Glial Swelling During Extracellular Acidosis
In Vitro

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Intracellular and extracellular acidosis may determine the ultimate outcome for brain tissue in cerebral ischemia. An extracellular acidosis that occurs in the penumbra zone was investigated in vitro as to its role in the formation of cytotoxic cell swelling. For that purpose, C6 glioma cells or primary cultured astrocytes were suspended in normal isotonic medium in normoxia during acidification to a final pH of 6.2. The cell volume response was determined by flow cytometry using hydrodynamic focusing, which allows one to recognize changes in cell size of <1%. A threshold pH of 6.8 was found that had to be crossed to induce cell swelling by acidosis. Once pH fell below this threshold, the increase in cell size appeared to be an all-or-nothing phenomenon. The cells rapidly assumed a final cell size of 115% of normal in the case of C6 glioma or of 118% in the case of primary cultured astrocytes independent of the actual level of acidosis or the duration of exposure. Acidosis-induced glial swelling could be significantly attenuated by 1) addition of amiloride, 2) administration of acetazolamide, or 3) replacement of bicarbonate buffer against N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES). Replacement of extracellular Na+ by choline chloride led to complete prevention of the acidosis-induced cell swelling. Taken together, the findings strongly indicate a central involvement of Na+/H+ and Cl-/HCO3− exchange mechanisms in the development of cell swelling under these conditions. Activation of the Na+/H+ antiporter can be considered an attempt to maintain a normal intracellular pH at the expense of an abnormal cell volume. Stimulation of the Na+/H+ and Cl-/HCO3− antiporters in acidosis promotes cell swelling by increasing the intracellular Na+ and Cl− concentrations of the glial cells. It is suggested that swelling of glial elements in the border zone of an infarct in vivo, for example, in an ischemic penumbra, evolves along the above-described mechanisms. Effective therapeutic attempts to control the cerebral pH under clinical conditions might be considered to protect brain tissue in cerebral ischemia. (Stroke 1988;19:385–392)

The development of extracellular and intracellular acidosis resulting from enhanced lactic acid formation is probably one of the most important mechanisms of brain damage in cerebral ischemia, leading to formation of cytotoxic brain edema and, eventually, to irreversible cell death.1,2 Our understanding of these processes is, however, hampered by the complexity of brain tissue and by the fact that in vivo, under abnormal conditions like ischemia, many parameters are affected simultaneously. Apart from tissue acidosis, there is a massive release of excitatory transmitters, accumulation of K+ in the extracellular compartment, accumulation of Ca2+ in the intracellular compartment, and formation of autotoxic material such as free radicals, etc.3-4 The clear identification and quantification of a single pathomechanism on a molecular level of cell or tissue damage is virtually not possible under conditions as complex as cerebral ischemia in vivo. Alternative approaches must be pursued, therefore, to dissect and isolate the causative mechanisms from epiphenomena taking place in damaged brain tissue under these circumstances.

Our laboratory has established an in vitro model to investigate the pathomechanisms of cytotoxic cell swelling that occur in cerebral ischemia by using suspended C6 glioma as model cells with glial characteristics under defined conditions.5,6 Thereby, a variety of parameters can be easily monitored and modified according to the underlying experimental problem. Cell volume is accurately assessed by a flow cytometric method that allows recognition of alterations in cell size of <1%.7,8

Former studies on anoxia have demonstrated that C6 glioma cells are capable of maintaining a normal cell volume during complete interruption of the cellular energy metabolism induced by anoxia plus inhibition of glycolysis.6 It has been concluded from these observations that mere energy failure does not suffice to induce cell swelling, although this should be expected on the basis of the pump–leak model of the double Donnan equilibrium.9 Since breakdown of energy metabolism in ischemic brain in vivo almost always is associated with tissue acidosis, the significance of this process for cytotoxic cell swelling was analyzed using the model described above, with special emphasis on the function of the Na+/H+ antiporter.

