Pyruvate Administered After Severe Hypoglycemia Reduces Neuronal Death and Cognitive Impairment

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Hypoglycemia-induced brain injury is a significant obstacle to optimal blood glucose control in diabetic patients. Severe hypoglycemia triggers a cascade of events in vulnerable neurons that may culminate in cell death even after glucose normalization. A key event in this cascade is the activation of poly(ADP-ribose) polymerase-1 (PARP-1). Activated PARP-1 consumes cytosolic NAD, and because NAD is required for glycolysis, hypoglycemia-induced PARP-1 activation may render cells unable to use glucose even when glucose availability is restored. Pyruvate, however, can be metabolized in the absence of cytosolic NAD. Here we tested whether pyruvate could improve the outcome in rats subjected to insulin-induced hypoglycemia by terminating hypoglycemia with either glucose alone or glucose plus pyruvate. In the four brain regions studied—CA1, subiculum, dentate gyrus of the hippocampus, and piriform cortex—the addition of pyruvate reduced neuron death by 70–90%. Improved neuron survival was also observed when pyruvate delivery was delayed for up to 3 h. The improved neuron survival was accompanied by a sustained improvement in cognitive function as assessed by the Morris water maze. These results suggest that pyruvate may significantly improve the outcome after severe hypoglycemia by circumventing a sustained impairment in neuronal glucose utilization resulting from PARP-1 activation. *Diabetes* 54:1452–1458, 2005

Hypoglycemia-induced brain injury is a significant obstacle to optimal blood glucose control in patients with diabetes. Tight blood glucose control can reduce the risk of diabetes complications but also increases the risk of hypoglycemic episodes. Severe hypoglycemia causes cell death in vulnerable neuronal populations: the neurons of CA1, subiculum and dentate granule cell areas of the hippocampus, cortical layer 2 and 3 of the cerebral cortex, and the dorso-lateral striatum (1,2). The hippocampal cell populations in particular are important for learning and memory, and impairment of cognitive abilities is the most common sequelae of hypoglycemic coma.

Neuronal death resulting from hypoglycemia is the result of a series of events triggered by reduced glucose availability, and the normalization of blood glucose levels does not necessarily block or reverse this cell death process once it has begun. Glutamate receptor activation and excitotoxicity has long been recognized as an upstream event in this cascade (3). A critical event downstream of glutamate receptor activation is poly(ADP-ribose) polymerase-1 (PARP-1) activation (4). PARP-1 has been established as a key mediator of glutamate excitotoxicity in several settings, including hypoglycemia (4–7). PARP-1 is one of a family of enzymes that use the ADP-ribose groups in NAD to form branched ADP-ribose polymers on specific acceptor proteins in the vicinity of DNA strand breaks or kinks (8,9). PARP-1 normally functions in DNA repair, but extensive PARP-1 activation can promote cell death through mechanisms involving NAD depletion and release of apoptosis-inducing factor (10–12).

Several lines of evidence indicate that the NAD depletion resulting from PARP-1 activation causes a sustained impairment in glycolysis, due to the obligatory coupling of glycolysis with cytosolic NAD at the glyceraldehydes phosphate dehydrogenase step. Specifically, cell culture studies have shown reduced glycolytic capacity but retained mitochondrial function for a limited time after PARP-1 activation (10,13). Cells that would otherwise go on to die after PARP-1 activation can be rescued by providing pyruvate, glutamine, α-ketoglutarate, and other energy substrates that are metabolized without a requirement for cytosolic NAD (10). These studies suggest that in brain, where glucose is normally the only significant energy substrate, the cytosolic NAD depletion induced by PARP-1 activation could lead to a sustained energy failure even after glucose availability is restored.

The blood-brain barrier normally transports pyruvate at a rate much slower than glucose, but prior work suggests that significant pyruvate entry to the brain can be achieved by elevating plasma pyruvate concentrations (14). The aim of this study was to determine whether pyruvate, when administered as an adjunct to glucose after hypoglycemia, could reduce neuronal death and long-term cognitive impairment. Results of these studies show a robust effect of pyruvate when used in this manner. Pyruvate is inexpensive, easily administered, and innocuous. Expedient clinical trials may be warranted.

RESEARCH DESIGN AND METHODS

Rat hypoglycemia. All animal experiments were approved by the animal studies committee of the San Francisco Veterans Affairs Medical Center.

