain injury could be quantitatively assessed and correlated. Preliminary findings of this study have been recently reported (Staub et al., 1989).

MATERIALS AND METHODS

Cell culture

C6 glioma cells were grown as monolayers in Petri dishes using Dulbecco's modified minimal essential medium with 25 m*M* bicarbonate. The medium was supplemented with 10% fetal calf serum (FCS). One hundred international units per milliliter of penicillin G and 50 μg/ml streptomycin were added to prevent bacterial infection. The cells were cultivated at 37°C in humidified room air with 5% CO_2. Subcultivation was carried out three times per week. Glial cells from primary culture were obtained from 3-day-old DB9 rats according to a modified method of Frangakis and Kimelberg (1984). In brief, six to eight rats were used per preparation. Cerebral hemispheres were removed from skulls and freed from meninges and choroid plexus under a dissecting microscope. The tissue was minced with scissors and then repeatedly exposed to dispase (Boehringer, Mannheim, F.R.G.) for 10 min. After sedimentation of remaining tissue pieces, single cells were harvested from the supernatant and seeded into Petri dishes (1029; Falcon, Oxnard, CA, U.S.A.). After 1 week without medium change the glial cells were transferred to standard Petri dishes (3003 Optilux; Falcon) and kept in culture for ≥3 more weeks. The methods of culturing and subcultivation were the same as those employed for C6 glioma cells.

Only confluent cultures obtained 2 days after subcultivation were used for the experiments. Dibutyryl-cyclic AMP in a final concentration of 0.5 m*M* was added to the medium of astrocytes from primary culture 24 h before harvesting to induce growth arrest and differentiation (Kempski et al., 1987*b*). For each experiment six Petri dishes were harvested with 0.05% trypsin/0.02% ethylenediaminetetraacetate in phosphate-buffered saline. The cells were subsequently suspended in medium containing FCS for inactivation of trypsin. Two washes in serum-free medium ensued to remove FCS. The cell suspension was then transferred into a Plexiglas incubation chamber equipped with electrodes for continuous measurements of pH, temperature, and Po_2. A gas-permeable silicon rubber tube in the chamber served as a membrane oxygenator supplying the cell suspension with a mixture of O_2, CO_2, and N_2. A magnetic stirrer prevented the cells from sedimentation. Further details have previously been published (Kempski et al., 1983, 1987*c*).

Experimental groups

A 45-min period preceded the experiments to obtain control values on normal cell size, viability, and density and to assess the medium osmolality. Preparations with unstable cell volume or an impaired cell viability during the control period were discharged. The mean of three volume measurements obtained during the last 15 min of the control period was taken as reference. The same procedure was employed for assessment of normal cell viability. Subsequently, acidosis was induced by addition of lactic acid (350 m*M*) to the suspended cells.

In the control group the extracellular pH was maintained at 7.4. In six different experimental groups the pH was lowered from initially 7.4 to 6.8, 6.2, 5.6, 5.0, 4.6, or 4.2. To compensate for a loss of CO_2 and thereby secondary normalization of pH, CO_2 was added, resulting in a Pco_2 of 80–100 mm Hg in the suspension medium. This level is comparable with respective in vivo findings on ischemic brain tissue (Smith et al., 1986; v. Hanwehr et

al., 1986). Three to six experiments were performed at each pH level. Cell volume and viability were monitored for 60 min during lactacidosis. Osmolality and measures of the acid-base state were frequently assessed in the control period as well as during acidosis. The volume response of C6 glioma cells to lactate ions (final concentration 20 m*M*) at normal pH (i.e., without acidosis) was analyzed in an additional group. Comparative studies on the swelling-inducing potency of sulfuric acid (150 m*M*) were made at pH 5.6. As before, the involvement of exchange mechanisms in the swelling process was assessed in experiments by employment of Na^+- and bicarbonate-free medium. Na^+ was replaced by choline, and bicarbonate by HEPES (10 m*M*) as buffer. In those experiments the medium of the incubation chamber was gassed with a N_2/O_2 mixture under omission of CO_2, yielding a Po_2 of 100 mm Hg as assessed by the Po_2 electrode of the chamber.

Measurement of cell volume and cell viability

Cell volume was determined by flow cytometry (Metricell) according to the Coulter principle. The Metricell volume analyzer uses hydrodynamic focusing, providing superior accuracy. Alterations in cell size of <1% can be recognized with this method (Kachel, 1976). The system is calibrated electrically as well as mechanically by using latex beads of known diameter (Chaussy et al., 1981). A single volume measurement takes ~30 s. Cell viability was also assessed by flow cytometry (Fluvo Metricell; HEKA-Elektronik, Lambrecht/Pfalz, F.R.G.; Kachel et al., 1977) using exclusion of propidium iodide (Pavlik et al., 1985; Rothe and Valet, 1988). Aliquots of 100 μl of the cell suspension were added with 2 μl of a solution containing 2 mg/ml propidium iodide in dimethylformamide (Rothe and Valet, 1988) and incubated at 37°C for 1 min. The wavelengths of maximum excitation of propidium iodide were at 330 and 470 nm (Crissman et al., 1979). Excitation was induced through a 500-nm short-pass filter. Maximum emission of propidium iodide at 630 nm was measured by using a 580-nm long-pass filter. A window integration system (Kachel, 1986) was employed for discrimination of propidium iodide–stained (dead) from non-fluorescing (viable) cells. The latter cells were identified by their electrical volume signal. Propidium iodide exclusion appeared to be advantageous to study cell viability in acidosis, since exclusion of trypan blue is highly pH dependent (Phillips, 1973).

