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Swelling and death of neuronal cells by lactic acid

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Summary

Lactacidosis occurring in cerebral ischemia or trauma is a major mechanism of cytotoxic brain edema and brain damage. Respective effects of lactacidosis were currently analyzed in vitro by employment of the murine neuronal cell line, Neuro-2A, in order to obtain a better understanding of specific mechanisms underlying cell swelling and cell death in comparison with glial cells. The cells were suspended in a physiological medium in the presence of lactic acid at increasing concentrations. Levels of acidosis reaching from pH 6.8–5.6 were obtained while other parameters, such as osmolarity and electrolyte concentrations, were maintained in the physiological range. Assessment of cell swelling and cell viability using exclusion of propidium iodide was made by flow cytometry with employment of an advanced Coulter system. Swelling of Neuro-2A cells commenced once the pH in the medium was lowered to 6.8 or below. From this level downward, cell swelling was a function of the severity of acidosis and duration of exposure. For example, lactacidosis of pH 6.8 or 5.6 lasting 90 min led to an increase in cell volume to 109.5% or 159.6% of normal, respectively. Viability of the neuronal cells was 85% under control conditions. It remained in this range down to pH 6.2. At pH 5.6, however, cell viability decreased in a time-dependent fashion. At 90 min, only 48.9% of the neuronal cells were viable at pH 5.6. The swelling response and impairment of viability of the neuronal cells was compared with that of C6 glioma cells. A 60 min exposure of the glial cells to either pH 6.2 or pH 5.6 led to swelling of only 55% or 65%, respectively, of the cell volume increase observed in the Neuro-2A cells. In addition, the glial cells were less vulnerable to lactacidosis as demonstrated by better maintenance of cell viability. After suspension for 1 h at pH 5.6, only 53.9% of the neuronal cells were alive, in comparison to 74.1% of the C6 glioma cells. Taken together, the present findings demonstrate, as former observations on glial cells, that lactacidosis is a powerful mechanism of cell swelling and cell death in a neuronal cell line. As in the glial cells, different pH thresholds could be identified, associated either with cell swelling or a decrease in cell viability. While cell swelling occurred already at relatively mild levels of acidosis (pH 6.8), viability of the Neuro-2A cells was decreasing only at pH 5.6, confirming different susceptibilities of cell swelling and cell death to acidosis. The level of acidosis found to destroy nerve cells in vitro has been observed in severe forms of cerebral ischemia in vivo, for example in hyperglycemia.

Key words: Cell swelling; Cell viability; Neuro-2A neuroblastoma cell; Lactacidosis; Glioma cell

Introduction

Brain edema is still one of the most important complications of neurological or neurosurgical disorders. The developement of edema after an ischemic or traumatic insult is highly significant for the outcome of patients. The cytotoxic type of edema, i.e., swelling of glial and nerve cells, is commonly found in cerebral ischemia, brain trauma but also in metabolic disorders (Baethmann 1978). Our laboratory utilizes an in vitro model for investigations of pathomechanisms of cytotoxic cell swelling under strictly defined conditions (Kempski et al. 1983). In recent studies mediators and mechanisms involved in the swelling of C6 glioma cells and astrocytes from primary culture have been examined. Acidosis, enhanced concentrations of glutamate, or of K^+ in the extracellular compartment were found to induce glial swelling (Kempski et al. 1988; Chan et al. 1990; Staub et al. 1990; Kempski et al. 1991). These findings indicated that glial swelling from acidosis, glutamate, or enhanced K^+ levels is not necessarily a manifestation of cell injury, but may reflect activation of a control mechanism to ensure survival and function of cerebral parenchyma.

While knowledge on volume regulation and swelling of glial cells has considerably grown during recent years, it is limited in the case of neuronal cells. We

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were therefore studying a neuronal cell line, mouse Neuro-2A neuroblastoma, to assess the effect of increasing levels of lactacidosis on cell volume and viability. Lactacidosis is common in acute lesions, e.g., from cerebral ischemia, severe head injury or seizures (Kraig et al. 1985; Smith et al. 1986; Andersen et al. 1988; Siesjö 1988), and it is considered as a major determinant of the formation of cytotoxic brain edema and, eventually, cell death (Kraig et al. 1987; Kempski et al. 1988; Siesjö 1988; Nedergaard et al. 1991).

