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Diabetic Stroke Severity: Epigenetic Remodeling and Neuronal, Glial, and Vascular Dysfunction

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We determined the mechanism of severity during type 1 diabetic (T1D) stroke (ischemia-reperfusion [IR] injury) that affects potential markers associated with epigenetics, neuronal, glial, and vascular components of the brain with regard to nondiabetic stroke. The study used male genetic T1D Ins2<sup>−/−</sup> Akita and wild-type (C57BL/6J) mice. The experimental mice groups were 1) sham, 2) IR, 3) shamAkita, and 4) IRAkita. Mice were subjected to middle cerebral artery occlusion for 40 min, followed by reperfusion for 24 h. Brain tissues were analyzed for inflammation, neuro-glio-vascular impairments, matrix metalloproteinase (MMP)-9 expression, and epigenetic alterations (DNA methyltransferase-3a [DNMT-3a]; DNA methyltransferase-1 [DNMT-1]; 5-methylcytosine [5-mC]; and 5-hydroxymethylcytosine [5-hmC]). Intracarotid fluorescein isothiocyanate-BSA infusion was used to determine pial-venular permeability. IRAkita mice showed more infarct volume, edema, inflammation, and vascular MMP-9 expression compared with IR and sham groups. ShamAkita mice showed the highest DNMT-1 and DNMT-3a levels compared with the other groups. Reduced tight and adherent junction expressions and severe venular leakage exemplified intense cerebrovascular impairment in IRAkita mice compared with the other groups. Interestingly, we found differential regulations (downregulated expression) of epigenetic (5-mC, DNMTs), vascular (endothelial nitric oxide synthase), glial (connexin-43, glial fibrillary acidic protein, CD11b), and neuronal (neuron-specific enolase, neuronal nitric oxide synthase) markers in IRAkita compared with the IR group. These findings suggest that IR injury in T1D is more severe because it intensifies differential epigenetic markers and neuro-glio-vascular changes compared with nondiabetic mice.

Type 1 diabetes (T1D) is a major risk factor for ischemic cerebrovascular disease that increases morbidity and mortality worldwide (1,2). Stroke occurs fivefold more often in patients with T1D and results in intense consequences compared with those in patients without diabetes; however, the mechanisms underlying stroke severity in patients with diabetes are unclear (3). Also, whether stroke is different in people with and without diabetes is unclear. Therefore, understanding these molecular changes and functional relationships with altered regulatory pathways can help explain the conceptual basis behind the severity of stroke in patients with diabetes. In addition, exploring the regulatory pathways and molecular mechanisms may be helpful in the future for designing preventive and therapeutic strategies.

The role of epigenetics was recently broadly characterized and found to be involved in the pathophysiology of stroke. Large clinical trials, such as the Diabetes Control and Complications Trial (DCCT) and Epidemiology of Diabetic Interventions and Complications Trial (EDIC), along with animal studies, have confirmed that hyperglycemia predisposes individuals to develop complications of diabetes, also referred to as the legacy effect (4,5). These reports suggest that hyperglycemia exposure mediates long-lasting epigenetic modifications that intensify global gene expressions by epigenetics modifications. Hence, exploring epigenetic factors underlying diabetic stroke is the need of the hour to better understand the basis of severity in patients with diabetes during stroke.

Epigenetic modifications, for example, DNA methylation, regulate phenotype and gene expression patterns and occur...
through the aid of enzymatic activity of DNA methyltransferases (DNMTs). Methylation usually occurs at the fifth carbon atom of cytosine residues and forms 5-methylcytosine (5-mC), which may be further oxidized to 5-hydroxymethylcytosine (5-hmC) (6,7). Studying DNMTs and global 5-mCs and 5-hmCs for their biological roles is of great scientific interest that may help in determining gene expression in the diseased and nondiseased state. Whereas 5-mC predicts a compacting chromatin inaccessible to transcription, 5-hmC determines efficient chromatin transcription. However, global 5-mC and 5-hmC levels in diabetic stroke have not been studied.

Specialized endothelial cells line the cerebral vessels and are enwrapped with pericytes and astrocytes that in turn connect to neurons providing nursing and maintenance. A number of different factors, including the extracellular matrix, tight junctions (TJs), pericytes, and astrocyte end-feet, together with adherens junctions, form junctional complexes and play a central role in the control of blood-brain barrier (BBB) integrity (8,9). Disruption in BBB integrity causes BBB permeability, which is manifested by the activation of matrix metalloproteinase-9 (MMP-9) that chops off junction barriers (10). Dysfunction of the BBB advances to stroke-like pathologies by increasing microvascular permeability that in turn affects the neuronal, glial, and vascular system. However, the roles of BBB and neuro-glio-vascular dysfunction in diabetic stroke pathology have not been studied much. The regulatory functions of vascular and neuronal integrity are essentially regulated by nitric oxide (NO) synthase (NOS) enzymes. The NOS enzymes produce NO, which regulates vascular tone, insulin secretion, and neuronal development via different isozymes, for example, endothelial NOS (eNOS) and neuronal NOS (nNOS). NOS regulation has been studied in ischemic injury, but an explanation is needed of its significance in diabetic stroke.