Preparation for scanning electron microscopy

Swelling of C6 glioma cells was also studied by scanning electron microscopy after 30-min exposure to lactic acid at pH 5.0. Two hundred microliters of the cell suspension obtained from the incubation chamber was mixed with cold 2% glutaraldehyde in phosphate buffer (4°C) for fixation of the cells. The cells were then dehydrated in graded ethanol, spontaneously sedimented, and critical point dried using CO_2 (CPD 020; Balzers Union, Fürstentum, Liechtenstein). Subsequently, the cells were coated with gold (SEM coating system; Polaron, Watford, Great Britain) and examined by a scanning electron microscope (JSM-35CF; Jeol, Tokyo, Japan) at 10 kV.

Other analytical procedures

The osmolality of the suspension medium was measured by freezing point depression (Osmomat 030; Gonotec, Berlin, F.R.G.). The acid-base state was assessed by measurements of pH, Po_2, and Pco_2 with respective electrodes from which bicarbonate concentrations and base excess were calculated. A micro–blood gas analyzer (BL 300; Radiometer, Copenhagen, Denmark) was used for that purpose. The results are expressed as means ± SD. The data were analyzed for statistical significance using the Kruskal-Wallis test for nonparametric one-way analysis of variance and multiple comparisons on ranks for unpaired samples (Theodorsson-Norheim, 1986). Experiments performed at pH 7.4 with addition of lactate (Table 1) were statistically evaluated by using the Quade test for paired samples (Theodorsson-Norheim, 1987).

RESULTS

During the 60-min control period at pH 7.4, the cell volume of C6 glioma was 791.4 ± 54.3 μm^3, and 89.1 ± 3.2% of the cell population were viable

TABLE 1. *Volume response of C6 glioma cells incubated at normal pH (7.4) in presence of 20 m*M *Na^+-lactate or during lactacidosis of pH 5.6 under omission of Na^+ ions*

	Control			Lactate 20 m*M*, pH 7.4				
Time (min)	−15	−10	−5	3	10	20	40	60
Volume (μm^3)	761.63	757.70	758.90	783.33[a]	785.02[b]	796.97[b]	792.40[b]	785.20[a]
SD	46.84	52.10	54.81	53.31	69.04	67.49	48.47	54.05
Volume (%)	100.32	99.77	99.90	103.16[a]	103.27[b]	104.85[b]	104.40[b]	103.42[a]
SD	1.50	1.67	0.70	3.14	3.12	2.64	2.81	3.03
	Control			Lactacidosis, pH 5.6, Na^+-free medium				
Time (min)	−15	−10	−5	3	10	20	40	60
Volume (μm^3)	702.42	703.92	705.95	725.33[a]	733.23[b]	732.48[b]	718.41[a]	731.48[b]
SD	32.66	34.14	34.74	24.54	31.13	35.85	47.76	54.77
Volume (%)	99.77	99.97	100.26	103.07[a]	104.19[b]	104.05[b]	102.00[a]	103.85[b]
SD	0.50	0.23	0.49	1.80	2.90	2.62	3.49	4.79

Means ± SD of cell volume obtained in four or six experiments are shown as μm^3 or % of normal, respectively. In both groups the differences during the experimental phase were statistically significant.

[a] $p < 0.05$ vs. control.

[b] $p < 0.01$ vs. control.

(Figs. 1 and 2). Incubations of up to 180 min under control conditions at pH 7.4 did not affect cell volume or viability; longer periods were not studied. When the pH was lowered from 7.4 to 6.8 by isotonic lactic acid, the cell volume increased (Fig. 1) to 105.5 ± 2.6% within 3 min and to 107.6 ± 0.5% ($p < 0.05$) within 30 min. Lowering of the pH to ≤6.2 led to more intensive swelling. At pH 6.2, cell volume increased within 1 min to 111.6 ± 2.3% and at pH 4.2 to 131.7 ± 3.8%. Exposure for 30 min at this level of acidosis led to marked enhancement of cell swelling. At pH 6.2 cell volume reached 123.4 ± 2.3%, and at pH 4.2 even 162.8 ± 8.0%. Cell swelling leveled off during the remaining period of lactacidosis except in experiments with pH 4.2. Under the latter conditions, the cell size increased within 60 min to 190.9 ± 17.0% (Fig. 1). Addition of lactic acid to the medium resulted in a decrease of bicarbonate buffer from initially 22.0 ± 0.5 m*M* at pH 7.4 to 5.7 ± 2.2 or 0.2 ± 0.08 m*M* at 60 min at pH 6.2 or 5.0, respectively (data not shown).

To investigate lactate-specific effects (without acidosis), Na^+-lactate was administered to the suspension medium in a final concentration of 20 m*M* at pH 7.4. This measure alone led to an increase, albeit moderate, in cell volume. Cell swelling was statistically significant within 3 min with a plateau at 104% after 15 min (Table 1). The kinetics of the cell volume response were similar to the cell volume increase from lactacidosis at pH 5.6 in Na^+-free medium (see below and Table 1).

