Diabetes increases brain damage caused by severe hypoglycemia

Adam J. Bree,1 Erwin C. Puente,1 Dorit Daphna-Iken,1 and Simon J. Fisher1,2

1Division of Endocrinology, Metabolism, and Lipid Research, Department of Medicine; and 2Department of Cell Biology and Physiology, Washington University, St. Louis, Missouri

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Bree AJ, Puente EC, Daphna-Iken D, Fisher SJ. Diabetes increases brain damage caused by severe hypoglycemia. Am J Physiol Endocrinol Metab 297: E194–E201, 2009. First published May 12, 2009; doi:10.1152/ajpendo.91041.2008.—Insulin-induced severe hypoglycemia causes brain damage. The hypothesis to be tested was that diabetes portends to more extensive brain tissue damage following an episode of severe hypoglycemia. Nine-week-old male streptozotocin-diabetic (DIAB; n = 10) or vehicle-injected control (CONT; n = 7) Sprague-Dawley rats were subjected to hyperinsulinemic (0.2 U·kg−1·min−1) severe hypoglycemic (10–15 mg/dl) clamps while awake and unrestrained. Groups were precisely matched for depth and duration (1 h) of severe hypoglycemia (CONT 11 ± 0.5 and DIAB 12 ± 0.2 mg/dl, P = not significant). During severe hypoglycemia, an equal number of episodes of seizure-like activity were noted in both groups. One week later, histological analysis demonstrated extensive neuronal damage in regions of the hippocampus, especially in the dentate gyrus and CA1 regions and less so in the CA3 region (P < 0.05), although total hippocampal damage was not different between groups. However, in the cortex, DIAB rats had significantly (2.3-fold) more dead neurons than CONT rats (P < 0.05). There was a strong correlation between neuronal damage and the occurrence of seizure-like activity (r2 > 0.9). Separate studies conducted in groups of diabetic (n = 5) and nondiabetic (n = 5) rats not exposed to severe hypoglycemia showed no brain damage. In summary, under the conditions studied, severe hypoglycemia causes brain damage in the cortex and regions within the hippocampus, and the extent of damage is closely correlated to the presence of seizure-like activity in nonanesthetized rats. It is concluded that, in response to insulin-induced severe hypoglycemia, diabetes uniquely increases the vulnerability of specific brain areas to neuronal damage.

Fluoro-Jade; insulin; seizure; streptozotocin

HYPOGLYCEMIA IS THE MOST PREVALENT clinical complication in the daily management of insulin-treated people with diabetes, and hypoglycemia continues to be the limiting factor in the glycemic management of diabetes (15). Since severe hypoglycemia affects 40% of insulin-treated people with diabetes (49), concern regarding the hazardous potential for severe hypoglycemia to cause “brain damage” continues to be a very real barrier in striving to fully realize the benefits associated with intensive glycemic control (14). Animal models have unambiguously demonstrated that acute episodes of severe hypoglycemia [blood glucose (BG) <18 mg/dl] reproducibly induce neuronal damage, especially in the vulnerable neurons in the cortex and hippocampus (2, 9, 33, 44, 45, 54). Deficits in learning and memory have been shown to be a direct consequence of this severe hypoglycemia-induced hippocampal neuronal damage (2, 44, 45). However, clinical studies in patients with diabetes have yielded variable results, since episodes of severe hypoglycemia have been shown to alter brain structure (37) and have been reported to cause significant cognitive damage in many (7, 16, 18, 23, 31, 34, 35, 39) but not all (4, 20, 25, 41, 43, 55) studies.

The presence of diabetes and associated hyperglycemia has been shown to worsen the extent of neuronal damage following other forms of central nervous system insults (i.e., stroke) in both preclinical (24, 26, 30) and clinical (5, 11) settings. This study was undertaken to determine whether neuronal damage induced by severe hypoglycemia would be similarly augmented due to the presence of diabetes per se.