Our goal in the current study was to address two aspects: first, to explore whether ischemia-reperfusion (IR) injury in T1D severely disturbs the neuro-glio-vascular unit by causing intense inflammation, global alteration in epigenetic markers, and high MMP-9 activation, and second, to compare IR injury in T1D with non-T1D to find out the basis of IR injury severity in diabetes.

RESEARCH DESIGN AND METHODS

Animals

All animal procedures were conducted at the University of Louisville Health Sciences Center, were in compliance with guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the University of Louisville Institutional Animal Care and Use Committee. Male wild-type (WT; C57BL/6J) and Akita (genetic T1D mice, C57BL/6-Ins2Akita/J) mice (8–10 weeks old) were obtained from The Jackson Laboratory (Bar Harbor, ME). The experimental mice groups were 1) sham, 2) IR, 3) sham-Akita, and 4) IR-Akita. Akita mice with a glucose level >400 mg/dL were used in the study. We assessed inflammatory (tumor necrosis factor-α [TNF-α], interleukin-6 [IL-6]) and anti-inflammatory (IL-10) cytokines and glial markers (glial fibrillary acidic protein [GFAP], integrin-α M [CD11b]) using real-time quantitative (q)-PCR. Neuronal (neuronal nuclei [NeuN], neuronal-specific enolase [NSE], nNOS), vascular (zona occluden-1 [ZO-1], claudin-5, eNOS), and epigenetic (DNMT-1 and DNMT-3a) markers were determined using Western blot. MMP-9, vascular (vascular endothelial [VE]-cadherin, occludin), glial (connexin-43 [Cx-43], GFAP), and neuronal (NeuN) markers were quantified using immunohistochemistry (IHC) analysis.

Animal Surgical Procedure

All mice were allowed free movement in the cage (4–5 mice/cage) and free access to water and food. The mice were maintained at 12/12-h day-night cycle at ~23°C room temperature. Mice were fasted overnight with free access to water just before the day of surgery. At the day of surgery, the mice were transferred to the surgery room, anesthetized with pentobarbital (50 mg/kg body weight), and operated on within 1 h. Anesthetized mice were orally intubated, mechanically ventilated, and the body temperature was maintained at 37 ± 1°C during surgery.

The common carotid artery was exposed through a midline neck incision and dissected free of the surrounding nerves. Lysine-coated monofilament (2-cm long; 5-0 or 6-0 [11,12]) was inserted into the left external carotid artery, advanced into the internal carotid artery, and wedged into the cerebral arterial circle to obstruct the origin of the middle cerebral artery. After 40 min of surgery, the filament was withdrawn to allow reperfusion for 1 day. The same anesthesia and surgical procedures, except the insertion of filament, were performed in sham groups of mice (13).

Mice were assessed with neurobehavioral tests after IR injury to determine ischemia severity. Neurological deficit scores were graded 0–12 (normal score = 0, maximum score = 12) after the mice performed several behavioral tests, including posture relax test, forelimb placing test, circling, and motor coordination tests. Only mice with a high-grade neurological deficit (≥9) were used for the study (14,15).

Microvascular Leakage Assessment

Microvascular permeability was measured as described earlier (10). Mice were anesthetized with pentobarbital sodium (50 mg/kg i.p.). A heating pad was used to maintain body temperature of the mice at 37 ± 1°C. A 14-mm hole was made in the skull using a high-speed microdrill (Fine Scientific, Foster City, CA). Fluorescein isothiocyanate (FITC)-conjugated BSA (BSA-FITC-albumin, 300 μg/mL) was infused through carotid artery cannulation. In vivo imaging with an BX61WI fluorescent microscope (Olympus, Tokyo, Japan) was used to examine the exposed area of the skull. Venules were identified by the topology of the pial circulation and blood flow direction. Selected third-order venular segments were recorded and used as the baseline. After the baseline reading was obtained, images of the venular segments were recorded. The lamp power
and camera gain settings were held constant during the experiments. Data were interpreted with the software provided with the instrument and Image-Pro Plus 6.3 software (Media Cybernetics, Bethesda, MD). Leakage of FITC-BSA was assessed by changes in the ratio of fluorescence intensity in the interstitium to that inside the vessel. The results were averaged and presented.

**Collection of Brain Samples**

Brain samples were collected for Western blot, q-PCR, and IHC analysis. The brain tissue samples were harvested from the experimental mice groups, washed with 50 mmol/L PBS (pH 7.4), and stored at −80°C until use.

**SDS-PAGE and Western Blotting**

Equal quantities of brain extracts (40 μg) were run on 10–15% polyacrylamide gel under reducing condition, and separated proteins were transferred to polyvinylidene fluoride membrane using an electrophoretic apparatus (Bio-Rad, Hercules, CA). After blocking with 5% nonfat dry milk for 1 h, the membranes were probed overnight with a primary antibody (eNOS, nNOS, claudin-5, ZO-1, DNMT-3a, DNMT-1, NSE, NeuN) at 4°C. The next day, the blots were probed with appropriate secondary antibody for 2 h at room temperature and were developed using the ChemiDoc XRS+ Molecular Imager (Bio-Rad). The images were recorded in the chemi-program of a gel documentation system (Bio-Rad). The membranes were stripped and reprobed with monoclonal anti-GAPDH antibody (Millipore, Billerica, MA) as a loading control. Each band density was normalized with the respective GAPDH density using Image Lab densitometry software (Bio-Rad).