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EEG, electroencephalogram; H&E, hematoxylin and eosin; PARP-1, poly (ADP-ribose) polymerase-1.

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Hypoglycemia was induced with insulin as described by Auer et al. (15) with minor modifications (4). In brief, male Sprague-Dawley rats weighing 250–350 g were fasted overnight, and hypoglycemia was induced by intraperitoneal injection of 15 units/kg of regular insulin (Novolin-R; Novo Nordisk, Clayton, NC). The extent of brain injury after severe hypoglycemia is tightly correlated with the duration of electroencephalogram (EEG) isoelectricity (15). For the present studies, hypoglycemia was terminated after 30 min of EEG isoelectricity to generate a reproducible brain injury of moderate severity (4,15). Hypoglycemia was terminated with a 25% glucose infusion for 3 h (1.5 ml/h i.v.) to maintain blood glucose at 5–10 mmol/l. The rats also received an intraperitoneal injection of sodium pyruvate, α-ketoglutarate, or α-ketobutyrate (500 mg/kg) either simultaneously with glucose or as designated later. Controls received an osmolarity-matched NaCl solution (104.5 mg/kg) intraperitoneally. After recovery from hypoglycemia, the rats are able to ambulate and feed normally. With severe brain injuries, there can be seizures and death in the posthypoglycemic interval; however, no rats exhibited seizures or death in the present study. Sham hypoglycemia rats were fasted overnight, injected the same amount of insulin, and then immediately injected with glucose to prevent hypoglycemia.

Histological evaluations of neuronal injury. Animals were anesthetized with isoflurane (3%) 7 days after hypoglycemia and intracardially perfused with 200 ml 0.9% saline followed by 4% paraformaldehyde for 5 min. The brains were postfixed for 1 h and immersed in 30% sucrose. Cryostat sections (20 μm) were mounted on superfrosted coated slides (Fisher Scientific, Pittsburgh, PA). Fluoro-Jade B staining was performed as described by Schmued and Hopkins (4,16). In brief, the slides were immersed in a basic alcohol solution followed by 0.06% potassium permanganate. The slides were then immersed in 0.0004% Fluoro-Jade B (Histo-Chem, Jefferson, AR) for 20 min and washed in distilled water. Sections were photographed with a Leica confocal laser-scanning microscope with blue (450–490 nm) excitation light and a barrier filter wavelength of 515 nm.

Hematoxylin and eosin (H&E) staining was used to quantify neuronal injury at the 7-day and 7-week time points. Brains were removed under deep anesthesia and frozen for cryostat sectioning (20 μm thickness). The sections were thawed, dried, postfixed in 70% ethanol for 24 h, and then conventionally stained with H&E (17). Degenerating neurons were identified by their eosinophilic pyknotic appearance (4). Five coronal sections were collected from each animal by starting 4.0 mm posterior to Bregma and collecting every third section until five sections were in hand. These sections were then coded and given to a second, blinded experimenter who counted the number of degenerating neurons in the hippocampal CA1, subiculum, dentate gyrus, and perirhinal cortex regions of both hemispheres. The total number of degenerating neurons in the region was averaged over the five sections from each brain.

Behavioral assessments. Rats were assessed using the Morris water maze method (18) to evaluate spatial learning and memory 6 weeks after hypoglycemia or sham hypoglycemia as described previously (4). Rats were housed individually in the testing room beginning 3 days before testing to minimize the effects of social influences on behavior. As a first step, the novel open field test was used to assess for any differences in motor function or exploratory activity. Rats were individually placed in a Plexiglas enclosure (40 × 40 inches) equipped with two rows of infrared photocell panels interfaced with a computer (Hamilton Kinder, San Diego, CA). The lower row detected horizontal exploratory activity and the higher row detected vertical exploratory activity or rearing. Light path breaks were recorded and assessed for the number of movements, active times, path length, and rearing events. On each of 3 consecutive days, open field activity was recorded for 10 min after an initial 1-min adaptation period.

