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ORIGINAL PAPER

Blockade of Acid-Sensing Ion Channels Attenuates Recurrent Hypoglycemia-Induced Potentiation of Ischemic Brain Damage in Treated Diabetic Rats

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Abstract

Diabetes is a chronic metabolic disease and cerebral ischemia is a serious complication of diabetes. Anti-diabetic therapy mitigates this complication but increases the risk of exposure to recurrent hypoglycemia (RH). We showed previously that RH exposure increases ischemic brain damage in insulin-treated diabetic (ITD) rats. The present study evaluated the hypothesis that increased intra-ischemic acidosis in RH-exposed ITD rats leads to pronounced post-ischemic hypoperfusion via activation of acid-sensing (proton-gated) ion channels (ASICs). Streptozotocin-diabetic rats treated with insulin were considered ITD rats. ITD rats were exposed to RH for 5 days and were randomized into Psalmotoxin1 (PcTx1, ASIC1a inhibitor), APETx2 (ASIC3 inhibitor), or vehicle groups. Transient global cerebral ischemia was induced overnight after RH. Cerebral blood flow was measured using laser Doppler flowmetry. Ischemic brain injury in hippocampus was evaluated using histopathology. Post-ischemic hypoperfusion in RH-exposed rats was of greater extent than that in control rats. Inhibition of ASICs prevented RH-induced increase in the extent of post-ischemic hypoperfusion and ischemic brain injury. Since ASIC activation-induced store-operated calcium entry (SOCE) plays a role in vascular tone, next we tested if acidosis activates SOCE via activating ASICs in vascular smooth muscle cells (VSMCs). We observed that SOCE in VSMCs at lower pH is ASIC3 dependent. The results show the role of ASIC in post-ischemic hypoperfusion and increased ischemic damage in RH-exposed ITD rats. Understanding the pathways mediating exacerbated ischemic brain injury in RH-exposed ITD rats may help lower diabetic aggravation of ischemic brain damage.

Keywords Cerebral blood flow · Psalmotoxin1 · APETx2 · Store-operated calcium entry · Acidosis · Vascular smooth muscle cells

Introduction

Cerebral ischemia is a pathological condition marked by severe reduction in cerebral blood flow, resulting in gross metabolic derangements associated with cerebral hypoxia (Hossmann 1997). Post-ischemic reperfusion results in initial reactive hyperemia (transient increase in cerebral blood flow) followed by a relatively sustained period of delayed post-ischemic hypoperfusion (Ginsberg et al. 1978; Hossmann et al. 1973; Levy et al. 1979). Pharmacological agents decrease cerebral ischemia-induced neurological deficits and hippocampal CA1 neuronal death by lowering the extent of post-ischemic hypoperfusion (Lin et al. 2010; Steen et al. 1983). Besides, an increase in the extent of ischemic brain injury is seen with a progressive increase in post-ischemic hypoperfusion caused by longer duration of ischemia (Matsumoto et al. 1990). Thus, post-ischemic hypoperfusion plays an important role in ischemic brain injury (Hossmann 1997; Hosomi et al. 2007; Hossmann and Zimmermann 1974).

Diabetes is a chronic metabolic disease affecting 425 million people worldwide (International Diabetes Federation 2018), and cerebral ischemia-induced brain injury is

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prevalent in diabetic subjects (Almdal et al. 2004; Jorgensen et al. 1994; Kissela et al. 2005; Ottenbacher et al. 2004). Mortality associated with ischemic heart disease and stroke is one of the most serious outcomes of diabetes (Mozaffarian et al. 2016; Morrish et al. 2001). Heart disease-induced mortality was higher than that produced by stroke in diabetic subjects (Centers for Disease Control and Prevention 2011). Available anti-diabetic therapy increases the risk of hypoglycemia in diabetics (Cryer 2007; van den Berghe et al. 2001, 2006; Yuan et al. 2015). Continuous blood glucose monitoring studies showed that the patients with type 1 diabetes daily experience hypoglycemia for a period of 1–1.5 h (Tamborlane et al. 2008). Anti-diabetic therapy in patients with type 2 diabetes also increases the risk of hypoglycemia (Donnelly et al. 2005; Gehlaut et al. 2015). Severe hypoglycemia exerts detrimental effects on brain energetics and also induced injury as discussed previously (Suh et al. 2007; Vannucci and Vannucci 2001; Languren et al. 2013). We have previously shown that prior exposure to recurrent hypoglycemia (RH) of moderate intensity worsens ischemic brain damage in diabetic rats (Dave et al. 2011b; Shukla et al. 2018) and oxygen/glucose deprivation-induced damage in hippocampal organotypic slices (Dave et al. 2011a). In addition, we previously observed that intra-ischemic acidosis mediates ischemic brain damage in RH-exposed insulin-treated diabetic (ITD) rats (Rehni et al. 2018). However, the mechanisms by which increased ischemic acidosis increases ischemic brain injury in RH-exposed ITD rats are unknown. Low pH during acidosis activates acid-sensing (proton-gated) ion channels (ASICs) (Waldmann et al. 1997). ASICs are expressed in the neurons and non-neuronal cells in the brain and cerebral vasculature (Alvarez de la Rosa et al. 2003; Kellenberger and Schild 2015; Lin et al. 2014; Nakamura et al. 2009; Meng et al. 2009). ASICs are proposed to mediate acidosis-induced cell death in vitro (Li et al. 2010; Sherwood et al. 2011) and ischemic brain injury in vivo (Sherwood et al. 2011; Xiong et al. 2004). Store-operated calcium entry (SOCE) into pulmonary vascular smooth muscle cells (VSMCs) via ASIC results in acute hypoxic pulmonary vasoconstriction (Jernigan et al. 2009, 2012; Nitta et al. 2014).