**Quantitative Gene Expression Analysis**

Total RNA from the brain tissue was isolated using TRIzol reagent (Invitrogen, Grand Island, NY), following the manufacturer’s instructions. After the quantity and purity of total RNA was confirmed using NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA), the RNA was reverse transcribed to cDNA according to the manufacturer’s protocol (ImProm-II, Invitrogen). cDNA samples were amplified for the given genes using gene-specific primers (GFAP, CD11b, TNF-α, IL-6, IL-10; sequences given in Supplementary Table 1) using Stratagene Mx3000p (Agilent Technologies, Santa Clara, CA). CT values were determined after baseline and threshold adjustments, and the results were expressed in fold expression. The transcript levels of given genes were normalized with Rn18s.

**IHC Analysis**

Frozen brain blocks, prepared with optimal cutting temperature media (Triangle Biomedical Sciences, Durham, NC), were cut into 20-μm sections using a Leica CM cryostat (Leica Microsystems, Buffalo Grove, IL). After removing the mounting matrix and fixing with methanol for 10 min, tissues were blocked with blocking solution (0.1% Triton X-100 TBS, 0.5% BSA, and 10% normal donkey serum) for 1 h at room temperature. The sections were incubated with primary antibody (MMP-9, occludin, VE-cadherin, NeuN, GFAP, Cx-43) overnight at 4°C. After incubation with the appropriate fluorescence secondary antibodies for 60 min at room temperature, the sections were stained with DAPI (1:10,000) and mounted with antifade mounting media. Images were acquired using a FluoView 1000 laser scanning confocal microscope (Olympus, Allentown, PA), and the data were analyzed with Image-Pro Plus image analysis software.

**Quantification of the 5-mC and 5-hmC Levels**

Genomic DNA was isolated (Sigma-Aldrich, St. Louis, MO) from the brain sample, and total 5-mC and 5-hmC levels were determined using ELISA kits (Epigentek, Farmingdale, NY), according to the manufacturer’s instructions.

**Fluoro-Jade C Staining**

Fluoro-Jade C (FJC) stain (Sigma-Aldrich) was used to determine the presence of neuronal damage. Brain sections were processed according to the method described previously (16). Briefly, paraffin-embedded sections were deparaffinized, rehydrated, and transferred to 0.06% potassium permanganate solution for 10 min. Afterward the sections were incubated in a 0.0001% solution of FJC for 20 min and mounted with DPX. Images were captured, and the data were analyzed with Image-Pro Plus image analysis software.

**Determination of Infarct Volume**

Coronal sections (2 mm) were cut using a mouse brain slice matrix (Harvard Apparatus, Holliston, MA). The slices were stained for 20 min at 37°C with 2% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich) and postfixed with 4% paraformaldehyde. Infarct area (pale white) of each brain section was determined with Image-Pro Plus image analysis software.

**Cerebral Edema**

The cortical parts of the brain ipsilateral area were dried for 3 days in a drying oven at 100°C. The absolute water content was calculated as water content (%) = [(wet weight − dry weight)/wet weight] × 100.

**Statistical Analysis**

All values are expressed as mean ± SEM. Statistical comparisons between two groups were performed by the Student t test. One-way ANOVA was used for more than two groups. A P value of <0.05 was considered to be statistically significant.

**RESULTS**

**IR^Akita^ Developed Larger Infarct Size, Edema, Inflammation, and Cell Death**

IR^Akita^ mice showed more infarct size (Fig. 1A and B), cerebral edema (Fig. 1C), proinflammatory cytokines (TNF-α and IL-6), and reduced anti-inflammatory cytokines (IL-10; Fig. 1D) compared with IR and sham Akita groups. Cresyl violet staining demonstrated significant degradation of the cellular constituents as indicated by cell swelling (blubbing), decreased cell size (shrinkage), and extensively decreased
cell number in the IRAkita group compared with the sham, IR, and shamAkita groups (Fig. 1E). These results indicate severe IR-injury in diabetic mice compared with non-diabetic mice.

**Diabetic Brain With Stroke Exhibited Intense Epigenetic Remodeling**

To address potential markers associated with epigenetics, mice brains were evaluated for levels of DNMTs and global %5-mC and %5-hmC levels. Western blots exemplified the highest protein expression of DNMT-1 and -3a in the shamAkita group. The IR group exhibited a significant increase in DNMT-1 and -3a protein levels compared with the sham group, and the IRAkita group showed a considerable decrease in DNMT-1 and -3a compared with the shamAkita group (Fig. 2A and B). Furthermore, the IR group showed an increase in global 5-mC levels and a decrease in global 5-hmC levels compared with the sham group, and the IRAkita group showed a decrease in global 5-mC and 5-hmC levels compared with the shamAkita group (Fig. 2C and D). Hence, DNMTs and 5-mC levels were increased in the IR group and decreased in IRAkita group compared with the sham and shamAkita groups, respectively. These results represented differential
epigenetic remodeling after IR injury in diabetic versus nondiabetic mice.

