Glucose but Not Lactate in Combination With Acidosis Aggravates Ischemic Neuronal Death In Vitro

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**Background and Purpose**—Hyperglycemia aggravates brain damage in clinical stroke and in experimental in vivo models of cerebral ischemia. Elevated preischemic glucose levels, lactate production, and intracerebral acidosis correlate with increased brain damage. We have developed a murine hippocampal slice culture model of in vitro ischemia (IVI), suitable for studies of the mechanisms of neuronal death. In this model we investigated the individual contribution of glucose, pH, lactate, and combinations thereof as well as ionotropic glutamate receptor activation to the development of hyperglycemic ischemic cell death.

**Methods**—Murine organotypic hippocampal slice cultures were exposed to IVI in a medium with an ionic composition similar to that of the extracellular fluid in the brain during ischemia in vivo. Cell death was assessed by propidium iodide uptake. Ionotropic glutamate receptor blockade was accomplished by D-2-amino-5-phosphonopentanoic acid (D-APV) or 2,3-dihydro-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX).

**Results**—The combination of high glucose levels and acidosis (pH 6.8), but not acidosis per se or the combination of lactate and acidosis during IVI, exacerbated damage. Cell death after hyperglycemic IVI was not diminished by blockade of ionotropic glutamate receptors.

**Conclusions**—Aggravation of brain damage by hyperglycemia in vivo can be reproduced in hippocampal slice cultures in vitro. Our results demonstrate that glucose per se, but not lactate, in combination with acidosis mediates the detrimental hyperglycemic effect through a mechanism independent of ionotropic glutamate receptors. (Stroke. 2004;35:753-757.)

**Key Words:** acidosis ■ cell culture ■ hippocampus ■ hyperglycemia ■ ischemia

It has long been known that preischemic hyperglycemia aggravates the development of damage after transient global or focal ischemia in experimental animals.1–3 Clinical studies on acute ischemic stroke have confirmed these findings.4,5 Thus, a high blood glucose level on admission predicts a poor outcome after stroke.6 In addition, a clear relationship between hyperglycemia, lactate production, and conversion of penumbra tissue to infarction has recently been demonstrated in stroke patients.7

Although the phenomenon of aggravated ischemic damage by hyperglycemia has been studied extensively in experimental models in vivo, these models are less suitable for studies of the individual contributions of potential harmful factors. Dissociated neuronal cultures and organotypic tissue cultures have been used extensively to investigate the mechanisms and pharmacology of ischemic brain injury. However, in these models studies of the harmful effect of hyperglycemia have been discouraging since the addition of glucose during oxygen deprivation is generally neuroprotective.8–10 We recently reported that incubation of organotypic hippocampal slice cultures in an anoxic medium with an ionic composition similar to that seen in the extracellular space in the brain during ischemia resulted in delayed and selective cell death. This cell death was aggravated by the addition of glucose to the medium.11

In the present investigation we used this model of in vitro ischemia (IVI) to investigate the contributions of glucose, acidosis, lactate, and combinations thereof as well as glutamate receptor activation to the development of cell death after hyperglycemic IVI.

**Materials and Methods**

**Hippocampal Organotypic Tissue Cultures**

All animal experiments were approved by the Malmoe/Lund ethical committee (M108-01). Hippocampal organotypic slice cultures, 250 μm thick, were prepared from 6-day-old Balb/c mice, plated onto Millicell culture inserts (0.4 μm Millicell-CM, 12 mm in diameter, Millipore Corp), 1 slice per insert, and cultured at 35°C, as previously described11 with the modification that after the first week of culture B27 was omitted from the medium. This modification was introduced because we observed that when the culture period was extended to 3 weeks, the continuous presence of B27 frequently caused central CA1 damage, while a complete removal of B27 led to loss of neurons in the CA2/CA3 region. The presence of B27 during the first week prevented the cell loss in CA2/CA3, while subsequent
omission led to a gradual thinning of the slices and preservation of the CA1 cells (Figure 1A to 1C). To make the culture conditions more physiological, the glucose level in the culture medium was decreased from 40 mmol/L (Figures 2 and 3) to 20 mmol/L (Figures 4 to 6). This change did not affect the viability of the slices, while a further decrease to 10 or 5 mmol/L glucose severely affected both morphology and viability. Patch-clamp recordings of CA1 cells from cultures grown in 20 and 40 mmol/L glucose showed no difference in basic electrophysiological properties such as resting membrane potential and input resistance. There was also no difference in the excitatory synaptic transmission measured as the decay time constant of evoked excitatory postsynaptic currents. Generally, when the glucose dependence of a process was studied, equiosmolarity of the medium was maintained by the addition of sucrose.