RESEARCH DESIGN AND METHODS

Animals. Nine-week-old male Sprague-Dawley rats (Charles River Laboratories) were housed individually in a temperature- and light-controlled environment, maintaining each animal’s diurnal cycle (12:12-h light-dark), and with an ad libitum diet. All studies were done in accordance with and approved by the Animal Studies Committee at the Washington University School of Medicine. Implantation of arterial and venous catheters. Anesthesia was induced with an intraperitoneal injection of 87 mg/kg ketamine and 2.6 mg/kg xylazine. A Micro-Renathane brand (Braintree Scientific, Boston, MA) catheter was inserted into the left common carotid artery, and two catheters were implanted into the right jugular vein. To prevent clotting, catheters were filled with a 40% polyvinylpyrrolidone (Sigma, St. Louis, MO) in heparin (1,000 USP U/ml) solution (Baxter Healthcare, Deerfield, IL).

Induction of diabetes. After 3 days of recovery from surgery, rats received intraperitoneal injections of either streptozotocin (STZ; 65 mg/kg, n = 10; Sigma) to induce diabetes (DIAB) or vehicle 0.1 mM sodium citrate (Fisher Scientific) buffer (n = 7) as a control (CONT). One DIAB rat required a second injection of STZ to ensure random BG >300 mg/dl. Following STZ or vehicle injection, measurements of their body weight were made, and random fed BG was measured from the tail vein by Ascensia Contour BG monitors (Bayer HealthCare, Mishawaka, IN).

Hypoglycemic hyperinsulinemic clamp. The clamp experiment was performed 1 wk after the date of STZ or citrate buffer injection. After an overnight fast, vascular catheters were externalized and attached to syringes. After a basal period to allow the rats to acclimate, insulin (Humulin R; Eli Lilly, Indianapolis, IN) was administered intravenously with a bolus (6 U/kg) followed by the constant infusion of a lower dose of insulin (0.2 U·kg−1·min−1) into awake, unrestrained rats. At the start of insulin infusion, control rats also received intravenous glucose (50% dextrose; Hospira, Lake Forest, IL) to match the rate of BG decline observed in diabetic rats. Throughout the clamp, BG was measured from the arterial cannula every 15 min using Ascensia Contour BG monitors. On the basis of pilot experiments that determined the nadir hypoglycemia necessary to reproducibly cause neuronal damage, the target for severe hypoglycemia was glucose levels <15 mg/dl. Once the BG dropped to <15 mg/dl, the clock was reset and hypoglycemia maintained at 10–15 mg/dl for 1 h by adjusting the rate of continuous intravenous glucose infusion. This technique allowed both groups to be matched precisely for duration (1 h) and depth of hypoglycemia in unrestrained animals, without the confounding effects of anesthesia. One CONT and one DIAB rat were...
not included in statistical analysis because they did not reach this level of hypoglycemia. Seizure-like activity was quantified by counting the number of characteristic, brief (<5 s), myoclonic convulsions. After an episode of severe hypoglycemia was induced on day 0, diabetic rats failed to gain weight throughout the experiment and had significantly lower body weights than control rats throughout the experiment. *P < 0.05.

**RESULTS**

Rats injected with STZ displayed a diabetic phenotype with elevated BG levels from 3 days postinjection until the end of the experiment when compared with control rats injected only with buffer (CONT 79 ± 2 mg/dl vs. DIAB 381 ± 17 mg/dl, *P < 0.05; Fig. 1A). Consistent with a diabetic phenotype, DIAB rats failed to gain weight throughout the experiment and had significantly lower body weight than CONT rats throughout the experiment (final body weight: CONT 345 ± 26 g vs. DIAB 297 ± 9 g, *P < 0.05; Fig. 1B).

During the hypoglycemic hyperinsulinemic clamp, both groups experienced the same degree of hypoglycemia for 1 h [CONT 12 ± 0.2 mg/dl, DIAB 11 ± 0.5 mg/dl, *P = not significant (NS); Fig. 2]. During the 1 h of severe hypoglycemia, rats became unresponsive, lost their righting reflexes, and displayed brief episodes of seizure-like activity. Immediately following this period of severe hypoglycemia, glucose was infused so that BGs were rapidly (within 15 min) returned to (H & E; Sigma) and examined with epifluorescent microscope (Zeiss) using fluorescein isothiocyanate (for FJB).