Therefore, we hypothesized that increased intra-ischemic acidosis in RH-exposed ITD rats leads to pronounced post-ischemic hypoperfusion by activation of ASICs. The potential role of ischemic acidosis-induced ASIC activation in mediating RH-induced increase in ischemic brain injury in treated diabetic rats is unknown. Therefore, during the present investigation, we tested this hypothesis. Considering higher mortality in diabetics due to heart disease compared to stroke, we employed a rodent model of global cerebral ischemia in the present study (Centers for Disease Control and Prevention 2011).

Materials and Methods

Animals

Experiments on animals were conducted as per the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and protocols approved by the Animal Care and Use Committee of the University of Miami.

Induction of Diabetes

Male Wistar rats (Charles River Laboratories International, Inc., Wilmington, MA) were made diabetic using a single intraperitoneal dose of streptozotocin (58 mg × kg^{-1}) (Sigma-Aldrich, St Louis, MO). Streptozotocin solution was made in citrate buffer immediately before administration. Induction of diabetes was confirmed by measuring blood glucose levels in samples obtained from tail pricking and use of a portable glucose meter between 9 a.m. and noon (FreeStyle Freedom, Abbott Diabetes Care, Inc., Alameda, CA) (Dave et al. 2011b). After induction of diabetes, blood glucose levels were monitored twice a week. The data shown in Fig. 1b for groups of diabetic animals are last readings of untreated diabetes, i.e., levels of blood glucose just prior to insulin pellet implantation. Animals having blood glucose levels > 310 mg × dl^{-1} after streptozotocin injection were considered diabetic.

Insulin Treatment

Approximately 2 to 3 weeks after the induction of diabetes, insulin pellet(s) (Linplant; LinShin, Toronto, Canada) were implanted subcutaneously to correct diabetic hyperglycemia. The same frequency of monitoring blood glucose levels was continued post-insulin pellet implantation. During this period, if the levels of blood glucose were out of the target range, the amount of implanted insulin pellet(s) was/were adjusted. These animals were considered as "insulin-treated diabetic rats" (Dave et al. 2011b). The data shown in Fig. 1b for ITD groups are the last blood glucose levels recorded at the time of cerebral ischemia surgery.

Induction of Recurrent Hypoglycemia

RH exposure was carried out after 2 to 3 weeks of insulin treatment. At the time of hypoglycemia induction, food was removed from the cage to avoid the fluctuations in blood glucose levels during the period of hypoglycemia. Hypoglycemia was induced using a supplementary injection of insulin (Novolog Insulin aspart, Novo Nordisk, A/S, Bagsvaerd,