Induction of In Vitro Ischemia

IVI experiments were performed according to Rytter el al. Three-week-old cultures were washed in glucose-free medium, transferred to the anaerobic incubator (Elektrotek Ltd), which had an atmosphere of 10% H₂, 5% CO₂, and 85% N₂, and placed in wells with anoxic IVI medium. After 12 minutes of IVI, slices were returned to oxygenated culture medium and to the culture incubator. The IVI medium, ischemic cerebrospinal fluid (iCSF), contained the following (in mmol/L): 0.3 CaCl₂, 70 NaCl, 5.25 NaHCO₃, 70 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 40 sucrose (pH 6.8). In hyperglycemic IVI, sucrose was replaced by 40 mmol/L glucose. When sodium-DL-lactate was added, a corresponding amount of sodium chloride was removed. Sodium bicarbonate was used to set pH, which was routinely measured in a standard microsampler blood gas monitor (ABL 50, Radiometer Copenhagen). The N-methyl-D-aspartate (NMDA) receptor antagonist D-2-amino-5-phosphonopentanoic acid (D-APV) and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor blocker 2,3-dihydro-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX) were from Tocris Cookson.

Quantification of Cell Death

Cell death was detected with the fluorescent cell death marker propidium iodide (PI) present in the medium from 24 hours before experiment and throughout the recovery period. If PI uptake was detected before the experiments, slices were discarded (10% to 20%). Cell death was assessed by measuring mean fluorescence intensity (MFI) in a standardized area in the CA1 region and in a hexagon placed in an undamaged area outside the CA2/CA3 cell band (background) (Figure 1). Cell death was calculated by subtracting the MFI in the background area from the MFI measured in CA1. Unsectioned slices were stained with NeuN to visualize the distribution of neurons. Neuronal death was detected by Fluoro-Jade staining.

Statistical Analysis

Experimental groups to be compared were run simultaneously inside the anaerobic incubator. All groups contain data from at least 3 separate experiments. Data are expressed as mean±SEM. Two-way ANOVA with the Scheffé post hoc test was used to evaluate differences between groups. Repeated-measures ANOVA with the Scheffé post hoc test was used to compare the amount of cell death at different time points within

Figure 1. A, NeuN-stained slice after 3 weeks in culture. B, Inverted fluorescence image of PI-stained culture with central CA1 damage after 3 weeks in culture in the presence of B27. C, Transmission image of a 3-week-old slice grown in the absence of B27. D, PI intensity was measured as MFI in a standardized area in the CA1 region. Background MFI was measured in a standardized hexagon outside the CA2/CA3 region. E, Fluoro-Jade-stained slice 72 hours after hyperglycemic in vitro ischemia; the CA1 region is shown in higher magnification (F). Bars in A through E=400 μm; bar in F=25 μm.

Figure 2. Temporal development of cell death after IVI with and without addition of glucose in the medium. A, Inverted fluorescent images of PI-stained hippocampal slices showing damage after IVI in glucose-free iCSF and glucose-supplemented (40 mmol/L) iCSF (iCSF+G). B, PI MFI in cultures exposed to IVI in iCSF (white bars) and iCSF+G (black bars). *P<0.05, significant differences between the 2 experimental paradigms at the same time point (ANOVA with Scheffé post hoc test). Data are mean±SEM; n=27 to 28 derived from 5 independent experiments.
one experimental group in Figures 2, 3, and 5. Variability between experimental dates was compensated for by including the date of the experiment as a factor. For statistical analyses, the commercial software Statview 4.0 (Abacus Concepts Inc) was used.

Results

A High Level Of Glucose During IVI Led to Delayed and Aggravated Cell Death

Slices, cultured in 40 mmol/L glucose, were exposed to IVI in media with or without 40 mmol/L glucose. In both groups marked cell death developed in the CA1 region, but with a different rate and intensity (Figure 2A). After 24 hours of recovery, cell death was seen in both groups but was significantly less with glucose present (Figure 2A and 2B). There was no further increase in cell death in slices subjected to glucose-free IVI. In contrast, when glucose was present, cell death increased between 24 and 48 hours and was at 48 hours more pronounced than that in the glucose-free group. Staining with Fluoro-Jade confirmed that damage in the hyperglycemic group was neuronal (Figure 1E and 1F).

The Aggravating Effect of Glucose Was Concentration Dependent

Slices, cultured in 20 mmol/L glucose and exposed to IVI in the presence of 20 mmol/L glucose, developed significantly less damage at 24 and 48 hours ($P<0.01$) than those subjected to glucose-free IVI (Figure 3A). When glucose was increased to 40 mmol/L during IVI, damage at 24 hours was still less pronounced than in the glucose-free group but was similar at 48 hours. However, damage at 48 hours was significantly more severe with 40 mmol/L glucose than with 20 mmol/L glucose. Furthermore, a 1-hour preincubation in 40 mmol/L glucose before glucose-supplemented IVI (Figure 3B) reproduced the pattern with delayed and aggravated cell death, seen in Figure 2B.