**IRAkita Brains Showed Severe Vascular Injury and BBB Disruption**

Intracarotid FITC-BSA infusion exemplified the highest macromolecular pial venular permeability in the IRAkita group. The IR and shamAkita groups also exhibited considerable high venular permeability compared with the sham group (Fig. 3A and B). We further evaluated endothelial junction proteins because these actively regulate selective barrier functions across the vessel walls. IHC analysis using endothelial junction proteins (occludin and VE-cadherin) determined drastically reduced expression in the cortical vessels of the IR and IRAkita groups compared with their respective sham groups (Fig. 3C and D). Similarly, Western blot analysis using TJs (ZO-1 and claudin-5) confirmed remarkably decreased expressions in the IRAkita group compared with the sham, IR, and shamAkita groups (Fig. 3E and F). Protein expressions of ZO-1 and claudin-5 were also considerably reduced in the IR and shamAkita groups compared with the sham group (Fig. 3E and F). Because the vascular impairment is exacerbated with MMP-9 activation, we further determined vascular MMP-9 expression using IHC analysis. The highest elevation in vascular MMP-9 expression among the different groups was observed in the IRAkita group. However, significant enhancement in vascular MMP-9 was also observed in the IR and shamAkita groups compared with the sham group (Fig. 3G and H).

**IRAkita Showed Disrupted Glia After Stroke**

To address glial markers, we determined transcript levels of GFAP and CD11b using q-PCR analysis. Whereas the IR group showed significantly increased GFAP and CD11b mRNA levels, the IRAkita group showed a noticeable decrease in GFAP and CD11b mRNA compared with their respective shams. GFAP and CD11b were also significantly high in the shamAkita mice compared with the sham mice (Fig. 4A). To address the vascular connection to glia, we evaluated Cx-43, an astral-gap junction marker, through IHC analysis of cerebral vessels. Extremely reduced immunoreactivity of Cx-43 was observed in the cortical vessels of the IRAkita mice compared with the shamAkita mice. Conversely, increased Cx-43 immunoreactivity was observed in the IR group compared with the sham group (Fig. 4B and C). Furthermore, IHC analysis confirmed reduced GFAP immunoreactivity in the hippocampus region of IR-injured Akita brains compared with the other brains, and significant amplification in the GFAP immunoreactivity was observed in shamAkita and IR brains compared with sham brains (Fig. 4D and E). These results showed differential regulation of glial activation after IR injury in diabetic versus nondiabetic conditions.

**Neuronal Loss After IR Injury in IRAkita Mice**

NeuN staining confirmed notable loss of neurons in the hippocampus region after ischemic injury in IR and IRAkita brains compared with their respective shams. Diabetic Akita brains also exhibited decreased NeuN expression compared with sham brains (Fig. 4D and E). Furthermore,
Western blot analysis confirmed the lowest expression of NeuN in IR\(^{Akita}\) brains (Fig. 5A and B). Western blot analysis illustrated reduced expression of NSE, another neuronal-related marker, in the IR\(^{Akita}\) group compared with the sham\(^{Akita}\) group. Interestingly, increased expression of NSE was also observed in the IR group compared with the sham group (Fig. 5A and B). We performed FJC staining to address neuronal loss, and the highest degenerating neurons were observed in IR\(^{Akita}\) brains. FJC staining confirmed neurodegeneration in IR and sham\(^{Akita}\) brains compared with sham brains (Fig. 5C and D). These results suggested intense neuronal damage during IR injury in diabetes.

**NOS Regulation in IR Injury**

We determined eNOS and nNOS levels that impart main roles in regulating vascular tone and glia and neuronal integrity. Western blot analysis confirmed decreased protein expression of eNOS and nNOS in the IR\(^{Akita}\) group compared with the sham\(^{Akita}\) group, while increased eNOS and nNOS expression was observed in the IR group compared with the sham group (Fig. 6A and B). The sham\(^{Akita}\) group also represented a remarkable increase in nNOS with respect to the sham group (Fig. 6A and B). These data further suggest differential IR injury outcomes in diabetic versus nondiabetic brains.
DISCUSSION

Our results suggest that IR injury in T1D is severe in infarct volume, intense inflammation, cell death, remodeling of global epigenetic markers, and intense vascular MMP-9 activation. Interestingly, the severity of IR injury in T1D is exacerbated by differential regulations of global epigenetic, vascular, neuronal, and glial functions compared with IR injury in non-T1D.

