

Modification of Hypoxia-Induced Injury in Cultured Rat Astrocytes by High Levels of Glucose

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Background and Purpose: Preexisting hyperglycemia exacerbates central nervous system injury after transient global and focal cerebral ischemia. Increased anaerobic metabolism with resultant lactic acidosis has been shown to cause the hyperglycemic, neuronal injury. The contribution of astrocytes in producing lactic acidosis under hyperglycemic/ischemic conditions is unclear, whereas the protective role of astrocytes in ischemic-induced neuronal injury has been documented. The ability of astrocytes to maintain energy status and ion homeostasis under hyperglycemic conditions could ultimately reduce neuronal injury. Therefore, we determined the effects of increased glucose concentrations on glucose utilization, lactate production, extracellular pH, and adenosine triphosphate concentrations in hypoxia-treated astrocyte cultures.

Methods: Primary astrocytes were prepared from neonatal rat cerebral cortices. After 35 days in vitro, cultures were incubated with 0–60 mmol/L glucose and subjected to hypoxic conditions at 95% N₂/5% CO₂ for 24 hours. In addition, under high-glucose conditions (30 mmol/L), astrocytes were exposed to up to 72 hours of hypoxia. Determination of lactate dehydrogenase efflux, adenosine triphosphate concentrations, and extracellular lactate concentrations defined astrocyte status. Equiosmolar levels of mannitol were added in place of high glucose concentrations to distinguish hyperosmotic effect.

Results: When physiological concentrations of glucose (7.5 mmol/L) or lower concentrations were used, significant cell damage occurred with 24 hours of hypoxia, as determined by increased efflux of lactate dehydrogenase and loss of cell protein. When higher glucose concentrations (15–60 mmol/L) were used, efflux of lactate dehydrogenase was similar to that observed in normoxic cultures, despite an increased utilization of glucose. Lactate concentrations in the media at low or normal glucose concentrations exceeded normoxic levels, but higher glucose concentrations (15–30 mmol/L) failed to increase lactate levels further. Values of adenosine triphosphate for hypoxic astrocytes treated with high glucose concentrations were significantly higher than those of astrocytes with zero or low glucose levels. In cultures exposed to hypoxia and high glucose levels (30 mmol/L), no cellular injury was observed before 48 hours of hypoxia. Lactate concentrations in the media increased during the first 24 hours of hypoxia and reached steady state. The pH of the media decreased to 6.4 after 24 hours and to 5.5 at 48 hours. The latter pH was concomitant with a marked increase in extracellular lactate dehydrogenase activity. Hyperosmotic mannitol failed to protect cultured astrocytes against hypoxia.

Conclusions: Hypoxic injury to mature astrocytes was reduced by the presence of 15–60 mmol/L glucose in the medium during 24–30 hours of hypoxia. Injury occurred when the pH of the medium was <5.5. This protection was not afforded by the hyperosmotic effect of high glucose concentrations, nor was the hypoxic injury at later time periods with 30 mmol/L glucose mediated solely by lactate accumulation. (*Stroke* 1993;24:855–863)

KEY WORDS • astrocytes • glucose • hypoxia

Preischemic hyperglycemia appears to exacerbate the injury produced with transient cerebral ischemia. The effects of high glucose levels under ischemic conditions include increased cellular edema,¹ increased neuropathology,^{2,3} reduction in energy metabolites,^{4,5} and increased lactic acidosis.^{3–8} However,

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Supported in part by National Institutes of Health grants NS-20692 (G.A.G.), NS-14543 (P.H.C.), NS-25372 (P.H.C.), and AG08938 (P.H.C.).

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other investigators using an irreversible focal ischemic model observed that preischemic hyperglycemia had a beneficial effect on neurological outcome.^{9,10} Evidence suggests that during the postischemic phase and during oxygen reperfusion there is increased neural pathology.^{7,11} Systemic reperfusion with high glucose concen-

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Received August 3, 1992; final revision received December 28, 1992; accepted January 25, 1993.

trations after ischemic insult^{2,12} either reduced or did not exacerbate the infarct size compared with similarly treated normoglycemic animals. Similarly, insulin-induced hypoglycemia reduced postischemic necrosis compared with dextran-fed animals alone.¹³ Contrary effects have been reported with postanoxic hyperglycemia, which caused further brain damage.¹⁴

Hyperglycemia during incomplete ischemia is reported to enhance ischemic brain damage by causing severe lactic acidosis.^{4,15} Tissue lactate values >15 or 25 $\mu\text{mol/g}$ tissue were found to be detrimental to brain tissue.^{7,16} After reperfusion, lactate values may remain high,⁵ possibly as a result of hypermetabolism and the return of nearly normal blood flow.^{17,18}