Figure 3. Dose dependence of glucose toxicity. Organotypic hippocampal slices were subjected to IVI in glucose-free iCSF (white bars), iCSF supplemented with 20 mmol/L (gray bars), and 40 mmol/L (black bars) glucose (A) or preincubated in 40 mmol/L glucose before hyperglycemic IVI (black bars) (B). $*P<0.01$ compared with iCSF group; $\#P<0.01$ compared with 40 mmol/L glucose-supplemented iCSF group (2-way ANOVA with Scheffé post hoc test). Data are mean±SEM; $n=18$ derived from 3 separate experiments.

Figure 4. Effect of lactate. Organotypic hippocampal slices were subjected to IVI in glucose-free iCSF (white bars), preincubated in 40 mmol/L glucose and subjected to IVI in iCSF with 40 mmol/L glucose (gray bars), or preincubated in 40 mmol/L glucose and subjected to IVI in iCSF with 40 mmol/L lactate (gray bars). $*P<0.01$ compared with glucose-free group; $\#P<0.05$ compared with group supplemented with 40 mmol/L glucose at the same time point (ANOVA with Scheffé post hoc test). Data are mean±SEM; $n=18$.

Figure 5. Effect of pH on cell death after IVI and hyperglycemic IVI. A, Organotypic hippocampal slices were subjected to IVI in iCSF with pH 6.8 (white bars) or pH 6.3 (gray bars). B, Organotypic hippocampal slices were subjected to IVI in iCSF with 40 mmol/L glucose at pH 6.8 (black bars) or pH 7.4 (gray bars) or in glucose-free iCSF, pH 7.4 (white bars). $*P<0.05$ compared with cultures exposed to iCSF with glucose at pH 6.8 at the same time point (ANOVA with Scheffé post hoc test). Data are mean±SEM; $n=18$ derived from 3 experiments.
Lactate or Acidosis Did Not Reproduce the Detrimental Effect of Hyperglycemia

The addition of 40 mmol/L lactate to the iCSF medium during IVI did not significantly affect cell death (Figure 4), although there was a tendency toward a decrease in damage. Damage was not affected when slices were preincubated in 40 mmol/L lactate for 1 hour and then exposed to IVI with 40 mmol/L lactate (data not shown). We have shown earlier that decreasing pH during IVI from 7.4 to 6.8 reduces damage. A further decrease in pH from 6.8 to 6.3 during IVI did not affect cell death (Figure 5A).

Aggravation of Cell Death by Glucose Required Acidosis

At pH 7.4, the delayed time course of cell death and the aggravating effect of hyperglycemia were abolished (Figure 5B). Hence, there was no difference in the development or amount of cell death between slices subjected to IVI at pH 7.4 with or without the addition of glucose. Moreover, in both these groups cell death developed more quickly compared with slices exposed to glucose-supplemented IVI at pH 6.8 but was less extensive at 48 and 72 hours of recovery.

Blockade of Ionotropic Glutamate Receptors Did Not Protect Against Hyperglycemic IVI

APV (50 μmol/L) was added to the slices from 1 hour before IVI, during IVI, and throughout recovery. In slices subjected to glucose-free IVI, APV diminished damage at both 24 (data not shown) and 48 hours (Figure 6A). In contrast, in the glucose-supplemented groups, APV did not diminish damage. Likewise, addition of NBQX (100 μmol/L) had no effect on damage after glucose-supplemented IVI at 24 (data not shown) or 48 hours (Figure 6B). Neither APV nor NBQX induced cell death in control slices.

Discussion

We earlier demonstrated a detrimental effect of glucose in an in vitro ischemia paradigm using organotypic hippocampal slices cultured in 40 mmol/L glucose. In the present investigation we confirm this finding and in addition show that the hyperglycemic cell death is delayed and dependent on acidosis. If instead slices were cultured in 20 mmol/L glucose, a preincubation in 40 mmol/L glucose was needed to reproduce this pattern. In contrast, a moderate level of glucose, ie, 20 mmol/L, during IVI had a clearly protective effect, similar to previous observations in cell culture systems. Thus, the glucose toxicity was dose dependent, which is in concordance with in vivo observations. Admittedly, the glucose levels employed during hyperglycemic IVI in our experiments appear high compared with those observed in vivo. However, such discrepancies are inherent in any cell and tissue culture model. The high level required to induce glucose toxicity must be viewed in regard to the basal level, 20 mmol/L, needed to maintain viable slices.