Spatial learning and memory were tested in a circular pool (180 cm in diameter, 50 cm deep) filled with opaque water of constant temperature (24°C). The pool was divided into four quadrants and the platform was always placed in the center of the designated quadrant. The rats were trained first to locate a visible platform (days 1–2) and then to locate a hidden platform (days 3–5). During the visible training, the platform was rotated clockwise to a new quadrant in each session. The platform remained in the same quadrant throughout all the sessions during hidden platform training. The rats received two training sessions per day for 5 consecutive days. Each session consists of three 1-min trials with a 10-min inter-trial interval. The interval between the two daily sessions was 3 h. Once the rats located the platform, they were allowed to remain on it for 10 s. Rats that failed to find the platform within 1 min were manually placed on the platform for 15 s. Time to reach the platform (latency), distance traveled, and swim speed were recorded with an EthoVision video tracking system (Noldus Information Technology, Leesburg, VA) set to analyze two samples per second. Two rats were excluded from the analysis because of slow swim speed (<10 cm/s) and significant periods of passive floating. Both of these rats had been randomized to the sham hypoglycemia group.

Statistical analysis. Data are expressed as means ± SE. The water maze data (swim speed and time to platform) were analyzed by mixed model regression using SAS version 9 (SAS Institute, Cary, NC) and Proc MIXED, followed by post hoc tests adjusted for multiple comparisons when appropriate. All other data were assessed by one-way ANOVA followed by either the Tukey-Kramer test for multiple comparisons between groups or the Dunnett's test for comparisons of multiple groups against a control group.

RESULTS

Pyruvate and α-ketoglutarate reduced neuronal death after severe hypoglycemia. Hypoglycemia was induced with insulin and maintained until the EEG was isoelectric (flat line) for 30 min, at which time plasma glucose was normalized with an infusion of 25% glucose (4,15). This protocol produces a reproducible pattern of neuronal death that mimics the pattern seen in human brain after severe hypoglycemia. Neuronal injury was assessed 7 days after hypoglycemia with Fluoro-Jade B staining, which produces bright green fluorescence in degenerating neurons (16). The Fluoro-Jade B staining showed widespread neuronal death in all four vulnerable brain regions examined and no neuronal death in rats subjected to sham hypoglycemia (Fig. 1*A*).

Fluoro-Jade B staining provides a selective unambiguous marker of neuronal degeneration, but conventional H&E staining remains more useful for quantifying neuronal death because it also allows visualization of normal surviving neurons and it is not subject to photobleaching during cell counting. Brain sections stained with H&E (Fig. 1*B*) showed the same pattern of neuronal death observed with Fluoro-Jade B staining, consistent with prior reports (4).

Compared with rats receiving glucose alone, rats that received glucose plus pyruvate (500 mg/kg i.p.) at the end of the hypoglycemic period showed a marked reduction in neuronal death in each of the regions evaluated (Fig. 1). As quantified in Fig. 2, rats receiving glucose plus pyruvate had 70–90% less neuronal death than the rats given glucose plus vehicle only (saline). Glucose was maintained at 5–10 mmol/l until the effects of insulin were no longer significant (6 h) in each of these experimental conditions. Increasing the plasma glucose to 10–20 mmol/l during this period had no additional protective effect (data not shown). α-Ketoglutarate, like pyruvate, does not require cytosolic NAD for energy metabolism, and α-ketoglutarate also produced significant reductions in neuronal death. By contrast, α-ketobutyrate, which is structurally similar to α-ketoglutarate but is poorly metabolized, had no significant neuroprotective effect (Figs. 1 and 2).

Effects of delayed administration of pyruvate. For these studies, blood glucose was normalized after 30 min of isoelectric EEG and pyruvate was injected at 1, 3, or 6 h later. As shown in Fig. 3, pyruvate administered 1 h after hypoglycemia showed a robust neuroprotective effect in all four brain regions; however, a delay until 3 h after hypoglycemia achieved significant neuroprotection only in the dentate granule cell and cortical areas. No effect was seen with a delay of 6 h.

Long-term effects of pyruvate supplementation. Assessment of brains 7 weeks after the hypoglycemic insult revealed atrophy and loss of neurons in the CA1 region of the hippocampus (Fig. 4). These changes were substan-