Because of the heterogeneity of cells in brain tissue, many investigators have used cell cultures to determine cell-specific injury. Hypoxic neuronal damage *in vivo* and *in vitro* contrasts sharply with that observed for other brain cells, specifically astrocytes.⁸ For example, astrocytes tolerate longer periods of hypoxia than neurons,¹⁹ and in addition, astrocytes increased the neuronal survival in coculture after 4 hours of anoxia.²⁰ In these studies, glucose had a minimal effect on the survival of neurons and mixed neuronal/astrocyte cultures. Furthermore, astrocyte-enriched neuronal cultures were less susceptible to glutamate toxicity than astrocyte-poor neuronal cultures.^{21,22} Glutamate toxicity is implicated in mediating ischemic/hypoxic damage, both *in vivo*²³ and *in vitro*.^{24,25} However, other investigators believe that the protective benefit of astrocytes on anoxic neurons is unrelated to glutamate metabolism by the glial cells.²⁰ How astrocytes implement neuronal protection under hypoxic conditions is unclear. Answers to this question may lie in the specific metabolic differences between astrocytes and neurons that make the former less susceptible to hypoxic injury. Therefore, determination of factors that are involved in the resistance of astrocytes to hypoxia may be of significance. Recently, developing glial cultures were shown to be resistant to 24 hours of hypoxia when high glucose concentrations were present.²⁶

In the present study, we assess the effectiveness of high glucose levels in incubation medium on differentiated astrocytes exposed to a period of hypoxia. The older astrocytes were used to determine the effect of glucose and hypoxia at a time when brain is less resistant to hypoxic insult.²⁷ Glucose concentrations capable of reducing hypoxic injury in astrocytes were then used to analyze anaerobic metabolism and glucose loss from the media and to determine the extent of resistance to increasing periods of hypoxia. We also investigated the role of osmolarity in the protective effect of high glucose concentrations.

Materials and Methods

Cell Culture

Primary cultures of cerebral astrocytes were prepared by the method of Booher and Sensenbrenner²⁸ as modified by Yu et al²⁹ using newborn Sprague-Dawley rats (Bantin & Kingman, Freemont, Calif.). The cerebral hemispheres were removed from the skull aseptically, and the meninges, olfactory bulbs, basal ganglia, and hippocampi were discarded. The neopallium was placed in modified Eagle's minimum essential tissue

culture medium (MEM) containing 2.2 g/L NaHCO_3 that contained 20% fetal calf serum (FCS) (Sterile Systems, Logan, Utah). Tissue was dissociated by pressing it through a Nitex sleeve, then passing it through two sterile tissue Collector sieves (Bellco Glass Inc., Vineland, N.J.) with pore sizes of 120 μm (first sieving) and 10 μm (second sieving). One thirtieth of each brain-cell suspension was placed in a 60-mm Falcon tissue culture dish (Becton Dickinson, Oxnard, Calif.), and sufficient fresh culture medium (with 20% FCS) was added to bring the final volume to 3 mL. Cultures were incubated at 37°C with 95% air/5% CO_2 (vol/vol) and a humidity of 95%. The medium was first changed 3 days after the cell seeding and then twice weekly. After 2 weeks *in vitro*, confluent cultures were treated with 0.25 mmol/L dibutyryl cyclic AMP (Sigma Chemical Co., St. Louis, Mo.) to induce differentiation into cells that resemble mature astrocytes morphologically and biochemically.³⁰ After 4–6 weeks in culture, the cells were used for normoxic and hypoxic studies.

Hypoxic Studies

The cultures were washed with glucose- and serum-free medium, and 3 mL of fresh medium containing 0, 1.5, 3.0, 4.5, 6.0, 7.5, 15.0, 30.0, or 60.0 mmol/L glucose was added to 60-mm plates. Plates were placed in a gas-tight chamber (Billups-Rothenberg Co., Inc., Del Mar, Calif.) and flushed with 95% N_2 /5% CO_2 (15 L/min) for 15 minutes to reduce the environmental oxygen concentration to zero.¹⁹ The atmosphere was at least 95% saturated with water throughout the study. The chambers then were sealed and placed in a 37°C incubator for 24 hours. Normoxic controls were similarly washed with medium, and 3 mL of fresh medium that contained similar glucose concentrations was added. Culture plates then were placed in a 95% air/5% CO_2 environment.

For timed studies on the effects of high glucose concentrations, cultures were washed in glucose- and serum-free Eagle's MEM, and 3 mL medium containing 30 mmol/L glucose was added to each 60-mm culture plate. Plates were put in hypoxic chambers or kept under normoxic conditions for 24, 30, 48, or 72 hours. In all cases triplicate plates were used for each time point, and the experiments were repeated at least twice with separate cell preparations.

Enzyme and Metabolite Determinations

After the designated incubation period, the culture medium was decanted and aliquots taken. The cells were washed three times with ice-cold medium and scraped off the plate into 1 mL of NaOH (1N). Protein from cell preparations was determined by the method of Lowry et al.³¹ Lactate dehydrogenase (LDH) activities in the media were determined using Sigma LDH-Kit (Sigma Diagnostics, St. Louis, Mo.), usually within the same day. Lactate concentrations by the method of Henry³² (Sigma Diagnostics) and glucose concentrations by the method of Carroll et al³³ (Sigma Diagnostics) were measured within a week of the experiment from frozen aliquots. Measurements of intracellular LDH were made from washed cells scraped into 2 mL glucose-free medium and homogenized using a Physchotron microhomogenizer (Bio-medica Ltd., Osaka,

Japan). These determinations were made on the day of the experiment.

