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Recurrent Hypoglycemia Exacerbates Cerebral Ischemic Damage in Diabetic Rats via Enhanced Post-Ischemic Mitochondrial Dysfunction

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Abstract

Diabetes significantly increases the risk of stroke and post-stroke mortality. Recurrent hypoglycemia (RH) is common among diabetes patients owing to glucose-lowering therapies. Earlier, we showed that RH in a rat model of insulin-dependent diabetes exacerbates cerebral ischemic damage. Impaired mitochondrial function has been implicated as a central player in the development of cerebral ischemic damage. Hypoglycemia is also known to affect mitochondrial functioning. The present study tested the hypothesis that prior exposure of insulin-treated diabetic (ITD) rats to RH exacerbates brain damage via enhanced post-ischemic mitochondrial dysfunction. In a rat model of streptozotocin-induced diabetes, we evaluated post-ischemic mitochondrial function in RH-exposed ITD rats. Rats were exposed to five episodes of moderate hypoglycemia prior to the induction of cerebral ischemia. We also evaluated the impact of RH, both alone and in combination with cerebral ischemia, on cognitive function using the Barnes circular platform maze test. We observed that RH exposure to ITD rats leads to increased cerebral ischemic damage and decreased mitochondrial complex I activity. Exposure of ITD rats to RH impaired spatial learning and memory. Our results demonstrate that RH exposure to ITD rats potentially increases post-ischemic damage via enhanced post-ischemic mitochondrial dysfunction.

Abbreviations

CA	cornus ammonis
ETC	electron transport chain
ITD	insulin-treated diabetic
MMP	mitochondrial membrane potential
MPTP	mitochondrial permeability transition pores
RH	recurrent hypoglycemia
ROS	reactive oxygen species
STZ	streptozotocin
s.c.	subcutaneous
T1D	type 1 diabetes
T2D	type 2 diabetes
TNF-α	tumor necrosis factor α

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Introduction

The prevalence of diabetes is rapidly increasing worldwide. The International Diabetes Federation and the CDC estimate that 415 and 29 million people suffer with diabetes globally and in the USA, respectively [13, 50]. Diabetes is a chronic disease that requires continuous medical care to prevent acute complications and reduce the risk of long-term complications [2]. Diabetes significantly increases the risk of stroke/cerebral ischemia and also increases mortality following stroke/cerebral ischemia [7].

Earlier studies reported that management of the disease with intensive glycemic control can limit, delay, or even prevent the chronic complications of diabetes [28, 76]. However, intensive glycemic control could increase the risk of hypoglycemia in both type 1 (T1D) and type 2 (T2D) diabetics, consequently increasing the risk of hypoglycemic brain injury [19, 25, 31, 32, 62]. Diabetics frequently experience episodes of symptomatic and asymptomatic hypoglycemia [31, 53, 84, 93, 100]. Recurrent exposure to hypoglycemia may lead to an impaired awareness of hypoglycemia [36, 105]. Diabetics with impaired hypoglycemia awareness experience much higher incidences of both asymptomatic and severe hypoglycemia than those with normal awareness [37, 91, 93]. Studies involving continuous glucose monitoring systems detect a high frequency of previously unrecognized hypoglycemic episodes in individuals with both T1D and T2D [8, 67, 99, 102].

The presence of oxidative stress during hypoglycemia is well documented [1, 83, 97, 98]. Using in vivo and in vitro models, earlier studies demonstrated that hypoglycemia leads to an increase in mitochondrial reactive oxygen species (ROS) production and a decrease in mitochondrial membrane potential (MMP) [24, 51, 56, 66]. The production of ROS and oxidative stress is involved in recurrent hypoglycemia (RH)-induced dendritic damage in the hippocampus [103]. Repetitive moderate hypoglycemia in the developing brain causes selective impairment of synaptic plasticity in the absence of hippocampal neuronal death and without complete disruption of basal synaptic transmission [104]. We earlier demonstrated that ROS generation is increased in isolated mitochondria from the hippocampus of insulin-treated diabetic (ITD) rats exposed to RH compared to the control groups [24]. Also, we observed that the most sensitive populations of neurons, the cornus ammonis (CA)1 hippocampal neurons survived least in the ITD + RH group compared to the control groups [24]. Increased ROS production is also observed during and following an acute ischemic stroke [21]. Thus, oxidative stress is an important mediator of tissue injury in both hypoglycemia and acute ischemic stroke.

Mitochondria have long been known to play a critical role in the pathogenesis of cerebral ischemia/reperfusion injury via ROS generation, mitochondrial failure or dysfunction, and mitochondrial-mediated apoptosis [14, 35]. Impaired function of respiratory chain complexes and ATP synthase after ischemia are potential major causes of enhanced mitochondrial ROS production [72, 78]. The excess production of ROS during and after ischemia causes mitochondrial dysfunction as well as induces the release of cytochrome c from mitochondria through the opening of the mitochondrial permeability transition pore (MPTP), which in turn initiates the apoptotic cascade [9, 48, 59, 79, 108]. Impaired mitochondrial energy metabolism is the most immediate result of mitochondrial dysfunction in cerebral ischemia [11, 107].

The present study tested the hypothesis that exposure to RH in diabetic conditions exacerbates cerebral ischemia-induced CA1 hippocampal neuronal death via aggravated post-ischemic mitochondrial dysfunction.

Experimental Procedures

Animals

All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by an institutional animal care and use committee. Efforts were made to minimize the number of animals used and their suffering.

Induction of Diabetes Male Wistar rats were made diabetic by intraperitoneal injection (i.p.) of the β cell toxin streptozotocin (STZ; (58-mg/kg body weight; Sigma-Aldrich, St. Louis, MO) [24]. After STZ injection, blood glucose levels were measured (using portable glucose meter: Freestyle Freedom, Abbott Diabetes Care Inc., Alameda, CA, USA; detection range 20–550 mg/dL) in non-fasting rats by tail pricking twice a week. Blood glucose values of > 550 mg/dL were assigned a value of 550 mg/dL for statistical analysis.

Insulin Treatment of Diabetic Rats Two to three weeks after the induction of diabetes, insulin pellets (LinShin Canada, Inc., Ontario, Canada) were implanted subcutaneously (s.c.) [24]. Blood glucose levels were monitored in the following week and if the level was > 220 mg/dL, an additional insulin pellet (or part of the pellet) was implanted to keep the glucose level in the target range (≤ 220 mg/dL). This group of animals was considered ITD rats.