Materials and methods

Cell culture

Mouse Neuro-2A neuroblastoma cells were grown in plastic culture flasks using Eagle's minimal essential medium (MEME) with 25 mM bicarbonate. The medium was supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acids (Flow Laboratories, Meckenheim, Germany), 100 IU/ml penicillin G, and 50 μg/ml streptomycin. The cells were cultivated at 37°C in humidified air supplied with 5% CO_2. Subcultivation was carried out once a week. The cells were harvested for the experiment 7–8 days after subcultivation when reaching confluency. After detachment with 0.05% trypsin/0.02% EDTA, the cells were resuspended in MEME with FCS for inactivation of trypsin. Two washes with serum-free medium followed to remove FCS thereafter. The C6 glioma cells were cultivated in Petri dishes, using Dulbecco's modified minimal essential medium (DMEM) with 25 mM bicarbonate, 10% FCS, 100 IU/ml penicillin G, and 50 μg/ml streptomycin. The cells were subcultivated every other day. Methods of culturing and preparation of the glial cells for the experiment were those employed for the Neuro-2A cells (cf., above). As a final step, the cells were resuspended in serum-free medium and introduced into a plexiglas incubation chamber. Physiological parameters, such as temperature, pH and PO_2, were continuously monitored by respective electrodes in the chamber. A permeable silicone rubber tube served as a membrane oxygenator supplying the cell suspension with CO_2, O_2, and N_2. Cell sedimentation was prevented by a magnetic stirrer. Further details of the incubation chamber have been published (Kempski et al. 1983, 1988).

Analytical procedures

The cell volume was determined by flow cytometry (Metricell®) based on the Coulter principle with hydrodynamic focusing (Kachel 1976). Accuracy of the method allows detection of cell size alterations of ~ 1%. The system is electrically and also mechanically calibrated by latex beads of known diameter. Cell viability was also assessed by flow cytometry (Fluvo Metricell®, HEKA-Elektronic, Lambrecht/Pfalz, Germany) (Kachel et al. 1977) using exclusion of propidium iodide (Rothe and Valet 1988). For that purpose, aliquots of the cell suspension (100 μl) 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. Indicator fluorescence was excited through a 500 nm short-pass filter by a high-pressure mercury arc lamp. Maximum emission of propidium iodide at 630 nm was measured by using a 580 nm long-pass filter. A window integration system was employed for discrimination of propidium-positive (dead) from propidium-negative (viable) cells. The osmolality of the suspension medium was measured by freezing point depression (Osmomat 030, Gonotec, Berlin, Germany).

Results are expressed as mean ± SEM. The data were analyzed for statistical significance using the Kruskal-Wallis test for non-parametric one-way analysis of variance and multiple comparisons on ranks for unpaired samples (Theodorsson Norheim 1986).

Induction of lactacidosis

The experiments were performed after a 45-min control period utilized for measurements of the cell volume, cell viability, and medium osmolality under normal conditions. Preparations with unstable cell volume or an impaired cell viability were discharged. The mean of 3 cell volume measurements obtained during the last 15 min of the control period was taken as reference. In the control group, the extracellular pH of the medium was maintained at 7.4 ($n = 6$). In 3 different groups, the pH was lowered from initially 7.4 (control) to either 6.8, 6.2 or 5.6 by addition of isotonic lactic acid (350 mM) to the suspended cells ($n = 4$–5 experiments/group). CO_2 was added to the incubation chamber during acidosis to compensate for its loss from buffering. Thereby, a pCO_2 of 80–100 mm Hg was obtained in the suspension medium. This level is comparable with respective in vivo findings in ischemic brain tissue (Smith et al. 1986). Cell volume and viability were monitored for 90 min during lactacidosis. Osmolality was frequently assessed under control conditions as well as after induction of lactacidosis. For comparison, studies on cell swelling and cell damage from lactacidosis were also performed with C6 glioma cells at pH 6.2 or 5.6, respectively ($n = 4$ per group). The measurements of the cell volume and viability were carried out during acidosis for 60 min.