The intracellular concentration of adenosine triphosphate (ATP) was determined by the method of Kauppinen and Nicholls³⁴ with modification by Huang et al.³⁵ Cultures were exposed to 0, 7.5, 15, and 30 mmol/L glucose and made hypoxic for 24 hours. Control cultures contained 7.5 mmol/L glucose and were maintained under 95% air/5% CO₂. At the end of the hypoxic period, the cultures were washed twice with cold phosphate-buffered saline and quickly scraped into 2 mL of ice-cold 50 mmol/L tris(hydroxymethyl)aminomethane (Tris) HCl (pH 7.2), and 0.5 mL of 2.1N perchloric acid was added. Similarly, standard ATP dilutions were made to give a final concentration of 0.3–5.0 nmol in 2 mL of the same Tris buffer. The standard and samples were centrifuged for 15 minutes at 5,000g. To 2 mL of the supernate was added 0.5 mL of ethylenediaminetetraacetic acid (0.105 mol/L) and 0.3 mL KOH/triethanolamine (2N/1.5 mol/L), and the mixture was vortexed and then centrifuged at 5,000g for 15 minutes. Then, 200 μ L of luciferin-luciferase (Sigma Chemical) in a 25-fold dilution with buffer (50 mmol/L K₂HPO₄ and 20 mmol/L MgSO₄, pH 7.4) was added to a scintillation vial and allowed to sit for 3 minutes; a 200- μ L aliquot of standard or sample was added every minute, and chemiluminescence was measured over the same time period on an LS 2000 Beckman scintillation counter. A preset program gave a measurement every 5 seconds using a single-photon detector. The counts-per-minute determinations for the standards gave a linear curve. The mean \pm SEM for normoxic samples for the four experiments was 6.7 ± 1.8 nmol/mg protein.

Hyperosmotic Studies

To determine whether high glucose concentrations protected cells by increasing extracellular osmolarity, 0, 4.5, 7.5, 15, or 30 mmol/L mannitol was added to medium containing 7.5 mmol/L glucose under normoxic and hypoxic conditions. After 24 hours the medium was decanted, and LDH activity, lactate concentrations in the media, and protein concentrations in the cells were determined as described previously.

Statistical Analysis

Data were analyzed by one-way analysis of variance using STATVIEW SE+ (Abacus Concepts, Inc.). Where appropriate, the unpaired *t* test was used for statistical significance, with a value of *p* < 0.05 considered significant. Data are presented as mean \pm SEM.

Results

Cell injury caused by hypoxic insult was evaluated by measuring the activity of LDH released from the astrocytes into the culture medium. At low glucose concentrations (0–3 mmol/L) there was maximal LDH release of >300 units/L after 24 hours of hypoxia (Figure 1A). Greater glucose concentrations during the hypoxic insult reduced the LDH efflux significantly. At initial glucose concentrations of 15–60 mmol/L, extracellular LDH activity was comparable to that in normoxic conditions in which the initial glucose concentration was 7.5 mmol/L (control).

Cell death, signified by loss of total cell protein, was most significant at initial glucose concentrations of 0–6

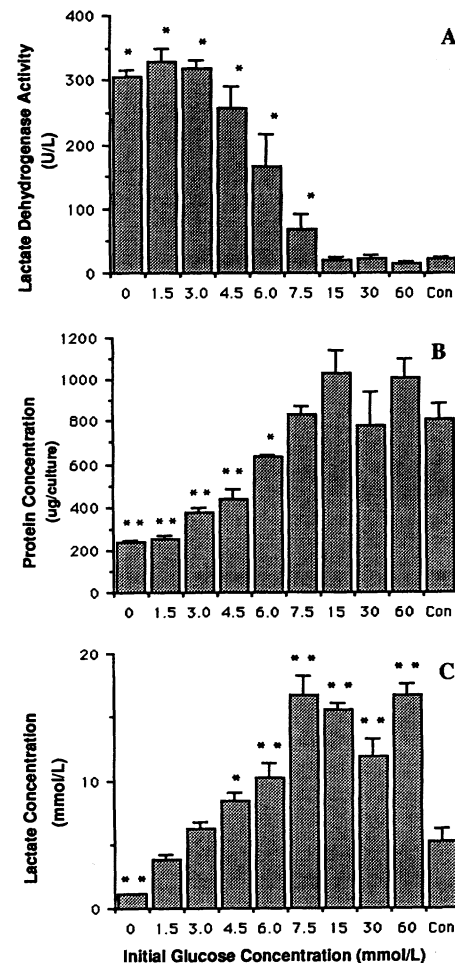


FIGURE 1. Bar graphs showing (mean \pm SEM) (panel A) lactate dehydrogenase (LDH) activity in culture medium during hypoxia and with increasing glucose concentration. Astrocyte cultures in modified Eagle's minimum essential medium containing 0–60 mmol/L glucose were made hypoxic for 24 hours, except controls (Con), which were maintained in 7.5 mmol/L glucose in 95% air/5% CO₂ for 24 hours. All bars represent two separate cell preparations and experiments using triplicate plates per condition (n=6). Units are defined as micromoles of NADH produced per minute under 3-mL assay conditions. **p* < 0.01 different from control. Panel B: Final concentration of cell protein with increased initial glucose concentration. **p* < 0.05, ***p* < 0.01 different from control. Panel C: Lactate concentration of culture medium after hypoxia/normoxia and increasing initial glucose concentration. **p* < 0.05, ***p* < 0.01 different from control.

mmol/L (Figure 1B). Glucose concentrations of 7.5–60 mmol/L maintained protein concentrations at normoxic levels.