Subjects and Methods

Cell Culture

C6 glioma cells (courtesy of H. Thoenen, Max-Planck-Institute of Biochemistry, Martinsried, F.R.G.) were cultivated as monolayers in Petri dishes using Dulbecco’s modified minimal essential medium...
Glial cells from primary culture were prepared from 3-day-old DB9 rats according to a method modified from the procedure described by Frangakis and Kimelberg. In brief, cerebral hemispheres were isolated and freed from meninges under a dissecting microscope. The tissue was minced with scissors and then repeatedly exposed to dispase (Boehringer Mannheim, Mannheim, F.R.G.) for 10 minutes. Single cells were harvested from the supernatant by centrifugation and seeded into Petri dishes (1029, Falcon, Oxnard, California). After 1 week of cultivation the glial cells were transferred to Petri dishes (3003 Optilux, Falcon) and kept in culture for another 3 weeks. Culture and subcultivation conditions were identical to those described for C6 glioma cells (see above).

For the experiments, only confluent cultures obtained 2 days after subcultivation were used. The cells were harvested and suspended in serum-free medium as previously described.²⁴ The cell suspension was transferred to an acrylic incubation chamber furnished with respective electrodes for continuous control of pH, temperature, and Po₂. Permeable silicone rubber tubing inside the chamber served as a membrane oxygenator supplying the cell suspension with CO₂, O₂, and N₂ in any desired mixture. Cell sedimentation was prevented by a magnetic stirrer. Further details of the incubation chamber have been published.⁵

**Experimental Groups**

The experiments were performed after a 45-minute control period necessary for measurements of the normal cell volume, medium osmolality, and cell viability. Only preparations with a stable cell volume and a cell viability of >90% were used for the subsequent acidosis experiments. The mean of three cell volume measurements obtained during the last 15 minutes of the control period were taken as normal reference values.

Acidosis or alkalosis, respectively, were induced in C6 glioma cell suspensions by addition of 150 mM sulfuric acid or 160 mM NaOH to the medium. Both solutions were made isotonic by the addition of NaCl to avoid changes in osmolarity of the suspension medium. Using 25 mM bicarbonate as buffer, the pH of the medium was titrated from 7.4 (normal) to 8.2, 7.8, 7.2, 7.0, 6.8, 6.6, 6.4, or 6.2. During acidosis, the CO₂ supply through the membrane oxygenator was maintained at 5%. Four to six experiments were performed at each pH level; cell volume was monitored for 60 minutes.

In other experiments, 0.1 mM amiloride or 0.01 mM acetzolamide were administered to C6 glioma cell suspensions during the control period 15 minutes before induction of acidosis (pH 6.2).

In another experiment with C6 glioma cells, bicarbonate as buffer was replaced with 15 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), and Na⁺ ions were exchanged against choline as extracellular cations in studies at pH 6.2 in HEPES-buffered media.

Additional experiments evaluated the swelling of glial cells from primary culture using bicarbonate and HEPES as buffers and the addition of 0.1 mM amiloride.

**Cell Volume Measurements**

Cell volume was determined by flow cytometry using “Metricell,” which is based on the Coulter principle and uses hydrodynamic focusing. The accuracy of this method allows detection of cell size alterations of <1%. The system is calibrated electrically as well as mechanically by the use of latex beads of known diameter. A single measurement takes about 30 seconds.

**Analytical Procedures**

The osmolality of the medium was examined by freezing-point depression (Knauer, Berlin, F.R.G.). Cells in the suspension in the incubation chamber were repeatedly counted using a conventional Neubauer chamber. Exclusion of trypan blue by living cells was employed to assess viability.