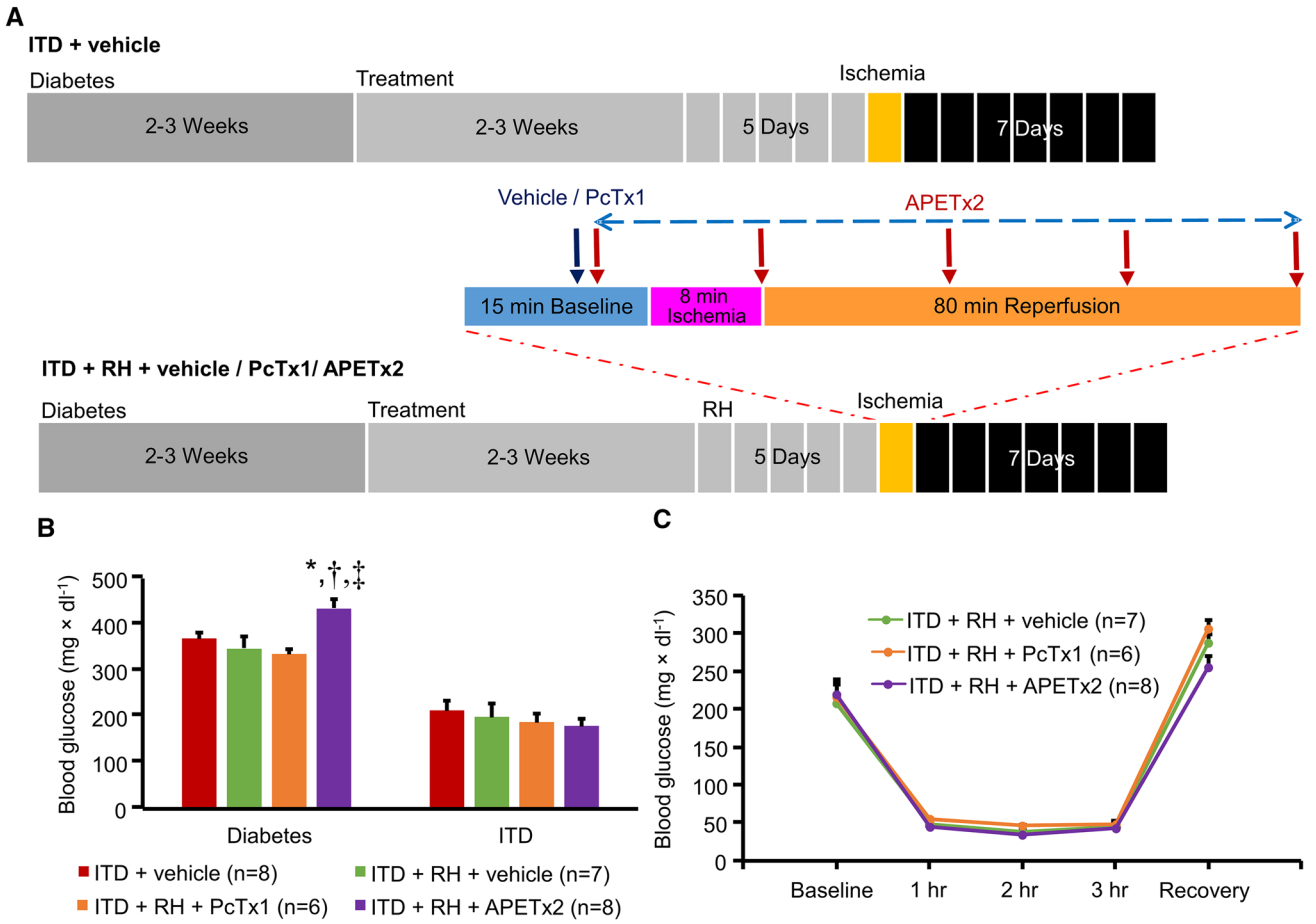

Fig. 1 **a** Synopsis of time course and experimental design of the study. All animals were subjected to cerebral ischemia and were euthanized at 7 days of reperfusion for histological analysis. **b** Blood glucose levels after diabetes induction and insulin treatment, and **c** blood glucose levels before and during induction of hypoglycemia and after recovery. ITD + vehicle, ITD + RH + vehicle, ITD + RH + PcTx1, and ITD + RH + APETx2. *$p < 0.05$ versus ITD + vehicle, $^{\dagger}p < 0.05$ versus ITD + RH + vehicle and, $^{\ddagger}p < 0.05$ ITD + RH + PcTx1

Denmark). An episode of moderate hypoglycemia was induced for a period of 3 h every day for five consecutive days. Blood glucose levels were monitored (as explained above) immediately prior to additional insulin dose and after every 1 h until the completion of intended 3-h period of hypoglycemia. A subcutaneous injection of dextrose and replacement of food was done to correct hypoglycemia. Euglycemia was ascertained by measuring blood glucose levels 30 min post-dextrose administration. Hypoglycemia was defined by the blood glucose levels below 70 mg × dl^{-1} (ADA Workgroup on Hypoglycemia 2005). The data shown in Fig. 1c for ITD + RH groups are the blood glucose values of treated diabetic rats immediately before, during, and after recovery from hypoglycemia.

Induction of Global Cerebral Ischemia

Global cerebral ischemia was induced overnight after completion of the last episode of RH. Rats were anesthetized with isoflurane in a mixture containing 33% oxygen and 67% nitrous oxide, paralyzed, and artificially ventilated. Physiological parameters (body temperature, head temperature, blood pH, partial pressure of carbon dioxide: pCO_2 in blood, partial pressure of oxygen: pO_2 in blood and mean arterial blood pressure: MABP) were monitored and maintained within normal range. An incision was made in the anterior neck region and the carotid arteries were isolated from the adjacent tissue. Ligatures (Polyethylene-10 tubing) were placed around the carotid arteries and secured with a flexible bilumen tube. Global cerebral ischemia was induced by tightening the carotid ligatures on the common carotid arteries with concurrent hypotension (blood pressure of ~ 50 mmHg was maintained by controlled outflow of blood using a syringe connected to a cannulated femoral artery). At the end of 8 min of cerebral ischemia, the carotid ligatures were loosened and removed, and the withdrawn blood was injected back into the body circulation. The arteries were physically examined to confirm reflow of blood.

The skin was sutured back, and appropriate post-operative care was provided.

Assessment of Cerebral Blood Flow

To quantify cerebral blood flow, a burr hole of 2 mm^2 area was made over the right aspect of the skull 1.5 to 3.0 mm posterior and 1.5 to 4.0 mm lateral to the bregma. Using a stereotaxic apparatus, a laser Doppler blood flow probe was mounted onto the right cortex. Cerebral blood flow was measured by laser Doppler flowmetry using a fiber-optic probe (PeriFlux System 5000, PF 5010: LDPM unit, Perimed, Järfälla, Sweden) (Della-Morte et al. 2011). Cerebral blood flow was recorded continuously at a frequency of 0.3 Hz from 30 min before the onset of cerebral ischemia to 80 min of reperfusion using PeriSoft for Windows software.

Administration of ASIC Inhibitors

For injection in the two lateral ventricles, two burr holes of 2 mm^2 area were made over the left and right aspect of the skull 0.5 mm to 1.0 mm posterior and 1.0 mm and 2.0 mm lateral to the bregma. Using a stereotaxic apparatus, the needle of the syringe containing the treatment solution was inserted into the lateral ventricles of both hemispheres (3.5 mm deep). Psalmotoxin1 (PcTx1) (ASIC1a inhibitor, 0.75 ng per ventricle over 5 min) was injected as a solution in Ringer's solution (147 mM NaCl, 4 mM KCl, and 1.3 mM $CaCl_2$) 10 to 15 min before the onset of cerebral ischemia (Pignataro et al. 2007). APETx2 (ASIC3 inhibitor, 75 ng per ventricle over 5 min—repeated every 20 min), dissolved in Ringer's solution, was injected from 10 to 15 min before the onsetof cerebral ischemia to 80 min of reperfusion. Based on the sum of total volume of the rat brain ventricles and of cerebrospinal fluid (CSF), and flow rate of CSF (Davson and Segal 1970; Levinger 1971; Pardridge 2011; Tajima et al. 1993; Davson 1969), we estimated that the i.c.v. injections of PcTx1 and APETx2 used in the study are expected to result in an approximate concentration of 3.55 nM and 366 nM in CSF, respectively. These concentrations were three to five times the IC_{50} concentration of PcTx1 and APETx2 (Diochot et al. 2004).

Histological Assessment

After 7 days of reperfusion, animals were anesthetized with isoflurane, sternotomy was performed, the apex of the left cardiac ventricle was incised, and a polyethylene catheter was inserted through the ventricle into the root of the aorta and was then ligated in place. The tip of the right atrium was incised to permit egress of the perfusate. The rats were then transcardially perfused (at a pressure of 120 mmHg) with saline until the blood was completely washed out of the body. This was followed by perfusion with a mixture of formaldehyde, glacial acetic acid, and methanol (in a ratio of 1:1:8) for 18–20 min, and brain samples were then isolated. 10-µm-thick coronal sections (200 µm apart) of processed brain samples were collected from 2.8 to 4.0 mm posterior to bregma. Hematoxylin and eosin staining was performed on the sections. Evaluation was carried out using a Nikon microscope (Nikon Microphot-SA; Nikon Corporation, Tokyo, Japan) and a computer system (MCID Elite 6.0 software; InterFocus Imaging Ltd., Cambridge, UK). Ischemic brain damage was computed in terms of the number of normal neurons on CA1 hippocampus at a magnification of ×40. Normal neurons were counted at fields in sequence along the medial to lateral part of the CA1 hippocampus on both sides on three consecutive sections containing hippocampus at the level of ~ − 3.6, ~ − 3.8, and ~ − 4.0 mm from bregma. The total count of neurons was added from the two brain hemispheres and the resulting values obtained from three sequential slides were averaged to compute the number of normal neurons in CA1 hippocampus.