Glucose remaining in the culture media was determined after 24 hours of hypoxia and increasing initial glucose concentrations. Cultures initially containing 7.5 mmol/L glucose had negligible glucose in the media after 24 hours of hypoxia (Table 1). Cells treated with high glucose concentrations (15–60 mmol/L) and with 24 hours of hypoxia maintained a glucose concentration in the media of ≥ 18.0 μ mol per culture dish. Calculated values for loss of glucose were similar for astrocytes with initial glucose concentrations of 7.5–30 mmol/L.

TABLE 1. Disappearance of Glucose From Culture Medium After 24 Hours of Normoxia or Hypoxia

	Normoxia (control)		Hypoxia		
Initial glucose concentration (mmol/L)	7.5	7.5	15.0	30.0	60.0
(μ mol per culture)	22.5	22.5	45.0	90.0	180.0
Final glucose content of cultures (μ mol per culture)	14.3 \pm 0.6	0.0 \pm 0.3	18.0 \pm 1.7†	64.2 \pm 1.6‡	102.0 \pm 1.9†
Glucose loss* (μ mol per culture)	8.2	22.5	27.0	25.8	78.0

Values are mean \pm SEM from three separate cell preparations and experiments using duplicate or triplicate plates for each condition ($n=6-9$).

*Values are calculated in reference to the loss of glucose from the medium after treatment.

† $p<0.01$, ‡ $p<0.05$ different from hypoxic values with initial glucose concentration of 7.5 mmol/L.

Lactate concentration in the media was measured to determine its correlation with glucose loss from the media. Figure 1C shows that lactate increased with initial glucose concentrations up to 7.5 mmol/L, although there was no significant difference between control (normoxic) and hypoxic cultures containing 1.5 or 3.0 mmol/L glucose. The lactate concentration was not increased beyond that observed for 7.5 mmol/L glucose, although higher initial glucose concentrations were used.

Cell injury and viability were further investigated by determining cellular ATP concentrations with increasing glucose concentrations and 24 hours of hypoxia (Figure 2). At glucose levels associated with cell death after hypoxic treatment, the ATP levels were <10% of the control value (normoxia). At 15 mmol/L glucose the ATP value after hypoxic treatment was 61% that of control. Higher levels of glucose (30 mmol/L) never completely prevented the ATP loss caused by hypoxic insult.

Under high-glucose conditions (30 mmol/L), cells were undamaged by <48 hours of hypoxia, as determined by LDH efflux measurements (Figure 3). Cultures treated with high glucose concentrations maintained LDH concentrations at normoxic levels and survived for an extended period of hypoxia. Astrocytes treated with normal glucose concentrations were dam-

aged by 24 hours of hypoxia (Figure 1A). Hypoxic cultures exposed to 30 mmol/L glucose removed 30% of the available extracellular glucose from the media within 24 hours (Figure 4A). At 24, 30, and 48 hours, hypoxic treatment caused greater glucose loss than normoxic treatment, with a difference of ≈ 3 mmol/L between the two conditions at each of the three time points (Figure 4A). Lactate production with high glucose concentrations correlated well with glucose uptake up to 48 hours of hypoxia. Production of lactate under hypoxic conditions was 7–8 mmol/L greater than at similar normoxic time points, i.e., twofold the amount of glucose used (Figure 4B).

The pH of the media for cultures in a high-glucose environment decreased progressively with increasing durations of hypoxia and was 5.5 ± 0.1 at 48 hours. The pH under normoxic conditions was >6.0 (Figure 4C). These data show that a pH value <6.0 is coincident with the induction of cell death; this was first observed with 48 hours of hypoxia (Figure 3).

Determination of intracellular LDH activity showed a dramatic decrease in cells exposed to both 30 mmol/L glucose and 48 hours of hypoxia, with a remaining activity <30% of the 24-hour value (Table 2). The intracellular LDH values for normoxic cells were never significantly different from the initial (0-hour) value.

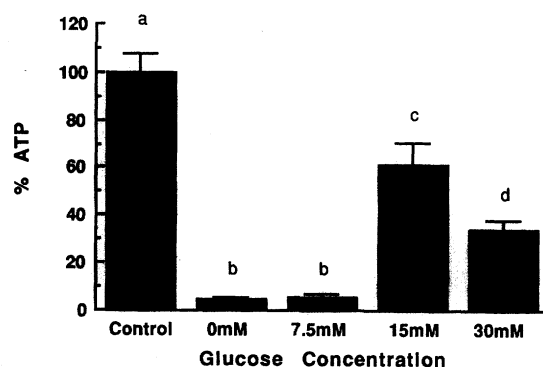


FIGURE 2. Bar graph shows percent adenosine triphosphate (ATP) of hypoxic astrocytes under conditions of increasing glucose concentration. Control represents normoxic cells with 7.5 mmol/L glucose included in the medium. Astrocytes in medium containing 0–30 mmol/L glucose were made hypoxic for 24 hours. Values represent mean \pm SEM from four separate cell preparations ($n=12-14$). a, b, c, d indicate significantly variable groups ($p<0.03$).

