Prevention of Severe Hypoglycemia-Induced Brain Damage and Cognitive Impairment With Verapamil

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People with insulin-treated diabetes are uniquely at risk for severe hypoglycemia-induced brain damage. Because calcium influx may mediate brain damage, we tested the hypothesis that the calcium-channel blocker, verapamil, would significantly reduce brain damage and cognitive impairment caused by severe hypoglycemia. Sprague-Dawley rats (10 weeks old) were randomly assigned to one of three treatments: 1) control hyperinsulinemic ($200 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$)-euglycemic (80–100 mg/dL) clamps ($n = 14$), 2) hyperinsulinemic-hypoglycemic (10–15 mg/dL) clamps ($n = 16$), or 3) hyperinsulinemic-hypoglycemic clamps, followed by a single treatment with verapamil (20 mg/kg) ($n = 11$). Compared with euglycemic controls, hypoglycemia markedly increased dead/dying neurons in the hippocampus by 16-fold and cortex by 14-fold. Verapamil treatment strikingly decreased hypoglycemia-induced hippocampal and cortical damage, by 87% and 94%, respectively. Morris Water Maze probe trial results demonstrated that hypoglycemia induced a retention, but not encoding, memory deficit (noted by both abolished target quadrant preference and reduced target quadrant time). Verapamil treatment significantly rescued spatial memory as noted by restoration of target quadrant preference and target quadrant time. In summary, a one-time treatment with verapamil after severe hypoglycemia prevented neural damage and memory impairment caused by severe hypoglycemia. For people with insulin-treated diabetes, verapamil may be a useful drug to prevent hypoglycemia-induced brain damage.

Hypoglycemia is the rate-limiting treatment barrier for people with insulin-treated diabetes (1). With an increased emphasis on glycemic control, the risk of severe hypoglycemia for insulin-treated patient rises markedly (2). Severe,

life-threatening hypoglycemia occurs, on average, once per year for patients with insulin-treated diabetes (3). The increased morbidity resulting from severe hypoglycemia is mediated by brain glucose deprivation leading to seizures, coma, and brain damage (4). In clinical and preclinical studies, hypoglycemia-induced brain damage results in significant cognitive impairments characterized by deficits in spatial and orientation memory (4–7).

Hypoglycemia induces a surge of stimulatory signals (excitotoxicity) leading to an inability of neurons to repolarize and control calcium influx (8–10). Excess calcium influx from the endoplasmic reticulum and extracellular space initiates a positive feedback loop that leads to brain cell necrosis (9,11). Consistent with a major role of calcium influx in mediating other types of brain damage, treatment with the calcium-channel blocker, verapamil, has been shown to reduce neural damage by up to 96% in other models of brain injury (12,13).

To test the hypothesis that verapamil administration (during the recovery period immediately after an episode of severe hypoglycemia) may prevent brain damage, experimental rats were subjected to an episode of insulin-induced severe hypoglycemic (10–15 mg/dL), followed by treatment with or without verapamil. Results demonstrate that a one-time treatment with verapamil prevented ~90% of the hypoglycemia-induced neural damage and completely prevented the hypoglycemia-induced cognitive impairment.

RESEARCH DESIGN AND METHODS

Animals

Male Sprague-Dawley rats (225–250 g) from Charles River Laboratory were housed in a temperature- and light-controlled

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environment and fed ad libitum with standard rat chow diet and water. Studies were in compliance with the University of Utah Institutional Animal Care and Use Committee (17-09002).

Cannula Implantation Surgery

Cannulation surgery was performed in all rats under general anesthesia (75 mg/kg ketamine i.p., 5 mg/kg xylazine i.p., and 5 mg/kg Rimadyl s.c.), as previously described (7,14).