Experimental Protocol

Rats were randomly assigned to various treatment groups in the following experiment (Fig. 1a):

Experiment 1 the effect of prior exposure of RH on post-ischemic hypoperfusion and ischemic brain injury in ITD rats. Groups included ITD + vehicle (control) and ITD + RH + vehicle.

Experiment 2 the effect of ASIC inhibition on RH-induced increase in post-ischemic hypoperfusion and ischemic brain injury in ITD rats. Groups included ITD + vehicle (control), ITD + RH + vehicle (control), ITD + RH + PcTx1, and ITD + RH + APETx2. Animals employed in the experiment 1 served as control animals for experiment 2.

Measurement of Store-Operated Calcium Entry (SOCE) in Vascular Smooth Muscle Cells

Freshly grown A7r5 cells (from rat aorta; CRL-1444: American Type Culture Collection, Manassas, VA) were loaded with the Ca^{2+}-sensitive fluorescent indicator Fluo-4 AM (8 µM) (Life Technologies, Carlsbad, CA) in the presence of pluronic acid (0.05%) in calcium medium (135 mM NaCl, 5.9 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 11.5 mM glucose, and 11.6 mM HEPES, pH 7.4) and incubated for 40 min at 37 °C and then for an additional 20 min at room temperature (Brueggemann et al. 2005; Jernigan et al. 2009). SOCE was measured at three pH values (6.0, 6.5, and 7.4) using Fluo-4 AM loaded cells superfused with Ca^{2+}-free medium (135 mM

NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 11.5 mM glucose, and 11.6 mM HEPES) with respective pH containing 50 µM diltiazem (Enzo Life Sciences, Farmingdale, NY) (to prevent Ca^{2+} entry through L-type voltage-gated Ca^{2+} channels), 10 µM cyclopiazonic acid (Enzo Life Sciences, Farmingdale, NY) (the sarco/endoplasmic reticulum Ca^{2+}–ATPase inhibitor, to deplete intracellular Ca^{2+} stores and prevent Ca^{2+} reuptake), and 3 mM EGTA (to chelate any residual Ca^{2+}). The changes in $[Ca^{2+}]_i$ (SOCE) were quantified in terms of change in Fluo-4 AM fluorescence (Ex: 488/Em: 520 nm) from baseline upon repletion of extracellular Ca^{2+} (1.5 mM) in the presence of diltiazem and cyclopiazonic acid over 30 min. Experiments were performed in triplicate for six observations in the presence of PcTx-1 (21.30 nM), APETx2 (300 nM), or vehicle control.

Statistical Analysis

Statistical analysis was carried out by Graph Pad prism software version 5. Statistically significant outlier data points were identified by Grubbs' test and excluded from additional analysis. Animals having levels of blood glucose outside the expected range, and measures of physiological parameters during surgical procedures to be outside the normal range, were excluded from the study. When data from more than two groups were compared, then one-way ANOVA followed by post hoc Tukey's test for multiple comparisons was used. Comparison between two groups was done using Student's *t* test. p value < 0.05 was considered statistically significant. The results are expressed as mean ± SEM.

Results

The details of study design are presented in Fig. 1a. We observed a minor yet significant difference in blood glucose levels prior to insulin pellet implantation in the APETx2 treatment group when compared to ITD + vehicle control, ITD + RH + vehicle control, and PcTx1 treatment groups. However, post-insulin treatment blood glucose values were not statistically different among all experimental groups (Fig. 1b). Blood glucose levels, in ITD groups, were maintained slightly above euglycemia to avoid any unwanted hypoglycemia. No statistically significant differences in blood glucose levels were observed during hypoglycemia in all RH-exposed ITD groups (Fig. 1c). Physiological parameters such as body weight, body temperature, head temperature, pCO_2, pO_2 and MABP were measured. A minor yet significantly lower value of blood pCO_2 level was observed in the ITD + RH + APETx2 group during ischemia when compared to the respective values in ITD + vehicle and ITD + RH + vehicle groups. However, there was no other statistically significant difference between the physiological parameters in all other experimental groups (Table 1).

RH Exposure Increases Post-ischemic Hypoperfusion in ITD Rats via ASIC Activation

Because we have previously observed that intra-ischemic acidosis in RH-exposed ITD rats is of greater extent, we hypothesized that increased intra-ischemic acidosis in RH-exposed ITD rats leads to pronounced post-ischemic hypoperfusion via ASICs. Animals belonging to the ITD + vehicle and the ITD + RH + vehicle groups demonstrated hyperemia