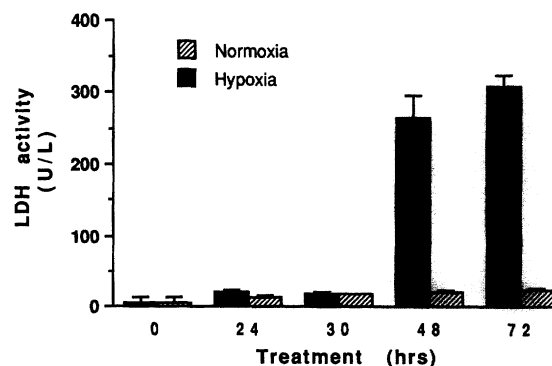


FIGURE 3. Bar graph shows lactate dehydrogenase (LDH) activity in culture medium with high initial glucose concentration and an increased period of hypoxia. The initial glucose concentration of the medium was 30 mmol/L; cells were exposed to hypoxia for 0–72 hours. Control normoxic cultures were exposed to 30 mmol/L glucose concentrations and 0–72 hours of incubation. Each bar represents mean \pm SEM from a minimum of four separate cell preparations and experiments, using triplicate plates for each condition ($n=12-19$).

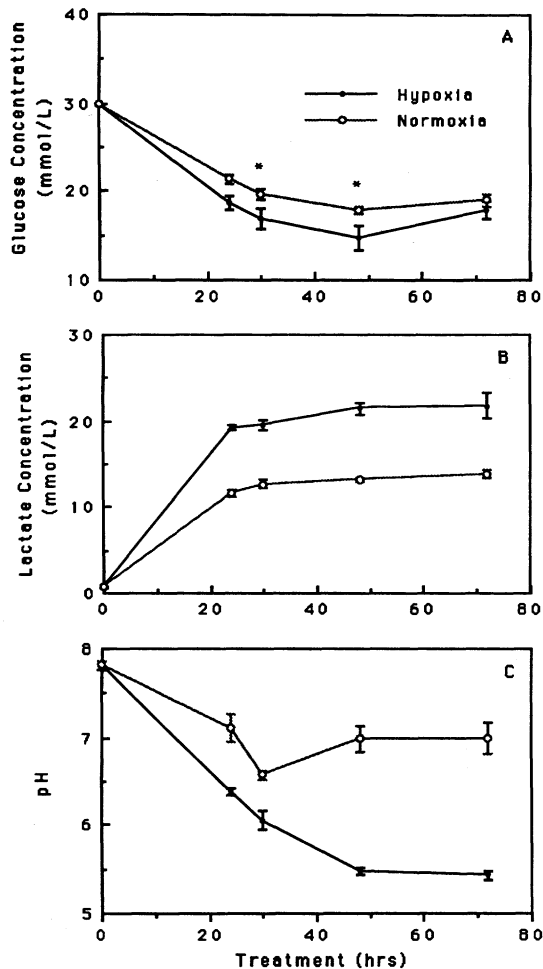


FIGURE 4. Line graphs show effects of high glucose concentrations under normoxic and hypoxic conditions. Cells in 30 mmol/L glucose were made hypoxic or were kept in 95% air/5% CO₂ (normoxic) for up to 72 hours. The medium was removed and tested for final glucose concentration (panel A), lactate concentration (panel B), and pH (panel C). Each point represents mean \pm SEM from at least two separate cell preparations and triplicate or quadruplicate culture plates ($n=8-12$). * $p<0.05$ different from normoxia.

Increased osmolarity caused by mannitol had no protective effect on hypoxic cultures, nor did it affect the LDH release from normoxic cells (Figure 5). The final glucose concentration of the media and the protein concentration of the cells treated with 30 mmol/L mannitol and 24 hours of hypoxia were similar to values observed for hypoxic cultures and glucose alone (data not shown).

Cells were kept under normoxic conditions in medium containing either 7.5 or 30 mmol/L glucose and at a pH of either 7.5 or 5.4 to determine the effect of high glucose levels on acidosis (Figure 6). If the pH was brought to 5.4 with 1N lactic acid compared with 1N HCl, then greater injury occurred earlier. The presence of high glucose concentrations did not exacerbate or prevent the injury for cells at pH 5.4 in lactic acid or HCl.

Discussion

Previous work by our laboratory has shown that mature astrocyte cultures were killed after 24 hours of

TABLE 2. Intracellular Levels of Lactate Dehydrogenase Activity in Astrocytes Exposed to 30 mmol/L Glucose and up to 72 Hours of Hypoxia or Normoxia

Hours	Lactate dehydrogenase activity (units/L)	
	Hypoxia	Normoxia
0	465.7 \pm 28.0	465.7 \pm 28.0
24	546.7 \pm 20.8	408.0 \pm 17.7
30	492.5 \pm 42.5	466.6 \pm 43.9
48	159.6 \pm 41.5	447.6 \pm 54.3
72	20.1 \pm 0.1	340.4 \pm 33.8

Cultures were changed to modified Eagle's minimum essential medium containing 30 mmol/L glucose and were either kept in normoxic incubator or changed to 95% N₂/5% CO₂ for up to 72 hours. Astrocyte cultures were immediately washed and then homogenized in 2 mL glucose-free medium. Before incubation, cells were homogenized and lactate dehydrogenase of cells measured (0 hours for both hypoxia and normoxia). Values represent mean \pm SEM of lactate dehydrogenase activity from three different cell preparations and triplicate plates ($n=9$).

hypoxia in the presence of 7.5 mmol/L glucose.¹⁹ Shorter periods of exposure to hypoxia were not injurious to these cells. The present results demonstrate that with high-glucose conditions (15–60 mmol/L), injury to astrocytes occurred after 30 hours of hypoxia when the pH of the media was <6.0 . Cell death occurred despite >10 mmol/L glucose remaining in the media.

