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Inhibition of Toll-Like Receptor-4 (TLR-4) Improves Neurobehavioral Outcomes After Acute Ischemic Stroke in Diabetic Rats: Possible Role of Vascular Endothelial TLR-4

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Abstract

Diabetes increases the risk of occurrence and poor functional recovery after ischemic stroke injury. Previously, we have demonstrated greater hemorrhagic transformation (HT), edema, and more severe functional deficits after stroke in diabetic animals that also presented with cerebral vasoregression and endothelial cell death in the recovery period. Given that Toll-like receptor 4 (TLR-4) activation in microvascular endothelial cells triggers a robust inflammatory response, we hypothesized that inhibition of TLR-4 signaling prevents endothelial cell death and improves outcomes after stroke. Animals were treated with vehicle or TLR-4 inhibitor TAK242 (3 mg/kg; i.p.) following middle cerebral artery occlusion (MCAO). Neurobehavioral deficits were measured at baseline and day 3 after ischemic stroke. Primary brain microvascular endothelial cells (BMVECs) from diabetic animals were subjected to oxygen glucose deprivation re-oxygenation (OGDR) and treated with 0.1 mM iron(III)sulfate hydrate (iron) (to mimic the post-stroke bleeding) and TLR-4 inhibitors. Ischemic stroke increased the expression of TLR-4 in both hemispheres and in the microvasculature of diabetic animals. Cerebral infarct, edema, HT, and functional deficits were greater in diabetic compared to control animals. Inhibition of TLR-4 significantly reduced the neurovascular injury and improved functional outcomes. OGDR and iron reduced the cell viability and increased the expression of TLR-4 associated proteins (RIP3, MyD88, phospho-NF-kB, and release of IL-6) in BMVECs from diabetic animals. In conclusion, TLR-4 is highly upregulated in the microvasculature and that beneficial effects of TLR-4 inhibition are more profound in diabetes. This suggests that inhibition of vascular TLR-4 may provide therapeutic benefits for stroke recovery in diabetes.

Keywords Diabetes · Stroke · Hemorrhagic transformation · Neurovascular injury · Brain vascular endothelial cells · TLR-4 · Inflammation

Introduction

While mortality from acute ischemic stroke declined in the past decade, stroke remains a leading cause of adult disability with limited treatment options [1]. Diabetes, a major comorbidity for stroke, contributes to stroke-related disability by not only increasing the risk of having a stroke but also impairing the recovery process [2]. Understanding the mechanisms by which diabetes worsens recovery is likely to lead to new therapeutic targets and strategies.

It is well established that there is a rapid angiogenic response following ischemic stroke, which is believed to contribute to neurovascular repair and improvement of functional outcomes [3–5]. Our recent studies showed that while control animals are able to promote this so-called reparative angiogenesis, diabetic animals undergo cerebral rarefaction after stroke and these animals fail to improve their neurological deficits [6]. A follow-up study showed that increased endothelial cell death as a result of excessive free radical formation contributes to this finding [7]. It is also well known that diabetes augments secondary bleeding into the brain (hemorrhagic transformation (HT)), and this is associated with poor

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outcomes. Although the mechanisms by which bleeding influences functional recovery are not known, it has been shown that free iron is deposited into the parenchymal brain tissue in intracerebral hemorrhage (ICH) models and it is neurotoxic [8, 9]. Iron can induce oxidative stress, but its role in endothelial cell death after ischemic stroke injury in diabetes remains unclear.

Activation of Toll-like receptors (TLRs) in brain cells including microglia, neurons, astrocytes, and endothelial cells mediates innate immune responses upon ischemic injury [10]. TLR-4 signaling has been reported to contribute to acute neuronal injury following ischemia as well as to poor recovery [11, 12]. Moreover, treatment of diabetic animals with tissue plasminogen activator (tPA) increased the brain hemorrhage, failed to improve functional outcomes, and this was accompanied with increased TLR-4 expression [13]. Furthermore, clinical findings in patients with ICH agree with experimental reports showing that TLR-4 is associated with poor functional outcomes and greater residual volumes [14]. TLR-4 activation in microvascular endothelial cells triggers a robust inflammatory response [15]. A recent study demonstrated that inhibition of TLR-4 signaling reduces neuronal cell apoptosis and improves neurological outcomes in diabetic animals [16]. However, the role of TLR-4 in amplified vascular injury in diabetes remains unexplored. Understanding the role and mechanisms by which TLR-4 activation impacts vascular integrity may identify novel targets for vasoneuronal protection in diabetes. Accordingly, this study was designed to test the hypotheses that (1) inhibition of TLR-4 reduces HT and improves functional recovery after ischemic stroke in diabetes, and (2) extracellular iron contributes to vascular endothelial cell death in a TLR-4-dependent manner.