Rats belonging to the ITD group were randomized into three groups: (1) ITD ($n = 19$), (2) ITD + RH ($n = 29$), and (3) ITD + RH + glucose ($n = 26$). Rats belonging to ITD + RH or ITD + RH + glucose groups were exposed to a total of five episodes of RH (hyperinsulinemic hypoglycemia) or RH + glucose (hyperinsulinemic euglycemia) over 5 consecutive days (1 episode/day), respectively.

Exposure of ITD Rats to RH RH was induced 2–3 weeks after insulin pellet implantation, with the aim of maintaining blood glucose levels in the moderate hypoglycemia range for 3 h, by s.c. injection of Novolog (insulin aspart, Novo Nordisk, AIS, Denmark) [24]. Duration, frequency, and total number of hypoglycemia episodes were decided based on earlier studies [45, 68, 70, 103]. Glucose levels were measured at baseline and every hour during hypoglycemia. Additional insulin or 50% dextrose solution was administered by s.c. injection if the target glucose level was not achieved within the first hour of insulin injection. After 3 h of hypoglycemia, the rats were

infused s.c. with 50% dextrose solution to raise the blood glucose to pre-hypoglycemia levels. Blood glucose levels were also measured 30-min post-dextrose injection (recovery) confirming termination of the hypoglycemia episode.

Animals belonging to the ITD + RH + glucose group were treated with insulin similar to the ITD + RH group. However, their glucose levels were maintained close to baseline by s.c. injection of 50% dextrose. Similar to the ITD + RH group, their blood glucose levels were measured at baseline, at every hour for 3 h following insulin + glucose injection, and at 30 min post-recovery.

Rats belonging to four experimental groups were included in the study: (1) Naïve ($n = 17$), (2) ITD ($n = 19$), (3) RH ($n = 29$), and (4) RH + glucose ($n = 26$). A schematic diagram detailing the experimental groups is presented in Fig. 1.

Induction of Ischemia

After the last exposure to hypoglycemia, on the next day, the rats were subjected to sham or global cerebral ischemia surgery (Fig. 1) [23]. Rats were first anesthetized with 4% isoflurane in a mixture of 70% nitrous oxide and 30% oxygen. Both femoral arteries were cannulated for blood pressure measurements, controlled hemorrhage, and for arterial sampling of blood gases and glucose. The femoral vein was cannulated for rocuronium infusion. Temperature probes were inserted into the rectum and the left temporalis muscle, and separate thermostat-regulated heating lamps were used to maintain rectal and temporalis muscle temperature between 36.5 and 37.5 °C throughout the experiment. The rats were endotracheally intubated and mechanically ventilated during the surgery procedure. During ventilation, the rats were immobilized with rocuronium (10 mg/kg). Blood glucose levels were measured before the induction of ischemia. Arterial blood gases (ABL80, Radiometer, Westlake, OH) were measured intermittently throughout the experiment. The goal was to maintain blood gases in the physiological range. Both common carotid arteries were exposed by a midline ventral incision and were gently dissected free of surrounding nerve fibers. Ligatures of polyethylene (PE-10) tubing, contained within double-lumen silastic tubing, were passed around each carotid artery. To induce cerebral ischemia, blood was gradually withdrawn from the femoral artery into a heparinized syringe to reduce systemic blood pressure to 50 mmHg, then the carotid ligatures were tightened bilaterally. Loosening the carotid ligatures and slowly reinfusing the shed blood into the femoral artery terminated the ischemic insult. The carotid arteries were inspected to verify that recirculation was reestablished. The duration of ischemia was 8 min. Arterial and venous catheters were then removed with
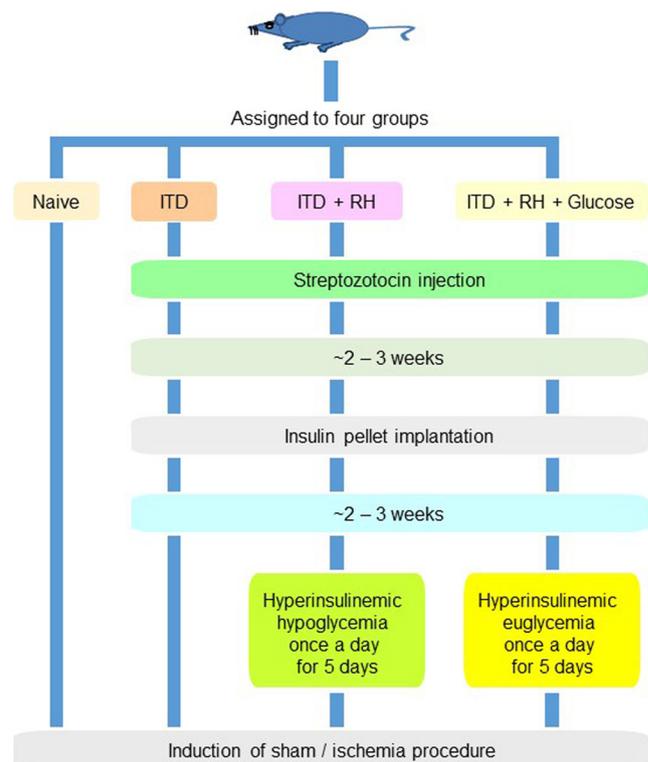

Fig. 1 Schematic diagram of experimental groups

appropriate caution. After 45–60 min, the animals were transferred to an individual cage. Sham animals were treated the same except for induction of cerebral ischemia.

Behavior Assessments

All rats used for behavior assessment were handled for 3 days (15 min per session, last session on the day before surgical procedure) prior to sham ischemia surgery. Rats were subjected to behavioral assessment starting on day 4 post-surgery. The details of the behavioral test schedule appear in Fig. 2. Behavioral assessments included the Barnes circular platform maze and the open field test to evaluate spatial learning and memory, and locomotor activity, respectively [17].

