Recurrent Hypoglycemia Exacerbates Cerebral Ischemic Damage in Streptozotocin-Induced Diabetic Rats

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Background and Purpose—Stroke and heart disease are the most serious complications of diabetes accounting for >65% of mortality among diabetics. Although intensive insulin therapy has significantly improved the prognosis of diabetes and its complications, it is associated with an elevated risk of recurrent hypoglycemia (RH). We tested the hypothesis that RH exacerbates cerebral ischemic damage in a rodent model of diabetes.

Method—We determined the extent of neuronal death in CA1 hippocampus after global cerebral ischemia in control and streptozotocin-induced diabetic rats. Diabetic animals included an insulin-treated streptozotocin-diabetic (ITD) group and a group of ITD rats exposed also to 10 episodes of hypoglycemia (ITD+recurrent hypoglycemia: RH). Hypoglycemia (55 to 65 mg/dl blood glucose) was induced twice daily for 5 consecutive days.

Results—As expected, uncontrolled diabetes (streptozotocin-diabetes, untreated animals) resulted in a 70% increase in ischemic damage as compared with the control group. Insulin treatment was able to lower ischemic damage by 64% as compared with the diabetic group. However, ITD+RH rats had 44% more damage when compared with the ITD group. We also observed that free radical release from mitochondria is increased in ITD+RH rats.

Conclusions—This is the first report on the impact of RH in exacerbating cerebral ischemic damage in diabetic animals. Our results suggest that increased free radical release from mitochondria may be responsible for observed increased ischemic damage in ITD+RH rats. RH thus may be an unexplored but important factor responsible for increased ischemic damage in diabetes. (Stroke. 2011;42:00-00.)

Key Words: brain ischemia ■ cardiac arrest ■ diabetes ■ free radicals ■ glucose ■ mitochondria ■ stroke

Diabetes is a devastating disease of epidemic proportions. It is estimated that 220 million patients are affected by diabetes worldwide.1 Stroke and heart disease are the most serious complications of diabetes, because they account for approximately 65% of mortality among diabetics.2 Epidemiological studies suggest that long-term diabetes increases the risk of cerebral ischemia as well as cardiovascular disease by 2 to 4 times as compared with the nondiabetic population.3–5 The incidence of cerebral ischemia is greater in patients with Type 2 diabetes mellitus than Type 1 diabetes mellitus (T1DM).3 Furthermore, cerebral infarction after ischemia is more extensive and common in diabetics, who also display slower recovery and worse survival rates than nondiabetic subjects.6 Animal models corroborate these clinical observations and provide mechanistic insights into the pathophysiology of the effect of diabetes on cerebral ischemia.7,8 It has been recognized that a high plasma glucose level is a key factor for the poor outcome observed after cerebral ischemia in diabetics.9

Attaining tight glycemic control is a desirable goal for both patients with T1DM and Type 2 diabetes mellitus. Aggressive therapeutic interventions able to normalize glycohemoglobin A1c can halt the progression of long-term complications of diabetes.10 However, daily glycemic excursions remain difficult to maintain tightly in the normal range even by means of intensive treatment, which is associated with an increased risk of hypoglycemia.11 The risk of experiencing severe hypoglycemia is significantly higher under intensive insulin compared with conventional treatment.12 Recent antecedent hypoglycemia causes defective glucose counterregulation to subsequent hypoglycemia leading to hypoglycemia unawareness and ultimately results in a vicious cycle of recurrent hypoglycemia (RH) in diabetics on glycemia management treatment.13 Most studies reporting the incidence of hypoglycemia in diabetics are based on discontinuous blood sugar monitoring and/or incidents that required emergency assistance. These approaches underestimate the number of mild (55 to 65 mg/dL) and asymptomatic, unreported hypoglyc-
mia events. The use of continuous glucose monitoring systems revealed that the incidence of hypoglycemia (<65 mg/dL) during the daytime was 0.9 events per patient per day (median duration, 32 minutes) in young subjects with T1DM despite excellent glycohemoglobin A1c and preprandial glucose levels.\textsuperscript{14} Another study observed that the mean duration for which ≥25-year-old subjects with T1DM are hypoglycemic (≤70 mg/dL) ranges from 60 to 89 minutes/day.\textsuperscript{15} These studies give some idea of the relatively high frequency of asymptomatic-to-moderate hypoglycemic episodes in patients with T1DM.