From experiments in vivo, it has been hypothesized that the detrimental effect of hyperglycemia is due to the excessive formation of lactate with a resultant tissue acidosis, ie, lactic acidosis. Whether acidosis per se aggravates ischemic cell damage in vivo is still a matter of controversy since hypercapnia-induced acidosis has been shown to increase cell damage after global ischemia but not after transient focal ischemia. It has been reported that lactic acidosis is toxic to neurons in cultures, while a corresponding acidosis induced by low bicarbonate is not. Whether lactate per se has a detrimental influence on outcome after experimental stroke has not been investigated.

When we replaced glucose with lactate during IVI at pH 6.8, the delayed and aggravated cell death pattern was lost. The lactate-supplemented cultures displayed a cell death pattern very similar to that seen with glucose-free IVI. In hyperglycemic ischemia in vivo, the high extracellular lactate level is the result of increased intracellular production. However, lactate is actively transported into neurons, and a 1-hour preincubation with 40 mmol/L should, through the activation of monocarboxylate transporters, be sufficient to raise intracellular lactate levels. We conclude that lactate combined with acidosis does not appear to aggravate damage after IVI.

We next studied whether acidosis was an independent mediator of hyperglycemic cell death. During cerebral ischemia in normoglycemic rodents, the pH of extracellular fluid decreases from 7.3 to approximately 6.6 to 6.8. Preschismic hyperglycemia aggravates this acidosis to levels between 6.1 and 6.4. It has been shown earlier that acidosis (pH 6.8) diminishes rather than enhances cell death in vitro after a combination of oxygen and glucose deprivation. In this study we found that a further decrease in pH to 6.3 did not aggravate cell death, which demonstrates that moderate acidosis by itself does not cause cell death during IVI.

However, acidosis was a prerequisite for the neurotoxic effect of hyperglycemia. When pH during IVI in the presence of 40 mmol/L glucose was increased to 7.4, the delayed and aggravated cell death pattern seen at pH 6.8 was lost. Consequently, it appears that “glucose acidosis” is the perpetrator of hyperglycemic ischemic cell death.

What is the mechanism behind the harmful effect of glucose acidosis? Glutamate toxicity is a prominent mechanism in brain ischemic cell death, and inhibition of NMDA and AMPA receptors is protective in cell and tissue cultures exposed to oxygen and glucose deprivation. Additionally, during IVI in iCSF, glutamate receptor blockade was highly protective. In contrast, here we show that neither NMDA nor AMPA receptor blockade was protective during hyperglycemic IVI. These results indicate that hyperglycemia-aggravated brain damage is not mediated via ionotropic glutamate receptors, suggesting that a different cell death mechanism is dominating during glucose.
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acidity. This is interesting in view of the failures of stroke trials in which glutamate receptor antagonists were used.

One possible cell death mechanism during hyperglycemic ischemia may be that lactate induces swelling and osmolyis.

This would, however, cause a rapidly developing cell death and not the delayed cell death pattern that we observed in our hyperglycemic cultures. Hyperglycemia in vivo appears to target both neuronal and nonneuronal cells since it aggravates selective neuronal damage and if prolonged leads to status epilepticus, glial swelling, and massive pannecrosis of the tissue.

We report an increase in neuronal death as described in vivo by Li et al., but we do not exclude the possibility that glucose acidosis also affects astrocytes. Astrocytic dysfunction could lead to the loss of metabolic and trophic support to vulnerable neurons and to secondary delayed neuronal degeneration.

Yet another possible explanation of glucose toxicity during ischemia is the formation of advanced glycation end products (AGEs). Interestingly, a role for AGEs in focal ischemia has been shown in rats. Even though the formation of AGEs is a slow process, the initial Schiff-based formation between lysine residues on proteins and glucose in the aldehyde form is fast.

Modification of the lysines may cause dysfunction of proteins important for cell survival, such as Cu-Zn superoxide dismutase, or cytostatcal or mitochondrial proteins or may enhance oxidative stress.

Chronic hyperglycemia can cause diabetic sensory neuropathy. In this condition dorsal root ganglion cells degenerate in a caspase-3- and reactive oxygen species–dependent fashion.

Interestingly, the in vitro concentrations used to induce hyperglycemic damage in these studies were ≥45 mmol/L. The hypothesis that the hyperglycemic cell death described in our study follows a programmed cell death pathway is supported by the fact that it is delayed and occurs under circumstances in which the metabolic stores are not completely depleted.

In conclusion, we have demonstrated that the aggravation of ischemic neuronal damage by hyperglycemia, which is well characterized in vivo and in clinical studies, can be reproduced in vitro. We propose that the combination of glucose and acidosis, rather than lactic acidosis, mediates the harmful effect of hyperglycemia after stroke and that this mechanism is glutamate receptor independent. Glucose toxicity may be an important factor to consider in the design of future stroke treatment and can readily be studied in this in vitro model.

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References


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