Barnes Circular Platform Maze Test Rats from each experimental group were subjected to the Barnes maze test on day 4 post-ischemia for a total of eight trials, one trial per day up to day 11 post-ischemia. The Barnes maze test was performed as described earlier [17]. In brief, the apparatus consisted of a circular platform with 18 escape holes located along the circumference. Only one of the escape holes was connected to the escape box. To start the test, rats were placed in the center of the platform and covered with a black box for 15 s. Rats were given 4 min to find the escape box following removal of the black box. Bright lights (noxious stimuli) were used to increase the incentive in finding the escape box. For each trial, the Barnes maze platform and escape box were thoroughly

Fig. 2 Behavioral test schedule
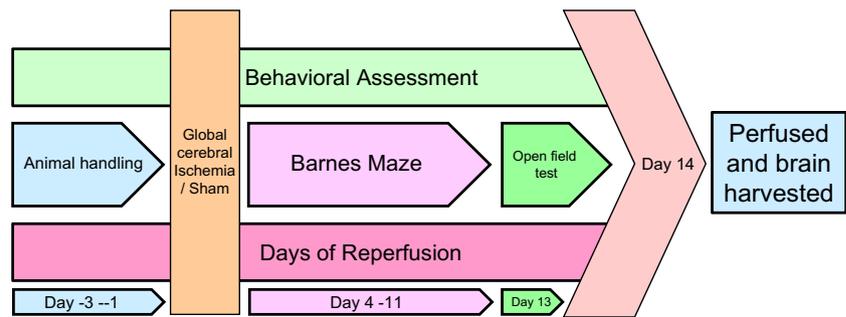
cleaned with 70% ethanol solution and dried with paper towels. Each day, the maze was rotated 90° in order to disperse residual odorant cues on the maze. All trials were recorded using an EQ 610 Polestar II Everfocus camera. The distance traveled until the rat first located the escape tunnel (known as a primary measurement, a potentially more accurate measure of learning [42] and the latency to the rat located the escape box (primary measure) were quantified using Ethovision 8.5 video tracking software (Noldus, Leesburg, VA, USA). The primary search strategy (random or systematic) used by each rat was also evaluated (details are in [17]).

Open Field Test The rats from each experimental group were subjected to an open field test on day 13 post-ischemia using an open field chamber (17″ L × 17″ W × 12″ H; Med Associates Inc., St. Albans, VT) [87]. To start the trial, rats were placed into the center of the open field and allowed to explore the apparatus for 30 min. For each trial, the open field box was thoroughly cleaned with 70% ethanol solution and afterward by dry paper towels. The analysis included the distance traveled by the rat over the arena in 30 min.

Histological Studies

The number of normal neurons was counted as described earlier [22]. In brief, on day 14 post-ischemia/sham surgery, rats were perfused using FAM (a mixture of 40% formaldehyde, glacial acetic acid, and methanol, 1:1:8 by volume) under isoflurane anesthesia for histological analysis. Coronal sections were made from paraffin-embedded brains and stained with hematoxylin and eosin. In sections containing hippocampus at the level of − 3.6, − 3.8, and − 4.0 mm from bregma, the number of surviving normal neurons within the entire CA1 region of hippocampus was counted using 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). The counts at the three bregma levels were averaged.

Isolation of Mitochondria and Determination of Mitochondrial Function

Mitochondria were isolated 24 h after sham/ischemia surgery. In brief, rats were decapitated under isoflurane anesthesia. The hippocampus was removed immediately and mitochondria were isolated as described earlier [24, 60]. The entire procedure was completed within 1 to 1.5 h. All procedures were carried out at 4 °C or on ice. Polarographic measurement of substrate oxidation rates and spectrophotometric measurements of the specific activities of complexes I–IV were performed as described earlier [23, 24].

Statistical Analysis

All statistical analyses were done using either GraphPad prism software version 5 or SAS version 9.4 (SAS Institute Inc., Cary, NC). Multiple comparison analysis among the groups for open field test, mitochondrial respiration rate, and mitochondrial respiratory chain complex activity were done by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Rats that did not respond on the Barnes maze, animals with abnormal ischemic injury (absence, unilateral, or severe), and significant outlier data points as identified by the Grubbs' test were excluded from analysis. Linear mixed-effect models for repeated measurements were used to assess the effects of treatment on spatial learning and memory with repeated Barnes maze tests over time. The chi-square test was used to compare the search strategy results among the groups. All data are expressed as mean ± SEM. A p value of less than 0.05 was considered statistically significant.

Results

Blood Glucose Levels

To confirm the induction of diabetes and control of blood glucose levels in ITD groups, the blood glucose levels were measured for rats belonging to each group before (after STZ injection) and after insulin pellet implantation (at the time of

ischemia/sham surgery). No significant differences in blood glucose level (Fig. S1) were observed in all six diabetic groups (i.e., ITD + sham: $n = 8$, ITD + ischemia: $n = 11$, ITD + RH + sham: $n = 14$, ITD + RH + ischemia: $n = 15$, ITD + RH + glucose + sham: $n = 12$, and ITD + RH + glucose + ischemia: $n = 14$).

To confirm hypoglycemia in rats, the glucose levels were measured at baseline (before insulin injection), at 1, 2, and 3 h post-insulin injection, and after half an hour of dextrose infusion (recovery) in rats belonging to the ITD + RH + sham ($n = 14$), ITD + RH + ischemia ($n = 15$), ITD + RH + glucose + sham ($n = 12$), and ITD + RH + glucose + ischemia ($n = 14$) groups (Fig. S2). Our results confirm that we were able to maintain glucose levels in the moderate hypoglycemia range in both ITD + RH groups (sham and ischemia). Our results also indicated that we were able to maintain glucose levels close to euglycemia in the ITD + RH + glucose groups (sham and ischemia).