**Statistics**

The results were evaluated statistically by the Kruskal-Wallis test using a Commodore-128 computer (Braunschweig, F.R.G.) and a basic program.¹⁴

**Results**

The normal modal C6 glioma cell volume at pH 7.4 was 834.8 ± 29.5 μm³ under control conditions in bicarbonate-buffered medium. Prolonged incubation of the cells for up to 120 minutes at pH 7.4 did not induce any significant cell volume changes (Figure 1). Cell volume also remained normal after decreasing the pH to 7.2 or 7.0. When the pH was lowered to 6.8, cell volume increased to 113% of normal (p < 0.001), but further reductions of the pH were not associated with additional increases in cell size above the value found at pH 6.8 (Figures 1 and 2). Cell swelling at pH 6.8 or below occurred within 1–3 minutes. At this pH, there was an initial steep increase of the cell volume to 107% during the first 5–10 minutes followed by more gradual swelling to 113% during the subsequent 2 hours (Figure 1). Immediate lowering of the pH to 6.2 led to a faster increase of cell volume, that is, within 10–15 minutes compared with the swelling kinetics at pH 6.8. At pH 6.2, no further cell swelling was observed after the cell volume had reached approximately 113% of normal (Figure 1). Lowering the pH of the medium to 6.2 led to an immediate reduction of the bicarbonate level by approximately half.

Induction of an extracellular alkalosis (pH 8.2) led to slight shrinking of the C6 glioma cells to 95.6% of normal, which was significant only during the first 30 minutes of exposure (p < 0.01, Figure 2). After prolonged periods at pH 8.2, cell shrinking became...
somewhat attenuated. The cell volume at pH 7.8 was not significantly different from the control values at pH 7.4.

Amiloride was added to the C6 glioma cell suspension medium to investigate the function of the Na\(^+\)/H\(^+\) antiporter in acidosis-induced cell swelling. At pH 7.4 using bicarbonate buffer, addition of amiloride did not influence cell volume during a control period of 15 minutes. The mean modal cell volume was 852.1 ± 13.1 \(\mu\)m\(^3\). The swelling response after titration of the medium to pH 6.8 or 6.2, however, was significantly diminished in the presence of amiloride (Figure 3). Addition of acetazolamide to inhibit carbonic anhydrase was also found to significantly reduce swelling of the glial cells at pH 6.2 (Figure 3). Since amiloride and acetazolamide were effective in attenuating acidosis-induced cell swelling at a pH as low as 6.2, studies at pH 6.8 were omitted.

Further, swelling of C6 glioma cells could be partially inhibited by replacement of bicarbonate by HEPES (Figure 4). The inhibition was complete after an additional exchange of Na\(^+\) in the medium against choline chloride (Figure 4). This procedure had no effect on cell volume under control conditions at pH 7.4. In this group of experiments, the normal cell volume was 837.3 ± 25.1 \(\mu\)m\(^3\). The removal of Na\(^+\) from the medium against choline, however, was associated with a significant reduction of the cell volume to 598.8 ± 6.5 \(\mu\)m\(^3\) \((p < 0.01)\) during the control period at pH 7.4. Cell viability was hardly affected by decreasing the pH of the suspension medium to as low as 6.2, whereas survival of the cells deteriorated markedly at pH 8.2 as concluded from the rapidly decreasing number of glial cells remaining in suspension. Cell volume studies at more pronounced levels of alkalosis were, therefore, not possible.

The data obtained with C6 glioma cells were confirmed by experiments using glial cells from primary culture. Figure 5 illustrates the degree and time course of glial cell swelling using bicarbonate-buffered medium. Just as glioma cells, the primary culture glial cells increased in volume when pH was lowered to 6.2. After 10 minutes, a stable plateau was reached at about 118% of control. The swelling response was attenuated again by the addition of amiloride to the medium. In HEPES-buffered medium, cell volume remained completely normal after induction of acidosis, even at pH 6.2.
Discussion

Our experiments continue in vitro studies of this laboratory on the molecular mechanisms of cytotoxic glial swelling. C6 glioma cells were again used as model cells with properties specific for glial cells. Glial-specific markers as well as transmitter uptake systems and enzymes characteristic for glia have been demonstrated in the cell line. C6 glioma cells were previously found to possess powerful volume regulatory mechanisms, for example, during anisotonic exposure or induction of energy failure by anoxia and inhibition of glycolysis. Maintenance of a normal glial cell volume under the latter conditions is in contrast with respective observations on rapid and extensive glial swelling during focal cerebral ischemia in vivo and suggests that the breakdown of cellular energy production alone is not sufficient to induce swelling, as might be concluded from the pump–leak model of the double Donnan equilibrium.