The effect of previous moderate (55 to 65 mg/dL) RH episodes on outcome after cerebral ischemia in diabetics is unknown. In the present study, we tested the hypothesis that RH renders the brain of diabetic individuals more sensitive to cerebral ischemia. To test our hypothesis, we used a rat model of streptozotocin-induced diabetes. A preliminary study was presented earlier in abstract form.\textsuperscript{16} We tested the additional hypothesis that RH-induced impaired mitochondrial function makes brains of diabetic individuals more sensitive to cerebral ischemic damage.

Methods

Animals

All animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health under protocols approved by the Animal Care and Use Committee of the University of Miami.

Induction of Diabetes

Diabetes was induced in male Wistar rats (Charles River Laboratories International, Inc, Wilmington, MA) by injection (intraperitoneally) of the β-cell toxin streptozotocin (Sigma-Aldrich, St Louis, MO) freshly dissolved in citrate buffer at the concentration of 60 mg/kg.\textsuperscript{17} Diabetes onset was monitored using a portable glucose meter (Ascensia; Bayer) on nonfasting blood obtained by tail pricking. Animals were considered diabetic if nonfasting glycemic values increased and remained >350 mg/dL after diabetes induction.

Insulin Treatment

To correct hyperglycemia, insulin treatment consisted of subcutaneous insulin pellets (Linplant; LinShin, Toronto, Canada) releasing approximately 2 U/24 hour/implant used on the 4th day after injection of streptozotocin. Nonfasting blood glucose was measured at Day 16 to 18 after streptozotocin injection (Day 2 to 4 after insulin pellet implant). If the blood glucose was >220 mg/dL, an additional quarter to half insulin pellet was implanted to keep the glucose levels in the target range (≤220 mg/dL). These animals were considered as “insulin-treated diabetic” (ITD) rats.

Induction of Hypoglycemia

Hypoglycemia in rats was induced by hyperinsulinemic–hypoglycemic clamping as described earlier.\textsuperscript{17} The hypoglycemia protocol was started on Day 23 to 27 after streptozotocin injection (Day 9 to 13 after insulin pellet implant in the ITD group). One day before the onset of hypoglycemic treatment, all rats were implanted with catheters into the right femoral artery and vein under isoflurane anesthesia. Both arterial and venous catheters were connected to an automated syringe pump and heparinized saline was injected at the rate of 1 μL/min throughout the experiment to prevent clogging of catheters. Rats underwent 5 days of hypoglycemia episodes (twice a day). Hypoglycemia was initiated by injecting 0.25 U/kg body weight Novolog Insulin aspart (Novo Nordisk, AIS, Denmark) into a femoral vein. Hypoglycemia was maintained for 30 minutes by continuous injection of 0.02 U/kg body weight $1\times10^{-4}$ of insulin. To maintain clamped blood glucose values in the target hypoglycemic range (55 to 65 mg/dL glycemia), 10% dextrose solution was also injected as needed. The blood glucose was monitored using a portable glucose meter every 5 minutes on blood obtained from a femoral artery. At the end of 30 minutes, the glucose level was brought to 100 to 120 mg/dL by injecting dextrose. Blood glucose was monitored for the next 30 minutes and if needed, additional dextrose was injected. For the control group (hyperinsulinemic–normoglycemic clamp), animals received an insulin dose equal to that in the hyperinsulinemic–hypoglycemic clamp group. However, blood glucose was maintained at preclamp levels by infusing 10% dextrose solution.

