Glycolytic Inhibition by 2-Deoxyglucose Reduces Hyperglycemia-Associated Mortality and Morbidity in the Ischemic Rat

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SUMMARY Numerous laboratories have shown that hyperglycemia increases cerebral ischemic damage. This presumably results from increased lactate production and accumulation during ischemia. Although increased tissue lactic acidosis is associated with increased ischemic brain damage, this damage has not been directly linked to glycolytic flux. Because 2-deoxyglucose (2-DG) is a competitive inhibitor of glycolysis we tested its ability to reduce hyperglycemia-exacerbated ischemic brain damage. Severe forebrain ischemia was produced by the four-vessel occlusion model in rats. Four rats received 3 g/kg glucose and saline while a second group (n = 5) was injected with 3 g/kg glucose plus 1.6 g/kg 2-DG. A third group (n = 5) was treated with 1 g/kg glucose plus saline and a fourth group (n = 5) received 1 g/kg glucose and 1.6 g/kg 2-DG. All rats were injected i.p. 10 minutes prior to the ischemic insult with the same volume/kg body weight. All rats receiving the high dose of glucose alone (3 g/kg) were dead within 24 hours posts ischemia. Rats who received 2-DG in addition to 3 g/kg glucose showed only 40% mortality (p = 0.119 Fisher's Exact). 2-DG completely eliminated convulsions during the initial two hours of recovery which was significant (p = 0.008), however, all rats in both groups showed some convulsions by 24 hours posts ischemia. Among rats receiving the low glucose dose (1 g/kg), none of the rats receiving 2-DG died or convulsed by 24 hours posts ischemia. There was 80% mortality and 100% incidence of convulsions by 24 hours posts ischemia among rats receiving glucose alone which were statistically worse (p = 0.024 and 0.004 respectively by Fisher's Exact test) than the 2-DG treated group. Since 2-DG did not eliminate the hyperglycemia in either experiment, we conclude that augmented ischemic damage during hyperglycemia is a consequence of glycolytic flux because 2-DG, which decreases glucose uptake and glycolytic flux, decreased mortality and morbidity.

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THE PRIMARY SUBSTRATE of the brain under normal conditions is glucose. However, the presence of high blood glucose prior to a cerebral ischemic event substantially increases the brain damage produced by the ischemic event. Presumably the reason for the increased ischemic damage associated with hyperglycemia is that under primarily anaerobic conditions there is increased accumulation of lactate due to increased production and decreased blood flow. Yet, no studies have demonstrated that lactate is the damaging agent rather than the hyperglycemia itself. If direct inhibition of glucose metabolism, independent of changes in blood glucose levels, could be shown to be protective in hyperglycemia-aggravated ischemic damage, then the hypothesis that glycolytic flux, and the build-up of tissue lactate, is the damaging agent rather than the hyperglycemia itself, is supported. Because 2-deoxyglucose (2-DG) is a glucose analogue which competitively inhibits glucose uptake and glycolytic flux, this inhibition occurs by competition for membrane carriers and by inhibition of phosphohexose isomerase. In large doses (1 to 3 g/kg) symptoms resembling those of insulin-induced hypoglycemia can be produced. If the damaging mechanism of hyperglycemia is attributable to increased glycolytic flux, then we hypothesized that 2-DG, by inhibiting this flux, should attenuate hyperglycemia-aggravated ischemic damage.

Materials and Methods

Cerebral Ischemia Experiments

Male Wistar rats (Charles River) weighing 210–280 grams were maintained in 12 hour light/dark cycle quarters on standard rat chow and water and used for all experiments. All husbandry and experimental procedures conformed to the standards in “The Guide for the Care and Use of Laboratory Animals.” We used the four-vessel occlusion (4-VO) model of forebrain ischemia developed by Pulsinelli and Briery and the modified snare technique of MacMillan. As described previously, rats were anesthetized with sodium pentobarbital (65 mg/kg i.p.) and supplemented as required through i.p. or i.v. injections. Both common carotid arteries were isolated and a snare was placed around each vessel and underlying muscles. The snares were exteriorized via a 2.5 cm guide tube (PE50). The vertebral arteries were electrocoagulated at the alar foramen and EEG leads were placed on the dorsal surface of the skull. All rats received 0.9% NaCl (0.5 ml/kg) to provide post-surgical hydration.
and were placed in individual cages with free access to water; the cages were placed in a room with a 12 hour light/dark cycle. All rats were fasted until the cerebral ischemic insult 22–26 hours after the last supplement of sodium pentobarbital.