Results

Under control conditions at pH 7.4, PO_2 of 80–100 mm Hg, and 37°C, volume and viability of the Neuro-2A neuroblastoma cells remained stable for up to 3 h

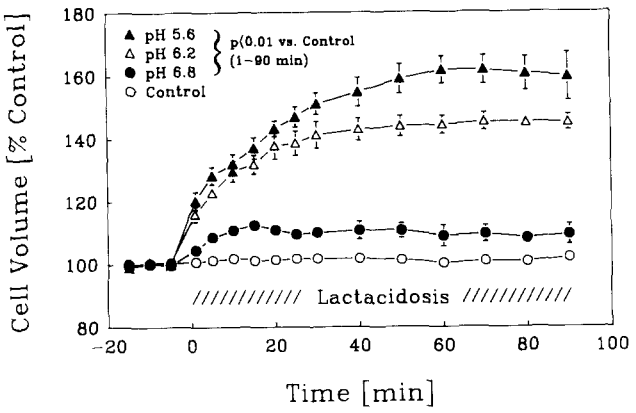

Fig. 1. Cell volume response of Neuro-2A cells suspended at pH 7.4 or at increasing levels of acidosis down to pH 5.6. Cell volume is given in percent of the normal value obtained during the last 15 min of the control period. The medium was titrated by addition of isotonic lactic acid, leading to a dose-dependent volume increase of Neuro-2A cells. 10^4–10^5 cell were used for a single measurement. The symbols represent mean ± SEM of 4–6 experiments. Absence of bars (as in the other figures) indicates SEM being smaller than the symbol size.

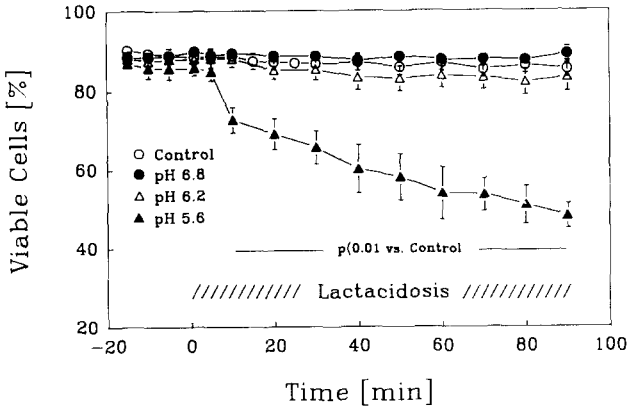

Fig. 2. Viability of Neuro-2A cells during administration of lactic acid at different pH levels. Viable cells (propidium iodide negative) are shown in percent of the total cell population. Viability remained normal for 90 min after lowering pH to 6.8 or 6.2; at pH 5.6 the number of viable Neuro-2A cells started to significantly decrease. Four to six experiments were performed at each pH level.

(Figs. 1 and 2); longer periods were not studied. The average cell volume was 1532.4 ± 17.9 μm^3, and 88.5 ± 0.8% of the Neuro-2A cell population were viable, i.e., propidium iodide-negative. Titration of the suspension from pH 7.4 to 6.8 by isotonic lactic acid led to a significant increase of the cell volume, to 104.5 ± 0.6% of control within 1 min, or 110.9 ± 1.4% within 10 min ($P < 0.01$, Fig. 1). The volume increase found at pH 6.8 was maintained for the remaining observation period. Lowering of the pH to 6.2 or 5.6, respectively, led to more intensive cell swelling. Exposure for 1 min at pH 6.2 increased the cell volume to 116.0 ± 2.5% of control, at pH 5.6 to 120.3 ± 2.9% ($P < 0.01$). At this level of acidosis, swelling of Neuro-2A cells was enhanced with continuing exposure. After 40 min, the cell volume was increased to 143.1 ± 3.6% (pH 6.2), while to 154.9 ± 4.6% at pH 5.6. At pH 6.2, cell swelling leveled off then for the remaining observation period, whereas at pH 5.6 the cell size increased further to approximately 160% of control (Fig. 1). The viability of the Neuro-2A cells remained normal after lowering pH to 6.8 (Fig. 2). At pH 6.2, cell viability was moderately affected after exposure for 40 min. In contrast, the number of viable cells started to significantly decrease to 72.6 ± 3.3% ($P < 0.01$) at pH 5.6 already 10 min after addition of lactic acid. Thereafter, cell viability continued to decline, and only 48.1 ± 3.2% of the Neuro-2A cells were alive at 90 min at this level of acidosis (Fig. 2).