In another set of experiments, hypoglycemia was induced by intraperitoneal injection of insulin 8 U/kg body weight with the aim of maintaining blood glucose levels between 55 to 65 mg/dL for 30 minutes.\textsuperscript{18} An additional 1 to 2 U of insulin was given if the target glucose level was not achieved within 30 minutes. An additional 0.25 to 0.5 mL of a 10% dextrose solution was administered through oral gavage if the glucose level was lower than the target range. After 30 minutes of hypoglycemia, the rat’s blood glucose concentration was raised to 100 to 120 mg/dL by administering 3 mL of 10% dextrose through gavage. For the control group, 4 injections of insulin (2 U/kg body weight) were given over a 40-minute period along with 1 mL of 10% dextrose through gavage.

Induction of Global Ischemia

Global cerebral ischemia was induced the day after the last hypoglycemia treatment as described in detail earlier.\textsuperscript{19} Rats with the different treatments were fasted overnight and then anesthetized with 4% isoflurane. Rats were paralyzed and artificially ventilated during the procedure. All physiological parameters (Supplemental Table I; http://stroke.ahajournals.org), brain temperature, and body temperature were maintained in the normal range. Before ischemic insult, blood was gradually withdrawn from the femoral artery into a heparinized syringe to reduce systemic blood pressure to 50 mm Hg. Cerebral ischemia was then produced by tightening the carotid ligatures bilaterally. The duration of the ischemic insult was 8 minutes.

Histological Assessment

At the end of 7 days of reperfusion, rats were perfused with a mixture of formaldehyde, glacial acetic acid, and methanol.\textsuperscript{19} Coronal brain blocks were processed and serial sections (10 μm thick, 200 μm apart) from 2.8 to 4.0 mm posterior to bregma were collected. The sections were then stained with hematoxylin and eosin. We counted the number of surviving normal neurons within the whole CA1 region of hippocampus as an index of ischemic damage. The number of normal neurons was quantified in an unbiased, systematic manner using the optical fractionator technique (stereology module of MCID Elite 6.0 software; InterFocus Imaging Ltd, Cambridge, UK) attached to a Nikon microscope (Nikon microphot-SA; Nikon Corporation, Tokyo, Japan), a Sony 3CCD color video camera (Sony Corporation, Tokyo, Japan), and an LEP motorized stage (Ludl Electronic Products Ltd, Hawthorne, NY). In brief, the CA1 hippocampus was contoured using a 20× objective. Using a 5000 μm$^2$ to 10 000 μm$^2$ counting frame, normal neurons were counted in 5% of randomly placed sampling sites within the contoured CA1 hippocampus using a 40× objective. The total number of normal neurons present in the CA1 hippocampus was estimated using MCID Elite 6.0 software. The results are expressed as number of normal neurons/mm$^3$ of CA1 hippocampus.

Isolation of Mitochondria

Mitochondria were isolated the day after the last hypoglycemia treatment according to the procedure described previously.\textsuperscript{20} In brief, homogenate of hippocampal formation was prepared in cold (4°C) isolation medium, which consisted of 250 mmol/L sucrose, 10 mmol/L HEPES (pH 7.4), 1 mg/mL bovine serum albumin (fraction V), 0.5 mmol/L ethylenediaminetetraacetic acid, and 0.5 mmol/L ethylene glycol bis (β-aminopropyl ether)-N,N,N′,N′-
tetraacetic acid dipotassium salt. Mitochondria were isolated according to the procedure described previously. The resulting mitochondrial pellet was placed inside a nitrogen cell bomb and pressurized at 1200 psi for 7.5 minutes. The samples obtained after nitrogen compression were centrifuged at 16,000 g for 15 minutes. The pellet was then suspended in 0.25 mol/L sucrose and used as the source of mitochondria for iTRAQ (isobaric tag for relative and absolute quantitation) experiments. The nitrogen bomb step was avoided for mitochondria used in the rest of the experiments.

Determination of Mitochondrial Functions

The substrate oxidation rates and phosphorylating capacities of isolated mitochondria were determined polarographically. Measurements of the specific activities of Complexes I and IV were performed spectrophotometrically as described earlier. The rate of hydrogen peroxide production was measured using an Amplex Red hydrogen peroxide assay kit (Invitrogen, Carlsbad, CA) per the manufacturer’s instructions.