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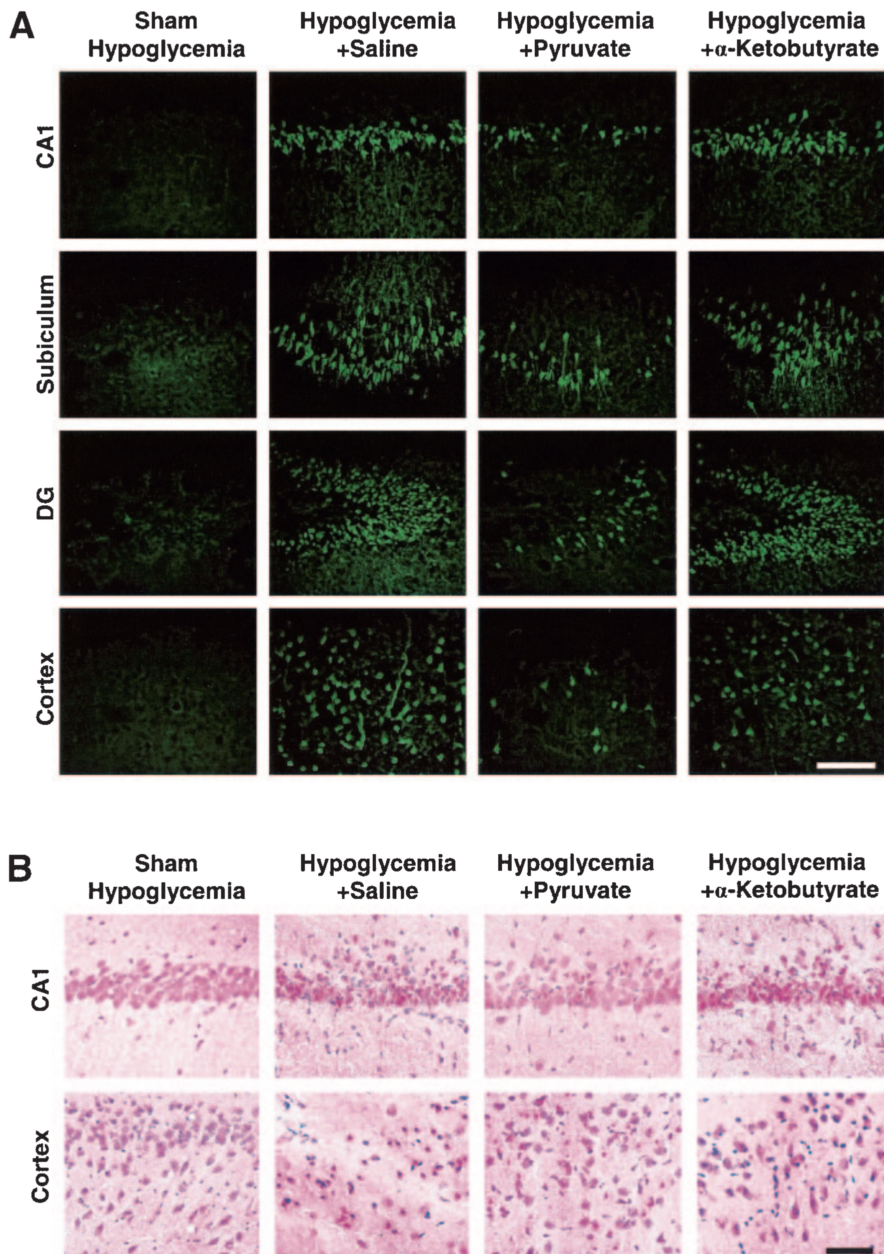

FIG. 1. *A*: Confocal fluorescence images of Fluoro-Jade B–stained brain sections harvested 7 days after hypoglycemia. Fluoro-Jade B staining shows numerous degenerating neurons (green fluorescence) in the hippocampal CA1, subiculum, and dentate gyrus (DG) and in the perirhinal cortex. Sections from sham hypoglycemia brains showed no degenerating neurons. Rats given 500 mg/kg pyruvate with glucose at the end of the hypoglycemic interval showed fewer degenerating neurons, whereas the poorly metabolized agent α-ketobutyrate (500 mg/kg) had no apparent effect. *B*: Conventional H&E-stained brain sections harvested 7 days after hypoglycemia. Dead neurons are identified by their shrunken, pyknotic morphology, and eosinophilic staining. The pattern of neuronal death and response to pyruvate and α-ketobutyrate is the same as seen with the Fluoro-Jade B staining. Photos are representative of seven to nine rats in each treatment condition. Scale bar = 100 μm.

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tially attenuated in the rats treated with pyruvate after hypoglycemia, suggesting neuronal death is prevented rather than simply delayed by this intervention. Pyruvate treatment also showed the same long-term protective effects in subiculum and dentate gyrus of hippocampus and in piriform cortex (data not shown).