Each rat received two i.p. injections 10 minutes prior to 4-VO and the total injection volume, on a per kilogram basis, was the same for all animals. The treatment groups were: (1) 3 g/kg glucose (17% solution) and saline (isovolumetric to 2-DG treatment); (2) 3 g/kg glucose (17% solution) and 1.6 g/kg 2-DG (17% solution); (3) 1 g/kg glucose (5.67% solution) and saline (isovolumetric to 2-DG treatment); (4) 1 g/kg glucose (5.67% solution) and 1.6 g/kg 2-DG (17% solution).

Approximately 5 minutes before 4-VO, the EEG leads were connected to an oscillograph and a thermistor probe (YSI 402) inserted into the rectum. Body temperature was maintained at control levels by heat lamp throughout the ischemic insult and for two hours after. To induce 4-VO the rats were restrained by hand and the snares tightened to occlude the vessels. Any rat that did not show isoelectric EEG for the duration of the 20 minute ischemic event was eliminated from the study as were any rats that died during 4-VO. About three minutes before the end of ischemia, 2% lidocaine was injected into the wound area around the guide tubes. At 20 minutes of 4-VO, the guide tubes were removed and the wound reopened to verify the return of flow in the carotid arteries. The wound was then quickly reclosed with wound clips and the rats monitored for two hours prior to returning them to their cages. Patency of the carotid arteries was rechecked under halothane anesthesia at 24 hours postischemia or postmortem in order to exclude from the study any animal with evidence of a clotted carotid artery.

**Blood Concentrations of Glucose and Beta-hydroxybutyrate Experiments**

Non-operated male Wistar rats (200–250 g, fasted weight), maintained in 12 hour light/dark cycle quarters, were fasted 22–26 hours prior to pretreatment as described above for the 4-VO protocol. Blood samples were taken from conscious, restrained rats using the tail snip method at time points corresponding to a control period, the start of 4-VO, the end of 4-VO, and the end of the two hour monitoring period. Blood samples were collected and immediately deproteinized by adding one part of blood to three parts 1M perchloric acid (on ice). The mixture was vortexed, centrifuged, and the supernatants neutralized with 5M potassium carbonate. The neutral extracts were stored at −70°C until analysis.

Determinations of blood levels of beta-hydroxybutyrate were carried out by the method of Williamson and Mellanby, modified for fluorometry. Spectrophotometric determinations of glucose were carried out on a Gilford 3500 Computer Directed Analyzer using the procedure of Cooper Biomedical Company (Statzyme Glucose Reagent) based on the coupled enzyme method of Slein using hexokinase and glucose-6-phosphate dehydrogenase, with modifications by Bondar and Mead.

Fluorometric determinations of glucose were carried out according to Lowry and Passonneau using hexokinase and glucose-6-phosphate dehydrogenase, with the following changes in the final concentrations in the assay medium: 93mM Tris Buffer, pH 7.6; 2 μg/ml glucose-6-phosphate dehydrogenase (from Leuconostoc mesenteroides, Boehringer); 1.85 μg/ml hexokinase (from yeast, Boehringer). Glucose standards (Preciset Glucose) were purchased from Boehringer. Assayed normal and abnormal human sera (Ortho Diagnostic Systems Inc.) were used as quality control samples.

**Interference by 2-deoxy-D-glucose in the Determination of D-glucose in Blood**

The presence of 2-DG in blood represents a potential source for interference in the determination of D-glucose using hexokinase/glucose-6-phosphate dehydrogenase (HK/G-6-PDH). The reported Kₘ's of hexokinase (from yeast) for D-glucose and 2-DG are 1 × 10⁻⁴ M and 3 × 10⁻⁴ M, respectively, with similar relative rates of reaction. 2-Deoxy-D-glucose is a structural analogue of glucose which competitively inhibits hexokinase in the brain. Horton et al. have determined glucose in mouse blood by a fluorometric procedure similar to the method described in this work, and have found the phosphorylation of glucose to be quantitative in the presence of a 10-fold excess of 2-DG. In the present work, we have assessed the experimental conditions for both fluorometric and spectrophotometric determinations of glucose in the presence of 2-DG.

**Dose-response Study**

Approximately 20 ml of rat blood were obtained by decapitation and collected in a heparinized container on ice. To aliquots of blood was added either 0.9% saline (control, baseline) or increasing amounts of 2.05 M 2-DG dissolved in 0.9% saline, and the samples deproteinized and neutralized as described above. The neutralized extracts were analyzed for glucose content both by spectrophotometric and fluorometric determinations.