Exposure to RH in ITD Rats Exacerbates Post-Ischemic Neuronal Damage in the CA1 Hippocampus

The goal of this experiment was to confirm our earlier results which demonstrated that prior exposure to RH exacerbates cerebral ischemic damage in ITD rats. The groups included were (1) naïve + sham ($n = 9$), (2) naïve + ischemia ($n = 8$), (3) ITD + sham ($n = 8$), (4) ITD + ischemia ($n = 5$), (5) ITD + RH + sham ($n = 14$), (6) ITD + RH + ischemia ($n = 8$), (7) ITD + RH + glucose + sham ($n = 12$), and (8) ITD + RH + glucose + ischemia ($n = 7$). The number of normal neurons in the CA1 region of hippocampus was evaluated for each experimental group on day 14 post-ischemia. Neither RH nor RH + glucose treatment resulted in any cell death as the number of normal neurons in sham-operated rats belonging to all experimental groups was not different. Due to this reason, all sham groups were pooled into one group, and further comparisons are made against this common sham group. We found a significant decrease in normal surviving neurons in the naïve (31%, $p < 0.001$), ITD (39%, $p < 0.001$), ITD + RH (61%, $p < 0.001$), and ITD + RH + glucose (35%, $p < 0.001$) groups after ischemia when compared with the sham group (Fig. 3). When further analysis was done between ischemic groups, we found a significant reduction in the number of normal neurons in the ITD + RH + ischemia group compared to three ischemia groups; i.e., naïve (44%, $p < 0.001$), ITD (37%, $p < 0.05$), and ITD + RH + glucose (40%, $p < 0.01$) (Fig. 3). These results confirm our earlier findings that the reduction in normal surviving neurons was more severe in the ITD + RH + ischemia group compared to the naïve, ITD, and ITD + RH + glucose ischemic groups. Thus, our results demonstrate that RH in a rat model of ITD exacerbates cerebral ischemic damage.
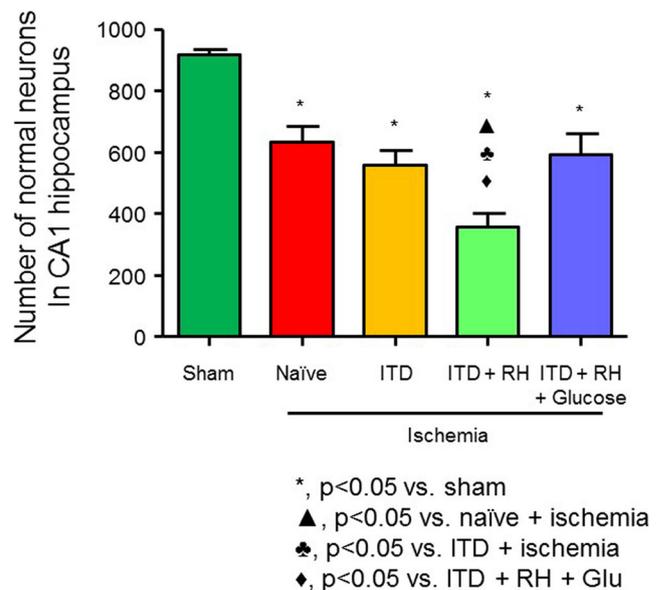

Fig. 3 Normal neurons were counted post-ischemia/sham surgery in rats belonging to (1) sham ($n = 41$), (2) naïve + ischemia ($n = 8$), (3) ITD + ischemia ($n = 5$), (4) ITD + RH + ischemia ($n = 7$), and (5) ITD + RH + glucose + ischemia ($n = 8$). The results are presented as mean ± SEM. Asterisk, $p < 0.05$ vs sham; triangle, $p < 0.05$ vs naïve + ischemia; club, $p < 0.05$ vs ITD + ischemia; diamond, $p < 0.05$ vs ITD + RH + glucose

Prior RH Exposure Induces No Significant Changes in Locomotor Activity in ITD Animals Subjected to Cerebral Ischemia

We observed a significant reduction in number of normal neurons in RH-exposed diabetic rats subjected to cerebral ischemia. Before determining the effect of RH on cognitive outcomes, first we confirmed absence of any motor deficits in our experimental groups specifically in RH treated group as motor deficits may affect results of cognitive test used in our study. We measured distance traveled by animals during the test period of 30 min in an open field chamber. Groups used for this experiment were same as the one used for histology study (sham: $n = 41$, naïve + ischemia: $n = 8$, ITD + ischemia: $n = 5$, ITD + RH + ischemia: $n = 7$, ITD + RH + glucose + ischemia: $n = 8$). The mean distance traveled by animals belonging to the groups subjected to cerebral ischemia was not significantly different from that of their respective sham groups (Fig. 4). Also, no significant difference in mean distance traveled was noticed among all experimental groups (Fig. 4). Overall, our results suggest that prior exposure of ITD rats to RH does not have any effect on locomotor activity in either control (sham) or ischemia groups.

RH Exposure Alone Impairs Spatial Learning and Memory in ITD Rats

To determine the effect of RH exposure to ITD rats on spatial learning and memory, we evaluated rats belonging to naïve +
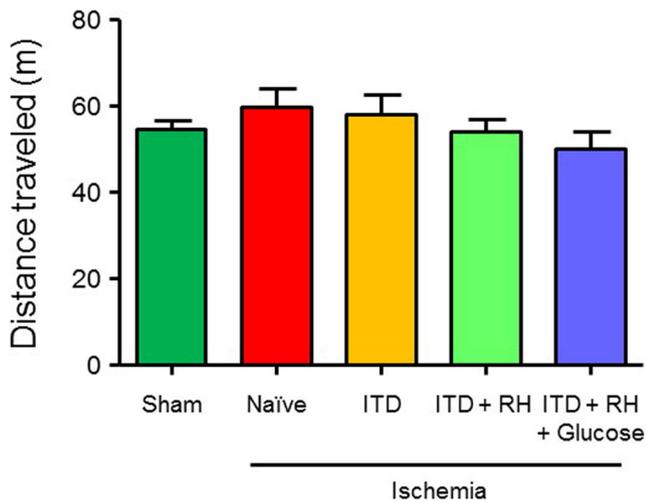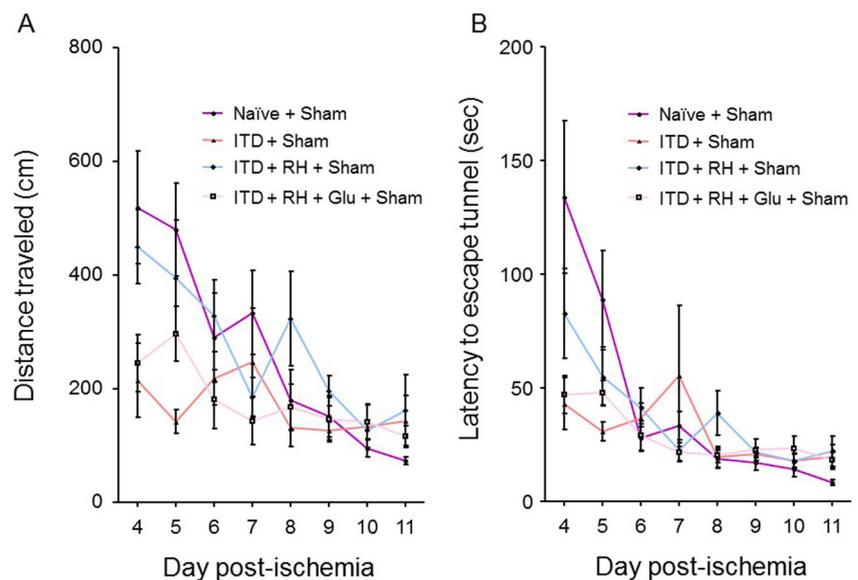
Fig. 4 The effect of RH exposure on distance traveled during an open field test in ITD rats subjected to cerebral ischemia. Distance traveled by rats belonging to (1) sham ($n = 41$), (2) naïve + ischemia ($n = 8$), (3) ITD + ischemia ($n = 5$), (4) ITD + RH + ischemia ($n = 7$), and (5) ITD + RH + glucose + ischemia ($n = 8$). The results are presented as mean ± SEM