In agreement with these long-term histological differences, pyruvate administration after hypoglycemia was also found to improve cognitive function as assessed by the Morris water maze test 6 weeks after hypoglycemia. Rats that had been subjected to hypoglycemia were slower than sham-hypoglycemia rats to find the platform during the visible platform trial of the water maze and were slower to learn the position of the submerged platform during the hidden platform trial (Fig. 5*A*). These differences in water maze performance were not determined by differences in swimming ability because there were no overall differences within the treatment groups with respect to swim speed during either phase of the test (Fig. 5*B*). By contrast, rats in which hypoglycemia was terminated with pyruvate in addition to glucose did not show any significant deficit in their ability to locate the platform compared with the sham-treated animals during either the visible or hidden platform trials. To further determine whether the impairments in sensorimotor function or reduced activity levels could have contributed to the differences in water maze performance, rats were also tested for differences in spontaneous open field activity. As shown in Fig. 6, comparable total path lengths, active times, and rearing events indicated comparable horizontal and activity levels and habituation in the three groups.

DISCUSSION

These results show that pyruvate administered as a supplement to glucose reduces the neuronal death and cognitive impairment that would otherwise result from severe hypoglycemia. Pyruvate significantly reduced hypoglyce-

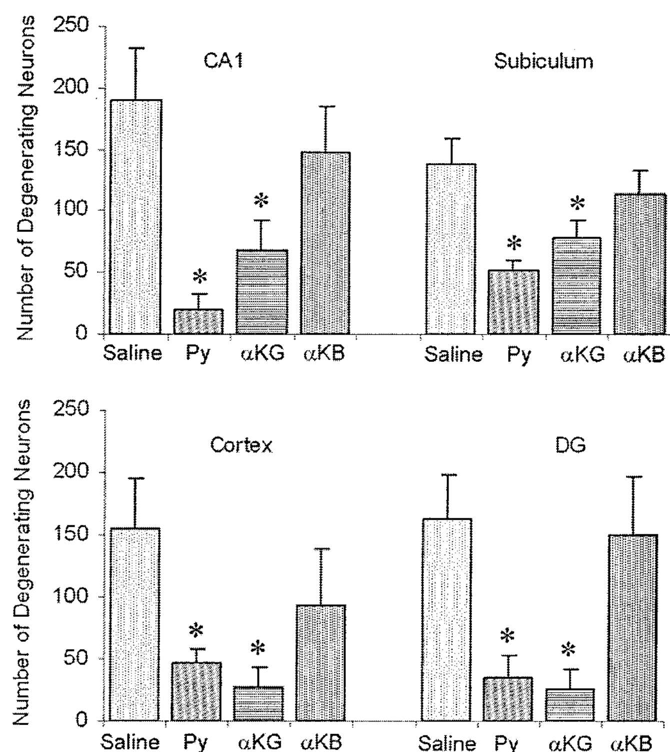

FIG. 2. Hypoglycemic neuronal death is reduced by pyruvate or α-ketoglutarate administered after hypoglycemia. Hypoglycemia was terminated with intravenous glucose plus intraperitoneal injections of pyruvate (Py, 500 mg/kg), α-ketoglutarate (αKG, 500 mg/kg), α-ketobutyrate (αKB, 500 mg/kg), or saline vehicle. *$P < 0.05$ vs. the saline group ($n = 7–9$ in each group).