Although T1D is much less common than type 2 diabetes, the symptoms and injuries are equally abrupt and sometimes more severe. To address the basis of severity in T1D, we performed our studies in the well-established genetic T1D Akita mouse model. Akita mice closely mimic human T1D because they have a genetic defect in the insulin 2 (Ins2+/−) gene and therefore induce hyperglycemia naturally. Besides that, these mice show higher diabetic traits (blood glucose, 27.3 ± 5.3 mmol/L for males and 13.6 ± 3.8 mmol/L for females), with decreased reactive immunologically detectable insulin (20.7 to 9.1% in males and 45.9 to 49.6% in females) (17). Akita mice have been used for studying the effects of diabetes on cerebral vasculature (18), sexual dimorphism during diabetes (17,19,20), myelinated fiber loss (21), peripheral neuropathy, and memory performance (22). Akita mice also showed adverse inflammatory and epigenetic remodeling in the heart (23) and impaired vascular density in the brain (18). However, there are no reports of these mice being used to study the effects and mechanisms of ischemic injury. After creating IR injury in Akita hyperglycemic (>400 mg/dL) mice, we found larger cerebral infarcts, more edema, increased cell death, intense inflammation, and reduced anti-inflammation compared with IR-injured nondiabetic mice. We also observed high functional neurological deficits in IRAkita mice compared with IR mice. A study of a T1D BioBreeding rat model described the association of diabetic-IR injury with
increased infarct size even at tight blood glucose control (24). Similarly, a clinical study that used X-ray computed tomography in 104 patients also confirmed the correlation between hyperglycemia and cerebral infarct size in patients with stroke (25).

The cumulative results of large clinical trials (DCCT, EDIC) and animal studies confirmed the abnormality of the cells under hyperglycemia and invokes phenomenon of altered epigenetics in perpetuating diabetic complications (4,5). Understanding epigenetic modifications may help in exploring novel epigenetic mechanisms that could be targeted for early standpoint or therapeutic aspects against intense ischemic injury during diabetes. We therefore addressed potential epigenetic markers (global %5-mC, %5-hmC, and methylation enzymes). DNMT-1 (maintenance methylation) and DNMT-3a (de novo methylation) were found to be highest in the sham Akita group. Our results further showed that DNMT-1, DNMT-3a, and global 5-mC levels were decreased in diabetic brains and increased in nondiabetic brains after ischemic insult. Stroke in the hyperglycemic state adversely affects potential epigenetic markers that increase the stroke severity in diabetic mice. These results certainly suggest that differential epigenetic alterations can be associated with intense IR injury outcomes in diabetic mice compared with nondiabetic mice. Similar to global %5-mC levels, global %5-hmC levels were also decreased in IR-injured diabetic brains, suggesting sheared and inactive chromatin status that can be associated with intense cellular damage in diabetic brains after ischemia. In agreement with our findings, previous studies have reported an increase in methyltransferases levels in middle cerebral artery occlusion and Akita mice models (23,26,27), and a study of the hippocampus region of 10 patients with Alzheimer’s disease found decreased levels of 5-mC and 5-hmC (28). Hence, the decrease of 5-mC and 5-hmC in the study of patients with Alzheimer’s disease and in our study suggests that these epigenetic modifications are
activated by in MMP-9, which is the hallmark for cellular junctions, and worsening the IR injury outcomes during diabetes.

We further reported functional BBB integrity loss associated with neuro-glia-vascular dysfunction that manifests with intense inflammation and global epigenetic remodeling after IR injury in diabetic mice. Extensive BBB disintegration in IR Akita mice increases the chances of blood proinflammatory molecules entering the brain and worsening the IR injury outcomes during diabetes. MMP-9, which is the hallmark for cellular junctions, is activated by inflammation and by creating IR injuries, and we have shown that MMP-9 activation chops off vascular junction proteins and raises BBB permeability (10). In the current study, we observed more fold change of MMP-9 in IR mice compared with IR Akita mice, although the basal level of MMP-9 was high in sham Akita compared with WT mice. In agreement, we have reported from our laboratory that the basal expression level of MMP-9 is high in diabetic mice compared with the control (31,32). We observed the highest vascular MMP-9 expression and cerebrovascular permeability in IR-injured diabetic mice, suggesting that persistent hyperglycemia might accelerate brain vasculature and BBB damage. However, controversy remains for subjects with diabetes: on the one hand, sustained BBB integrity was reported (33), whereas on the other hand, increased BBB damage was revealed during diabetes (34).

In our study, we found increased BBB permeability in diabetic Akita mice. Previous studies have demonstrated that the ischemic brain in db/db mice (type 2 diabetes) and streptozotocin-induced mice and rats (T1D) also exhibits increased cerebrovascular dysfunction (35,36).

A cascade of events is mediated after ischemic brain injury, yielding Ca2+-dependent activation of the NOS isoforms nNOS and eNOS (37). The role of NOS isoforms in cerebral ischemia damage was described in the study performed in transgenic mice lacking expression of nNOS or eNOS and in in vitro and in vivo models of cerebral ischemia. The study suggested that nNOS plays key roles in neurodegeneration, whereas eNOS has a prominent role in maintaining cerebral blood flow and preventing neuronal injury (38). By looking at eNOS expression, which serves as a major weapon against different vascular diseases, differential regulation was observed during IR injury in diabetic versus nondiabetic mice, indicating its involvement in enhancement of IR pathology in diabetes.