sham ($n = 9$), ITD + sham ($n = 7$), ITD + RH + sham ($n = 14$), and ITD + RH + glucose + sham ($n = 12$) using the Barnes maze. The average distance traveled (cm), latency (sec), and strategy (random or systematic) used to locate the escape box over the 8-day period of trial were measured in animals belonging to each experimental group (same as histology study). We observed significant group (difference among groups, $p < 0.005$), day (groups improved over time, $p < 0.001$) and day × group (treatment by time interaction, $p < 0.05$) effects among the four experimental groups for total distance traveled (Fig. 5a). The rate of learning (calculated from distance traveled) in the ITD + RH + sham group was significantly lower when compared to other two

diabetic groups (ITD $p < 0.005$ and ITD + RH + glucose + sham $p < 0.005$) (Fig. 5a). However, no significant difference was observed in the rate of learning (distance traveled) between ITD + RH + sham and naïve + sham groups (Fig. 5a). Also, no significant differences were observed for latency between ITD + RH + sham and three control groups (Fig. 5b). ITD + RH + sham animals used random verses systematic strategy more frequently (45% of the time) compared to the ITD + sham (25% of the time, $p < 0.05$) and ITD + RH + glucose + sham (28% of the time, $p < 0.05$) groups (two diabetic groups) (Fig. 6). However, no significant difference was observed in the frequency of random strategy use between ITD + RH + sham (45% of the time) and naïve + sham (40% of the time) groups (Fig. 6). Our results indicate that RH exposure to ITD leads to impaired spatial learning and memory.

RH Exposure Does Not Affect Spatial Learning and Memory in ITD Rats Subjected to Cerebral Ischemia

To examine whether increased CA1 hippocampal neuronal injury observed in RH-exposed ITD rats following cerebral ischemia correlated with spatial learning and memory deficit, we evaluated rats belonging to four experimental groups (naïve, ITD, ITD + RH, ITD + RH + glucose) with sham or ischemia surgeries using Barnes maze testing (naïve + sham: $n = 9$, naïve + ischemia: $n = 8$, ITD + sham: $n = 7$, ITD + ischemia: $n = 5$, ITD + RH + sham: $n = 14$, ITD + RH + ischemia: $n = 8$, ITD + RH + glucose + sham: $n = 12$, ITD + RH + glucose + ischemia: $n = 7$). We observed day (groups improved over time) effect ($p < 0.01$ for distance and $p < 0.005$ for latency) but did not observe group and day × group effects among the four ischemia groups (naïve, ITD, ITD + RH, and ITD + RH + glucose) for these two parameters

Fig. 5 The groups include (1) naïve + sham ($n = 9$), (2) ITD + sham ($n = 7$), (3) ITD + RH + sham ($n = 14$), and (4) ITD + RH + glucose + sham ($n = 12$). The results are presented as mean ± SEM. A significant group (difference among groups, $p < 0.005$), day (groups improved over time, $p < 0.001$), and day × group (treatment by time interaction, $p < 0.05$) effects among the four experimental groups for total distance traveled was observed. The rate of learning (calculated from distance traveled) in the ITD + RH + sham group was significantly lower when compared to the two other diabetic groups (ITD $p < 0.005$ and ITD + RH + glucose + sham $p < 0.005$)
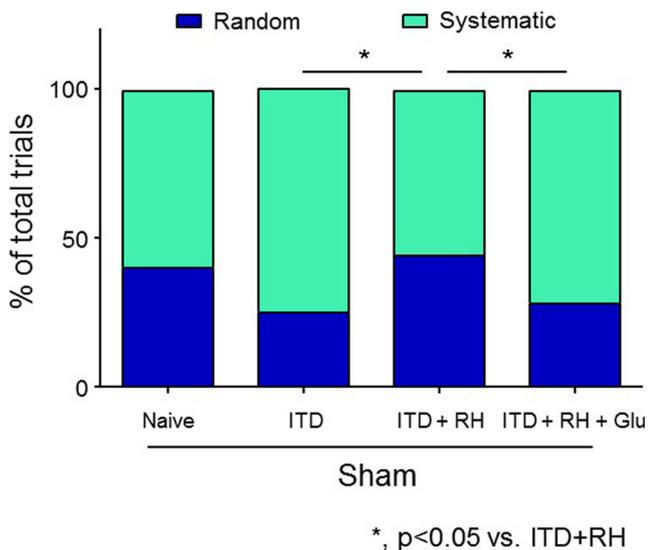
Fig. 6 The search strategy used during the Barnes maze test. The groups include (1) naïve + sham ($n = 9$), (2) ITD + sham ($n = 7$), (3) ITD + RH + sham ($n = 14$), (4) ITD + RH + glucose + sham ($n = 12$). The results are presented as mean ± SEM. Post hoc analysis revealed that the percentage use of random search strategy was significantly more by ITD + RH + sham rats compared to ITD + sham (chi-square, $p < 0.05$) and ITD + RH + glucose + sham rats (chi-square, $p < 0.05$)