Cell viability remained normal after lowering the pH to 6.8 or 6.2 (Fig. 2). Even at pH 5.6 cell viability was not affected during the first 30 min. However, the number of viable cells started to significantly decrease thereafter, falling to 73.4 ± 4.4% after 60 min. When the pH was lowered to ≤5.0, cell viability began immediately to decline. Approximately 50% of the glial cells died, i.e., became propidium iodide positive within 30 min of suspension at pH 5.0, 4.6, or 4.2. A maximum of cell killing from lactacidosis was found at pH 4.2. Only 21.1 ± 18.0% of the glial cells were alive after 1 h at this pH level. Thus, a dose-response relationship could be established between the decrease in viability and the level of acidosis and duration of exposure, respectively, once a threshold of pH 5.6 had been passed.

When sulfuric acid was administered for induction of acidosis, the degree of cell swelling at a given pH was much more restricted as compared to the experiments with lactic acid (Fig. 3). At pH 5.6, the increase in cell size from sulfuric acid was only half of what was found when using lactic acid (Fig. 3). On the other hand, both acid species had nearly identical effects on the decrease in cell viability, once the pH fell to ≤5.6. Exposure of the glial cells to lactacidosis of pH 5.6 for 60 min resulted in a viability of 73.4 ± 4.4% as compared with 78.5 ± 6.4% when sulfuric acid was used.

The swelling of C6 glioma cells from acidosis could be almost completely inhibited by replacement of Na^+ ions and of bicarbonate by choline chloride and HEPES (Fig. 4; Table 1). In those experiments a somewhat smaller cell volume (704.1 ± 33.7 μm^3) was already found during the control period at pH 7.4. Lactacidosis of pH 5.6 under omission of Na^+ ions and bicarbonate resulted in cell swelling of no more than 104.2 ± 2.9%, while in the presence of Na^+ ions and bicarbonate, glial swelling from pH 5.6 was 140%. Inhibition of cell swelling from acidosis under these conditions notwithstanding, cell viability was more severely affected in Na^+-free medium as compared with the experiments conducted at the same pH and a normal extracellular Na^+ concentration (Fig. 4).

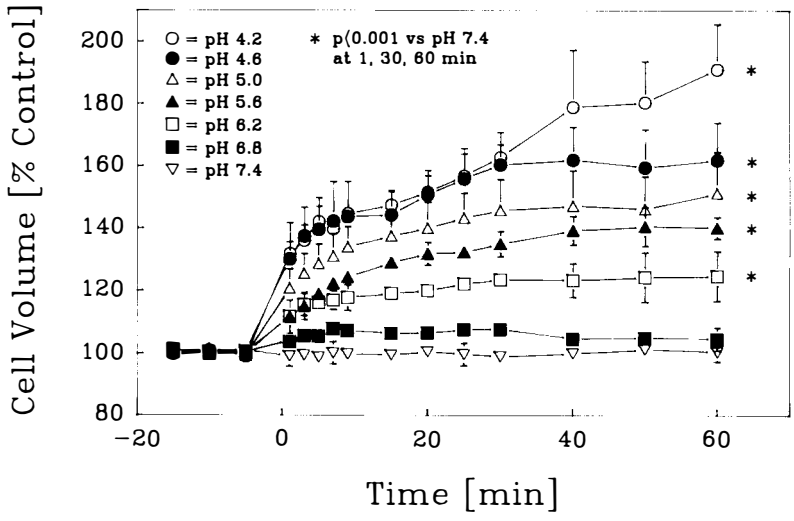

FIG. 1. Cell volume response of C6 glioma cells suspended at pH 7.4 or at different levels of acidosis down to pH 4.2. Cell volume is given as percentage of the normal value obtained during the last 15 min of the control period. The medium was titrated at time 0 by addition of isotonic lactic acid. The symbols represent means ± SD of three to six experiments. Absence of SD bars (as in other figures) indicates a smaller SD than the size of the symbol. 10^4–10^5 cells were utilized per single volume measurement.

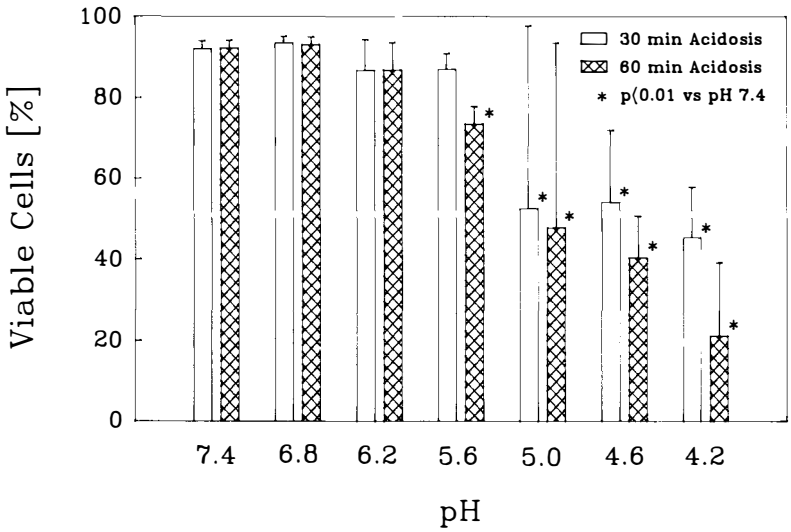

FIG. 2. Viability of C6 glioma cells during administration of lactic acid at different pH levels. Viable cells (propidium iodide negative) are shown as percentage of the total cell population. The height of a column symbolizes the mean ± SD after 30- or 60-min exposure. Three to four experiments were performed at each pH level.