Euglycemic and Hypoglycemic Clamps

After 7 days' recovery from surgery, animals were fasted overnight and underwent hyperinsulinemic ($200 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$; Humulin R; Eli Lilly, Indianapolis, IN) and variable 50% dextrose (Hospira, Lake Forest, IL) glycemic clamps (7,14). Contemporaneous with insulin infusion, dextrose was infused to achieve predefined glucose targets. In one group, euglycemia (EU; 80–100 mg/dL) was targeted for 90 min. In the other two groups, glucose infusion was adjusted to target severe hypoglycemia (SH; 10–15 mg/dL) (7,14). Arterial blood glucose was measured every 15 min with Ascensia Contour Blood Glucose Meter (Bayer Healthcare, LLC, Mishawaka, IN). Seizure-like activity was quantified by counting the number of characteristic movements: tonic stretching ($>5 \text{ s}$), uncontrolled limb movements, or spontaneous spinning (7). After the 90-min glycemic clamps, the rats received an injection of verapamil (VER; 20 mg/kg i.p.; Cayman Chemicals, Ann Arbor, MI) (SH+VER group) suspended in 1 mL saline or just saline (SH and EU groups). After the treatment with verapamil or saline, the glycemic clamps were terminated with increased glucose administration and allowing the rodents free access to chow (7,14) (Fig. 1). ELISA assays were used to measure epinephrine (Abnova, Taipei, Taiwan) and insulin (Crystal Chem, Downers Grove, IL).

Histology

Seven days after the glycemic clamp, one cohort of rats (EU, $n = 7$; SH, $n = 8$; SH+VER, $n = 5$) was sacrificed and perfused as previously described (7,14). Four predetermined

brain sections, collected at 25 μm from -1.23 and -0.48 posterior of the bregma, were stained with Fluoro-Jade B (FJB; Chemicon International, Temecula, CA), and the total FJB⁺ cells were quantified in the hippocampus and the entire cortex with an Olympus fluorescence microscope (FLUOVIEW FV1000) using fluorescein isothiocyanate (FITC) excitation by a blinded observer.

Behavioral Testing

Consistent with other studies (7,15,16), a second, identically treated cohort of rats (EU, $n = 7$; SH, $n = 8$; SH+VER, $n = 6$) underwent sensorimotor and behavioral testing 6 weeks after the glycemic clamp.

Grip-Strength Test

To confirm that severe hypoglycemia did not impair muscle strength or dexterity, three grip-strength trials per animal were performed by measuring the amount of time the animal was able to suspend itself on a wire.

Morris Water Maze

Spatial/orientational learning and memory were assessed by the Morris water maze (MWM) test using previous published methods (7,15,16). A computerized tracking program (EthoVision Color-Pro 3.1.16; Noldus Information Technology, Amsterdam, the Netherlands) recorded the swim-path length and time required to find the platform. After two cue trials and a recall trial, four place trials per day for 5 days were conducted to assess spatial learning. Memory retention was assessed by a probe trial, conducted 24 h after the last place trial. To minimize potential directional bias and ensure the reliance of the rats on visual cues, each of the four daily trials was initiated from a different quadrant (in pseudorandom order), and the average latency to reach the platform was determined.

Statistical Analyses

All data are expressed as mean $\pm$ SEM. Statistical significance was determined by one-way and two-way ANOVA

Figure 1—Experimental protocol. Arterial and venous catheters were implanted into Sprague-Dawley male rats (225–250 g) 1 week before the glycemic clamp. Rats were randomized 1 week later to one of three treatments: 1) control (saline) hyperinsulinemic-euglycemic (EU, 80–100 mg/dL) clamps ($n = 14$), 2) control (saline) hyperinsulinemic-hypoglycemic (SH, 10–15 mg/dL) clamps ($n = 16$), or 3) similar SH clamps followed by a single treatment with verapamil (20 mg/dL; SH+VER) ($n = 11$). One cohort of rats was sacrificed 7 days after the glycemic clamps to assess neuronal damage by FJB. A second cohort of similarly treated rats underwent sensorimotor and cognitive testing 6 weeks after the glycemic clamp.

using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA).