Table 1 Physiological parameters

Group	Time point	Body weight (g)	Body temperature (°C)	Head temperature (°C)	pH	pCO_2 (mmHg)	pO_2 (mmHg)	MABP (mmHg)
ITD + vehicle ($n=8$)	Before	352 ± 8	37.0 ± 0.0	36.8 ± 0.1	7.43 ± 0.02	38 ± 1	120 ± 4	97 ± 2
	During		37.0 ± 0.0	36.6 ± 0.1	7.34 ± 0.02	50 ± 2	115 ± 5	50 ± 0
	After		37.0 ± 0.0	36.8 ± 0.1	7.44 ± 0.01	35 ± 2	136 ± 8	101 ± 3
ITD + RH + vehicle ($n=7$)	Before	353 ± 9	37.0 ± 0.0	36.8 ± 0.1	7.47 ± 0.04	38 ± 1	118 ± 6	103 ± 6
	During		36.9 ± 0.1	36.6 ± 0.1	7.34 ± 0.01	45 ± 2	118 ± 7	49 ± 1
	After		37.0 ± 0.0	36.7 ± 0.1	7.43 ± 0.01	39 ± 1	115 ± 6	100 ± 7
ITD + RH + PcTx1 ($n=6$)	Before	339 ± 6	36.9 ± 0.1	36.4 ± 0.1	7.40 ± 0.02	37 ± 2	134 ± 8	103 ± 4
	During		36.8 ± 0.1	36.5 ± 0.2	7.27 ± 0.02	53 ± 3	124 ± 6	49 ± 0
	After		36.9 ± 0.1	36.5 ± 0.2	7.38 ± 0.02	36 ± 2	142 ± 9	113 ± 5
ITD + RH + APETx2 ($n=8$)	Before	353 ± 19	37.1 ± 0.1	36.6 ± 0.1	7.47 ± 0.01	37 ± 1	132 ± 9	103 ± 1
	During		36.9 ± 0.1	36.5 ± 0.1	7.44 ± 0.01	34 ± 2*,†	126 ± 8	49 ± 0
	After		37.4 ± 0.1	36.9 ± 0.1	7.47 ± 0.01	34 ± 1	141 ± 5	113 ± 2

*$p < 0.05$ versus ITD + vehicle control, †$p < 0.05$ versus ITD + RH + vehicle control

immediately after ischemia. However, the extent of cerebral ischemia-induced hypoperfusion was greater (25% to 46%) in RH-exposed ITD rats from 3 to 4 min of ischemia and 23 to 62 min of reperfusion ($p < 0.05$) when compared to ITD rats (Fig. 2). These data suggest that prior RH exposure leads to severe post-ischemic hypoperfusion in ITD rats.

To evaluate the potential role of ASIC activation in increased post-ischemic hypoperfusion in RH-exposed ITD rats, next we sought to evaluate the effect of a selective ASIC1a inhibitor PcTx1 (Escoubas et al. 2000, 2003; Salinas et al. 2006) and the ASIC3 inhibitor APETx2 (Diochot et al. 2004; Karczewski et al. 2010), on ischemia-induced hypoperfusion in brains of RH-exposed ITD rats. We did not investigate the role of ASIC2 as it is activated at a much lower pH ($pH_{0.5}$ 4.9), which is not normally observed during cerebral ischemia, when compared with ASIC1a ($pH_{0.5}$ 6.8) and ASIC3 ($pH_{0.5}$ 6.6) (Benson et al. 2002; Wemmie et al. 2006). PcTx1 treatment to RH-exposed ITD rats significantly decreased (46 to 95%) the extent of post-ischemic hypoperfusion when compared to ITD + RH + vehicle control rats from 3 to 7 and 22 to 80 min after cerebral ischemia ($p < 0.05$, Fig. 3). Further, APETx2 treatment to RH-exposed ITD rats also significantly decreased (27 to 54%) the extent of post-ischemic hypoperfusion when compared to its respective vehicle control group from 2 to 4 and 25 to 66 min after cerebral ischemia ($p < 0.05$, Fig. 3). Overall, our results indicate that prior exposure of ITD rats to RH leads to severe post-ischemic cerebral hypoperfusion, possibly via ASIC1a and ASIC3 activation.

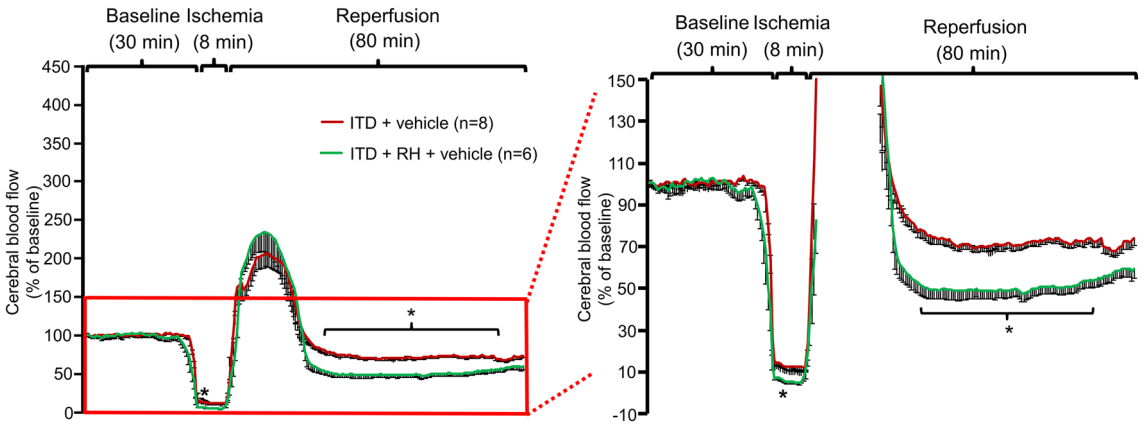

Fig. 2 The effect of prior RH exposure to ITD rats on ischemia-induced decrease in percentage change in cerebral blood flow. Percentage change in cerebral blood flow versus time curve of rats belonging to (1) ITD + RH + vehicle and (2) ITD + vehicle. *$p < 0.05$ versus ITD + vehicle