As seen in Fig. 3, the lactacidosis induced swelling of Neuro-2A cells was more severe at a given pH than the swelling response of C6 glioma cells. For instance, at pH 6.2 the increase in cell volume of Neuro-2A cells was 144.4 ± 3.0% after 60 min. This is about twice of what was found under corresponding conditions with C6 glioma cells (i.e., 124.5 ± 4.6%, $P < 0.01$, data not

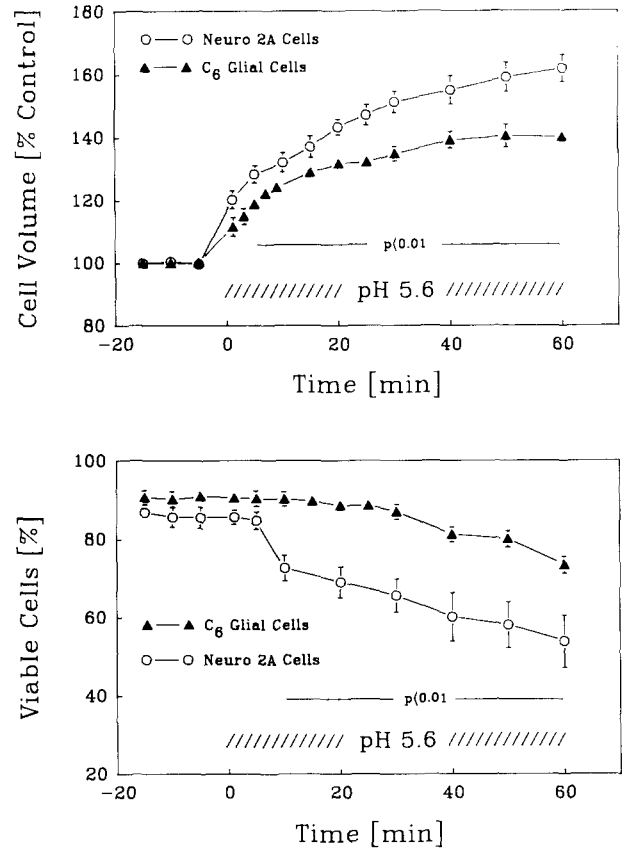

Fig. 3. Volume (top) and viability (bottom) of Neuro-2A cells as compared to C6 glioma cells subjected to lactacidosis of pH 5.6. The increase in cell volume is given in percent of control. Addition of lactic acid led to significantly more swelling of Neuro-2A cells. Viability of the neuronal cells was also more affected by pH 5.6 as compared to C6 glioma cells. Symbols represent mean ± SEM of 4–5 experiments per group.

shown). Exposure of the neuronal cells to lactacidosis of pH 5.6 for 1 h led to cell swelling of 161.8 ± 4.3% as compared to 139.9 ± 2.1%, when C6 glioma cells were studied ($P < 0.01$, Fig. 3). Moreover, lactacidosis caused a more severe decrease of viability in Neuro-2A cells than in the C6 glioma cells. Viability of the Neuro-2A cells was found to decrease already after 10 min exposure at pH 5.6, a time period when the viability of C6 glioma cells still remained in a normal range ($P < 0.01$). At 60 min, 53.9 ± 6.7% of Neuro-2A were alive as compared to 74.1 ± 2.2% of C6 glioma cells (Fig. 3).

Discussion

The current experiments expand former in vitro investigations of this laboratory, using C6 glioma cells and astrocytes from primary culture, to uncover mechanisms underlying cytotoxic brain edema in vivo (Kempski et al. 1988; Staub et al. 1990). Neuronal swelling and irreversible damage was currently analyzed, since only limited information is available on respective changes of this cell type under corresponding pathophysiological conditions in vivo, as e.g., compared to astrocytes (Klatzo 1967; Baethmann 1978). A neuronal cell line having properties of nerve cells in situ was utilized for that purpose. Employment of an established cell line was considered advantageous as compared to using nerve cells from primary culture on account of the present experimental requirements. These made necessary availability of a high yield of cells for the extended experimental observation periods with frequent sampling of aliquots from the suspension medium. Thereby, the dynamics of cell swelling and of viability changes could be studied with excellent temporal resolution. The analytical system of this laboratory allows us to examine cell volume and cell viability changes almost continuously, and with accuracy hardly available when studying the brain in situ, for example in ischemia or trauma. The availability of an established cell line with defined cell biological properties, such as the cell size facilitated standardization of the experimental conditions. Further, physiological variables of the suspension medium, such as PO_2, temperature, ion concentrations, osmolality and other parameters could be controlled and maintained during the course of the experiment.