Materials and Methods

Animals All ischemic stroke experiments were performed on 13-week-old male Wistar rats ($n = 6$–8 in each group) procured from Envigo, Indianapolis, IN. Animals were housed at the Augusta University animal care facility that is approved by the American Association for Accreditation of Laboratory Animal Care. The study was conducted in accordance with the National Institutes of Health guidelines for the care and use of animals in research, and all protocols were approved by the institutional animal care and use committee. Animals were fed standard rat chow (4% fat content) or high-fat diet (45% fat content) and tap water ad libitum, and were maintained at 12-h light/dark cycle.

Induction of Diabetes Animals were procured at 4 weeks of age and were immediately put on a high-fat diet (45% fat; D12451, Research Diets, New Brunswick, NJ). A low dose of streptozotocin (30 mg/kg body weight; STZ; Cayman Chemicals, Ann Arbor, MI) was injected intraperitoneally at 6 weeks of age. Animals were monitored for 7 weeks. Blood glucose levels were measured before noon (10 am–noon) twice a week from tail vein samples using a commercially available glucometer (Freestyle, Abbott Diabetes Care, Inc.; Alameda, CA). Animals were not fasted for this measurement. Control (vehicle treated) and diabetic animals did not show any difference in weight gain throughout the experimental period. However, blood glucose levels significantly increased within 3 days of STZ injection in the diabetic group and remained significantly elevated compared to control animals. Representative blood glucose measurements just prior to initiation of stroke surgery are shown on Table 1.

Stroke Surgery Animals were subjected to cerebral ischemia at 13 weeks of age using a nylon suture as previously described [17]. All surgeries were performed before noon. Briefly, 60 min of MCAO was performed under 2% isoflurane anesthesia. A midline cervical incision was made to expose common, external, and internal carotid arteries. A rounded tip 3-0 monofilament nylon suture was inserted into the external carotid artery and advanced into the internal carotid artery to occlude the origin of the MCA. The occlusion suture was secured with a silk suture at the external carotid artery. After 60 min of occlusion, the suture was gently removed to allow reperfusion. Laser Doppler imaging with a scanning laser Doppler (PIM3, Perimed; North Royalton, OH) was used to confirm successful occlusion and ensure similar levels of blood flow reduction in all groups. With the exception of control+TAK242 group ($n = 5$), other groups included 10 rats/group.

In Vivo TLR-4 Inhibition TAK242 (cat. no. 614316, Calbiochem), a cell-permeable cyclohexenecarboxylate was used to inhibit the TLR-4. It inhibits the interaction of TLR-4 with adaptor molecules TIRAP and TRAM via direct binding to the TLR4 intracellular Cys747 residue. Animals were

Table 1 Measure of body weight (gm) and blood glucose (mg/dl) level of control and diabetic animals before stroke surgery

	Control ($n = 10$)	Control+TAK242 ($n = 5$)	Diabetic ($n = 10$)	Diabetic + TAK242 ($n = 9$)
Body weight (g)	416 ± 24.7	386 ± 17	394 ± 18.7	383 ± 20.0
Blood glucose (mg/dl)	87 ± 2.64	88 ± 1.73	312.8 ± 15.37#	346.8 ± 24.38*

*$p < 0.001$ vs control animals. Data expressed as mean ± SEM

injected with TAK242 (3 mg/kg; i.p.) just after reperfusion, 24 and 48 h after ischemic stroke surgery. Animals were randomly selected from control and diabetic groups for the treatment. Dose and route of administration of TLR-4 inhibitor TAK242 was determined based on previous reports in mice model of cerebral ischemic injury [10, 16]. Due to its low molecular weight and liposolubility, it can cross the blood-brain barrier (BBB) after intraperitoneal injections and a single dose of 3 mg/kg TAK242 can be detected in brain tissue, plasma up to 24 h. In order to maintain the continued inhibition of TLR-4, we repeated the treatment after 24 and 48 h after ischemic injury.

Infarct Size, Edema Ratio, HT, and Hemoglobin (Hb) Analysis All analyses were performed by an investigator blinded to experimental groups. After 72 h of ischemic stroke, animals were put into deep anesthesia using isoflurane, and intracardiac perfusion was performed with cold PBS to flush out the blood cells from cerebral vessels. Brains were isolated and sliced into seven coronal sections (A–G) of 2-mm thickness. Infarct size was measured after 2,3,5-triphenyltetrazolium chloride (TTC) staining, and edema was calculated as percent increase in ischemic hemisphere vs. the contralateral hemisphere as previously described [17]. Macroscopic HT was measured in sections B to E using a four-point rubric and total score for each animal was reported [18]. Hemoglobin content was measured in brain homogenates with Quanti-Chrom Hemoglobin (Hb) Assay (BioAssay Systems, Hayward, CA) [19]. It was reported as excess Hb (μgHb/mg total protein) in ischemic hemisphere. In some of the animals that were subjected to stroke surgery, brains were used to isolate microvessels. Hence, neurovascular injury measurements included $n = 5$/group.