**Microscopy and cell counting.** Every 10th section for a total of five coronal sections between 2.8 and 3.3 mm posterior to Bregma were collected and analyzed from each animal. H & E staining was used to help identify the different regions and identify cell morphology. For quantification purposes, the number of FJB-positive (FLB+) cells per high-power field was assessed by an observer blinded to the treatment conditions. The average number of FJB-positive cells per brain section are shown.

**Statistical analysis.** All data expressed as means ± SE. *P values were obtained by two-tailed Student *t*-test. Significance was determined by a *P* value <0.05.
euglycemic levels (Fig. 2). During the recovery period, rats regained consciousness and their righting reflexes, and their episodes of seizure-like activity ceased. Exogenous glucose was infused for 4 h until insulin’s effects wore off and glycemia could be maintained in the absence of glucose infusion. After 1 h of recovery, there was a trend for DIAB rats to have a higher BG than CONT rats (143 ± 38 vs. 67 ± 38 mg/dl, P = NS). At the end of recovery, both groups of rats reached similar levels of glycemia (DIAB 93 ± 24, CONT 67 ± 38, P = NS; Fig. 2). During recovery, each group received similar amounts of glucose (DIAB 3.2 ± 0.2 g, CONT 3.5 ± 0.3 g, P = NS).

One day following recovery, BGs were higher in the DIAB (422 ± 52 mg/dl) than in the CONT (79 ± 5 mg/dl) rats and remained elevated until the rats were euthanized (final glucose: CONT 84 ± 14 mg/dl vs. DIAB 414 ± 145 mg/dl, P < 0.05). Animals regained their previous weight 1 wk after the episode of severe hypoglycemia. Note that the negative control group of STZ-diabetic rats (n = 5) that were not subjected to an episode of severe hypoglycemia had a random glucose (479 ± 48 mg/dl) on the day of their euthanization not different from the DIAB rats that were exposed to severe hypoglycemia.

Rats that underwent a single episode of severe hypoglycemia had condensed morphology and pyknotic nuclei in the cortex and hippocampus (CA1 region, dentate gyrus, CA3 region) compared with rats that did not undergo severe hypoglycemia as identified by the nuclear H & E staining (Fig. 3). Brain sections were also stained for FJB, a specific marker for neuronal degeneration (40), which stained positively in cells of the cortex and hippocampus. FJB-positive (FJB+) cells were

Fig. 3. Neuronal damage as assessed by hematoxylin and eosinophilic staining. Compared with rats that did not undergo hypoglycemia, neuronal damage induced following 1 h of severe hypoglycemia was evidenced by hematoxylin and eosinophilic staining. The rats that underwent severe hypoglycemia show brain cells characterized by condensed shrunken morphology and pyknotic nuclei (red arrows) with eosinophilic staining compared with the nuclei of rats that did not undergo hypoglycemia. This brain damage morphology is most evident in the cortex and specific regions of the hippocampus [CA1 and dentate gyrus (DG)].

Fig. 4. Neuronal damage in the cortex. A: Fluoro-Jade B-positive (FJB+) staining in representative images taken at 3.1 mm posterior to Bregma in the lateral ectorhinial cortex superior to the rhinal fissure and viewed at ×100 magnification. A, insets: FJB+ cells viewed at ×400 magnification, with the bar indicating 100 microns. The 4 groups shown are nondiabetic control and STZ-diabetic rats that did not undergo hypoglycemia as well as nondiabetic control rats and STZ-diabetic rats that were subjected to severe hypoglycemia. B: there were no cortical FJB+ cells seen in rats that did not undergo hypoglycemia, whereas, in response to severe hypoglycemia, STZ-induced diabetes caused a 2.3-fold increase in the number of cortical FJB+ cells per brain section compared with nondiabetic controls. *P < 0.05. C: there was no difference in the amount of FJB+ cells in the left (bars with horizontal lines) vs. the right (cross-hatched bars) hemisphere of the brain in either control (n = 6) or diabetic (n = 9) rats.
counted and used as a marker to quantify the extent of brain cell damage (Figs. 4 and 5). It is important to note that, in the absence of severe hypoglycemia, the induction of diabetes with STZ itself did not result in the appearance of FJB+/H11001 cells in the cortex, hippocampus, or hypothalamus (Figs. 4A and 5A). In the rats that underwent severe hypoglycemia, particularly noteworthy was the significantly more ($P < 0.05$) FJB+/H11001 cells seen throughout the cortex of DIAB vs. CONT rats (Fig. 4B). DIAB rats had a 2.3-fold increase in the amount of FJB+/H11001 cells found in the cortex (CONT 262 ± 138 FJB+ cells vs. DIAB 603 ± 96 FJB+ cells, $P < 0.05$). The amount of FJB+ cells was assessed in both hemispheres of each section. There was no difference in the amount of cortical FJB+ cells in the left vs. the right lobe of the brain in either group (CONT 136 ± 75, 126 ± 68; DIAB 304 ± 65, 299 ± 50 FJB+ cells, left and right hemispheres respectively; Fig. 4C).