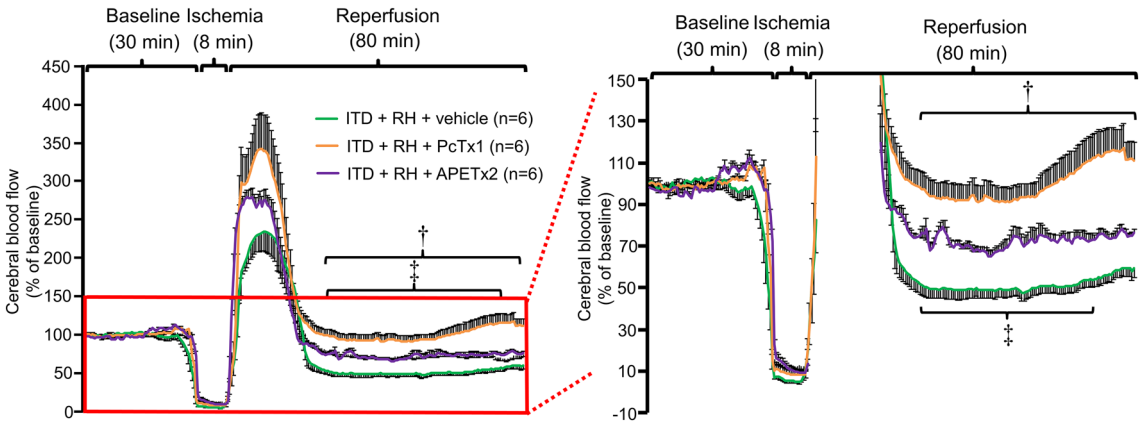

Fig. 3 The effect of PcTx1 and APETx2 on ischemia-induced decrease in percentage change in cerebral blood flow in ITD rats subjected to RH. Percentage change in cerebral blood flow versus time curve of rats belonging to ITD + RH + vehicle, ITD + RH + PcTx1, and ITD + RH + APETx2 groups. ITD + RH + vehicle trace is the same as in Fig. 2. †$p < 0.05$, ITD + RH + PcTx1 versus ITD + RH + vehicle and, ‡$p < 0.05$, ITD + RH + APETx2 versus ITD + RH + vehicle

ASIC Inhibition Attenuates RH-Induced Ischemic Brain Injury in ITD Rats

To address the question if ASIC-dependent severe hypoperfusion in RH-exposed ITD rats is responsible for exacerbation of ischemic brain damage, we evaluated the effect of ASIC inhibition on the extent of ischemic brain injury in these rats. We quantified the degree of ischemic brain injury in RH-exposed ITD rats treated with either PcTx1, APETx2, or vehicle. The number of normal neurons in CA1 hippocampus in RH-exposed ITD rats was significantly lower (58%, $p < 0.05$) than in ITD control rats. PcTx1 as well as APETx2 treatments prevented RH-induced increase in ischemic damage in CA1 hippocampus as the number of normal neurons were higher in these groups by 56 ($p < 0.01$) and 62% ($p < 0.01$) when compared to vehicle control group, respectively (Fig. 4). This result demonstrates that decreasing post-ischemic hypoperfusion in RH-exposed ITD rats by ASIC inhibition prevents exacerbated ischemic brain injury in RH-exposed rats.

Acidosis Leads to SOCE in Vascular Smooth Muscle Cells via ASIC3 Activation

Because earlier studies demonstrated the role of ASIC-dependent SOCE in VSMCs in causing pulmonary vasoconstriction (Jernigan et al. 2009, 2012; Nitta et al. 2014), we next determined if increased intra-ischemic acidosis leads to SOCE in VSMCs via activation of ASICs. This hypothesis was tested in vitro using VSMCs. We determined the contributions of ASICs in SOCE at different pH values by measuring SOCE in the presence and absence of ASIC1a or ASIC3 inhibitors. We observed that SOCE in the presence of APETx2 was lower by 40% ($p < 0.05$) when measured at pH 6.0 (Fig. 5a). However, SOCE at pH 6.5 and 7.4 was not affected in the presence of APETx2. We also did not observe any such effect of ASIC1a inhibition on SOCE at the three pH values that were tested (Fig. 5b). Therefore, our observations indicate that SOCE during pronounced ischemic acidosis is ASIC3- and not ASIC1a-dependent.

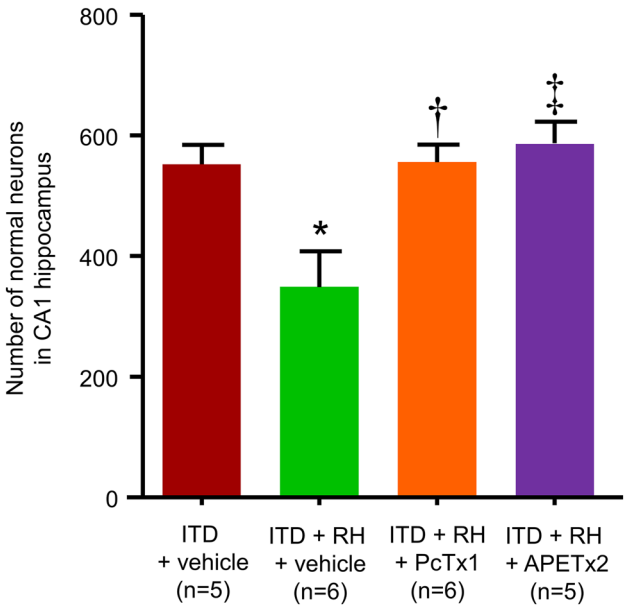

Fig. 4 The effect of PcTx1 and APETx2 on RH-induced increase in ischemic damage in hippocampus of ITD rats. The numbers of normal neurons in CA1 hippocampus of rats belonging to the ITD + vehicle, ITD + RH + vehicle, ITD + RH + PcTx1, and ITD + RH + APETx2 groups are shown. *$p < 0.05$ ITD + vehicle versus ITD + RH + vehicle, †$p < 0.05$ ITD + RH + PcTx1 versus ITD + RH + vehicle, and ‡$p < 0.05$ ITD + RH + APETx2 versus ITD + RH + vehicle