**Data Analysis**

Statistical analysis was performed with the aid of the Michigan Interactive Data Analysis System (MIDAS) on an Amdahl 5860 computer. Comparison of average differences between fluorometric and spectrophotometric measurements were assessed with one way analysis of variance. All average data are expressed as mean ± one standard error of the mean (SEM). The sample size (n) for all experiments is the number of animals. Exact p values are given for Student's t test and the Fisher's Exact test. Survival and morbidity data were compared with the Fisher's Exact test.

**Results**

All rats that received 3 g/kg glucose alone died within 24 hours of 4-VO. Only 40% of those rats which
received 2-DG in addition to the glucose died within 24 hours, however, this difference only reached a significance level of \( p = 0.119 \) by Fisher’s Exact Test (fig. 1). All rats receiving 3 g/kg glucose had convulsive activity by 24 hours postischemia, with the onset of convulsions markedly sooner in rats which received glucose only. All rats receiving glucose alone began to convulse during the initial two hour postischemic recovery period but none of the glucose plus 2-DG group of rats showed any convulsions during this time; this difference was significant \( (p = 0.008 \) Fisher’s Exact test).

By 24 hours postischemia 80% of the rats given 1g/kg glucose plus saline died but all of those given 1.6 g/kg 2-DG with the glucose were alive \( (p = 0.024 \) Fisher’s Exact Test) (fig. 1). The surviving rats were walking about the cage at 24 hours postischemia. Sixty percent of the rats receiving only glucose were convulsing by 2 hours postischemia and 100% were convulsing by 24 hours postischemia. None of the rats receiving 2-DG in addition to the glucose convulsed by either two or 24 hours postischemia. The difference in incidence of convulsive activity between the two groups at 24 hours postischemia was statistically significant \( (p = 0.004 \) Fisher’s Exact).

**Blood Glucose and Beta-hydroxybutyrate Analysis**

Since cross-reaction of 2-DG in the determination of glucose was a potential problem, glucose was determined in the presence of varying concentrations of 2-DG by both the spectrophotometric and the fluorometric techniques (fig. 2). Analysis of the dose-response data indicated that approximately 2% of the 2-DG present (calculated on an “as glucose” basis using MW = 180.2) contributes to the concentrations determined in the spectrophotometric method and 0.6% in the fluorometric method.

Our routine determination of glucose is carried out by an automated, spectrophotometric procedure. Utilizing samples from this study, this method was compared to the manual fluorometric procedure. The variable analyzed was the spectrophotometric result minus the fluorometric result. These differences were much less variable for different times within rats than for different rats \( (F \text{ with } 2 \text{ and } 26 \text{ degrees of freedom} = 0.42, \text{ significance level}\ p > 0.5) \). When the mean differences for non-treated and 2-DG treated rats were compared, the effect of treatment was also non-significant relative to the variability between rats \( (F \text{ with } 1 \text{ and } 15 \text{ degrees of freedom} = 0.085, \text{ significance level}\ p = 0.77) \). Moreover, the mean difference between the determinations by the two methods is actually less for the treated than for the non-treated samples \((1.0 \text{ mg/dl and } 1.6 \text{ mg/dl respectively})\). Based on both treated and non-treated samples from all rats \((n = 17)\), the upper one-sided 95% confidence limit on the mean difference between determinations is 3.2 mg/dl, which is less than the difference of 3.7 mg/dl at zero whole blood concentration of 2-DG in figure 2. Using only data from the nine rats treated with 2-DG, the corresponding limit is 3.9 mg/dl, which translates into a 95% confidence that the whole blood concentration of 2-DG is less than 1.6 mM.

Blood glucose rose after i.p. injection of glucose whether or not 2-DG was also given (fig. 3). At the 30 minute time point, the presence of 2-DG resulted in a statistically significant \( (p = 0.01, \text{ by Student’s}\ t\text{ test}) \) increase in blood glucose in the groups given either the low or high dose of glucose. This time corresponds to the time at which carotid blood flow was restored in rats exposed to 4-VO. Blood glucose levels 150 minutes following injections were approximately 300 mg/dl in rats receiving 2-DG while they were only slightly elevated in the rats receiving glucose alone. In the same group of blood samples the concentration of the beta-hydroxybutyrate was high for the control value, reflecting, presumably, the effects of fasting (fig. 4). In the rats receiving the high dose of glucose, blood beta-hydroxybutyrate fell rapidly upon glucose pretreatment. The reduction in blood beta-hydroxybutyrate was less severe in the group given the low glucose dose. 2-DG did not appear to effect blood ketone concentrations.

**FIGURE 2.** *Interference study for glucose in the presence of 2-DG.* Lines and equations represent linear least square regression analysis.