(Fig. S3). The rate of learning (distance traveled and latency) in ITD + RH + ischemia group was not significantly different from naïve, ITD, and ITD + RH + glucose animals subjected to ischemia. The rates of learning (distance traveled) in ITD + ischemia ($p < 0.05$) and ITD + RH + glucose + ischemia ($p < 0.05$) groups were significantly impaired when compared to the respective sham groups (Fig. S3B and D). We also analyzed the strategy used to find the escape box during the Barnes maze test (Fig. S4). We did not observe any significant difference in frequency of random strategy use among the four ischemia groups. When we compared ischemia groups with their respective sham groups, we observed that ITD + ischemia animals used random strategy

(48% of the time) more frequently compared to ITD + sham animals (25% of the time, $p < 0.05$) (Fig. S4). However, we did not observe any differences in use of random strategy among the other three sham groups when compared with their respective ischemia groups. These results indicate that prior RH exposure has no significant additive effect on spatial learning and memory in ITD rats subjected to cerebral ischemia.

Measurement of Mitochondrial Respiration Rate in RH-Exposed ITD Rats Subjected to Cerebral Ischemia

Hypoglycemia leads to an increase in mitochondrial ROS production and decrease in MMP [24, 56, 66]. This pre-existing mitochondrial dysfunction may further exacerbate cerebral ischemia-induced mitochondrial dysfunction. Thus, we next determined hippocampal mitochondrial function in our experimental conditions at 24-h post sham/ischemia surgery. The oxygen consumption rate was measured in the presence of different pairs of substrates: pyruvate and malate, succinate and glycerol-3-phosphate, and ascorbate and N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD). We observed that the rate of oxygen consumption in the presence of pyruvate and malate was lower in the ITD + RH ($n = 7$) ischemic group by 25 ($p < 0.05$), 46 ($p < 0.001$), and 33% ($p < 0.001$) compared to the naïve ($n = 6$), ITD ($n = 6$), and ITD + RH + glucose ($n = 7$) ischemic groups, respectively (Fig. 7a). No statistically significant differences were observed between ITD + RH and the other three control groups when oxygen consumption was measured in the presence of succinate + glycerol-3-phosphate (Fig. 7b) and ascorbate + TMPD (Fig. 7c) as substrates. Our results demonstrate that prior exposure to RH in ITD rats following cerebral ischemia leads to decreased rate of oxidation in presence of pyruvate and malate as substrates.
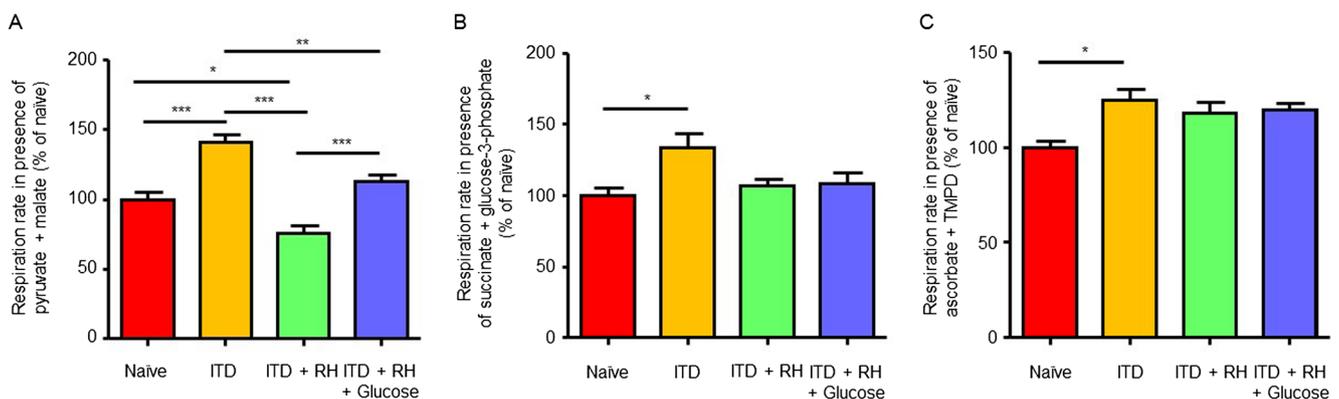

Fig. 7 Mitochondrial respiration rates in RH-exposed ITD rats subjected to cerebral ischemia. Substrate oxidation rates in isolated mitochondria from hippocampus of (1) naïve + ischemia ($n = 6$), (2) ITD + ischemia ($n = 6$), (3) ITD + RH + ischemia ($n = 7$), and (4) ITD + RH + glucose + ischemia ($n = 7$) were measured in the presence of different substrates: **a**

pyruvate + malate, **b** succinate + glycerol-3-phosphate, and **c** ascorbate + tetramethyl-p-phenylenediamine (TMPD). The results are presented as mean ± SEM. *, $p < 0.05$ ITD + RH + ischemia vs naïve; ***, $p < 0.001$ ITD + RH + ischemia vs ITD and ITD + RH + ischemia vs ITD + RH + glucose + ischemia

Measurement of Mitochondrial Respiratory Chain Complex Activity in RH-Exposed ITD Rats Subjected to Cerebral Ischemia

Because we observed a decreased rate of respiration in presence of pyruvate and malate as substrates, next we measured activities of complexes I, II, III, and IV of the mitochondrial electron transport chain (ETC). Similar to the decreased rate of substrate oxidation in ITD + RH + ischemia group, complex I activity was also lower in the ITD + RH + ischemia group ($n = 7$) by 36 ($p < 0.001$), 38 ($p < 0.001$), and 33% ($p < 0.01$) compared to the naïve ($n = 6$), ITD ($n = 6$), and ITD + RH + glucose ($n = 7$) ischemia groups, respectively (Fig. 8a). No significant difference in complex II, III, and IV activity was observed in the ITD + RH + ischemia group when compared to the naïve, ITD, and ITD + RH + glucose ischemia groups, respectively (Fig. 8b–d). Our results demonstrate that prior exposure to RH in ITD rats results in pronounced impairment in mitochondrial complex I activity following cerebral ischemia.