Multidimensional Protein Identification Technology/Isobaric Tag for Relative and Absolute Quantitation Proteomics Analysis

Mitochondrial fractions prepared as described previously were used for Multidimensional Protein Identification Technology in combination with isobaric tag for relative and absolute quantitation (iTRAQ) analysis. Mitochondria from 3 rats were pooled to make 1 sample. A total of 3 such samples was analyzed. Mitochondria of naive, ITD, ITD+RH intraperitoneal, and ITD+RH+glucose intraperitoneal groups were labeled with 117-iTRAQ, 118-iTRAQ, 119-iTRAQ, and 121-iTRAQ tags, respectively. In brief, dried 200 µg of protein was suspended in 40 µL of dissolution solution buffer (0.5 mol/L triethylammoniumbicarbonate at pH 8.5. The proteins in the resulting suspension were denatured and reduced using a final concentration of 0.1% sodium dodecyl sulfate and 5.5 mmol/L Tris-(2-carboxyethyl)phosphate HCl and incubating at 60°C for 1 hour. The processed samples were then digested overnight with Promega Sequencing Grade trypsin in 0.5 mmol/L triethylammonium bicarbonate, pH 8.5. Trpsyn-digested peptide samples were dried and reconstituted with 30 µL of iTRAQ dissolution buffer (0.5 mol/L triethylammonium bicarbonate at pH 8.5; Applied Biosystems, Foster City, CA). Peptide samples from each group were labeled with 1 of 4 iTRAQ reagents. Further reaction with the reagents was quenched as per manufacturer’s instructions (Applied Biosystems). The resulting samples were analyzed for Multidimensional Protein Identification Technology/iTRAQ analysis as described earlier. A combination of the tandem mass spectrometry data from all fractions were used for a Paragon “Sequence Temperature Value” algorithm contained in Protein Pilot software Version 3.0 (Applied Biosystems/MDS SCIEX) and the ProGroup algorithm for protein inference and grouping from tandem mass spectrometry spectral/peptide data. Tandem mass spectrometry data from 2-dimensional LC MALDI Multidimensional Protein Identification Technology experiments were analyzed using both Mascot and Protein Pilot software Version 3.0. For both algorithms, protein identification acceptance criteria were 95% CI (equal to a Protein Pilot Unused Score of 1.3). The relative quantities of mitochondrial proteins are expressed as averages of values from the independent experiments. The value of calretinin is from 1 experiment.

Electrophoresis and Immunoblotting

Electrophoresis and Western blotting were carried out on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described previously. Protein was transferred to an Immobilon-P (Millipore) membrane and incubated with the primary antibody anticomplex IV subunit Vla (COXVIa; 1: 3000; Mitosciences, Eugene, OR), anti-NADH-ubiquinone oxidoreductase 24 kDa subunit (NDUFV2; 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), antitranslocase of outer mitochondrial membrane 22 (1:2000; Santa Cruz Biotechnology), anticalbindin 2 (calretinin; 1:1000; Santa Cruz Biotechnology), or anticomplex IV subunit IV (COXIV; loading control; 1:4000; Mitosciences). Immunoreactivity was detected using an enhanced chemiluminescence (Amershampharmacia Biotech, UK). Western blot images were digitized and subjected to densitometric analysis.

Statistics

All data are expressed as mean ± SD. Statistical evaluation of the histology, blood glucose, and physiological parameter data was performed using analysis of variance followed by Bonferroni post hoc test. Statistical evaluation of the remaining data was performed using analysis of variance followed by the Student Newman-Keuls post hoc test.