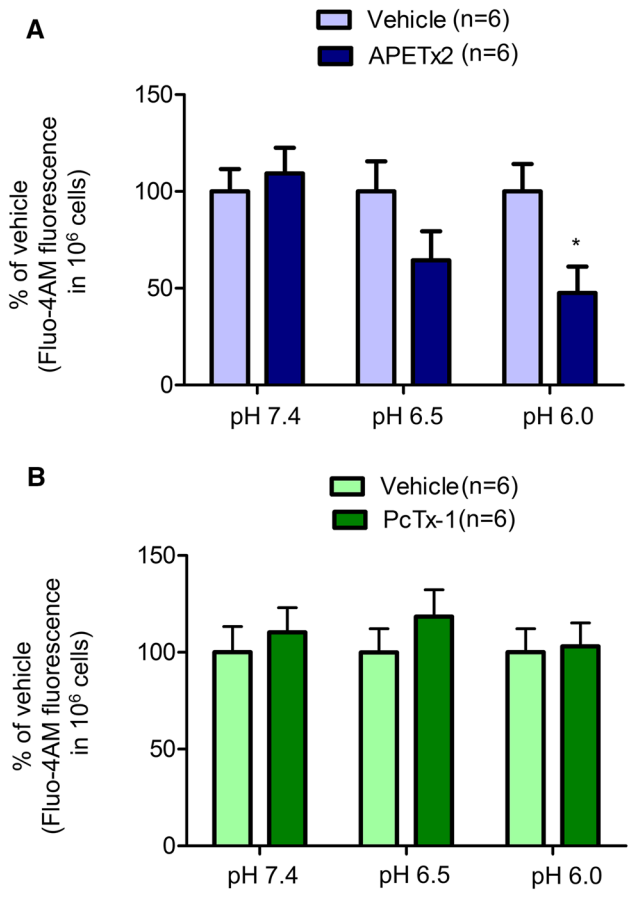

Fig. 5 The role of **a** ASIC3, and **b** ASIC1a in acidosis-induced SOCE in VSMCs. SOCE at pH 7.4, 6.5, and 6.0 in the presence of vehicle, APETx2, or PcTx1. *$p < 0.05$ versus respective vehicle control

Discussion

Cerebral ischemia causes substantial mortality and morbidity in the population worldwide and diabetes causes a further increase in ischemic brain injury (Benjamin et al. 2017; Kissela and Air 2006; Morrish et al. 2001; Preis et al. 2009). Therapeutic options available to treat diabetic hyperglycemia are unable to ensure tight round-the-clock maintenance of euglycemia, and unavoidably result in transient episodes of hypoglycemia (Nathan et al. 1993; Leese et al. 2003). Repeated episodes of hypoglycemia inhibit counter-regulatory mechanisms regulating blood glucose levels (Parekh 2009; McCrimmon et al. 2005; Mandal and Briski 2018) and thus increases the risk of RH (Cryer 2013; Leese et al. 2003). We have previously shown that prior exposure to RH aggravates ischemic brain damage in treated diabetic rats (Dave et al. 2011b; Shukla et al. 2018). However, the mechanism responsible for RH-induced exacerbation of ischemic brain injury in diabetic rats is not well understood. We earlier demonstrated the role of ischemic acidosis in mediating RH-induced increase in ischemic brain injury in ITD rats (Rehni et al. 2018). However, the downstream mechanisms responsible for the pronounced ischemic acidosis-induced increase in brain injury in RH-exposed ITD rats are unknown. ASIC1a, ASIC2a, and ASIC3 are expressed in VSMCs (Grifoni et al. 2008). ASIC1 are expressed in the central nervous system (Alvarez de la Rosa et al. 2003) and cerebral vasculature (Lin et al. 2014; Nakamura et al. 2009). A study has shown the presence of ASIC3 in many parts of the rat brain (Meng et al. 2009). An earlier study also confirmed the presence of ASIC3 in brain (including the hippocampus) using ASIC3 knockout mice (Drew et al. 2004; Wu et al. 2010). In addition, ASIC3 are also expressed in human brain (Babinski et al. 1999; Delaunay et al. 2012). In the present study, we showed that prior exposure of ITD rats to RH causes ASIC1a- and ASIC3-dependent post-ischemic hypoperfusion, which in turn increases ischemic brain damage. Our results corroborate earlier findings that hypoglycemia in combination with ischemia causes profound post-ischemic hypoperfusion and decreases hypercapnic reactivity in neonates (Kim et al. 1994). We studied the effect of ischemia on hippocampus, which is a vulnerable part of the brain (Bartsch et al. 2015; Kirino and Sano 1984; Petito et al. 1987; Schmidt-Kastner 2015; Schmidt-Kastner and Freund 1991; Schmidt-Kastner et al. 1990). However, future studies are needed to evaluate the effect of ASIC inhibition on ischemic damage in other parts of the brain in RH-exposed ITD rats.

Low pH during acidosis activates ASICs (Waldmann 2001; Waldmann et al. 1997). ASICs are located in the VSMCs in the cerebral vasculature and are responsible for facilitation of myogenic response and VSMC migration (Chung et al. 2010; Drummond et al. 2004; Grifoni et al. 2006, 2008; Jernigan and Drummond 2005; Lin et al. 2014). Post-ischemic hypoperfusion is a characteristic phenomenon of ischemia–reperfusion injury (Ginsberg et al. 1978; Hossmann et al. 1973; Levy et al. 1979; Snyder et al. 1975). Preventing cerebral ischemia-induced vasoconstriction, by MEK1/2 inhibition, lowers ischemic brain damage (Johansson et al. 2014). ASICs are extensively distributed in the nervous system and are also known to induce neuronal depolarization in response to drop in pH (Kellenberger and Schild 2002; Lingueglia 2007; Waldmann 2001). An ischemic acidosis-related increase in extracellular protons triggers neuronal necroptosis via ASIC activation (Wang et al. 2015). Overall, the activation of ASIC-sensitive proton-gated calcium currents and vascular changes may play a significant role in mediating acidosis-induced neuronal death (Sherwood et al. 2011) and ischemic brain damage (Xiong et al. 2004). Although prior exposure to RH leads to increased ischemia-induced acidosis and increased ischemic damage, the participation of ASIC in RH-induced increased ischemic damage was not known. We observed that both ASIC1a and ASIC3 inhibitors attenuated the cerebral blood flow deficits and brain injury after ischemia in RH-exposed ITD animals. Our results suggest that severe hypoperfusion observed after global cerebral ischemia in ITD animals exposed to RH may be due to the activation of ASICs.