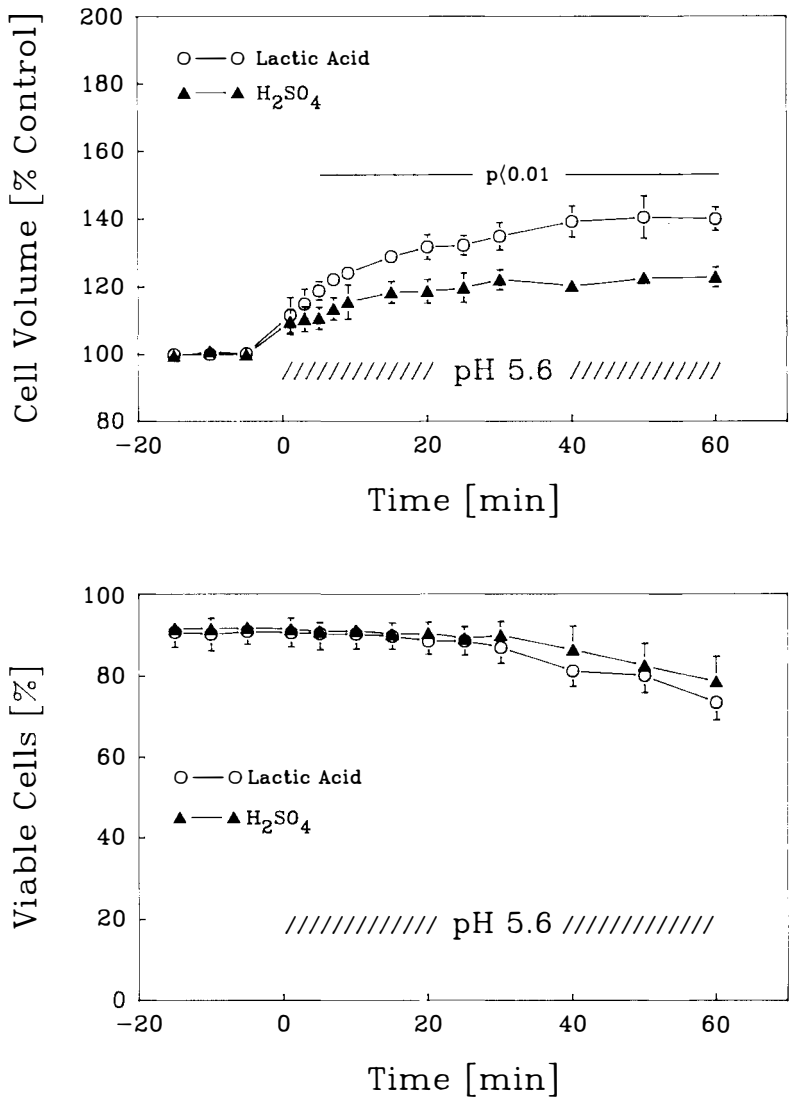

FIG. 3. Cell volume **(top)** and viability **(bottom)** of C6 glioma subjected to acidosis at pH 5.6 by addition of lactic or sulfuric acid, respectively. The increase in cell volume is given as percentage of control. Within a few minutes the addition of lactic acid led to significantly ($p < 0.01$) more pronounced cell swelling than sulfuric acid, while cell viability was not differently affected. Symbols represent means ± SD of three to four experiments per group.