A variety of pathophysiologically significant processes are initiated after interruption of cerebral blood flow in vivo. Many may be more relevant for cell swelling than energy deprivation. The development of brain tissue acidosis, to pH levels around 6.0 from enhanced lactic acid production in the ischemic focus and the surrounding penumbra, is a case in point. The significance of tissue acidosis for ischemic brain damage is underlined by observations on the role of the actual blood glucose level. It is now generally accepted that enhancement of a standard ischemic lesion by hyperglycemia must be attributed to an increase of tissue acidosis. Thus, the glial and nerve cells of an ischemic penumbra under conditions of a marginal energy supply not only must cope with a massive spillover of excitotoxic active transmitter compounds, like glutamate, into the interstitial fluid space and a marked increase of the extracellular K+ concentration, but also are threatened by extracellular acidosis.

Our in vitro findings quantify the degree of cell swelling at a level of acidosis that is observed in vivo under respective pathophysiologic circumstances. Moreover, the data provide pertinent information as to the underlying mechanisms. In-depth investigations of the damaging potential of acidosis on a cellular or molecular level are necessary since acidosis must be considered a common denominator not only in ischemia but also in other pathologic states, such as epileptic seizures. On the other hand, the maintenance of cellular functions under conditions as critical as...
acidosis require closely controlled mechanisms to avert cell damage. One such mechanism is regulation of the intracellular pH, which is accomplished not only by powerful intracellular buffering systems but also by transmembrane exchange processes, especially the Na⁺/H⁺ antiporter.

Na⁺/H⁺ antiporter systems have been described for many cell types including glial cells taken from...
primary culture as well as for C6 glioma cells. If H+ is transported out of the cell against extracellular Na+ by this system without violating electroneutrality. Due to the unfavorable transmembrane electrical potential, removal of intracellular H+ requires energy made available by the downhill influx of Na+. The price to pay, however, is their subsequent uphill extrusion involving a functional energy metabolism and the Na+ pump. Obviously, an energetically expensive mechanism is bound to fail under conditions of limited energy supply as in an ischemic penumbra. There, Na+ exchanged against protons for maintenance of a normal intracellular pH remains trapped within the intracellular compartment, leading to cytotoxic cell swelling.

Figure 6 illustrates the sequence of events by which extracellular acidosis of an ischemic penumbra might induce cell swelling. Accordingly, H+ accumulating in the extracellular compartment is buffered by bicarbonate forming carbonic acid, which immediately dissociates into CO2 and water. The newly generated CO2 enters the intracellular space, recombining there with water to form carbonic acid as catalyzed by carbonic anhydrase. Subsequently, the product dissociates into protons and bicarbonate, with the former being exchanged by the Na+/H+ antiporter against Na+ and the latter against Cl-. As seen in Figure 6, neither exchange process violates electroneutrality. The resulting net influx of Na+ and Cl- increases the osmotic solute concentration inside the cell as the basis for cytotoxic cell swelling unless both ions are actively discharged.

Evidence for this concept is available. Our experiments demonstrate attenuation of the acidosis-induced swelling of glial cells, by either amiloride or acetazolamide, which inhibit the Na+/H+ exchanger or carbonic anhydrase, respectively. Moreover, cell swelling was reduced or even completely prevented when bicarbonate was replaced by HEPES or extracellular Na+ by choline. Deletion of bicarbonate from the medium prevented formation of carbonic acid and, consequently, of CO2 on addition of H+ (Figure 6), whereas exchanging Na+ against choline, like amiloride, directly led to a failure of the Na+/H+ shuttle. Both procedures evidently interfered with an intracellular accumulation of Na+ and thereby with cell swelling. The specificity of the pathways leading to cell swelling by extracellular addition of H+ as discussed in Figure 6 could be further ascertained by administration of anion-exchange inhibitors, such as 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS) or others. Respective experiments are currently in progress in this laboratory.