Results

The experimental paradigm for each group is summarized in Figure 1. Before induction of cerebral ischemia, physiological variables were similar in all experimental groups (Supplemental Table I). Physiological parameters remained unchanged throughout the surgical procedure. Blood glucose levels at the time of cerebral ischemia induction were different among experimental groups. As expected, naive and diabetic groups had the lowest and highest blood glucose levels, respectively (Supplemental Table I). All ITD groups had blood glucose values higher than the naive group. However, these differences were not statistically significant. To avoid unwanted hypoglycemia in the ITD groups, we intentionally maintained blood glucose levels of all 10 ITD groups higher than the control group. The ITD groups and associated blood glucose levels in milligrams per deciliter at 1 week after initial insulin implant were: ITD: 199 ± 21, ITD+ischemia: 193 ± 37, ITD+RH (clamp): 203 ± 36, ITD+RH (clamp)+ischemia: 196 ± 32, ITD+RH+glucose (clamp): 204 ± 19, ITD+RH+glucose (clamp)+ischemia: 204 ± 39, ITD+RH (intraperitoneal): 205 ± 19, ITD+RH (intraperitoneal)+ischemia: 200 ± 114, ITD+RH+glucose (intraperitoneal): 216 ± 25, and ITD+RH+glucose (intraperitoneal)+ischemia: 195 ± 21. The blood glucose levels during the first induced hypoglycemia event for each hypoglycemia and respective control groups are shown in Supplemental Figure I.

Animals belonging to all cerebral ischemia groups were euthanized at 7 days of reperfusion. Although animals belonging to control groups were euthanized at the same time, they were not subjected to cerebral ischemia. We included these control groups to determine if the experimental conditions per se resulted in any neuronal death. We did not observe any significant differences among the nons ischemia groups (Supplemental Figure II). In animals subjected to cerebral ischemia, neurons exhibiting ischemic cell change (eosinophilic cytoplasm, dark-staining triangular-shaped nuclei, and eosinophilic-staining nucleoli) were present in the CA1 hippocampus. Besides the CA1 hippocampus, other brain areas such as the CA3 hippocampus, dentate gyrus, cingulate cortex, thalamus, and substantia nigra were examined. However, the subtle neuronal death we observed in these areas may be because we used a mild ischemic insult (8 minutes).

Presently, we counted number of normal neurons in CA1 hippocampus as an index of cerebral ischemic neuronal death. As expected, we observed that uncontrolled diabetes resulted in a 70% (P < 0.001) increase in ischemic neuronal death as compared with the control group (Figure 2). Ischemic neuro-
neural death was lower in the ITD group by 64% (P<0.001) as compared with the diabetic group. Neuronal death in the ITD group after ischemia was higher by 16% compared with that in the control nondiabetic+ischemia group. However, this difference was not statistically significant. The RH protocol in ITD rats induced by either clamp or intraperitoneal injection resulted in 44% (P<0.001) and 47% (P<0.001) more neuronal death as compared with the ITD group, respectively. However, neuronal death in control groups for ITD+RH induced either by clamp or intraperitoneal was significantly lower by 45% (P<0.001) and 47% (P<0.001) when compared with ITD+RH+glucose clamp and ITD+RH+glucose intraperitoneal groups, respectively. Neuronal death in these control groups was similar to that observed in the ITD group. The results of this study demonstrate that previous exposure of insulin-treated T1DM rats to RH exacerbates cerebral ischemic neuronal death.

For further studies, we used only 4 groups: naïve, representing the nondiabetic population; ITD, representing diabetics receiving insulin therapy; ITD+RH, ITD experiencing RH; and ITD+RH+glucose as a control for additional insulin injected to induce RH. We used an intraperitoneal method to induce hypoglycemia in the following studies. Earlier studies demonstrated that hypoglycemia leads to an increase in mitochondrial reactive oxygen species production and decrease in mitochondrial membrane potential.25,26 In view of this, we tested the hypothesis that RH-induced impaired mitochondrial function makes brains of diabetic individuals more sensitive to cerebral ischemic damage. To test this hypothesis, first we measured the relative quantities of mitochondrial proteins using Multidimensional Protein Identification Technology/iTRAQ analysis. This analysis identified >1700 proteins, of which 480 were identified with >95% confidence. Of these 480 proteins, the levels of 4 proteins (NADH-ubiquinone oxidoreductase 24 kDa subunit: NDUFV2, Complex IV subunit V1α: COXVIa, translocase of outer mitochondrial membrane 22: translocase of outer mitochondrial membrane 22, and calretinin) were altered by >20% in the ITD+RH group as compared with 3 control groups (ie, naïve, ITD, and ITD+RH+glucose). The summary of these results is presented in the Table.