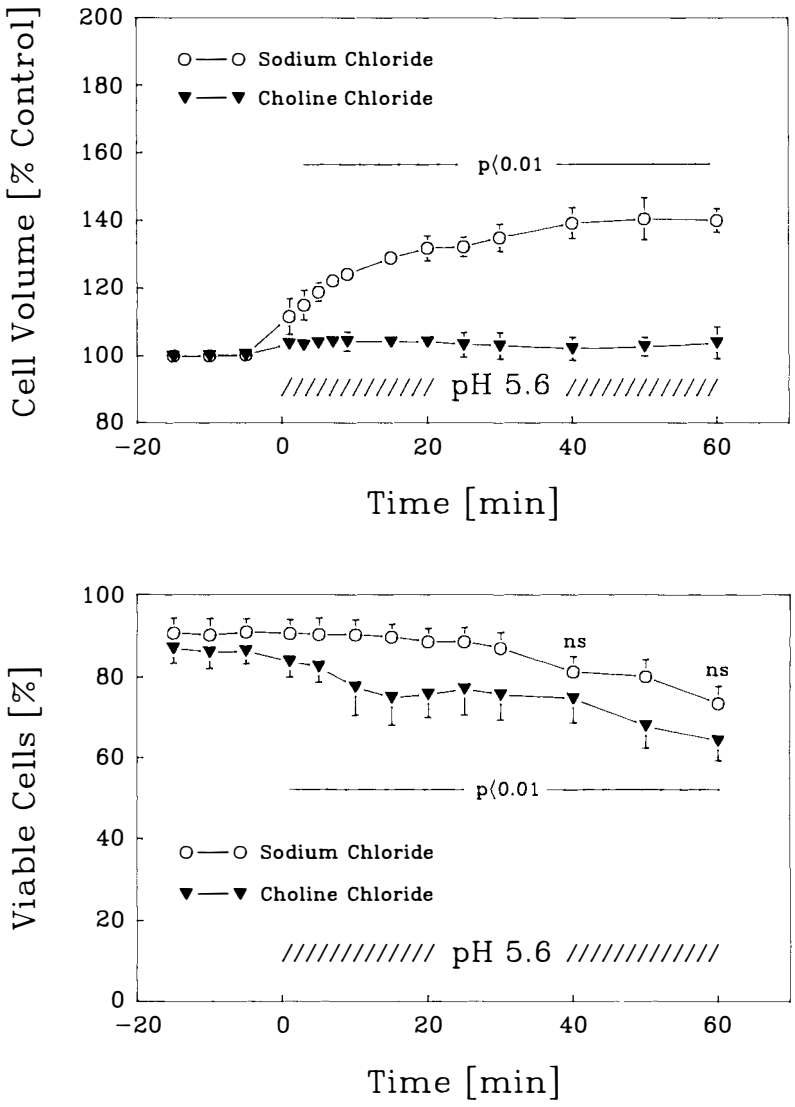

FIG. 4. Cell volume response and viability of C6 glioma during suspension in medium containing lactic acid at pH 5.6 with (○) and without (▲) a normal Na^+ and bicarbonate concentration. Na^+ and bicarbonate were replaced by choline chloride and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid. Control values were obtained prior to induction of acidosis. Cell swelling occurred only in the presence of Na^+ and bicarbonate **(top),** whereas cell viability was more severely affected in experiments with omission of Na^+ and bicarbonate **(bottom).**

Swelling of glial cells and a decrease in viability from lactacidosis were confirmed in experiments using astrocytes from primary culture (Fig. 5). Under control conditions at pH 7.4, noticeable changes in cell size or cell viability did not occur (Fig. 5). The astrocytes had a modal cell volume of 1,140.3 ± 89.1 μm^3, and the number of viable cells was 74.7 ± 2.5%. The viability of astrocytes under control conditions was somewhat below that of C6 glioma at pH 7.4. Titration of the medium down to pH 5.6 led to marked swelling of astrocytes. As in C6 glioma, lactic acid was more effective in inducing swelling than sulfuric acid at a given pH. However, contrary to the findings on C6 glioma cells, lactic acid (pH 5.6) in astrocytes led to a more pronounced decrease in viability than sulfuric acid (Fig. 5). When sulfuric acid was used to lower pH to 5.6, the viability of astrocytes was practically unchanged as compared with pH 7.4 (Fig. 5).

Scanning electron microscopy was employed for ultrastructural analysis of the morphological alterations at the cell surface of glial cells subjected to acidosis-induced swelling. At normal pH, the cell membrane of the C6 glioma cells appeared uniformly well preserved (Fig. 6). Microvilli extending into the medium were homogeneously distributed and appeared structurally normal. Acidosis of pH 5.0 by lactic acid led to a variable increase in cell size. A large fraction of the cell population had marked alterations at the cell surface. The microvilli seemed to be irregularly distributed and clumped. The cell membrane was frequently disrupted by large holes indicative of severe damage or even cell death (Fig. 6).