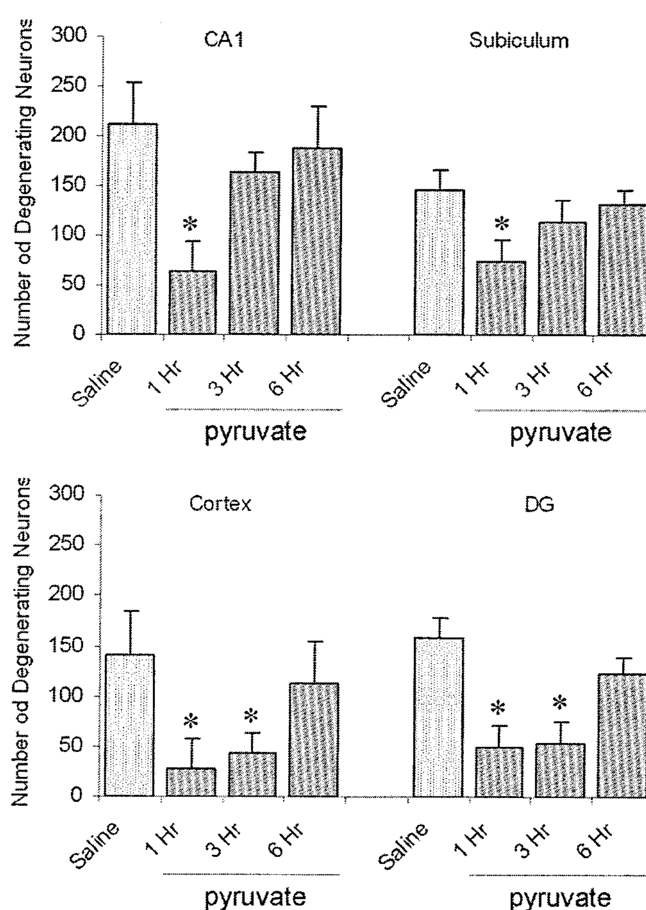

FIG. 3. Effects of pyruvate administration at time points after hypoglycemia. Pyruvate (500 mg/kg i.p.) was administered at time points of 1, 3, or 6 h after glucose infusion. All groups were compared with a control group receiving vehicle (saline) immediately after the glucose infusion. Neuron death was evaluated in the CA1, subiculum, and dentate gyrus (DG) of hippocampus and in the perirhinal cortex. *$P <$ 0.05 vs. the saline group ($n = 3–8$ in each group).

mia-induced neuronal death in the CA1, dentate granule cell, subiculum, and perirhinal cortex regions of the hippocampus. This improved neuronal survival was accompanied by improved cognitive performance assessed 6 weeks after the hypoglycemic insult.

The brain regions most vulnerable to hypoglycemia are important for learning and memory. Accordingly, patients who recover from severe hypoglycemia may be left with difficulties in cognition, particularly short-term memory, out of proportion to gross motor disability (19). The preservation of neuron cell bodies may not always be accompanied by normal synaptic activity and function (20). Cognitive assessments are thus an important adjunct to histological end points in animal models of brain injury. The rats subjected to severe hypoglycemia showed deficits in the Morris water maze test, a standard measure of learning and spatial memory; however, rats treated with pyruvate in addition to glucose exhibited better cognitive performance than rats treated with glucose alone. Together, the behavioral and histological studies performed 6–7 weeks after hypoglycemia suggest that the neuroprotection provided by pyruvate leads to long-lasting preservation of neurons and their function.

Mature brain is one of the few tissues that depends on glucose as its primary metabolic substrate. Once in the brain, glucose may be metabolized directly by neurons and glia; alternatively, glucose may be metabolized to lactate in glia and the lactate subsequently shuttled to neurons for oxidative metabolism (21–24). Lactate, pyruvate, and other ketone bodies are similar to glucose in their capacity to support neuron metabolism in vitro. However, the blood-brain barrier in mature brain has far greater transport capacity for glucose than for other energy substrates. Transport of pyruvate and other substrates does occur, however, and can be augmented by increasing the plasma concentrations of these compounds. The dose of pyruvate used in the present studies is estimated to create a plasma concentration of ~5 mmol/l, which is ~100-fold higher than normal. Because pyruvate crosses the blood-brain barrier by both facilitated transport and diffusion (25,26), this increase in plasma concentration should substantially increase pyruvate transport into brain. In support of this, pyruvate was found to extend the interval between severe hypoglycemia and onset of EEG isoelectricity (R.A.S., S.W.S., unpublished data), and in a prior study, pyruvate was found to reduce hippocampal ischemic injury using a dosing protocol similar to that used here (14).

Although pyruvate and α-ketoglutarate both have weak antioxidant effects (27), an antioxidant mechanism of neuroprotection is unlikely in the present studies because α-ketobutyrate, which also has antioxidant properties, had no effect on neuronal survival. α-Ketobutyrate, like pyruvate and α-ketoglutarate, is capable of directly neutralizing peroxides, but it is not an energy substrate. Pyruvate, α-ketoglutarate, and α-ketobutyrate all cross the blood-