To confirm the results of the iTRAQ experiment, we measured the levels of these 4 proteins by Western blotting. The results are presented in Figure 3. The level of NDUFV2 in the ITD+RH group was higher by 68% (P<0.005), 102% (P<0.005), and 111% (P<0.005) as compared with the naïve, ITD, and ITD+RH+glucose groups, respectively. The levels of COXVIa in the ITD+RH group were higher by 206% (P<0.001), 513% (P<0.001), and 164% (P<0.001) as compared with the naïve, ITD, and ITD+RH+glucose groups, respectively. Similarly, the level of COXVIa was higher in the ITD+RH group as compared with the 3 control groups. The level of COXVIa in the ITD group was higher by 206% (P<0.001), 513% (P<0.001), and 164% (P<0.001) as compared with the naïve, ITD, and ITD+RH+glucose groups, respectively. The levels of COXVIa in the ITD+RH group were lower by 84% (P<0.001) as compared with the ITD group. We could not detect any significant differences among groups for the levels of translocase of outer mitochondrial membrane 22. The level of calretinin in the ITD+RH group was increased by 61% (P<0.005), 63% (P<0.001), and 34% (P<0.005) as compared with the naïve, ITD, and ITD+RH+glucose groups, respectively. The results of this experiment demonstrate that levels of NDUFV2 and calretinin in the ITD+RH group were significantly different from the 3 control groups (ie, naïve, ITD, and ITD+RH+glucose).

Because we observed alterations in levels of NDUFV2 and COXVIa, next we measured the rate of substrate oxidation in mitochondria isolated from rats belonging to the naïve, ITD,
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RH, recurrent hypoglycemia.

isobaric tag for relative and absolute quantitation; ITD, insulin-treated diabetic; ip, intraperitoneal.

Table. Protein Expression Relative to the Naïve Group as Analyzed by MudPIT/iTRAQ

<table>
<thead>
<tr>
<th>Protein</th>
<th>ITD/Naïve</th>
<th>ITD+RH/Naïve</th>
<th>ITD+RH+Glucose/Naïve</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH-ubiquinone oxidoreductase</td>
<td>0.89</td>
<td>1.22</td>
<td>0.94</td>
</tr>
<tr>
<td>Complex IV subunit Vla</td>
<td>1.73</td>
<td>0.76</td>
<td>0.99</td>
</tr>
<tr>
<td>Translocase of outer mitochondrial membrane 22</td>
<td>0.88</td>
<td>1.20</td>
<td>0.99</td>
</tr>
<tr>
<td>Calretinin</td>
<td>0.91</td>
<td>1.72</td>
<td>1.38</td>
</tr>
</tbody>
</table>

MudPIT indicates Multidimensional Protein Identification Technology; iTRAQ, isobaric tag for relative and absolute quantitation; ITD, insulin-treated diabetic; RH, recurrent hypoglycemia.

Discussion

Achievement of tight glycemic control throughout the day still remains difficult using conventional insulin therapy, and the risk for long-term diabetic complications is not eliminated. The availability of improved insulin formulations, infusion pumps, and continuous glucose monitoring systems has changed the management of diabetes care in recent years, contributing to an enhancement of quality of life and to the reduction of the untoward side effects of conventional insulin therapy in patients with T1DM. However, subjects with erratic daily glycemic excursions, progressive complications, and hypoglycemia unawareness are highly susceptible to multiple severe hypoglycemic events. By the use of continuous glucose monitoring devices, it has been revealed that the mean duration for which patients with T1DM are hypoglycemic (asymptomatic/moderate) is quite extended, ranging over 60 to 89 minutes/day.

The effect of the frequency and duration of asymptomatic/moderate hypoglycemic events on the progression and outcome of diabetic secondary complications is not well studied in the clinical setting. The present study was designed to determine the effect of moderate RH on outcome after cerebral ischemia in chemically induced diabetic rats. The duration and frequency of hypoglycemia was selected based on earlier clinical studies. We observed that exposure to RH makes the diabetic brain more susceptible to subsequent cerebral ischemic damage. To the best of our knowledge, this is the first report demonstrating that RH episodes in an animal model of insulin-requiring diabetes exacerbate cerebral ischemia. The implications of this observation in the clinical settings are multifold.