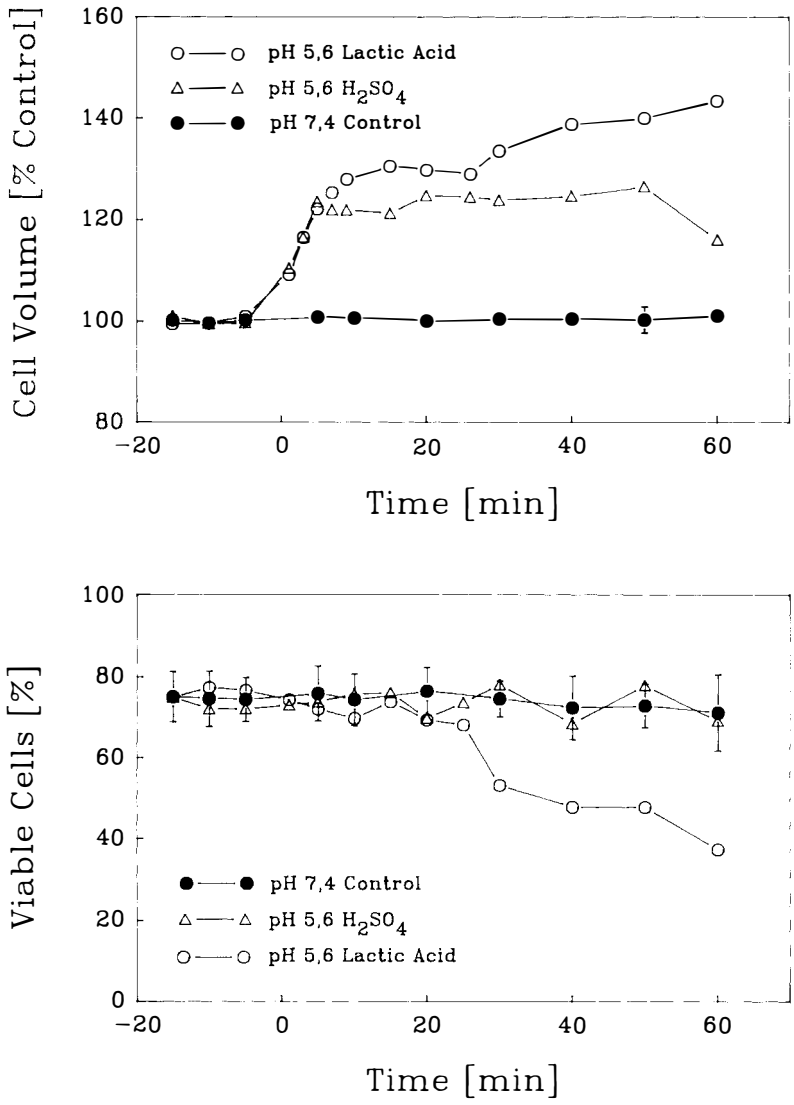

FIG. 5. Cell volume response **(top)** and viability **(bottom)** of astrocytes from primary culture to lactacidosis at pH 5.6. Cell volume is shown as percentage of the control level obtained at pH 7.4 (●). As in C6 glioma, lactic acid (○) in astrocytes led to more pronounced swelling than sulfuric acid (△). In astrocytes lactacidosis of pH 5.6 caused a decline in cell viability after 30 min.

DISCUSSION

Experimental model

The present studies assessed the effects of lactacidosis on glial cell swelling and viability. The experiments continued former attempts of this laboratory to uncover mechanisms underlying cytotoxic brain edema occurring, for example, in ischemia (Kempski et al., 1987*c*, 1988*a,b;* Kempski, 1989). The experimental model allows us to quantitate changes of cell volume and viability at a level of subtlety and accuracy hardly available when studying the brain in vivo, particularly under pathological conditions. A further asset is that important parameters, such as Po_2, temperature, ion concentrations, and osmolality, can be controlled and maintained during the course of the experiment. Thereby, a single phenomenon can be studied in isolation without interference by others. The current investigations underline the usefulness of C6 glioma as model cells to study pathophysiological mechanisms involved in cell swelling. A total of 55 experiments were conducted utilizing no less than 10^8 cells each. Extended experimental observation periods together with frequent sampling of suspension aliquots from the chamber required a high yield of cells. C6 glioma is a cell line with homogeneous properties, which rapidly grows in culture. Its homogeneous cell size distribution facilitates detection of even subtle volume changes. The validity of C6 glioma as model cells for astrocytes has been ascertained (see Kempski et al., 1988*a*). The cells express specific markers, such as glial fibrillary acidic and S-100 protein, specific enzymes, uptake systems for neurotransmitters, and ion carriers. C6 glioma cells have an active respiratory metabolism

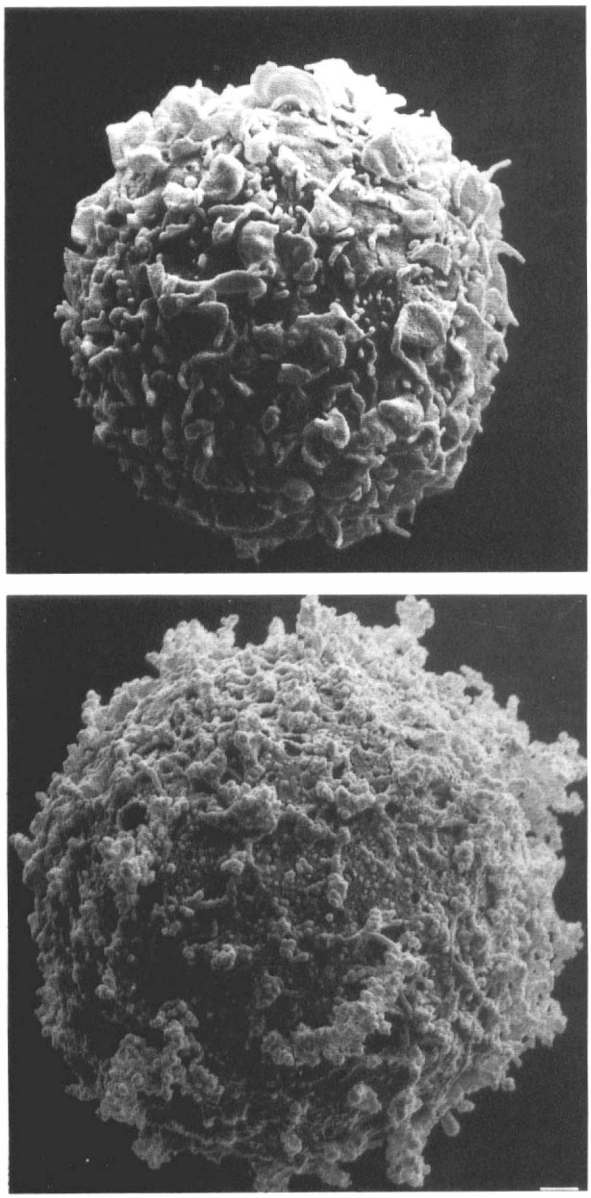

FIG. 6. Scanning electron micrographs of C6 glioma cells studied at pH 7.4 **(top)** and after 30 min of lactacidosis at pH 5.0 **(bottom).** At pH 7.4, the cell surface appeared normal with extension of numerous microvilli into the environment. Acidosis led to structural derangements of the cell surface with clumping of microvilli and formation of holes in the cell membrane. Bar, 1 μm.

that is comparable with that of the brain in vivo (Kempski et al., 1983). Astrocytes from primary culture were utilized in addition as a nontransformed glial cell type to control and compare our findings obtained with C6 glioma. As seen, the swelling response to acidosis was well comparable with that of C6 glioma cells. As a difference, a somewhat higher vulnerability of astrocytes to acidosis induced by lactic acid was found.