RESULTS

Insulin concentrations were not statistically different between the EU, SH, and SH+VER groups during the basal period (1.1 ± 0.8 , 2.7 ± 0.6 , and 2.5 ± 0.5 ng/mL) or during the clamp (139 ± 79 , 204 ± 40 , and 209 ± 32 ng/mL), respectively. The EU animals were clamped at euglycemia (88 ± 2 mg/dL), and the other groups were clamped at hypoglycemic levels (SH, 11 ± 0.2 mg/dL; SH+VER, 11 ± 0.2 mg/dL) for 90 min (Fig. 2A). Thus, by design, the SH and SH+VER glycemic levels were both significantly lower than EU group glycemic levels at all points throughout the clamp ($P < 0.0001$). Glucose infusion rates (GIR) during the clamp were significantly different

between EU and SH and SH+VER, with GIR of 49 ± 1.0 , 2.3 ± 0.3 ($P < 0.0001$ vs. EU), and 1.9 ± 0.4 mg $\cdot$ kg $^{-1}$ $\cdot$ min $^{-1}$ ($P < 0.0001$ vs. EU), respectively (Fig. 2B). To terminate the clamp, glucose was administered, and blood glucose levels increased to 283 ± 32 , 300 ± 28 , and 301 ± 23 mg/dL at 120 min for EU, SH, and SH+VER, respectively. Hypoglycemia induced an equal number of seizure-like episodes in SH and SH+VER (both $P < 0.05$ vs. EU) (Fig. 2C). Epinephrine concentrations, which were low and similar during the basal period, rose substantially in response to hypoglycemia in SH and SH+VER compared with EU (SH vs. EU, $P < 0.01$; SH+VER vs. EU, $P < 0.01$). In the hypoglycemic groups, epinephrine peaked when blood glucose dropped to <15 mg/dL (at time zero) and then tended to decrease throughout the clamps and into recovery (Fig. 2D).

Figure 2—Blood glucose, epinephrine, and seizure data. **A:** By design, blood glucose levels during the clamp period resulted in significantly different glucose levels between EU (●) and the two hypoglycemic groups, SH (■) and SH+VER (▲). At the termination of the 90-min glycemic clamps, treatment was given (black arrow), then glucose was administered, which resulted in a rapid increase in glucose concentrations before returning toward euglycemic levels (final). # $P < 0.0001$ EU vs. SH and SH+VER. **B:** As expected, the GIR during the glycemic clamp was significantly increased in EU compared with SH and SH+VER. # $P < 0.0001$. There was no difference in the GIR between SH and SH+VER. **C:** The number of episodes of seizure-like activity was significantly increased in SH and SH+VER compared with EU. * $P < 0.05$ vs. EU. The number of episodes of seizure-like activity during hypoglycemia was not different between SH and SH+VER. **D:** Basal levels of epinephrine were similar in all groups. When blood glucose fell to <15 mg/dL (time zero), as well as throughout the clamp, SH (■) and SH+VER (▲) rats had significantly increased epinephrine concentrations compared with EU (●) rats. # $P < 0.001$ SH and SH+VER vs. EU. The serum epinephrine levels decreased in SH and SH+VER throughout the duration of the hypoglycemic clamp until returning to similar concentrations once euglycemia was reestablished (final).

Seven days after the glycemic clamps, one cohort of animals was sacrificed for FJB⁺ staining, an established histology marker for dead/dying neurons (17). Representational images of the hippocampus and the cortex are shown in Fig. 3A. Compared with EU, the number of FJB⁺ cells in the SH group was significantly increased in all areas of the hippocampus and cortex ($P < 0.001$). VER treatment (SH+VER), compared with SH, resulted in markedly fewer FJB⁺ cells in all the areas of the hippocampus and cortex, an 87% and 94% reduction, respectively ($P < 0.001$ vs. SH) (Fig. 3B and C). The number of FJB⁺ cells was not statistically different between EU and SH+VER in any area of the hippocampus or the cortex. The blood glucose concentration at 120 min (i.e., 30 min into recovery) was positively correlated with FJB⁺ cells in the SH group ($r^2 = 0.52$, $P < 0.05$) (Fig. 3) but not in the EU (not shown) or SH+VER groups ($r^2 = 0.53$, $P = \text{NS}$).

The second cohort of identically treated animals was tested for sensorimotor deficits and spatial learning 6 weeks after the glycemic clamps. Sensorimotor testing by the grip-strength test demonstrated that the groups did not significantly differ in the amount of time gripping the wire (9 ± 1.2 , 10 ± 1.6 , and 14 ± 3.0 s for EU, SH, and SH+VER, respectively).