With regard to other areas of the brain, no appreciable Fluoro-Jade staining was noted in the glucose-sensing regions

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**Fig. 5.** Hippocampal and hypothalamic neuronal damage. A: FJB+ staining in the CA1, DG, and CA3 regions of the hippocampus and the hypothalamus viewed at ×100 magnification. A, insets: FJB+ cells viewed at ×400 magnification, with the bars indicating 100 microns. The pictures of the hypothalamus (HYP) were taken lateral to the 3rd ventricle (3V). The 4 groups shown are nondiabetic control and STZ-diabetic rats that did not undergo hypoglycemia, nondiabetic control rats that were subjected to severe hypoglycemia, and STZ-diabetic rats that were subjected to severe hypoglycemia. B: in response to severe hypoglycemia, both control ($n = 5$) and diabetic ($n = 8$) rats demonstrated no neuronal damage in the hypothalamus, but hippocampal neuronal damage was most evident in the DG and CA1 regions, with significantly less damage in the CA3 region. *$P < 0.05$. 

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of the hypothalamus (Fig. 5A). However, there was significant FJB staining in the different regions of the hippocampus [dentate gyrus (DG), CA1, CA3] in the rats that underwent severe hypoglycemia (Fig. 5A). In both CONT and DIAB rats that underwent severe hypoglycemia, the number of cells staining positive for FJB were most notable in the DG and CA1 regions, with significantly less damage in the CA3 region (Fig. 5B). There was not a significant difference in the amount of total hippocampal FJB + cells in DIAB (470 ± 181 cells) vs. CONT (532 ± 259 cells). Given that all blood sugars during the period of severe hypoglycemia were reproducible in a narrow range (10–15 mg/dl), the amount of FJB staining was not correlated to glycemia (P = NS). There was no correlation between the length of time it took for the glucose levels to drop to a value <15 mg/dl and the amount of FJB + cells in the cortex or hippocampus (P = NS). There was no significant difference in the amount of visible seizure-like activity between groups during the 1 h of severe hypoglycemia (CONT 2.5 ± 0.7 seizures, DIAB 2.4 ± 0.4 seizures; Fig. 6A). However, there was a strong correlation between the amount of FJB + cells in the cortex and the number of seizure-like episodes (CONT \( r^2 = 0.995 \), DIAB \( r^2 = 0.924 \); Fig. 6B).

**DISCUSSION**

Severe hypoglycemia reproducibly causes brain damage in animals. In the setting of other causes of brain damage (i.e., ischemia, stroke, traumatic brain injury), it has been observed that the presence of diabetes and hyperglycemia leads to more extensive neuronal damage (5, 11, 24, 26, 30). In this study, severe hypoglycemia was shown to cause brain damage in the cortex and the hippocampus, and the extent of damage was closely correlated to the presence of seizure-like activity. Under conditions studied, the results indicate that diabetes per se increases the sensitivity of the cortex to the damaging effects resulting from a single episode of severe hypoglycemia.