Intensive vascular injury affects vascular-glia interactions during IR injury in diabetes. Astrocytes, the major glial subtype, establish glial network and communicate through gap junctions. A number of studies reported that astrogliosis, a process of glial activation, increases after an ischemic injury to provide support to the neurons. However, we observed decreased GFAP immunoreactivity against IR injury in diabetic versus nondiabetic mice, indicating its involvement in enhancement of IR pathology in diabetes.
after ischemic stroke (44) and to confirm the evolving phase of infarction after middle cerebral artery occlusion (45). Furthermore, increased levels of neuronal NSE and nNOS in IR mice, whereas decreased levels in IR\textsuperscript{Akita}, also indicated differential regulation of the neuronal microenvironment after an IR insult in diabetic versus nondiabetic mice. The increase of the NSE level in IR mice is in agreement with previous findings (46,47), but the decrease of NSE in IR\textsuperscript{Akita} is suggestive of neuronal inability to transcribe NSE due to persistent hyperglycemia. In support, another study showed an increase in the NSE mRNA level in patients with diabetes, but a decrease occurred in subjects with diabetic neuropathy (48).

According to the 2014 National Diabetes Statistics Report, ~29.1 million people, or 9.3% of the U.S. population, have diabetes. In this population, 21.0 million people have been diagnosed with diabetes, and 8.1 million people (~27.8%) with diabetes are undiagnosed. Compared with diagnosed people who receive some treatment, the undiagnosed people face more risk of stroke because they do not receive any treatment. Our study can be helpful in that direction. To show the close resemblance of our mouse model with the population with diabetes receiving some treatment, we treated Akita mice with insulin and observed less stroke severity after creating the IR injury compared with untreated mice (Supplementary Figs. 1 and 2).

Although some reports suggest that DNA methylation levels have been found altered in T1D patients, the information about the effect of antidiabetic therapy on epigenetics is scarce. In concordance with our study, altered epigenetic changes have been observed in the kidney of db/db diabetic mice in a tissue-specific manner. The authors further reported aberrant DNA methylation, changes in histone modifications, and mRNA expression in the diabetic kidney and that these changes were resistant to pioglitazone (49). In another study by Ishikawa et al. (50), there was increase in the DNA methylation of the Ins1 (insulin 1) promoter in isolated islets from Zucker diabetic fatty rats. However, use of metformin treatment suppressed DNA methylation and upregulated insulin gene expression. Evans-Molina et al. (51) observed 15- and 7-fold increases in expression of Ins1/2 and GLUT2 in cultured islets harvested from mice after 6 weeks of oral pioglitazone therapy. These islets also showed increased altered epigenetic modifications, mRNA, and protein levels. Although there are few reports on the effect of antidiabetic therapy on epigenetic markers, literature suggests that with the progression of diabetes, the epigenetic changes are also progressive, although the changes are slowed with the use of antidiabetic therapy. The study data clearly indicate that the stroke effect in the Akita mice was significantly enhanced compared with the stroke effect in the WT mice. The results show strong evidence that epigenetics and neuro-glio-vascular markers are drastically changed in diabetic IR\textsuperscript{Akita} mice and contribute to the disease pathology. However, additional studies in this direction are warranted. On the basis of the results obtained, we have proposed a hypothesis that explains the severity in diabetic stroke (Fig. 7).

In summary, our study provides a novel insight in understanding the mechanistic basis of IR injury severity in T1D by comparing with nondiabetic injury. Through the current report we observed differential regulations of epigenetic markers and neuro-glio-vascular components that explain the reasons of IR severity in subjects with diabetes. The involvement of intense inflammation, severe inflammation and BBB damage, pronounced cell death, vascular dysfunction, glial loss, neurodegeneration, and severe BBB dysfunction.

![Figure 7](image_url)

**Figure 7** — The proposed hypothesis for diabetic stroke severity is shown. Stroke during T1D causes altered epigenetic markers and neuro-glio-vascular dysfunction that amplify the severity of the stroke.
potential markers associated with epigenetic alterations, and high vascular MMP-9 activation in diabetes profoundly intensify the injury outcomes. These findings pave the way for further studies that might be helpful in developing better preventive and therapeutic approaches for diabetic stroke.