Although the nerve cells were suspended in medium with virtually infinite volume and unlimited availability of Na^+, Cl^- and H_2O, the cell volume was kept constant in a narrow range under control conditions. Previous experiments with other cell types including primary cultured astrocytes confirm that inspite of a practically unlimited suspension volume, the cells are not liable to swell spontaneously (Kempski et al. 1983, 1988). The present experimental conditions, thus, are different from respective circumstances in vivo, e.g., cerebral ischemia. During interruption of cerebral blood flow the amount of fluid available for cell swelling is limited in the brain. It is the fluid present in the extracellular compartment. If, however, blood flow is re-established again after ischemia, cell swelling is markedly enhanced by the additional availability of electrolytes and water entering the brain by the perfusion (Hossmann et al. 1976).

As to the validity of murine neuroblastoma cells as model cells, Neuro-2A have morphological as well as functional properties also found in neurons of cerebral tissue. The cells are capable to form synaptic contacts (Spoerri et al. 1980), have an excitable cell membrane (De Laat and Van der Saag 1982), express glutamate receptors (Van der Valk and Vijverberg 1990), and high activities of specific neuronal enzymes, such as choline acetyltransferase, tyrosine hydroxylase or glutamate decarboxylase (De Laat and Van der Saag 1982; Thompson et al. 1982).

Lactacidosis was studied, since accumulation of lactic acid is an important metabolic response in the brain in cerebral ischemia or head injury, among others (Kraig et al. 1985; Smith et al. 1986; Andersen et al. 1988). Lactacidosis has been shown to play a significant role in the formation of cytotoxic brain edema and cell damage under these circumstances (Kraig et al. 1987; Staub et al. 1990), and it is also likely to enhance other adverse mechanisms, such as free radical formation (Siesjö et al. 1985). Lactic acid produced in ischemic or traumatic tissue necrosis can be expected to leak into the perifocal brain (penumbra), resulting there in an interstitial acidosis (Nedergaard et al. 1990). In cerebral infarction the penumbra is characterized as still viable tissue, yet with blood flow too low to support normal neuronal function, but not low enough for the development of irreversible infarction. The penumbra zone is in jeopardy from secondary disturbances of blood flow, e.g., due to formation of cytotoxic cell swelling by acidosis and other mechanisms (Astrup and Symon 1981).

The clinical significance of brain tissue acidosis is underscored by therapeutical attempts with buffering in severe head injury (Rosner and Becker 1984; Yoshida and Marmarou 1991). Administration of thromethamine in animals with fluid percussion injury of the brain was found to decrease production of lactate and brain edema, promoting recovery of energy metabolism (Yoshida and Marmarou 1991). Therapeutical efforts to control brain tissue pH in patients with ischemia or trauma might salvage tissue at risk, for example the penumbra zone by interfering with cytotoxic cell swelling from acidosis.

The present findings provide quantitative information on dose-effect relationships between cell swelling,

or decrease in cell viability on the one hand and the severity of lactacidosis on the other. As seen, swelling of Neuro-2A cells commenced as soon as the pH of the suspension was reduced to 6.8 by the administration of lactic acid. Below this pH level, cell swelling occurred in a dose-dependent manner (Fig. 1).

A novel observation is that neuronal cells had a greater susceptibility to swelling at a given pH and that in severe acidosis the cells were more vulnerable than glial cells (Fig. 3). In accordance with results obtained in C6 glioma cells, swelling is interpreted as a response which is activated to maintain or restore, respectively the normal intracellular pH (pH_i) by exchange of H^+ against Na^+, leading to intracellular accumulation of Na^+ ions and, consequently, of water (Kempski et al. 1988). Activation of the Na^+/H^+ antiporter is a major regulatory mechanism of pH_i in acidosis (Grinstein and Rothstein 1986; Chow et al. 1991). It was recently demonstrated, however, that neither neuronal nor glial cells are capable of defending pH_i, if the external pH (pH_e) is decreased below 6.8 (Nedergaard et al. 1991; Mellergård and Siesjö 1991). The fact that pH_i is following pH_e in acidosis, however, does not rule out an increased H^+ extrusion of the cells as an attempt of pH_i regulation. It has indeed been shown that proton transport rates increase exponentially with the degree of intracellular acidification (Molenaar 1986). The more extensive swelling of neuronal cells in the present study is explained by the hypothesis that H^+ ion exchange mechanisms were running at a higher level than in the glial cells. This conclusion is supported by acidosis experiments of Nedergaard et al. (1991) demonstrating that neuronal cells are capable of gradual recovery of pH_i after initial decrease, whereas pH_i in glial cells does not recover under respective circumstances. Obviously, the better recovery of pH_i in these cells during acidosis has its price, namely an enhanced influx of Na^+ ions causing more severe swelling from acidosis as confirmed by the present findings. It was previously demonstrated by ourselves that activation of the Na^+/H^+ and Cl^-/HCO_3^- antiporter systems leading to intracellular accumulation of Na^+ and Cl^- ions and water must be considered as final pathway of cell swelling from acidosis (Kempski et al. 1988; Staub et al. 1990).