Since severe hypoglycemia is not defined clinically by a specific value, but rather as a low-blood sugar event that requires the assistance of another person, a marked variability exists in the severity of hypoglycemia in clinical reports and the observation that cognitive damage is seen in some (7, 16, 18, 23, 31, 34, 35, 39) but not all (4, 20, 25, 41, 43, 55) studies. Unlike retrospective clinical studies, severe hypoglycemia induced in prospective studies with laboratory rats has reproducibly caused brain damage (2, 9, 33, 44, 45, 54). Consistent with the notion that hypoglycemic seizures are a marker of severe hypoglycemia, the extent of neuronal damage experienced by each rat was strongly correlated \( (r^2 > 0.9) \) to the number of events of seizure-like activity. These correlations do not necessarily imply causality, since in this setting of profound hypoglycemia, vulnerable brain regions may be susceptible to both damage and seizure activity (2, 9, 33, 44, 45, 54). As in the real-world setting, seizures in these experiments were defined clinically. In the absence of EEG monitoring, we cannot rule out the possible presence (or effect) of subclinical seizures (i.e., not associated with any obvious motor activity) to the observed brain damage. Clinical studies corroborate this finding since neurocognitive deficits are not necessarily associated with severe hypoglycemia but are associated with witnessed hypoglycemic seizures (23, 39). Our novel findings indicate that seizure-like activity may serve as a clinical biomarker to identify those subjects who are at the highest risk for brain damage and those who would most likely benefit from neuroprotective therapy (2, 9, 33, 44, 45, 54).

With the clamp technique, all groups were precisely matched for duration and nadir of hypoglycemia (10–15 mg/dl). Given this very narrow range, seizures and neuronal damage were not correlated with blood glucose levels. Thus, increased seizure-like activity and more extensive brain damage were due not to more profound hypoglycemia but rather biological variability in being able to cope with (or respond to) the hypoglycemic episode. Although there is some evidence that diabetes per se can alter the glycemic thresholds for hypoglycemic comas (48), the current results do not indicate whether diabetes alters thresholds for hypoglycemic seizures because all blood sugars were lowered to the same degree. However, the difference in the regression curves demonstrates that although diabetic rats did not have more seizure-like activity, they experienced greater neuronal damage per seizure episode compared with control rats (Fig. 6B). These findings indicate that diabetes does not alter susceptibility to hypoglycemia-induced seizure-like activity but does render brain regions, specifically the cortex, more vulnerable to damage.

Consistent with previous reports, it was noted that hypoglycemic brain damage preferentially occurs in the CA1 and dentate gyrus regions of the hippocampus as well as the cortex (2, 9, 33, 44, 45, 54). Although hypoglycemia-induced neuronal injury to the hypothalamus has been reported as present (50), absent (51), or variable (3), in the current experiments, no hypothalamic brain damage was noted (Fig. 5). Hippocampal damage due to severe hypoglycemia has been associated with learning and memory deficits (2, 44, 45). The cortex, however, encompasses a diverse region responsible for auditory, olfac-

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**Fig. 6.** A: correlation between seizure-like activity and neuronal damage. Both groups experienced the same number of seizure-like events (control 2.5 ± 0.7, diabetic 2.4 ± 0.4, \( P = NS \)). B: the amount of damage incurred in the cortex strongly correlates with the amount of seizure-like activity occurring during the 1 h of severe hypoglycemia [control \( r^2 = 0.996 \) (■), \( n = 6 \); diabetic \( r^2 = 0.945 \) (▲), \( n = 9 \)].
ory, vision, sensory, and motor pathways. It remains unclear why diabetes uniquely increases the cortex’s susceptibility to hypoglycemic damage. Previous studies have indicated that levels of chronic hyperglycemia similar to those achieved in our model cause morphological/biochemical changes that appear to differentially affect the cortex compared with the hippocampus. Specifically, STZ diabetes preferentially decreases dendritic neuronal length in the cortex compared with the hippocampus (28). Additionally, the induction of STZ diabetes alters neuronal cell density differently in the hippocampus compared with the cortex (27). Also, the cortex of STZ-diabetic rats experiences a preferential downregulation of N-methyl-d-aspartate receptors (6), which may be particularly important given the putative role of excess glutamate in mediating hypoglycemia-induced neuronal damage (2, 47). Taken together, this differential regional effect of diabetes on cortical vs. hippocampal brain morphology and biochemistry may have contributed to the enhanced susceptibility of the cortex to damage induced by hypoglycemia. Consistent with these findings of preferential damage to the cortex are clinical observations that severe hypoglycemia in people with diabetes leads to cortex-dependent deficits in motor speed, psychomotor efficiency, verbalization, and visual reasoning (7, 16, 39).