The role of high glucose concentrations in preventing injury to astrocytes during extended periods of hypoxia is unclear. Preliminary results by our group showed that 30 mmol/L glucose had a negligible effect on diminishing hypoxic injury in pure neuronal cultures, even at short time periods (authors' unpublished observations). Astrocytes have been shown to reduce anoxic²⁰ and hypoxic³⁶ injury in mixed neuronal cultures. In vivo experiments have shown that hypoxic-induced astrocyte damage correlates with increased neuronal injury.⁸ Re-

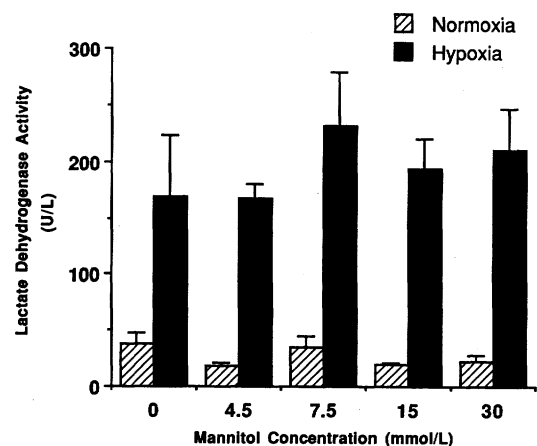


FIGURE 5. Bar graph shows lactate dehydrogenase activity in culture medium after hypoxia and increasing mannitol concentrations. The culture medium initially contained 7.5 mmol/L glucose. Samples were made hypoxic, except control (0), which contained no mannitol and was kept in 95% air/5% CO₂ for 24 hours. All bars represent mean \pm SEM data from two experiments done in triplicate ($n=6$). Units are defined as micromoles of NADH produced per minute under 3-mL assay conditions.

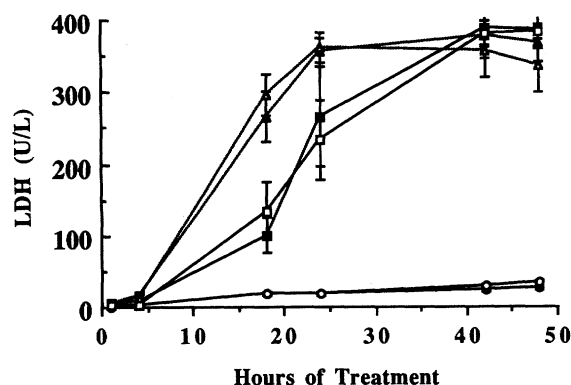


FIGURE 6. Line graph shows lactate dehydrogenase (LDH) activity of astrocytes under normoxic, normal-glucose, or high-glucose conditions. pH was brought to 5.4 with either 1N HCl or 1N lactic acid. An aliquot of medium was removed from each plate at 1, 4, 18, 24, 42, and 48 hours for LDH determination. High-glucose conditions (30 mmol/L) are represented by solid symbols and normal-glucose conditions (7.5 mmol/L) by open symbols. Astrocytes treated with lactic acid are designated by triangles, HCl-treated cells by squares, and controls at pH 7.5 by circles. Points represent the mean \pm SEM of three separate experiments using triplicate or quadruplicate plates ($n=9-11$).

cently, numerous studies have demonstrated the ability of astrocytes to protect neurons in mixed cultures from possible mediators of ischemic injury, including excitatory amino acid neurotoxicity.^{21,22} As metabolically distinct neural cells, astrocytes have numerous features that could account for the ability to protect themselves and neurons from hypoxia. These features include considerable LDH activity,³⁷ large glycogen synthesis and storage capacity,³⁸⁻⁴¹ pyruvate carboxylase activity,⁴² and putative gluconeogenic capability.⁴³ Other astrocyte functions that may afford protection include a greater percentage of glucose cycling through the hexose monophosphate pathway than by neurons⁴⁴ and the predominantly astrocytic glutamine synthetase activity.⁴⁵ These metabolic differences may account in part for the ability of astrocytes to tolerate hypoxia and to use glucose without deleterious effect.

Measurement of glucose loss from the media showed that uptake/utilization was affected by oxygen availability. Glucose loss was nearly threefold greater as a result of hypoxia compared with normoxia (Table 1). However, the glucose loss from the media was not dependent on the availability of high glucose levels, because the losses from the media at initial glucose concentrations of 7.5, 15, and 30 mmol/L were nearly equivalent. Increased uptake of glucose under hypoxic conditions would counteract the depletion of high-energy phosphates when respiration reverts to anaerobic metabolism. Our laboratory has previously shown that flushing the chamber with a hypoxic gas mixture for 15 minutes removes all oxygen from the atmosphere, although a small oxygen tension still exists within the media after 12 hours.¹⁹ It is likely that the small concentration of available oxygen in the media is not sufficient to maintain mitochondrial ATP production, because KCN caused no further effect on hypoxic injury or metabo-

lism (data not shown). Indeed, lactate data indicate that more glucose is being used anaerobically under hypoxic conditions, since the difference between normoxic and hypoxic values for lactate released into the media (Figure 4B) is at least twice the difference between normoxic and hypoxic glucose loss (Figure 4A).