Studies of the volume response of C6 glioma during anisotonic exposure have previously shown the utility of the model (Kempski et al., 1983). In those experiments, C6 glioma cells revealed a potential for spontaneous normalization of cell volume (regulatory volume decrease) during hypoosmotic exposure with maintenance of a normal cell viability. Maintenance of a normal cell size and viability was also found secondary to induction of complete energy failure by anoxia in combination with inhibition of glycolysis by iodoacetate or after blocking of Na^+-K^+-ATPase by ouabain (Kempski et al., 1987*c*, 1988*b*). This is noteworthy, since the transcellular Na^+ and K^+ ion gradients were broken down. Resistance of glial cells to swelling under those conditions appears to be in contrast with observations on the development of cytotoxic brain edema and the swelling of glial and dendritic processes in cerebral ischemia in vivo (Van Harreveld, 1972; Garcia et al., 1974; Baethmann, 1978). According to the classical concept of the "pump leak" model as described by the Donnan equilibrium, the normal cell volume is interpreted to reflect a balance of a continuous passive influx of Na^+ ions together with chloride and water and their active extrusion afforded by Na^+-K^+-ATPase, fueled by cellular energy metabolism (Macknight and Leaf, 1977). Thus, a breakdown of the energy metabolism alone should suffice to induce cell swelling. The apparent conflict between this concept based on respective in vivo findings and the recently obtained in vitro observations might be resolved by assuming additional mechanisms that are contributing to the development of cytotoxic brain edema in vivo. One is a release of K^+ ions and of mediator compounds such as glutamate into the extracellular compartment, another the development of brain tissue acidosis (Baethmann et al., 1988, 1989; Kempski et al., 1988*b*; Siesjö, 1988).

Extracellular acidosis

A role of acidosis in manifestation of brain damage from cerebral ischemia, such as cell swelling and necrosis, has been assumed for quite a while (Siesjö, 1981). The extent of acidosis may determine whether an ischemic injury sustained by the brain is irreversible or not. Experimental findings on acidosis-induced swelling and injury of glial cells in vitro have recently been published (Kempski et al., 1988*b*; Goldman et al., 1989; Jakubovicz and Klip, 1989). The purpose of the current studies was to investigate the specificity of lactic acid and whether the resulting cell swelling can be attributed to lowering of the pH alone or to additional properties of the compound. A marked decrease of brain tissue pH has been demonstrated in cerebral isch-

emia, particularly in association with hyperglycemia, with an accumulation of lactic acid of up to 20–30 m*M* (Rehncrona et al., 1980; Harris and Symon, 1984; Kraig et al., 1985; Munekata and Hossmann, 1987). Accumulation of lactic acid in brain tissue is also found in seizures, spreading depression, and severe head injury (Mutch and Hansen, 1984; Siesjö et al., 1985; Andersen et al., 1988). As in former experiments with sulfuric acid (Kempski et al., 1988*a,b*), glial swelling commenced as soon as the pH fell to 6.8 or below by addition of lactic acid. The swelling response of the glial cells was dose dependent (Fig. 1). For example, the cell volume increased to ~120% of normal within 60 min at pH 6.2, but nearly to 200% at pH 4.2. Replacement of Na^+ and bicarbonate by choline and HEPES buffer was again effective to prevent swelling of the glial cells from lactacidosis as found when using sulfuric acid. This confirms activation of the Na^+/H^+ exchange as a central mechanism in the swelling process (Grinstein and Rothstein, 1986; Kempski et al., 1988*a;* Jakubovicz and Klip, 1989).

The following concept was developed on the basis of these findings. Addition of lactic acid to the medium with buffering by bicarbonate leads to formation of carbonic acid, which dissociates immediately into CO_2 and water. CO_2 enters the intracellular compartment, causing intracellular acidosis via formation of H_2CO_3 catalyzed by carbonic anhydrase. The resulting H^+ and bicarbonate ions are exchanged against extracellular Na^+ and Cl^- ions by activation of antiporters (see above). The cellular discharge of HCO_3^- makes available further buffer for lactic acid, thereby maintaining the above-described pathway of formation of CO_2 and subsequent intracellular acidification. As a result, net amounts of Na^+ and Cl^- are shuttled into the intracellular compartment, whereas CO_2, H^+, and HCO_3^- ions are continuously recycled into and out of the cell. Accumulation of Na^+ and Cl^- in the cell is the ultimate cause of glial swelling for osmotic reasons. Under normal conditions, cell swelling secondary to activation of the Na^+/H^+ antiporter and of anion-exchange mechanisms might be prevented or attenuated by the active Na^+ pump. This, however, is liable to fail in ischemic brain tissue on account of the low supply of metabolic fuel (see Kempski et al., 1988*a*). Moreover, experiments with glial cells conducted in this laboratory do not support a predominant role of the Na^+/K^+ pump (Na^+-K^+-ATPase) as a major volume control mechanism. Addition of ouabain to C6 glioma did not induce cell swelling (Kempski et al., 1988*b*) or prevent the regulatory volume decrease of these cells after hypotonic exposure associated with immediate cell swelling (Kempski et al., 1983). Further, ouabain was not found to affect the volume response of the glial cells to acidosis (data not shown). Thus, the active Na^+/K^+ pump does not appear to be of major significance for glial swelling from acidosis. On the other hand, it is conceivable that a Na^+/HCO_3^- cotransport into the cells contributed to cell swelling in acidosis. It has been shown that the cotransporter is involved in the regulation of the intracellular pH of oligodendrocytes of mice (Kettenmann and Schlue, 1988).