For patients with diabetes, severe hypoglycemia usually occurs following a high-dose, subcutaneous, single injection of insulin. However, in the laboratory setting, hyperinsulinemic hypoglycemic clamp methods were employed to control the absolute nadir and duration of hypoglycemia to reproducibly induce neuronal damage. A major confounding factor in laboratory severe hypoglycemia experiments is the use of anesthetic agents, which differs from real-world hypoglycemia because anesthesia 1) markedly reduces brain glucose uptake, which may increase brain damage, 2) abrogates seizure activity often associated with severe hypoglycemia, 3) induces hyperthermia, 4) impairs the increased blood pressure (Cushing) response to hypoglycemia, and 5) has been shown to be neurotoxic (1, 10, 21, 22, 32). To circumvent these technical problems and consistently recreate real-world hypoglycemic conditions, our experiments were conducted in awake, freely mobile rodents without the confounding effects of anesthesia. Previous studies have identified an EEG pattern of isoelectricity as a marker for severe hypoglycemia (2, 9, 44, 45, 54). Since brain glucose metabolism can be markedly altered by diabetes (8, 17, 36, 42, 52), and the glycemic threshold for hypoglycemic coma may be altered by diabetes (48), it was speculated that the glycemic threshold for eliciting an isoelectric EEG recording could be altered in diabetic rats. Thus, for protocol standardization, it was thought to be critically important to match groups for the degree and duration of hypoglycemia rather than a specific EEG pattern.

The separate group of STZ-diabetic rats that did not undergo an episode of severe hypoglycemia served as negative controls, and they did not have any observable brain damage. Thus, the observed brain damage was specific to hypoglycemia per se and not due to STZ, hyperglycemia, or hypoinsulinemia. Cannulation of the left common carotid artery did not appear to have any significant effects because there was equal damage in both left and right hemispheres in response to global severe hypoglycemia (Fig. 4C).

Consistent with other studies using an STZ-diabetic rat model, our rats were diabetic for a total of 14 days (12, 13). The STZ-diabetic rat is a model of poorly controlled diabetes characterized by hyperglycemia, relative insulin deficiency, and hyperlipidemia. One or all of these factors may have played a contributing role by which diabetes leads to increased neuronal damage in response to an episode of severe hypoglycemia, but the exact mechanism was not directly assessed in these studies. There are many potential mechanisms for increased neuronal damage due to diabetes. The greater net drop in glycemia experienced in the diabetic rats (i.e., from 381 to 11 mg/dl) may have exacerbated the brain damage. Diabetes is well known to decrease brain glucose transporters (52) and alter glucose transport and metabolism (17), which may have led to the increased susceptibility to brain damage in DIAB rats despite equivalent systemic hypoglycemia. Following the episode of hypoglycemia, the effects of chronic hyperglycemia may have been toxic to vulnerable neurons, causing increased brain damage (26, 30). Since insulin has been shown to be neuroprotective in other models of brain damage, the chronic relative insulin deficiency in STZ rats may have played a role in enhanced brain damage (19, 29, 38, 53). There is evidence that neuronal damage induced from an episode of severe hypoglycemia is actually initiated when high concentrations of glucose are infused during the recovery period (46). Although reintroduction of glucose may contribute to neuronal damage, on the basis of the fact that equal amounts of glucose were given to both groups (DIAB 3.2 ± 0.2 g, CONT 3.5 ± 0.3 g, P = NS), it is unlikely that the reintroduction of glucose contributed to the differential amount of hypoglycemia-induced cortical damage observed in our STZ-diabetic rats.

In summary, severe hypoglycemia in nonanesthetized rats causes brain damage in the cortex and regions within the hippocampus, and the extent of damage is closely correlated to the presence of seizure like activity. In conclusion, under our experimental conditions, diabetes uniquely increases the susceptibility of specific brain regions to brain damage induced by severe hypoglycemia.

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