The interaction of mechanisms proposed in Figure 6 might have been particularly effective under the current experimental conditions to induce glial swelling by an extracellular acidosis. The supply of bicarbonate from the medium provided for abundant production of CO2 secondary to addition of H+. Thereby, the Pco2 of the medium could be expected, even in the open system used, to have exceeded the intracellular Pco2, facilitating diffusion of CO2 into the cells and, hence, cell acidification. Although it might appear that a stoichiometric exchange of protons against Na+ and of bicarbonate against Cl- cannot increase the cellular solute concentration, one should be aware that protons and bicarbonate are recycled into and out of the cell, whereas Na+ and Cl- become trapped inside the cell and consequently accumulate unless the active Na+ pump is operative.

The molecular model of the Na+/H+ antiporter proposed by Grinstein and Rothstein may explain the phenomenon of a pH threshold that must be surpassed before glial cells begin to swell. As seen in Figure 2, glial swelling commenced only after the extracellular pH was <7.0, which is actually close to the intracellular pH. Experimental evidence considers the antiporter as a functional complex consisting of an operator, the engine that extrudes protons against Na+ in a 1:1 ratio, and a regulatory unit, which is allosterically controlled by the actual intracellular pH turning the operator on and off. The activation threshold of the regulatory unit of the antiporter appears to be near a pH of 7.0–6.8, that is, close to the normal intracellular pH. Our findings of a pH threshold of 6.8 that was associated with commencement of glial swelling is in perfect agreement with this notion.

As shown in Figures 1 and 2, cell swelling on acidification of the medium appeared to be an all-or-nothing phenomenon once the pH fell below 6.8 and
did not follow dose–response characteristics. The mechanism underlying this observation is not fully understood at the moment. It might be inferred that the influx of Na⁺ through the antipporter and its removal by Na⁺,K⁺-ATPase immediately attained an equilibrium that prevented further cell swelling. This hypothesis is based on the assumption that the Na⁺ pump was activated under these circumstances. This hypothesis was experimentally tested by combining exposure of glial cells to acidosis, with addition of ouabain to inhibit the active Na⁺ pump. In a preliminary group of experiments (not described here) using primary-culture glial cells, ouabain did not, however, increase the swelling response to acidosis as one would expect according to the above considerations. Alternatively, one could assume a higher activity of the Na⁺/H⁺-antiporter compared with that of the Cl⁻/HCO₃⁻ exchanger, which would result in accumulation of bicarbonate inside the cell, slowing down the carbonic anhydrase reaction and, consequently, the intracellular acid load. Finally, it might be proposed that with decreasing intracellular pH, formation of H⁺ and bicarbonate became attenuated as the pK of the HCO₃⁻/CO₂ equilibrium was approached. This could have limited the further supply of protons for the antipporter. The fact that cell volume increased only initially and did not continue to increase may be explained by exposure of the cells to acidosis in an open system. CO₂ produced by buffering with bicarbonate was eliminated eventually through the membrane oxygenator of the chamber. This might have accelerated attainment of a steady state of the intracellular-extracellular CO₂ distribution as a determinant of the degree of cellular acidosis and, hence, cell swelling. In summary, our results demonstrate significant swelling of glial cells on acidification of the extracellular suspension medium requiring Na⁺ and HCO₃⁻ in the medium. Activation of the Na⁺/H⁺ as well as the Cl⁻/HCO₃⁻ antiporters is proposed as a central mechanism of acidosis-induced cell swelling, which is supported by inhibition and replacement experiments using amiloride, bicarbonate-free, or Na⁺-free medium, respectively. Accordingly, cell swelling by acidosis results not only from intracellular but also from extracellular accumulation of H⁺ along the mechanisms discussed in Figure 6. Induction of extracellular rather than intracellular acidosis in our studies can be considered to reflect the conditions of an ischemic penumbra in vivo, where the extracellular environment of still-viable perifocal brain tissue is flooded with hydrogen ions leaking from the ischemic focus into surrounding areas. Consequently, effective control of the tissue pH under respective clinical conditions might improve cytotoxic brain edema in patients with ischemic stroke.

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References


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