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References

1. Borch-Johnsen K. The prognosis of insulin-dependent diabetes mellitus. An epidemiological approach. Dan Med Bull 1989;36:336–348
2. Dorman JS, Laporte RE, Kuller LH, et al. The Pittsburgh insulin-dependent diabetes mellitus (IDDM) morbidity and mortality study. Mortality results. Diabetes 1984;33:271–276
3. Hägg S, Thorn LM, Putsala J, et al.; FinnDiane Study Group. Incidence of stroke according to presence of diabetic nephropathy and severe diabetic retinopathy in patients with type 1 diabetes. Diabetes Care 2013;36:4140–4146
4. Miau F, Chen Z, Genueth S, et al.; DCCT/EDIC Research Group. Evaluating the role of epigenetic histone modifications in the metabolic memory of type 1 diabetes. Diabetes 2014;63:1748–1762
5. Reddy MA, Zhang E, Natarajan R. Epigenetic mechanisms in diabetic complications and metabolic memory. Diabetologia 2015;58:443–455
6. Chopra P, Papale LA, White AT, et al. Array-based assay detects genome-wide 5-mC and 5-hmC in the brains of humans, non-human primates, and mice. BMC Genomics 2014;15:131
7. Khare T, Pai S, Koncivicz K, et al. 5-hmC in the brain is abundant in synaptic genes and shows differences at the exon-intron boundary. Nat Struct Mol Biol 2012;19:1037–1043
8. Zikovic BV. The blood-brain barrier in health and chronic neurodegenerative disorders. Neuron 2008;57:178–201
9. Nitta T, Hata M, Gotoh S, et al. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. J Cell Biol 2003;161:653–660
10. Kalani A, Kumat PK, Familtseva A, et al. Role of microRNA29b in blood-brain barrier dysfunction during hyperhomocysteinemia: an epigenetic mechanism. J Cereb Blood Flow Metab 2014;34:1212–1222
11. Chiang T, Messing RO, Chou WH. Mouse model of middle cerebral artery occlusion. J Vis Exp 2011:2761
12. Lee S, Hong Y, Park S, Lee SR, Chang KT, Hong Y. Comparison of surgical methods of transient middle cerebral artery occlusion between rats and mice. J Vet Med Science 2014;76:1555–1561
13. Tyagi N, Oshidize N, Munjal C, et al. Tetrahydrocurcumin ameliorates homocysteinylated cytochrome c-mediated autophagy in hyperhomocysteinemia mice after cerebral ischemia. J Mol Neurosci 2012;47:128–138
14. Belaiev L, Alonso OF, Busto R, Zhao W, Ginsberg MD. Middle cerebral artery occlusion in the rat by intraluminal suture. Neurological and pathological evaluation of an improved model. Stroke 1996;27:1616–1622; discussion 1623
15. Eady TN, Khoutorova L, Anzola DV, et al. Acute treatment with docosahexaenoic acid complexed to albumin reduces injury after a permanent focal cerebral ischemia in rats. PLoS One 2013;8:e77237
16. Kalani A, Kumat PK, Givvimani S, et al. Nutri-epigenetics ameliorates blood-brain barrier damage and neurodegeneration in hyperhomocysteinemia: role of folic acid. J Mol Neurosci 2014;52:202–215
17. Yoshioka M, Kayo T, Ikeda T, Koizumi A. A novel locus, Mody4, distal to D7Mit189 on chromosome 7 determines early-onset NIDDM in nonobese C57BL/6 (Akita) mutant mice. Diabetes 1997;46:887–894
18. Givimmani S, Sen U, Tyagi N, Munjal C, Tyagi SC. X-ray imaging of differential vascular density in MMP-9–/–, PAR-1–/–, hyperhomocysteinemic (CBS–/–) and diabetic (Ins2–/–) mice. Arch Physiol Biochem 2011;117:1–7
19. Toyoshima M, Asakawa A, Fujiyama M, et al. Dimorphic gene expression patterns of anorexigenic and orexigenic peptides in hypothalamus account male and female hyperphagia in Akita type 1 diabetic mice. Biochem Biophys Res Commun 2007;352:703–708
20. Ueno N, Inui A, Karas PS, Karla SP. Leptin transgene expression in the hypothalamus enhances euglycemia in diabetic, insulin-deficient nonobese Akita mice and leptin-deficient obese ob/ob mice. Peptides 2006;27:2332–2342
21. Yaguchi M, Nagashima K, Izumi T, Okamoto K. Neuropathological study of C57BL/6Akita mouse, type 2 diabetic model: enhanced expression of alphaB-crystallin in oligodendrocytes. Neuropathology 2003;23:44–50
22. Cheeri C, Hewitt K, Durkin J, Simard CJ, Renaud JM, Messer C. Longitudinal evaluation of memory performance and peripheral neuropathy in the Ins2C96Y Akita mice. Behav Brain Res 2005;157:31–38
23. Chavali V, Tyagi SC, Mishra PK. Differential expression of dicer, miRNAs, and inflammatory markers in diabetic Ins2–/– Akita hearts. Cell Biochem Biophys 2014;68:25–35
24. Tongs TK, Hurn PD, Traysman RJ, Sieber FE. Estrogen decreases infarct size after temporary focal ischemia in a genetic model of type 1 diabetes mellitus. Stroke 2000;31:2701–2706
25. de Falco FA, Sepe Visconti O, Gubicci F, Caruso G. Correlation between hyperglycemia and cerebral infarct size in patients with stroke. A clinical and X-ray computed tomography study in 104 patients. Schweiz Arch Neurol Psychiatr 1993;144:233–239