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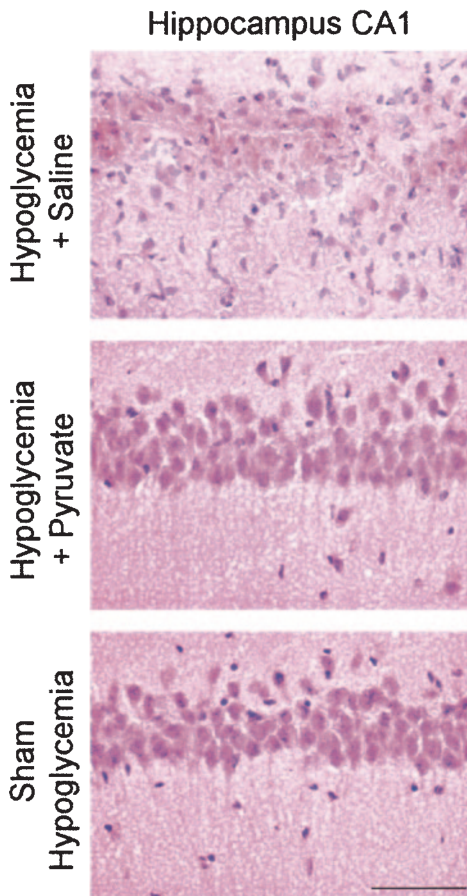

FIG. 4. Long-term neuroprotective effect of pyruvate after hypoglycemia. The H&E-stained brain sections were harvested 7 weeks after sham hypoglycemia, hypoglycemia terminated by intravenous glucose alone, or hypoglycemia terminated by intravenous glucose plus intraperitoneal pyruvate injection. Sections are representative of six to eight rats in each group. Scale bar = 100 μm.

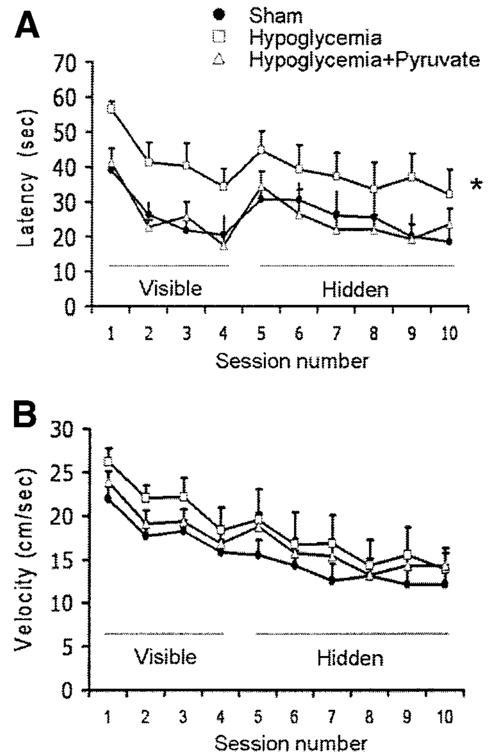

FIG. 5. Pyruvate reduces hypoglycemia-induced cognitive impairment. Rats were tested in the Morris water maze 6 weeks after hypoglycemia terminated by glucose alone, hypoglycemia terminated with glucose plus pyruvate (500 mg/kg i.p.), or sham hypoglycemia. *A*: Rats treated with glucose alone after hypoglycemia showed a significant impairment in their ability to locate the platform when compared with the sham hypoglycemia group. Performance of the rats treated with glucose plus pyruvate was significantly better than the glucose-only group ($P < 0.05$) and not significantly different than the sham hypoglycemia group. *B*: There was no overall difference in swim velocity between the three groups ($P = 0.33$). $n = 6–8$. *$P < 0.05$.

brain barrier by a carrier-mediated process and by simple diffusion (25,26).

The neuronal death that results from severe hypoglycemia is not a direct and immediate consequence of low brain glucose availability, but results instead from a cascade of events precipitated by the lack of energy substrate. Glutamate release, zinc translocation, and PARP-1 activation have been identified as key steps in this cell death pathway (3,28,29). Activated PARP-1 consumes cytosolic NAD. The depletion of cytosolic NAD prevents metabolism of glucose, and in settings where glucose is the chief metabolic substrate, this leads to mitochondrial dysfunction and cell death (13,30,31). Cell culture studies have shown that repletion of NAD after PARP-1 activation restores glycolytic capacity and prevents cell death (31,32). Similarly, providing nonglucose substrates such as pyruvate and α-ketoglutarate, which can be metabolized without the need for cytosolic NAD, also preserves cell viability, even when delivered hours after PARP-1 activation (10,30). Of note, metabolism of lactate for energy production requires NAD for conversion of lactate to pyruvate (the lactate dehydrogenase reaction). Thus, to the extent that glial-derived lactate is used for neuronal energy metabolism (23), this energy source may be unavailable to neurons after PARP-1 activation.