**FIGURE 1.** *Cross hatched bars indicate percent mortality in rats given glucose but no 2-DG. Open bars indicate percent mortality in rats given 2-DG in addition to glucose. Fisher’s Exact test was used for p values.*
Discussion

Numerous studies have focused on the association between high lactate concentrations in the brain and disruption of cerebral integrity in the presence of ischemia. Most often blood glucose was manipulated by glucose infusion or feeding versus fasting protocols and the brain tissue assayed for lactate. While these studies are strongly suggestive in implicating lactate as opposed to the hyperglycemia as the damaging factor, the role of the hyperglycemia itself was not ruled out as the damaging factor in the cerebral ischemic event.

Both the high and low dose glucose pretreatment produced hyperglycemia and high mortality (100% and 80% respectively). While it is possible that this mortality occurred as a result of extracerebral factors, the general physiological stability of the 4-VO preparation under these conditions and the specificity of the ischemic insult makes involvement of nonneural factors unlikely. In contrast, a previous study from our laboratory reported 20 minutes of 4-VO produced no mortality over the 48 hour period following ischemia, however, those animals were fasted but received no glucose injection or 2DG. Similarly, Pulsinelli et al demonstrated no mortality or convulsions by 24 hours after 20 minutes of 4-VO in the fasted rat. Despite the high mortality in the current study the data still demonstrate cerebral protection in rats treated with 2-DG. This protection occurred without eliminating the hyperglycemia. Thus, it seems likely that it is not the hyperglycemia itself which is deleterious to cerebral ischemic outcome, but rather some metabolic event precipitated by hyperglycemia. Since 2-DG is known to competitively inhibit glucose transport in the brain as well as glycolytic flux, 2-DG may have exerted its protective effect by limiting brain glucose uptake or by directly inhibiting glycolytic flux to lactate. While suppression of glucose uptake may account for some of 2-DG's action, Horton et al demonstrated that mice treated with 2-DG had significantly decreased brain lactate levels but had increased brain glucose concentrations. This would suggest that the inhibition of glycolytic flux was the dominant protective effect of 2-DG.

Since 2-DG reduced the onset and incidence of convulsive activity in this study, this may have contributed to the increased survival seen in 2-DG treated rats. Since there is little reason to suspect that 2-DG is an anticonvulsant per se, other reasons for 2-DG's effect on convulsive activity should be considered. A reduction in ischemic damage may have prevented seizure foci from developing or a reduction in cerebral metabolism may have prevented potential foci from becoming active. A reduction in ischemic damage seems more likely, particularly in the case of the low glucose group, since a single dose of 2-DG resulted in no convulsions or mortality over a 24 hour period. In the high dose glucose group, a transient reduction in cerebral metabolism produced by the 2-DG may have delayed the onset of convulsions. In any case, convulsive activity and mortality were reduced by 2-DG.

The blood levels of glucose measured in the unoperated rats were probably somewhat lower than in the

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**Figure 3.** Blood glucose in non-ischemic rats at key time points corresponding to procedure in ischemic 4-VO rats. 2-DG elevates blood glucose.

**Figure 4.** Blood beta-hydroxybutyrate in non-ischemic rats at key time points corresponding to procedure in ischemic 4-VO rats. 2-DG does not seem to alter blood ketone levels.
rats which actually underwent 4-VO since these animals did not have the stress of surgery or cerebral ischemia. Indeed, the blood glucose levels found by Pulsinelli et al\(^3\) in fasted rats treated with 3 g/kg ten minutes prior to 20 minutes of 4-VO were higher than the blood glucose levels seen in the high glucose animals in this study. Blood glucose was not measured in the rats exposed to ischemia because we wished to avoid the potential complication factor of hemorrhage in our 4-VO ischemic animals. It is reasonable to assume that the qualitative if not quantitative relationship between 2-DG and blood glucose still holds for the rat exposed to ischemia. The outcome which would have negated our conclusion of 2-DG’s protective effect would be if 2-DG produced a relative decrease in blood glucose among glucose-treated rats. This clearly was not the case (fig. 3) in that 2-DG treated rats not only had higher blood glucose concentrations but blood glucose remained high even 150 minutes after injection.

The presence of 2-DG in blood causes falsely elevated levels of D-glucose to be determined using the coupled enzymatic HK/G-6-PDH method (fig. 2). The interference is dose-related and, under the conditions of our assays, is about 3.3-fold greater in the spectrophotometric method than the fluorometric method. This is apparently due to the 50-fold higher dilutions of the samples in the latter procedure, which are necessitated by the greater sensitivity of the fluorometric determination.