26. Pandi G, Nakka VP, Dharap A, Roopra A, Venuganti R. MicroRNA miR-29c down-regulation leading to de-repression of its target DNA methyltransferase 3a promotes ischemic brain damage. PLoS One 2013;8:e58039
27. Endres M, Fan G, Meisel A, Dimagli U, Jaenisch R. Effects of cerebral isch-eemia in mice lacking DNA methyltransferase 1 in post-mitotic neurons. Neuroropept 2001;12:3763–3766
28. Chouliaras L, Mastroeni D, Delvaux V, et al. Consistent decrease in global DNA methylation and hydroxymethylation in the hippocampus of Alzheimer’s disease patients. Neurobiol Aging 2013;34:2091–2099
29. Liyanage VR, Jarmasz JS, Murugeshan N, Del Bigio MR, Rastegar M, Davie JR. DNA modifications: function and applications in normal and disease states. Biology (Basel) 2014;3:670–723
30. Shao B, Bayraktutan U. Hyperglycemia promotes cerebral barrier dysfunction through activation of protein kinase C-b. Diabetes Obes Metab 2013;15:993–999
31. Mishra PK, Chavali V, Metreveli N, Tyagi SC. Ablation of MMP9 induces survival and differentiation of cardiac stem cells into cardiomyocytes in the heart of diabetics: a role of extracellular matrix. Can J Physiol Pharmacol 2012;90:353–360
32. Chaturvedi P, Kalani A, Medina I, Familtseva A, Tyagi SC. Cardiosome mediated regulation of MMP9 in diabetic heart: role of mir29b and mir455 in exercise. J Cell Mol Med 2015;19:2153–2161
33. Dai J, Vrensen GF, Schlingemann RO. Blood-brain barrier integrity is unaltered in human brain cortex with diabetes mellitus. Brain Res 2002;954:311–316
34. Starr JM, Wardlaw J, Ferguson K, MacLullich A, Deary IJ, Marshall I. Increased blood-brain barrier permeability in type II diabetes demonstrated by gadolinium magnetic resonance imaging. J Neurol Neurosurg Psychiatry 2003;74:70–76
35. Chen J, Cui X, Zacharek A, Cui Y, Roberts C, Chopp M. White matter damage and the effect of matrix metalloproteinases in type 2 diabetic mice after stroke. Stroke 2011;42:445–452
36. Cui X, Chopp M, Zacharek A, Ye X, Roberts C, Chen J. Angiopoietin/Tie2 pathway mediates type 2 diabetes induced vascular damage after cerebral stroke. Neurobiol Dis 2011;43:285–292
37. Love S. Oxidative stress in brain ischemia. Brain Pathol 1999;9:119–131
38. Samdani AF, Dawson TM, Dawson VL. Nitric oxide synthase in models of focal ischemia. Stroke 1997;28:1283–1288
39. Jing L, Mai L, Zhang JZ, et al. Diabetes inhibits cerebral ischemia-induced astrocyte activation - an observation in the cingulate cortex. Int J Biol Sci 2013;9:980–988
40. Muranyi M, Ding C, He Q, Lin Y, Li PA. Streptozotocin-induced diabetes causes astrocyte death after ischemia and reperfusion injury. Diabetes 2006;55:349–355
41. Jing L, He Q, Zhang JZ, Li PA. Temporal profile of astrocytes and changes of oligodendrocyte-based myelin following middle cerebral artery occlusion in diabetic and non-diabetic rats. Int J Biol Sci 2013;9:190–199
42. Chang AS, Dale AN, Moley KH. Maternal diabetes adversely affects pre-ovulatory oocyte maturation, development, and granulosa cell apoptosis. Endocrinology 2005;146:2445–2453
43. Won SJ, Kim JH, Yoo BH, et al. Prevention of hypoglycemia-induced neuronal death by minocycline. J Neuroinflammation 2012;9:225
44. Liu F, Schafer DP, McCullough LD. TTC, fluoro-Jade B and NeuN staining confirm evolving phases of infarction induced by middle cerebral artery occlusion. J Neurosci Methods 2009;179:1–8
45. Huttner HB, Bergmann O, Salehpour M, et al. The age and genomic integrity of neurons after cortical stroke in humans. Nat Neurosci 2014;17:801–803
46. Bharosay A, Bharosay W, Varma M, Saxena K, Sodani A, Saxena R. Correlation of brain biomarker neuron specific enolase (NSE) with degree of disability and neurological worsening in cerebrovascular stroke. Indian J Clin Biochem 2012;27:186–190
47. Kim BJ, Kim YJ, Ahn SH, et al. The second elevation of neuron-specific enolase peak after ischemic stroke is associated with hemorrhagic transformation. J Stroke Cerebrovasc Dis 2014;23:2437–2443
48. Sandhu HS, Butt AN, Powrie J, Swaminathan R. Measurement of circulating neuron-specific enolase mRNA in diabetes mellitus. Ann N Y Acad Sci 2008;1137:258–263
49. Marumo T, Yagi S, Kawarazaki W, et al. Diabetes induces aberrant DNA methylation in the proximal tubules of the kidney. J Am Soc Nephrol 2015;26:2388–2397
50. Ishikawa K, Tsunekawa S, Ikeniwa M, et al. Long-term pancreatic beta cell exposure to high levels of glucose but not palmitate induces DNA methylation within the insulin gene promoter and represses transcriptional activity. PLoS One 2015;10:e0115350
51. Evans-Molina C, Robbins RD, Kono T, et al. Peroxisome proliferator-activated receptor gamma activation restores islet function in diabetic mice through reduction of endoplasmic reticulum stress and maintenance of euchromatin structure. Mol Cell Biol 2009;29:2053–2067