Recurrence of Hypoglycemia Exacerbates Cerebral Ischemic Damage in Streptozotocin-Induced Diabetic Rats

Kunjan R. Dave, PhD; John Tamariz, AS; Kushal M. Desai, AS; Frank J. Brand, BS; Annie Liu; Isabel Saul, BS; Sanjyot K. Bhattacharya, PhD; Antonello Pileggi, MD, PhD

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+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;42:1404-1411.)

Key Words: brain ischemia ■ cardiac arrest ■ diabetes ■ free radicals ■ glucose ■ mitochondria ■ stroke
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 prandial glucose levels. 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. 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. 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. 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 14th 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. 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 anesthesia. Both arterial and venous catheters were connected to an

**Induction of Global Ischemia**

Global cerebral ischemia was induced the day after the last hypoglycemia treatment as described in detail earlier. 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. Coronal brain blocks were processed and serial sections (10 μm thick) 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 μm² to 10 000 μm² 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/mm² of CA1 hippocampus.

**Isolation of Mitochondria**

Mitochondria were isolated the day after the last hypoglycemia treatment according to the procedure described previously. 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’-
Mitochondrial Functions

The substrate oxidation rates and phosphorylating capacities of isolated mitochondria were determined polarographically.\(^2^2\) Measurements of the specific activities of Complexes I and IV were 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.\(^2^2\) Measurements of the specific activities of Complexes I and IV were avoided for mitochondria used in the rest of the experiments.

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 naïve, 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 \(\mu\)g of protein was suspended in 40 \(\mu\)L of dissolution solution buffer (0.5 mol/L triethylammonium bicarbonate 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) phosphine 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. Tryptsin-digested peptide samples were dried and reconstituted with 30 \(\mu\)L 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.\(^2^3\) A combination of the tandem mass spectrometry data from all fractions was used for a Paragon “Sequence Temperature Value” algorithm\(^2^4\) 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 resulting quantities of mitochondrial proteins are expressed as averaged values of 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.\(^2^1\) Protein was transferred to an Immobilon-P (Millipore) membrane and incubated with the primary antibody anticompex IV subunit VIa (COXVIa; 1: 3000; Mitosciences, Eugene, OR), anti-NADH-ubiquinone oxidoreductase 24 kDa subunit (NDUFP2; 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 anticompex 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, naïve 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 naïve 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 nucleioli) 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-...
nal 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 VIa: COXVIa, translocase of outer mitochondrial membrane 22: translocase of outer mitochondrial membrane 22, and calretinin) were altered by 22. The level of calretinin in the ITD RH group was higher by 20% as compared with the 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 513% (P<0.005), 63% (P<0.005), and 513% (P<0.005) 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,
RH, recurrent hypoglycemia.

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</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 ischemic damage. The implications of this observation in the clinical settings are multipled.

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-

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.

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

**Figure 3.** Effect of RH on levels of NDUFV2, Complex IV subunit Via, Tom 22, and calretinin in hippocampal mitochondria of insulin-treated diabetic rats. Immunoblots of (A) NDUFV2, (B) Complex IV subunit Via, (C) Tom 22, and (D) calretinin in mitochondrial fraction of hippocampus harvested from rats belonging to naïve (n=6), ITD (n=5), ITD+RH ip (n=6), and ITD+RH+glucose ip (n=6) groups. Immunoblots (typical images are shown atop each bar) were subjected to densitometric analysis and the level of each protein is expressed as fold of naïve animals. *P<0.05 vs naïve; #P<0.05 vs ITD; and \#\#P<0.05 vs ITD+RH+glucose ip. RH indicates recurrent hypoglycemia; NDUFV2, anti-NADH-ubiquinone oxidoreductase 24 kDa subunit; Tom 22, translocase of outer mitochondrial membrane 22; ITD, insulin-treated diabetic; ip, intraperitoneal.

**Figure 4.** Effect of RH on rate of hydrogen peroxide production, rate of substrate oxidation, and Complex I and IV activities in hippocampal mitochondria of insulin-treated diabetic rats. A, Rate of substrate oxidation, (B) Complex I and IV activities, and (C) rate of hydrogen peroxide production in hippocampal mitochondria harvested from rats belonging to naïve (n=6), ITD (n=5), ITD+RH ip (n=6), and ITD+RH+glucose ip (n=6) groups. The left ordinate in B represents data for pyruvate plus malate and succinate plus G-3-P. The right ordinate in B represents data for ascorbate plus TMPD. *P<0.05 vs naïve; #P<0.05 vs ITD; and \#\#P<0.05 vs ITD+RH+glucose ip. RH indicates recurrent hypoglycemia; ITD, insulin-treated diabetic; ip, intraperitoneal.
We 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 cardioliopin 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 activating protective pathways after RH in diabetic rats is ruled out owing to the fact that ischemic preconditioning cannot be induced in diabetic patients or animal models. Earlier studies demonstrated that recurring 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 diabetics 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.

References


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SUPPLEMENTAL MATERIAL
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<td>40±2</td>
<td>144±18</td>
<td>7.47±0.04</td>
<td>118±6</td>
</tr>
</tbody>
</table>

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 2

![Graph showing the number of normal neurons per mm³ across different conditions: Non-diabetic, Diabetic, ITD, ITD + RH (clamp), ITD + RH + Glucose (clamp), ITD + RH + Glucose (i.p.).]