It is unclear why the lactate concentration did not increase throughout the hypoxic and high-glucose period, if anaerobic glycolysis is the major metabolic pathway for glucose utilization under hypoxic conditions. Petito and Babiak⁴⁶ have shown increases in astrocyte number, cell volume, and number of mitochondria after a short period of hypoxia and reperfusion. Other investigators have described increased glycogen stores⁴⁷ and pyruvate concentrations in mixed cultures³⁶ after ischemia. The effect of long periods of hypoxia and high glucose concentrations on these astrocyte-specific events is unclear. An increase in glycogenolysis⁴¹ and pyruvate production⁴⁸ could also promote neuronal survival.

Several *in vivo* studies, described earlier, indicate that the hyperglycemic exacerbation of ischemic brain injury is likely mediated through lactacidosis. This does not appear to be the case in astrocyte cultures under high-glucose conditions (30 mmol/L), because the lactate concentrations are $>15-18$ mmol/L and cells appear unharmed. After 48 hours of hypoxia, high glucose concentrations (30 mmol/L) no longer protected astrocytes. The increase in lactate accumulation between 30 and 48 hours of hypoxia was $<1\%$ of total value, whereas the pH fell from a nondeleterious value of 6.1 to a value of 5.5. The latter caused astrocyte death in primary cultures exposed to either 1N lactic acid for >1 hour⁴⁹ or millimolar amounts of lactic acid and 30-60 minutes of exposure time.⁵⁰ Astrocytes exposed to 15-50 mmol/L lactate and adjusted to the equivalent pH showed no difference in time of onset or degree of injury, implicating pH rather than lactate concentration as the major mediator of astrocyte injury.⁵⁰ Under high-glucose/hypoxic conditions, a pH of 5.5 also appears to be critical under our culture conditions, whereas lactate values of >15 mmol/L and pH values >5.5 had a minimal effect on astrocyte injury. The lactic acid formed by astrocytes and a glioma cell line could reduce intracellular acidosis by activating both Na^+/H^+ and the $\text{Cl}^-/\text{HCO}_3^-$ antiporters, although swelling occurs.^{51,52} Loss of ATP from the hypoxic cell would reduce the ability of the cell to transport ions and maintain homeostasis. Staub et al⁵³ demonstrated that astrocyte viability is affected by a pH of 5.6 in the media, independent of whether lactic acid or a nonorganic acid (H_2SO_4) was used. However, we observed that astrocytes exposed to lactic acid at pH 5.4 were injured earlier than those brought to the same pH with HCl. Ultimately, pH in the range of 5.4 causes astrocyte death. High glucose concentrations had no effect on cell survival in either case (Figure 6). A trade-off exists during anaerobic metabolism between the increased requirement for energy substrates and an increased risk of injury due to higher levels of deleterious anaerobic metabolites, e.g., H^+ and lactate. Our results suggest that this simple cell system may be weighted in the direction of increased requirement for glucose and ATP, whereas a heterogeneous cell system may be injured from lactic acid formation.

One possible explanation for observed differences between *in vivo* studies and our cell culture system might be the greater extracellular space present under culture conditions, which could greatly dilute extracellular lactate. Another difference could arise from the use of a single cell type; for example, neurons also produce lactate, which, if removed by astrocytes, would produce further astrocyte lactic acidosis and potentially increase injury to cells. Of note is that unlike the *in vivo* system of incomplete ischemia, the lack of tissue perfusion prevents removal of the lactate from the culture medium.

Astrocytes are thought to behave as regulators of pH in brain tissue, yet they are more susceptible to acidosis than neurons.⁴⁹ Neurons appear uninjured after periods of HCl or lactate exposure.^{49,54} In fact, an increase in H⁺ concentration reportedly protects neurons from glutamate toxicity⁵⁵ by noncompetitively modulating the *N*-methyl-D-aspartate channel.⁵⁶ One possible beneficial behavior of astrocytes under high-glucose/hypoxic exposure might be that they remove lactate and glutamate that might be injurious to neurons, whereas the subsequent release of H⁺ and increase in intracellular Na⁺ may be protective for neurons but at the expense of astrocytes. Kempinski et al⁵² suggest that astrocyte swelling is an attempt to maintain normal intracellular pH and tissue ion homeostasis under ischemic-induced acidosis.

In addition to lactate, pyruvate is another organic acid that might influence pH. The concentration of pyruvate is reported to rise after hypoxia in mixed cultures³⁶ and in cerebral cortex.⁵⁷ Further studies by our group should include pyruvate determination with astrocyte and with mixed astrocyte/neuron cultures. Another contributor to the lowering of pH might be ATP turnover.⁵⁸ Although not determined in our studies, the decrease of intracellular pH might be a good, early indicator of ATP hydrolysis during hypoxia.

During hypoxia, ATP values were lower than control values even with high glucose concentrations (15–30 mmol/L). Mitochondrial failure increases ATP loss during hypoxia, and an increased acidosis reduces the ability of brain to increase ATP production.^{59,60} Rehnrona et al⁴ suggest that “acidosis is detrimental only in conjunction with energy failure.” We believe that pH rather than lactic acidosis may affect ATP levels in astrocytes. It appears likely that there is an imbalance between production and utilization of ATP with the onset of anaerobic glycolysis, but the reduction in ATP to 60% of the normoxic value apparently was not detrimental to the astrocytes at 24 hours of hypoxia when high glucose concentrations were present. The ATP level at 60% of normoxic values after 24 hours of hypoxia and 15 mmol/L glucose is higher than expected from the meager increase in lactate production. The mechanism of this increase is unclear, but it is not unlikely from creatine phosphate, since the period of hypoxia was lengthy.