Cell culture studies also support a key role for neuronal zinc accumulation in this cell death pathway. Zinc is a trigger for PARP-1 activation (33), and zinc has also been shown to induce NAD depletion, impaired glycolysis, and cell death in cultured neurons (34). These effects of zinc are blocked by both PARP inhibitors and pyruvate (34). Studies in vivo show that hypoglycemia triggers neuronal zinc accumulation and that brain administration of a zinc chelator can block hypoglycemia-induced PARP-1 activation and neuronal death (29). These results together support a sequential series of events by which neuronal zinc release, PARP-1 activation, NAD consumption, and glycolytic blockade contribute to hypoglycemic neuronal cell death. Later, downstream events in this cascade likely involve mitochondrial dysfunction and induction of mitochondrial cell death programs (35–38).

In agreement with the cell culture studies, the present findings suggest that pyruvate promotes neuronal survival after hypoglycemia by bypassing a sustained impairment in glycolysis induced by PARP-1 activation. This idea is supported by the observation that α-ketoglutarate had an effect similar to that of pyruvate, whereas α-ketobutyrate and increased plasma glucose had no effect. α-Ketoglutarate, like pyruvate, is oxidatively metabolized without the participation of cytosolic NAD. Also like pyruvate, α-ketoglutarate prevents neuronal death after PARP-1 activation in vitro (10). The finding that pyruvate was neuroprotective when administered at 1–3 h after termination of hy-

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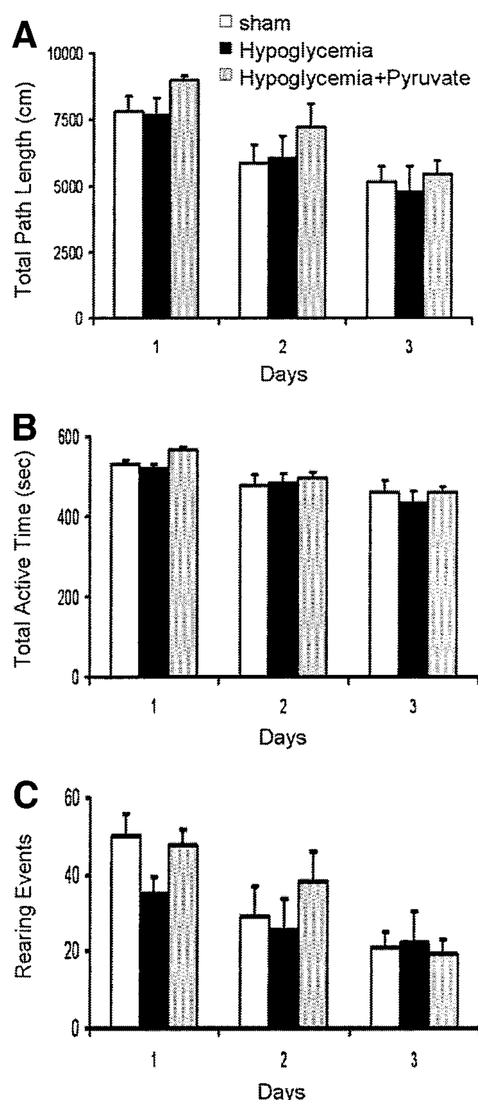

FIG. 6. Hypoglycemia did not affect spontaneous activity and exploratory behavior. Rats were tested in a novel open field for 3 consecutive days. In all groups, horizontal (*A* and *B*) and vertical activities (*C*) declined over the 3-day period, indicating normal habituation. There was no significant difference in path length ($P = 0.57$), total active time ($P = 0.42$), and rearing events ($P = 0.57$).

poglycemia further supports the idea that hypoglycemia causes a sustained impairment in glucose metabolism that persists after glucose reperfusion.

The results of the present studies are similar to those obtained in the same hypoglycemic brain injury model using PARP inhibitors, in which administration of PARP inhibitors at the termination of hypoglycemia substantially reduced neuronal death in vulnerable brain regions and prevented cognitive impairment (4). These parallel results provide additional evidence that pyruvate is acting on the PARP-1 cell death pathway and suggest that the effect of PARP-1 antagonists in this setting can be mimicked by pyruvate. Pyruvate is inexpensive, readily available, and unlike the PARP inhibitors, does not have the theoretical disadvantage of potentially impairing DNA repair (39). Results of this study suggest that pyruvate may be an effective intervention for patients with severe hypoglycemia.

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