Discussion

Stroke is one of the leading causes of death and physical disability worldwide and diabetes is a recognized risk factor for ischemic stroke [58]. Diabetes increases the risk of stroke and also enhances brain damage caused by cerebral ischemia [54, 55, 63, 101]. Insulin-induced hypoglycemia is the most frequent side effect of insulin-therapy in diabetes [20, 65].

Although studies demonstrate that diabetics frequently experience mild/moderate hypoglycemia, the effect of mild/moderate hypoglycemia on brain is not well explored. In the present, as well as in an earlier study, we demonstrated that prior exposure of ITD rats to RH increases cerebral ischemic damage. We also observed that the rate of learning in RH-exposed ITD rats was significantly lower when compared to other two diabetic groups and pronounced ischemia-induced impairment in mitochondrial complex I activity was observed in ITD rats exposed to RH.

It is evident that oxidative stress and mitochondrial dysfunction play an important role in tissue damage associated with cerebral ischemia. Mitochondria undergo morphological and functional changes which contribute to oxidative stress and cell death following cerebral ischemia [3, 11, 74, 96, 107]. Increased ROS production disrupts antioxidant defense and directly impairs mitochondrial homeostasis and energy production. Deficits of mitochondrial energy metabolism are the most immediate cause of mitochondrial damage and dysfunction in cerebral ischemia [11, 107]. Mitochondrial dysfunction is mainly manifested as decreased mitochondrial ATP synthesis, increased ROS production, dysregulation of intracellular lipid homeostasis, loss of the MMP, and induction of the MPTP [34, 49, 86]. Further, mitochondrial oxidative stress prevents metabolic recovery and promotes apoptosis [64, 75]. Hence, the sequence of post-ischemic events that damage mitochondria and lead to mitochondrial dysfunction, in turn, participate in cerebral ischemic damage. Thus, we tested the hypothesis that increased extent of

Fig. 8 Mitochondrial complex (I, II, III, and IV) activities in RH-exposed ITD rats subjected to cerebral ischemia. **a** Complex I, **b** complex II, **c** complex III, and **d** complex IV activities were measured in mitochondria isolated from hippocampus of (1) naïve + ischemia ($n = 6$), (2) ITD + ischemia ($n = 6$), (3) ITD + RH + ischemia ($n = 7$), and (4) ITD + RH + glucose + ischemia ($n = 7$). The results are presented as mean ± SEM. **, $p < 0.01$ ITD + RH + ischemia vs ITD + RH + glucose + ischemia; ***, $p < 0.001$ ITD + RH + ischemia vs naïve + ischemia and ITD + ischemia
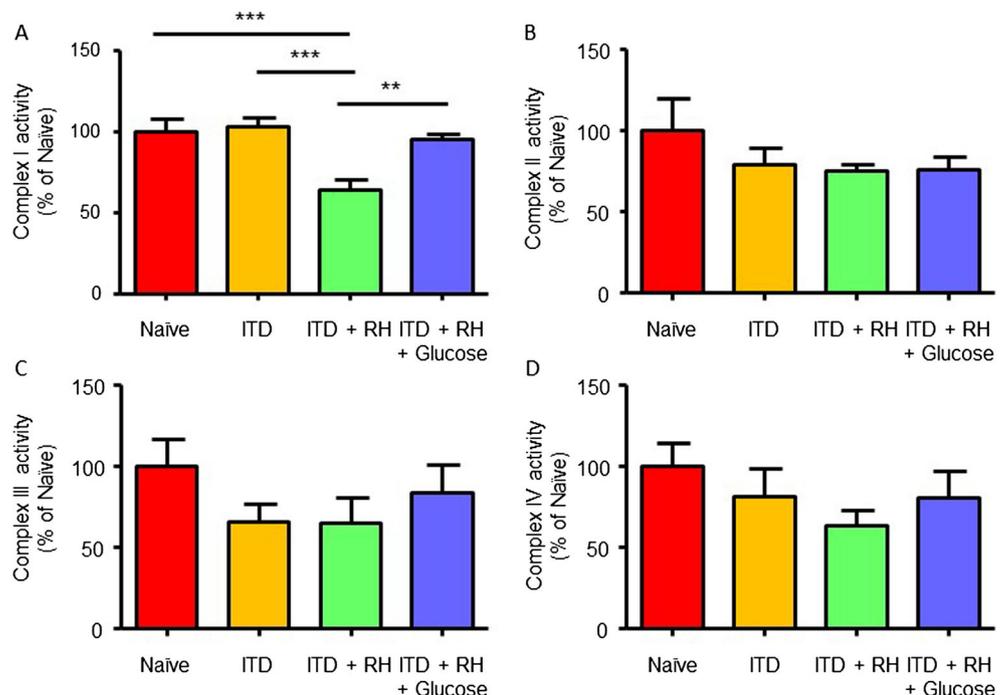
mitochondrial dysfunction plays a role in exacerbating cerebral ischemic damage in RH-exposed ITD rats.

In the present study, we observed that prior exposure of ITD rats to RH leads to more robust decrease in the rate of oxidation in the presence of pyruvate and malate as substrate. We also observed lower complex I activity in the ITD + RH group compared to the naïve, ITD, and ITD + RH + glucose groups. Mitochondrial complex I is the first and largest protein complex in the mitochondrial ETC, which has an essential role in maintaining mitochondrial function and integrity [38]. Prior studies observed that tumor necrosis factor-α (TNF-α) adversely affects mitochondria. Using an in vitro model, Higuchi et al. demonstrated that mitochondrial ETC complex I is inhibited following TNF-α treatment during the early phase leading to apoptosis via the mitochondrial apoptotic pathway [46]. Recently, Doll et al. observed rapid and profound mitochondrial dysfunction when HT22 hippocampal cells and mouse primary cortical neurons were exposed to TNF-α for a short duration (90 min) [30]. TNF-α induces significant mitochondrial dysfunction and activation of mitochondrial apoptotic responses [4, 30]. The increased level of TNF-α is associated with worsened clinical outcomes after stroke and exacerbation of infarct size in pre-clinical models [77, 81]. Moreover, intracerebroventricular administration of exogenous TNF-α significantly expands infarct volume [5]. In an earlier study, we observed a robust increase in TNF-α level in RH- exposed ITD rats compared to naïve and ITD rats at 24 h post-cerebral ischemia [95]. It is possible that increased post-ischemic TNF-α levels in hippocampus of RH-exposed ITD rats may be responsible for observed lower complex I activity.