Mannitol protects cerebral tissue from injury after focal ischemia.⁶¹ This protection was thought to be due to osmolar dehydration of the cells and plasma expansion. Therefore, to evaluate whether high glucose concentrations protected astrocytes through hyperosmotic effects, we replaced high glucose concentrations with mannitol. Adding equivalent concentrations of mannitol with 24 hours of hypoxia did not prevent LDH efflux from

cultures, indicating that the beneficial effects of high glucose concentrations were not due to hyperosmolarity.

It is apparent that protection of hypoxic mature astrocytes, like that of immature astrocytes, is influenced by glucose availability.²⁶ The differentiating astrocytes of Callahan et al²⁶ behaved in a manner very similar to that of our mature astrocytes under both normoglycemic and hyperglycemic conditions, but immature astrocytes are also more resistant to hypoxic injury than mature cells (D. Bull, G.A. Gregory, P.H. Chan, and J.A. Kelleher, unpublished observations).

The addition of glycolytic intermediate fructose-1,6-bisphosphate (FBP) to culture media protects astrocytes from 24 hours of hypoxic exposure.⁶² Adding this compound may provide additional information regarding the complex metabolism of astrocytes under hypoxic conditions. Previously we showed that adding FBP reduced the loss of ATP with 18–30 hours of hypoxia,⁶³ similar to the behavior of high glucose concentrations described in the present study. FBP was able to protect hypoxic-treated astrocytes at a lower concentration than that required for glucose, and because it is unlikely that FBP crosses the plasma membrane, FBP probably modulates energy metabolism, as it appears that high glucose concentrations do.

The results of our *in vitro* studies differ from studies done *in vivo*. *In vivo*, hyperglycemia exacerbates central nervous system injury after transient cerebral ischemia, as evidenced by increased neuropathology,^{2,3} reduced energy metabolites,^{4,5} and increased lactic acidosis.^{3–8} However, there are some reports showing that injury after focal ischemia is reduced by hyperglycemia.^{9,10} Our *in vitro* studies showed delayed injury to astrocytes exposed to high glucose concentrations during hypoxia. The reasons for this paradox are not readily apparent. One possible explanation is that hyperglycemia increases platelet aggregation, which induces patchy cerebral vascular obstruction and permanent ischemia of the areas subtended by these vessels.⁶⁴ This, as well as the fact that hyperglycemia reduces the volume of infarct in the penumbra of injury after focal ischemia, suggests that the injury may be due to altered distribution of cerebral blood flow after ischemia and not directly to the high glucose concentration per se. High glucose concentrations also disrupt the blood–brain barrier, which may allow toxic products to enter the brain and cause injury *in vivo*.⁶⁵ Another possible explanation for the differences in injury with high glucose concentrations *in vivo* and *in vitro* may be related to lactic acid production by nonastrocyte cells, including neurons, followed by uptake of the acid by astrocytes. This could reduce the pH of astrocytes to injurious levels, causing subsequent neuronal injury possibly as a result of the inability of astrocytes to remove toxic products from the perineuronal environment. Sher and Hu³⁶ have shown greater injury to astrocytes than to neurons by acidosis. Yet acidosis caused by glycolysis in astrocytes under high-glucose/hypoxic conditions was insufficient to injure astrocytes for at least 30 hours.

The new findings of our study are as follows: 1) High glucose concentrations delay the onset of injury to astrocytes, in part because the concentration of ATP is maintained at more normal levels, which would maintain membrane function and prevent or delay injury. 2)

There was an increase in lactic acid in the media up to 18.5 mmol/L that then plateaued for reasons that are not clear. One possibility is that lactic acid/lactate was metabolized or fixed by the astrocytes. 3) The protective effects of high glucose concentrations were not due to osmolarity, because similar osmolarities induced by mannitol were not protective.

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Editorial Comment

The effects of hyperglycemia on cerebrovascular injury after transient periods of cerebral ischemia are controversial. Some investigators have reported that the damaging effects of transient periods of cerebral ischemia appear to be augmented by hyperglycemia.^{1–4} In contrast, others have reported beneficial effects of hyperglycemia on neurological outcome after cerebral ischemia.^{5,6}

The purpose of the present study was to examine the effects of high glucose levels on differentiated astrocytes exposed to a period of hypoxia. The authors report that hypoxia produced significant cell damage, as determined by increased lactate dehydrogenase efflux and loss of cell protein, at concentrations of glucose ≤ 7.5 mmol/L. In contrast, hypoxic cell injury was reduced by incubation with higher concentrations of glucose (15–60 mmol/L).

Although the findings of the present study suggest that damage to astrocytes during hypoxia may be decreased by increasing concentrations of glucose, caution must be exercised in the interpretation of these findings. The authors used in vitro methodology to examine the effects of hypoxia on a specific cell type, i.e., astrocytes. Since periods of cerebral ischemia followed by reperfusion affect many cell types, it may be difficult to

extrapolate the findings of the present study to that which occurs in an intact model of cerebral ischemia.

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Modification of hypoxia-induced injury in cultured rat astrocytes by high levels of glucose.

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Stroke. 1993;24:855-863

doi: 10.1161/01.STR.24.6.855

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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