Spatial/orientational learning and memory were tested in a MWM (16). With sequential place trials, all treatment

groups demonstrated an equal and gradually decreased distance required to find the submerged platform (Fig. 4A), indicating equivalent spatial learning. Swimming velocities were not different between treatment groups (Fig. 4B). In the probe trial, EU animals spent significantly more time in the target quadrant, demonstrating target quadrant preference (Fig. 4C and F). SH animals did not demonstrate target quadrant preference (Fig. 4D and F) and had a significant reduction in the amount of time spent in the target quadrant ($P < 0.05$ vs. EU) (Fig. 4F), indicating a defect in memory retention. SH+VER rats did show target quadrant preference (Fig. 4E) and demonstrated a significantly increased amount of time spent in the target quadrant compared with SH rats ($P < 0.05$) (Fig. 4F). Time spent in the target quadrant was not significantly different between EU and SH+VER rats (Fig. 4F).

DISCUSSION

Severe hypoglycemia induces damage to the hippocampus, causing memory and cognition impairments (3–7,18). The mechanism(s) by which hypoglycemia causes brain damage remains incompletely understood, but increased intracellular calcium influx has been hypothesized to play a role (10,19). In this study we demonstrate the role of calcium channels in

Figure 3—Verapamil treatment prevented severe hypoglycemia-induced brain damage. **A:** Representational images show FJB staining of the dentate gyrus (DG) and cornu ammonis (CA) in one region of the hippocampus and the sensorimotor region of the cortex for each treatment group, EU ($n = 7$), SH ($n = 8$), and SH+VER ($n = 5$). Positive cells are apple green and brighter than the background. Scale bars = 100 μm . **B:** The number of FJB⁺ cells was quantified in all major areas of the hippocampus. There was a significant increase of FJB⁺ cells in all quantified areas of the hippocampus with SH treatment (gray bar) compared with EU (black bar). The amount of FJB⁺ cells in every quantified area of the hippocampus was significantly reduced in SH+VER (gray stripe bar) compared with SH. EU and SH+VER were not statistically different in any area of the hippocampus. * $P < 0.001$ SH vs. EU; # $P < 0.001$ SH+VER vs. SH. **C:** Cortex FJB⁺ cells refer to FJB⁺ cells lying within layers I–III of the cortex. Total FJB⁺ cells are the summed total of cortex and hippocampus FJB⁺ cells. SH significantly increased the amount of FJB⁺ cells in the total hippocampus, cortex, and total brain compared with EU. In the total hippocampus, cortex, and total brain, the number of FJB⁺ cells was markedly reduced in SH+VER compared with SH. EU and SH+VER were not statistically different in the total hippocampus, cortex, and total brain. * $P < 0.001$ SH vs. EU; # $P < 0.001$ SH+VER vs. SH. **D:** In the SH (■) group, the number of FJB⁺ cells was positively correlated with the blood glucose concentration at 120 min ($r^2 = 0.52$, $P < 0.05$). **E:** With SH+VER (▲), the number of FJB⁺ cells was not correlated with blood glucose levels at 120 min ($r^2 = 0.53$, $P = \text{NS}$ vs. a slope of zero).

mediating hypoglycemia-induced brain damage and, more importantly, that administration of verapamil immediately after an episode of hypoglycemia prevents brain damage and cognitive dysfunction caused by severe hypoglycemia.

After an episode of severe hypoglycemia, there is a marked increase in neurotransmitter and cytokines release that occurs during the glucose reperfusion period (9,20,21). This glucose reperfusion-induced excitotoxicity is associated with an elevation in intracellular calcium, leading to neuronal cell death (9,11). Consistent with the notion that the extent of glucose reperfusion after hypoglycemia plays a role in mediating neuronal damage, we note that the blood glucose concentrations attained during the glucose reperfusion period was positively correlated with the amount of brain damage in SH rats (Fig. 3D). Thus, a lesser extent of glucose reperfusion after hypoglycemia could conceivably result in less brain damage (19); however, we previously demonstrated that glucose reperfusion to euglycemia after hypoglycemia is also associated with brain damage and cognitive dysfunction (7). Particularly noteworthy was that this positive correlation was not observed in the EU or SH+VER groups even though they were

well matched for blood glucose levels during the reperfusion period (Fig. 2A). Our data are therefore consistent with the notion that severe hypoglycemia predisposes hippocampal and cortical neurons to ensuing glucose reperfusion-induced neural damage and that this effect appears to be mediated by calcium influx (because verapamil treatment abrogated associated brain damage).