In a normal subject exposed to hypoglycemia, a sequence of responses can be triggered consisting of suppression of insulin secretion by counterregulatory hormone release (namely, glucagon), release of epinephrine, cortisol, and norepinephrine that contribute to the generation of prodromic symptoms and awareness of hypoglycemia. After diabetes onset and recurrent hypoglycemia, diabetics develop hypoglycemia unawareness, which may expose them to life-

ITD+RH intraperitoneal, and ITD+RH+glucose intraperitoneal groups. We could not detect any significant changes in the rate of substrate oxidation when pyruvate plus malate, succinate plus glycerol 3 phosphate, or ascorbate plus TMPD were used as substrates (Figure 4A). We also measured activities of Complex I and IV in the 4 mentioned experimental groups because NDUFV2 and COXVIa are part of the mitochondrial respiratory chain Complex I and IV, respectively. Similar to the rate of substrate oxidation, we could not detect any statistically significant changes in Complex I and IV activity (Figure 4B).

Despite increased levels of NDUFV2 in the ITD+RH intraperitoneal group, we failed to detect any changes in substrate oxidation rate or Complex I activity (Figure 4). Because NDUFV2 is part of the FMN-containing NADH binding site of Complex I and is 1 of the major sites of superoxide production in Complex I, next we tested the hypothesis that increased NDUFV2 levels will lead to increased superoxide production. We measured this in terms of the rate of mitochondrial 

Figure 2. Effect of RH on ischemic damage in hippocampus of insulin-treated diabetic rats. A, Representative histological images and (B) number of normal neurons in the CA1 region of hippocampus in rats belonging to different experimental groups subjected to cerebral ischemia. Nondiabetic without ischemia (n=5), nondiabetic+ischemia (n=8), diabetic+ischemia (n=12), insulin-treated diabetic+ischemia (n=10); ITD+RH+ischemia (n=8), ITD+RH+glucose clamp+ischemia (n=7), ITD+RH ip+ischemia (n=7), ITD+RH+glucose ip+ischemia (n=7). *P<0.05 vs nondiabetic+ischemia; #P<0.05 vs ITD. RH indicates recurrent hypoglycemia; ITD, insulin-treated diabetic; ip, intraperitoneal.
threatening events. Epinephrine, cortisol, and norepinephrine responses are impaired, whereas glucagon responses to hypoglycemia are absent in patients with diabetes experiencing recurrent hypoglycemia.\textsuperscript{30–32} It is possible that cerebral ischemia-induced release of epinephrine, cortisol, and norepinephrine is lower in diabetic rats exposed to RH. However, it is unlikely that lower release of these hormones may be responsible for increased cerebral ischemic damage after RH exposure, because blockade of cerebral ischemia-induced epinephrine, cortisol, and norepinephrine release and/or blockade of their action after cerebral ischemia results in lower cerebral ischemia damage.\textsuperscript{33–36}

Apart from a systemic effect, hypoglycemia-induced limitation of mitochondrial substrate leads to higher reactive oxygen species production, and oxidative stress inhibits mitochondrial protein import.\textsuperscript{37} Because approximately 99% of mitochondrial proteins are encoded by nDNA, translated in the cytosol and then translocated to various mitochondrial subcompartments, any process affecting the mitochondrial protein import process will adversely affect several mito-
We could not observe any general decrease in protein import in the ITD+RH group, thus ruling out the possibility of impaired mitochondrial protein import. We observed that the level of NDUVF2 and the rate of mitochondrial superoxide production were higher in ITD+RH animals. It is possible that an altered ratio of mitochondrial respiratory chain Complex I subunits may be responsible for the observed increase in free radical production. We hypothesize that due to higher basal level, free radical generation during and after cerebral ischemia may be higher in ITD+RH animals. Increased oxidative stress in the ITD+RH group may result in more severe postischemic mitochondrial dysfunction. It is plausible that this ultimately may be responsible for increased ischemic damage in the ITD+RH group.