Based on the finding that there was no significant difference between the determinations by the two methods when comparing the 2-DG treated rats with the non-treated rats, we conclude that the endogenous levels of 2-DG were not elevated enough to constitute a significant interference and that the conditions chosen for analysis of D-glucose in this study, i.e. fluorometric analysis using highly-diluted samples, were such that any 2-DG present was diluted to such an extent that it no longer interfered in the assay.

This study lends strength to the argument that hyperglycemia-aggravated cerebral ischemic damage is the result of increased lactate build-up during ischemia due to increased glycolytic flux in the presence of increased glucose availability. The results are consistent with the observation in previous animal studies that high blood glucose levels before\(^{1,4}\) or after cerebral ischemia\(^{15-20}\) increase cerebral damage. Even post-ischemic treatment with 2-DG has been shown to decrease mortality in a gerbil study of unilateral common carotid ligation\(^{21}\) although these animals were not intentionally made hyperglycemic. Two recent retrospective clinical studies\(^{22-27}\) report a strong correlation between elevated blood glucose levels on admission to the hospital and poorer neurologic outcome from ischemic stroke or cardiac arrest. Although cause and effect relationships were not explored in these studies, when viewed together with the experimental laboratory work, they point to the possible clinical importance of close monitoring of blood glucose levels and the potential therapeutic advantage of limiting excessive brain glycolytic metabolism in the cerebral ischemic patient. The therapeutic potential of 2-DG in the control of glucose-induced damage in the cerebral ischemic patient is clearly suggested.

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Cerebral Glucose Metabolism During the Recovery Period After Ischemia — Its Relationship to NADH-Fluorescence, Blood Flow, ECoG and Histology

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SUMMARY Local cerebral glucose utilization (ICMRgl), NADH fluorescence, cerebral blood flow (CBF), electrocortical activity (ECoG) and histology were studied during a 4 hr recovery period following 2 hrs of left middle cerebral artery (MCA) occlusion in cats. Changes in relative reduced pyridine nucleotides and CBF were measured by fluororeflectometry, ECoG was obtained from the left middle ectosylvian gyrus (MEG), and ICMRgl was measured at the end of the recovery period autoradiographically with 14-C-2-deoxyglucose. A sham group was comprised of 4 cats. The ten animals subjected to the stroke were classified into 3 groups based on the mean amplitude of the ECoG at the end of the ischemic period. At the end of the recovery period, the relative reduced pyridine nucleotides showed a 22.5% oxidation (oxidation of NADH), a 66.2% reduction (reduction of NAD) and a 3.0% reduction compared to the sham group in the severe, moderate and mild groups, respectively. LCMRgl of the left MEG in the severe group was 64.2% of the corresponding sham value, whereas ICMRgl in the moderate and mild groups were 124.8% and 132.0% of the sham, respectively. CBF at the end of the recovery period ranged from 28.1% to 83.0% of the sham value, although there was no significant difference among these groups. Histologically, a large portion of the neurons in the left MEG in the severe group showed ischemic neuronal changes, while the damage was less severe in the moderate and mild groups. On the basis of these data, it is suggested that a relative substrate deficiency and/or a loss of mitochondrial enzymatic pool size may occur in the animals comprising the severe group. Conversely, anaerobic glycolysis may be activated in the moderate group, while the mild group exhibits an increase in glucose metabolism that is most likely aerobic. A gradient in the magnitude of changes in ICMRgl was noted from the central MCA territory to the surrounding brain regions in the ischemic hemisphere. In addition, there was a mild, but statistically significant (p < 0.05), depression in ICMRgl with no histological damage in the non-ischemic hemisphere of the severe group.

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UNDER NORMAL CONDITIONS, the energy production of brain tissue is almost totally dependent upon oxidative metabolism in the mitochondria. The mitochondrial mechanism of synthesizing energy-rich compounds in brain, however, seems to be very vulnerable to ischemia and has been demonstrated that the dysfunction of brain mitochondrial metabolism deteriorates further during recirculation after incomplete ischemia. In addition, histopathological studies have shown that structural mitochondrial alterations are the first sign of ischemic cellular damage in brain tissue. Since the pyridine nucleotide coenzyme, reduced nicotinamide adenine dinucleotide (NADH), stands at the negative end of the chain of mitochondrial respiratory components, the pyridine nucleotide fluorescence correlates well with the ability of mitochondria to carry out energy-linked functions such as the production of adenosine triphosphate (ATP) and the removal of reducing equivalents. The method of surface fluororeflectometry, which enables us to continuously monitor the alterations of pyridine nucleotide fluorescence, vascular volume and cerebral blood flow (CBF) in the same volume of tissue, has been recently applied to various animal studies and a significant reduction...
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