ASICs are known to induce vasoconstriction in cerebral vessels (Chung et al. 2010; Drummond et al. 2004; Jernigan and Drummond 2005; Lin et al. 2014). Contraction in the VSMCs results from calcium influx through the cell membrane, intracellular calcium release, and increased sensitization of calcium-dependent contractile proteins (Avila-Medina et al. 2018). ASIC activation is proposed to cause vasoconstriction by mediating SOCE (Jernigan et al. 2009). Our current data show that exposure to acidic pH leads to ASIC3-mediated SOCE in VSMCs. Therefore, it is plausible that increased ischemic acidosis may elicit vasoconstrictive effects on cerebral vessels via ASIC3-dependent SOCE in RH-exposed ITD rats. We did not observe any impact of ASIC1a inhibition on SOCE in the VSMCs at two acidic pH levels tested. However, we did observe lower ischemic damage in ASIC1a inhibitor-treated RH-exposed ITD rats. ASICs are expressed on brain neurons as well as on central and peripheral immune cells (Kellenberger and Schild 2015). Calcium influx in neurons is known as one of the central mechanisms regulating cell death (Wojda et al. 2008). Although ASIC currents are Na^+-selective, ASIC1a present in the cell membrane have an additional low permeability to Ca^{2+} ions as well (Kellenberger and Schild 2015; Sherwood et al. 2011; Xu et al. 2018; Zuo et al. 2018) and their blockade produces protective effects in several neurodegenerative

diseases including stroke (Pignataro et al. 2007; Xiong et al. 2004; Hu et al. 2011; Wong et al. 2008; Arias et al. 2008). It is plausible that ASIC1a on neurons may also participate in RH-induced increase in ischemic damage. Moreover, the role of acidosis (Combs et al. 1990; Kuyama et al. 1994; Nagao et al. 1996; Rehni et al. 2018) and ASIC activation (Pignataro et al. 2007; Xiong et al. 2004) in mediating ischemic brain injury is well-established. Since we did not observe impact of PcTx1 on SOCE, but observed lower extent of cerebral ischemia-induced hypoperfusion and lower cerebral ischemic damage in PcTx1-treated group, we hypothesize that ASIC1 activation in the neurons may indirectly regulate post-ischemic hypoperfusion in the brain. Future studies to understand neuronal control of post-ischemic cerebral blood flow in RH-exposed ITD rats are warranted.

Besides the presence of homomeric ASICs in brain, ASIC1a/2 heteromers are also present on the brain cells (Askwith et al. 2004; Sherwood et al. 2011). Heteromeric ASIC1a/2 have lower and higher proton sensitivities as compared to homomeric ASIC1a and ASIC2a, respectively (Hesselager et al. 2004; Joeres et al. 2016). Proton sensitivity of heteromeric ASIC2a/3 is higher than that of ASIC2a, ASIC2b, and ASIC3 (Baron et al. 2001; Benson et al. 2002). Heteromeric ASIC1a/ASIC2a are not inhibited by PcTx1, while heteromeric ASIC2b/1a are inhibited by PcTx1 (Escoubas et al. 2000). The inhibitory effect of PcTx1 on ASIC1a/ASIC3 channels is controversial (Escoubas et al. 2000; Gregory et al. 2018). However, PcTx1 slows the kinetics of desensitization and recovery of desensitization, and inhibits pH-dependent steady-state desensitization of ASIC1a/2/3 heteromeric channels (Gregory et al. 2018). The literature suggests that the effect of ASIC inhibitors observed in our studies may be due to inhibition of homomeric ASICs and its impact on properties of heteromeric ASICs. Future studies are required to determine the role of these ASIC heteromers in mediating pronounced ischemic acidosis-induced increase in brain injury in ITD rats previously exposed to RH. ASIC2 is upregulated after global cerebral ischemia and may contribute to ischemia-induced neuronal injury (Jiang et al. 2017; Johnson et al. 2001). Earlier study also demonstrated the role of ASIC2a in surface trafficking of ASIC1a in brain (Harding et al. 2014; Jiang et al. 2017). However, as mentioned above, we did not evaluate contribution of ASIC2 in our experimental conditions as ASIC2 are activated at much lower pH (usually not observed during cerebral ischemia) (Benson et al. 2002; Wemmie et al. 2006). The role of ASIC2, if any, in RH exposure-induced increased ischemic damage remains to be evaluated. Our proof-of-concept study demonstrates the neuroprotective effect of ASIC inhibition on RH-induced increase in ischemic brain injury. Further rigorous preclinical evaluation following the Stroke Therapy Academic Industry Roundtable (STAIR) recommendations may help test the therapeutic potential of inhibitors used in the present study (STAIR 1999).

Therefore, we conclude that severe post-ischemic hypoperfusion is responsible for RH-induced aggravation of ischemic brain damage, possibly mediated through the activation of ASICs. Nevertheless, further understanding of the mechanisms leading to ASIC-dependent changes in cerebral blood flow and other non-vascular alterations in the brain that cause RH-related increase in ischemic brain injury in ITD rats is required. More understanding on how increased extent of ischemia/reperfusion-induced cerebral hypoperfusion leads to increased damage is also needed. Our study demonstrates that post-ischemic acidosis increases ischemic brain injury in RH-exposed ITD rats via ASIC activation-mediated post-ischemic hypoperfusion. In addition, our data implicate that ASIC modulation may serve as an ameliorative approach for diabetes-induced increase in ischemic brain damage.

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Compliance with Ethics Standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical Approval All institutional and national guidelines for the care and use of laboratory animals were followed.

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