We also observed increased calretinin levels in ITD rats exposed to RH. Calretinin is a member of the calcium binding protein E-helix-loop-F-helix-hand family expressed differentially in neuronal subpopulations. Calretinin is one of the important intracellular calcium-buffering proteins. Calretinin also acts as a calcium-dependent regulator of enzymes and ion channels. Most calretinin in the cell is in the soluble compartment; however, approximately 27% is in the particulate fraction, including mitochondria. The role of calretinin in mitochondria is not well understood. It is plausible that increased levels of calretinin in mitochondria may result in a larger ischemia-induced influx of calcium into mitochondria. This larger increase in calcium may result in overstimulation of calcium-regulated mitochondrial processes such as reactive oxygen species production, cytochrome c release, mitochondrial permeability transition pore opening, and cardiolipin peroxidation, resulting in increased ischemic damage in the ITD+RH group.

Ischemic tolerance studies suggest the possibility that RH may render tissue more resistant to any metabolic stress. An earlier study using a model of cerebral hypoxia–ischemia preconditioning demonstrated that repeated brief cerebral hypoxia–ischemia events induced brain damage and not protection. The possibility of induction of tolerance or overstimulation of calcium-regulated mitochondrial processes cannot be induced in diabetic patients or animal models. An earlier study demonstrated that recurrent hypoglycemia protected the brain against severe hypoglycemia. Our results demonstrate that RH does not induce tolerance against cerebral ischemia but rather increased cerebral ischemic damage. The possibility that RH makes tissue in diabetic rats more resistant to any metabolic stress is thus ruled out.

In summary, the major side effect of intensive therapy in diabetes is increased risk of RH events. Our results demonstrate that previous exposure of T1DM rats to RH exacerbates cerebral ischemic damage. Similar results are obtained when RH was induced by a complex clamping method or by relatively simple intraperitoneal injections. RH thus may be an unexplored but important factor responsible for increased ischemic damage in diabetes. Our results also demonstrate that increased free radical production may be responsible for increased ischemic damage in insulin-treated T1DM rats exposed to RH.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL
Table 1: Physiological parameters.

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ITD: insulin-treated diabetic; RH: recurrent hypoglycemia; Clamp: hyperinsulinemic / hypoglycemic clamp or hyperinsulinemic / normoglycemic clamp; i.p.: intra-peritoneal; Before: before ischemia; After: 30 min of reperfusion. *, p<0.05 vs. naïve; #, p<0.05 vs. diabetic
**Supplemental Figure 1**

*Blood glucose levels during hypoglycemia.*

Animals belonging to RH groups were made hypoglycemic by hyperinsulinemic-hypoglycemic clamping or i.p injection of insulin. Control animals received an insulin dose equal to the respective hypoglycemia group. However, glucose was maintained to normoglycemia by dextrose administration. A) ITD+RH clamp (n=4), ITD+RH+Glucose clamp (n=3), ITD+RH (clamp)+ischemia (n=8), and ITD+RH+Glucose clamp+ischemia (n=7); B) ITD+RH i.p. (n=3), ITD+RH+Glucose i.p. (n=4), ITD+RH i.p.+ischemia (n=7), and ITD+RH+Glucose i.p.+ischemia (n=7).

**Supplemental Figure 2**

*Effect of RH on number of neurons in CA1 region of hippocampus in insulin-treated diabetic rats.*

The number of normal neurons in the CA1 region of hippocampus in rats belonging to different experimental groups. Non-diabetic (n=5), Diabetic (n=5), ITD (n=6); ITD+RH clamp (n=4), ITD+RH+Glucose clamp (n=3), ITD+RH i.p. (n=3), ITD+RH+Glucose i.p. (n=4). Neuronal death in these control groups was not statistically different among groups.
Supplemental Figure 1

A

B

Blood glucose (mg/dl)

Baseline

Time (min)

Blood glucose (mg/dl)

Baseline

Time (min)