The concept so far developed would suffice to explain swelling of glial cells from acidosis, irrespective of whether organic or inorganic acids were administered. The current studies, however, demonstrated specific swelling-inducing properties of lactic acid in addition, which seemed to be independent of the actual pH level. This follows from comparisons of the increase in cell size in response to either lactic or sulfuric acid at a given degree of acidosis (Fig. 3). In agreement with observations by others on lactic acid (Roos, 1975) or on other weak organic acids such as propionic acid (Boron, 1983; Grinstein, 1988), it is proposed that a fraction of lactic acid had entered the intracellular compartment as nonpolar, undissociated compound. The intracellular pH favors immediate dissociation of the compound on account of its low pK. Lactate anions thereby generated in the cell cannot freely penetrate the cell membrane as charged molecules. Nevertheless, evidence is available that lactate anions can be eliminated from glial cells by a specific transport system (Walz and Mukerji, 1988). This phenomenon apparently did not suffice under the current conditions to prevent an intracellular accumulation of lactate anions resulting from the continuous influx of undissociated lactic acid along its extra- to intracellular concentration gradient. Accumulation of lactate anions in the glial cells might therefore be considered to have contributed to an increase of the osmotic concentration, explaining why this compound was more effective than sulfuric acid in the development of glial swelling.

Further, a carrier-mediated transport of lactate into the cell has been considered (Kuhr et al., 1988), which is enhanced by an extracellular acidosis (Watt et al., 1988). Our data, however, suggest this mechanism to be of minor significance as concluded from the experiments conducted in Na^+-free medium and with employment of HEPES buffer. Under these conditions, glial swelling was largely prevented, although lactate was abundantly available for carrier-mediated transport into the cells (Figs. 1 and 3). Nevertheless, the small increment in glial cell size from lactacidosis at pH 5.6 during omission

of Na^+ ions, with lactic acid concentrations in the medium of 16.3 m*M*, might have resulted from a carrier-mediated transport of lactate. The same may be concluded from the findings obtained in experiments with addition of lactate (20 m*M*) under maintenance of a normal pH (Table 1). Taken together, glial swelling from lactacidosis can be attributed to both a net influx of Na^+ and Cl^- ions from activation of ion-exchange mechanisms in response to intracellular acidification and a cellular uptake of lactate as nonpolar compound, whereas a carrier-mediated transport might be of minor significance.

Viability of glial cells in acidosis

The present findings demonstrate a remarkable ability of glial cells to remain viable at advanced levels of acidosis and long periods of exposure. Maintenance of viability at low medium pH appeared to be associated with a successful protection of the intracellular pH in a normal or close-to-normal range. This is concluded from the corresponding findings on cell viability in experiments with acidosis under omission of Na^+ ions from the medium. Viability was significantly deteriorating there as compared with the experiments with a normal Na^+ concentration in the medium (Fig. 4). Absence of extracellular Na^+ ions rendered the Na^+/H^+ antiporter useless to eliminate intracellular H^+ ions to defend a normal intracellular pH (Jakobuvicz and Klip, 1989). Consequently, intracellular accumulation of H^+ ions ensued, eventually resulting in cell death (Siesjö, 1988).

In the presence of a normal Na^+ concentration in the medium, viability of the glial cells started also to decline, however at a more severe level of acidosis. pH 5.6 was found as the threshold at and below which viability deteriorated in a dose-dependent fashion within observation periods of 30 and 60 min. Such a pH level is found in vivo in the severest forms of ischemia, for example, in association with hyperglycemia (Kraig et al., 1985). The findings obtained by scanning electron microscopy (Fig. 6) suggest that acidosis (pH 5.0) inflicts direct structural damage on the cell membrane. The results are in good agreement with morphological studies conducted in cultured astrocytes and with in vivo findings (Norenberg et al., 1987). Lactacidosis of pH 5.5 led to morphological alterations, such as cell swelling, cytoskeletal abnormalities, chromatin clumping, and loss of polyribosomes, which became irreversible within 30–60 min (Norenberg et al., 1987). Further, microinjection of lactic acid into the parietal cortex of rats, lowering the tissue pH to 5.3, was found to cause a cerebral necrosis resembling ischemic infarction (Kraig et al., 1987).

Comparison of the swelling response and the resulting cell viability from acidosis induced with either sulfuric or lactic acid is particularly revealing. As seen, lactic acid at a given pH produced a significantly larger increase in cell size as compared to sulfuric acid, whereas the viability of the glial cells appeared to depend on the level of acidosis, regardless of the type of acid added. This would support the concept that irreversible cell damage must be attributed directly to the H^+ ion concentration and not to the specific properties of a given acid, as also concluded by Norenberg et al. (1987).

In conclusion, the current studies on acidosis-induced swelling of glial cells underline a special role of lactic acid in the development of cytotoxic brain edema as occurring in cerebral ischemia. The different effects of lactic and sulfuric acid on cell swelling and irreversible cell damage lend support to the hypothesis that the mechanisms underlying cytotoxic brain edema are different from those causing cell necrosis. The increase in cell size from acidosis might be the price the cell has to pay for maintenance of a normal intracellular pH in defense of its own survival.

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