These experiments did not directly examine the precise mechanism by which verapamil was able to prevent neural damage. Because verapamil crosses the blood-brain barrier and can reduce ischemic brain damage (12,13) and synaptic activation in neurons (13,22), verapamil's neuroprotective effect could be mediated via direct blockade of neuron calcium influx or indirectly through an unknown mechanism. Regardless of the mechanism, verapamil's effect was profound in completely preventing neural damage and cognitive dysfunction mediated by severe hypoglycemia.

Although there was a significant amount of hypoglycemia-induced damage to the cortex (Fig. 3C), no measurable difference was found among groups in sensorimotor coordination and strength after 6 weeks of recovery. Similar abilities in grip-strength testing and swimming speeds in all treatment groups

Figure 4—Verapamil treatment prevents severe hypoglycemia-induced decreased performance in the MWM. **A:** Shown are the swim path lengths required by the animal to locate the platform. There was no statistical difference between the EU (●; $n = 7$), SH (■; $n = 8$), and SH+VER (▲; $n = 6$) groups throughout the trials. All groups had similar escape path length during the cue and place trials. **B:** The average swim velocity of the animals was not different between treatment groups. **C–E:** Representative individual swim paths during the probe trial. The arrows represent the location of the starting point and the small circle in the opposite (target) quadrant indicates the previous location of the submerged platform. **F:** Mean time spent in each quadrant during the probe trial. The dashed horizontal line represents a random amount of time in the quadrant. EU and SH+VER (but not SH) animals spent significantly more time in the target quadrant, demonstrating target quadrant preference. SH rats spent less time in the target quadrant compared with EU. * $P < 0.05$ SH vs. EU. SH+VER rats spent significantly more time the target quadrant compared with SH. # $P < 0.05$ SH+VER vs. SH. Bar legends indicating treatment groups are as in Fig. 2. NE, northeast; NW, northwest; SW, southwest.

indicate no gross motor deficits that could have affected interpretation of cognitive function as assessed during the MWM.

Consistent with our previous findings (7), the SH animals developed reference memory deficits as noted during the probe trial by 1) a lack of target quadrant preference and 2) a reduced amount of time spent in the target quadrant compared with EU controls (Fig. 4C–F). In contrast, SH animals did not exhibit differences in learning compared with EU animals. A parsimonious interpretation for this difference may be that severe hypoglycemia selectively impairs long-term retention of reference memory but that this deficit may not compromise procedural learning and acquisition training due to the presence of proximal navigational cues (16). Of note, the retention memory deficits induced by hypoglycemia were completely reversed with verapamil, with a restoration of target quadrant preference and a significantly increased amount of time spent in the target quadrant (equal to that observed in the EU group). Thus, consistent with the brain damage data, verapamil treatment prevented the development of neurocognitive deficits induced by hypoglycemia.

An increased number of seizures (suggestive of a more profound central nervous system insult during severe hypoglycemia) was associated with poorer cognitive performances in SH rats (Figs. 2C and 4F, respectively) (23). Interestingly, even though an equivalent number of seizures occurred in both hypoglycemic groups before treatment (Fig. 2C), VER+SH rats did not demonstrate impaired cognitive function (Fig. 4F). By limiting ensuing brain damage and preventing cognitive deficits after an episode of hypoglycemia, verapamil treatment allowed for dissociation between the presence of hypoglycemic seizures and resultant cognitive impairment.

In summary, a one-time dose of the calcium-channel blocker, verapamil, significantly prevented brain damage and cognitive dysfunction in our rodent model of severe hypoglycemia. Verapamil may have potential use as a neuroprotective agent for people with type 1 diabetes recovering from an episode of severe hypoglycemia.

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