Studies have reported that mitochondria are the major source of cellular ROS involved in TNF-α-induced cell death/apoptosis [18, 39, 40, 92, 94]. Treatment of cells with TNF-α alters mitochondrial membrane permeability, inhibits respiratory chain complex I, induces mitochondrial swelling and clustering, and leads to cytochrome c release. Release of cytochrome c activates caspases leading to cell death [39, 46, 82, 89, 92]. Mitochondrial respiratory chain complex I inhibitor (i.e., rotenone)-induced apoptosis has been widely studied [6, 15, 52]. Complex I inhibition results in enhanced ROS production and depletion of ATP in cells, membrane depolarization, and mitochondrial permeability transition pore opening, all of which contribute to apoptosis [6]. Free radical overload results in damage of protein, DNA, and membrane phospholipids [41]. Our results indicate that severe inhibition of complex I activity may be, in part, responsible for exacerbating cerebral ischemic damage in ITD rats exposed to RH. However, our results warrant confirmatory studies to establish the role of complex I inhibition on increased cerebral ischemic damage in our experimental conditions.

Mitochondrial respiratory chain complex I catalyzes NADH oxidation and regenerates NAD^+. The role of oxidative stress and altered NAD^+ metabolism has been established in ischemic brain injury. We observed that the complex I activity was significantly reduced in ITD + RH rats following cerebral ischemia. It has been found that ischemia/reperfusion can induce significant decreases in NAD^+ levels in the brain [33], and NAD^+ administration can significantly decrease ischemic brain injury in an animal model of cerebral ischemia [106]. It is plausible that the reduced ability of complex I to oxidize NADH to NAD^+ in the ITD + RH group contributed to the exacerbation of cerebral ischemic damage.

We further investigated whether RH exposure, by itself or with cerebral ischemia, has any effect on memory and cognitive impairment. We observed that RH exposure in ITD rats (sham surgery group) leads to significant spatial learning and memory impairment compared to other two sham-operated diabetic groups (ITD and ITD + RH + glucose). Our findings are corroborated by an earlier study where diabetic rats exposed to recurrent moderate hypoglycemia showed significant cognitive impairment compared to non-diabetic rats [103]. Similarly, recently McNeilly and colleagues also reported that exposure of streptozotocin-diabetic mice to moderate RH results in impaired cognitive function when assessed using novel object recognition and spontaneous alteration tests [71]. Considering very few studies investigated the effect of brain with respect to its effect on cognition, the mechanism behind is not well understood. Recurrent moderate hypoglycemia is observed to impair hippocampal synaptic plasticity, suggesting its potential link to observed memory and cognitive deficits as hippocampal long-term potentiation is involved in memory and learning [104]. Uncontrolled blood glucose levels (hyper- or hypoglycemia) also affect brain structure and function, potentiating cognitive impairment [12]. Severe hypoglycemia induces hippocampal neuronal death and impairs learning and memory in rodents [12] and in patients with diabetes [44, 90]. Recurrent moderate hypoglycemia causes oxidative injury in hippocampal dendrites [103]. Repetitive episodes of moderate hypoglycemia induce synaptic injury in the hippocampus, and consequently may contribute to the development of cognitive impairment [16, 103]. An earlier study observed that exposure of diabetic rats to RH potentiated an increase in lipid peroxidation and a decrease in aconitase activity (both markers of oxidative damage) in hippocampal mitochondria [12]. Conversely, RH improved cognitive ability and preserved basic brain functions in diabetic and non-diabetic rats tested in a euglycemic state [69, 70]. However, under hypoglycemia, the animals performed worse compared to control animals. Severe hypoglycemia is observed to induce brain damage and related deficits in spatial learning and memory [88]. Our results

thus confirm previous reports that prior exposure to hypoglycemia results in impaired cognitive function.

Post-stroke cognitive decline is considered a major contributor to post-stroke disability [10, 61]. An earlier prospective clinical study observed that stroke survivors not only suffer from acute cognitive deficits but also experience accelerated and persistent cognitive decline [61]. Similarly, cognitive impairments are also observed in survivors of cardiac arrest [73, 85]. Post-stroke cognitive impairments are also evident in animal models of cerebral ischemia [43, 47, 57, 80]. However, we did not observe an effect of cerebral ischemia on cognitive function evaluated by means of a Barnes circular platform maze. It should be noted that since we anticipated increased ischemic damage in the RH-exposed group, we chose a relatively short duration of global cerebral ischemia (8 min). It is possible that we did not observe a sufficient impact of cerebral ischemia on cognitive function from such a mild ischemic insult. An earlier study that used 30 min (relatively short ischemia duration) of transient middle cerebral artery occlusion in mice also did not observe any impact on cognitive function when that was evaluated using a Morris water maze [29, 30]. We also did not observe an adverse effect of ischemia on cognitive function in RH-exposed rats compared to sham controls. It is plausible that the effect of ischemia on cognitive function in RH-exposed rats is masked by the effect of RH itself on cognitive function. Further studies using relatively sensitive test to evaluate cognitive deficits may help evaluate post-ischemia cognitive deficits in our experimental conditions.

Inclusion of only male rats is a limitation of our studies. Earlier studies established that response to hypoglycemia is different in normal and diabetic women compared to the respective male population. Hormonal changes in response to hypoglycemia (epinephrine, norepinephrine, and growth hormone) and endogenous glucose production response are lower in T1D women than in T1D men and in healthy womencompared with healthy men [26]. Another study demonstrated that women are less susceptible to the blunting of neuroendocrine counterregulatory responses following repeated exposure to hypoglycemia, compared to men [27]. Differential hormonal responses to hypoglycemia in men and women diabetic subjects warrant studies to determine the effect of prior hypoglycemia exposure on ischemic outcomes in female diabetic animals.

Overall, our findings suggest that prior RH exposure in ITD rats increases ischemic damage potentially via inhibition of mitochondrial complex I. The mechanism by which RH exposure inhibits post-ischemic mitochondrial complex I inhibition remains to be defined. Understanding the mechanism by which RH exposure increases cerebral ischemic injury may ultimately help lower cerebral ischemic damage in diabetics.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

Ethical Approval All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by an institutional animal care and use committee.

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