The present analytical system made possible a combined analysis of cell volume alterations together with impairments of cell viability to evaluate the significance of the former for the development of irreversible cell damage. The findings demonstrate a remarkable ability of Neuro-2A cells to remain viable at advanced levels of acidosis. Viability was in the control range during the entire observation period, even at more severe levels of acidosis, as for example pH 6.2. Cell viability deteriorated only when the pH of the suspension medium was lowered to 5.6 (Fig. 2). The increase of irreversibly damaged, propidium-iodide positive cells occurred then in a time-dependent fashion. Although brain tissue pH is usually not decreasing below 6.0 in cerebral ischemia, data are available that cerebral ischemia in combination with marked hyperglycemia causes far more severe acidosis than in normoglycemia (Nedergaard et al. 1990; Katsura et al. 1991). Under extreme conditions with a blood glucose level of 57 mM, brain tissue acidosis of pH 5.5 has been reported (Kraig et al. 1985). The currently observed pH threshold of 5.6 associated with impairment of cell viability might be relevant for the acute response of this parameter. It does not rule out that more moderate levels of acidosis also affect cell viability, however, with a delay of hours or even days (Nedergaard et al. 1991). Nevertheless, the presently obtained pH threshold related with the destruction of neuronal cells is in agreement with corresponding studies in astrocytes and neurons (Nedergaard et al. 1991) or respective findings in vivo (Kraig et al. 1987).

Whereas the mechanisms underlying cell swelling in acidosis are increasingly understood, it is not completely clear why higher levels of acidosis cause irreversible cell damage. A variety of factors might be involved, such as inhibition of the cellular energy metabolism, of protein synthesis, or adverse effects on intracellular Ca^{2+} buffering which all are sensitive to a loss of pH_i control (Trivedi and Danforth 1966; Busa and Nuccitelli 1984). Former investigations have shown that severe acidosis causes denaturation of proteins (Tanford 1968) and formation of free radicals (Siesjö et al. 1985). Each of these factors alone might suffice to inflict irreversible cell damage. These possible mechanisms notwithstanding, it would not explain why according to the present findings the neuronal cells were more vulnerable to lactacidosis than the glial cells (Fig. 3). It is conceivable, for example, that differences between both cell types to maintain energy metabolism at advanced levels of acidosis played a role. It has been shown that phosphofructokinase, the key enzyme controlling glycolysis, is inhibited in acidosis (Trivedi and Danforth 1966; Busa and Nuccitelli 1984). If the neuronal cells were more dependent on glycolysis than the glial cells to support energy metabolism, a higher vulnerability of the neuronal cell line might be attributable to a more severe inhibition of this metabolic pathway in acidosis as compared to that of the glial cells. Experiments of this laboratory have shown (Mackert et al. in preparation) that inhibition of glycolysis by iodoacetate (2.5 mM) in neuronal cells causes intracellular accumulation of Na^+ ions and a decrease of K^+, indicating inhibition of Na^+/K^+ ATPase and failure of energy metabolism. On the other hand, anoxia did not induce such changes, indicating that glycolysis is a pertinent pathway of energy production in Neuro-2A cells. An increasing vulnerability of the nerve cells

might, thus, be attributable to more severe inhibition of glycolysis at low pH.

Altogether, the present findings obtained by employment of a defined neuronal cell line confirm former observations and concepts, respectively, on the significance of acidosis as an important pathophysiological factor of cytotoxic cell swelling and cell damage under corresponding pathological conditions in vivo (Siesjö 1988). A better understanding of underlying mechanisms may provide a basis for the development of more specific methods of treatment. Respective efforts of inhibition of cell swelling from acidosis, however, must be selective to avoid interference with potentially protective mechanisms activated to ensure cell survival.

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