Evaluation of Neurobehavioral and Functional Outcomes Neurobehavioral tests were assessed, recorded, and scored at baseline before ischemic stroke surgery, day 1 and day 3 after ischemic stroke surgery in a blinded fashion [20]. Briefly, prior to behavior testing, animals were handled for 5–7 days to acclimatize for testing room. Bederson's score was obtained by using multiple parameters which include circling bias (no circling as 2, partial circling as 1, and continuous circling as 0), hind limb retraction (score 2 for healthy and score 1 or 0 according to animals ability to pull hind limb), and forelimb flexion score (score 2 for both forearm movement and score 1 or 0 according to animals ability to move arm). The resistance to push is also measured and scored as 1 or 0, depending on whether the animal is able to resist the push or not. Maximum score of 7 is given to a normal rat in above tests. Beam walk scores were obtained from beam walking ability and graded from 7 to 0 (7 as normal walking and 0 as inability to balance on beam). Total composite score was presented as sum of Bederson's score and beam walk score (maximum 14). Adhesive removal test (ART) was used to assess fine sensorimotor functions. Contact and removal latency of an adhesive paper dot was recorded on day 1 and day 3 after MCAO. For each day, the average was taken from three trials with a maximum removal latency of 180 s per trial. With the exception of control+TAK242 group ($n = 5$), other groups included $n = 9$–10/group.

Cerebral Microvessel Isolation Microvessels were isolated with slight modifications as previously described [21]. Briefly, animals were anesthetized and decapitated, and brain tissue was removed from the skull. Meninges and choroid plexuses were removed from brain tissue, and cerebral hemispheres were dissected out. The hemispheres were cut into small pieces using dissection scissors in a Petri dish. Then tissue is homogenized in a fivefold volume of phosphate-buffered saline (PBS) and centrifuged at 4000 rpm for 10 min at 4 °C. Supernatant was discarded, and the pellet was resuspended in 15 ml of PBS and mixed with an equal volume of 30% dextran, and centrifuged for 10 min at 4000 rpm at 4 °C. The top myelin containing dense white layer is removed. The pellet was resuspended in PBS and passed through a 100-μm mesh. The filtrate was collected by washing the mesh with PBS in a 50-ml tube followed by centrifugation for 10 min at 3000 rpm at 4 °C, and the resulting pellet was resuspended in 0.2 ml of RIPA buffer in a 1.5-ml centrifuge tube. Each tube was sonicated using a 3-s pulse three times on ice. Tubes were again centrifuged at 6000 rpm for 10 min at 4 °C. Supernatant was collected and prepared for protein estimation and Western blot analysis.

Oxygen Glucose Deprivation Reoxygenation Study (OGDR) Brain microvascular endothelial cells (BMVECs) isolated from spontaneously diabetic male Goto-Kakizaki (GK) rats [6, 7] were used. This was based on previous findings that (1) there is pathological cerebral neovascularization in these animals; (2) BMVECs isolated form GK rats have greater angiogenic properties (like tube formation and cell migration) compared to normal control rats under normal conditions reflecting diabetic phenotype; (3) VEGF mediates an angiogenic response in BMVECs from diabetic GK rats in peroxynitrite-dependent manner [6], whereas hypoxia/reoxygenation promotes peroxynitrite-mediated apoptosis in these cells [7]; (4) when an ischemic injury is overlayed on this pathology, animals develop greater hemorrhagic transformation (HT); (5) when animals that develop HT are followed for 14 days after stroke, there is significant loss of cerebral vascularization [5, 20]; and (6) while GK rats represent a lean model of diabetes, db/db obese mice model of type 2 develops similar cerebrovascular pathology [22]. When cells (passage 6–8) reached 80–90% confluency, media was replaced with glucose-free 1% serum-containing media and then placed in a hypoxic chamber (0.2% O_2, 5% CO_2) for 6 h, followed by 12 h of normoxia (21% O_2, 5% CO_2, and normal glucose) to

mimic ischemia and reperfusion injury. Some plates were treated with 0.1 mM iron(III)sulfate hydrate (iron; cat. no. 518212, Sigma Aldrich) at reoxygenation to mimic bleeding and free iron release after stroke in diabetes [23]. To inhibit TLR-4 activity, 10 μg/ml TLR-4 neutralizing antibody (SC 13591, Santa Cruz Biotechnology) or 30 μM TAK242 was added upon reoxygenation. One set of plates was treated with antioxidant *N*-acetyl cysteine (NAC, 1 mM).

Immunocytochemistry GK BMVECs grown on slides were subjected to OGDR followed by fixation with 4% paraformaldehyde for 15 min, and subsequently washed with TBS followed by treatment with 0.2% Triton X-100 for 3 min. After washing, cells were blocked by 5% BSA for 1 h at room temperature. Cells were then incubated with anti-TLR-4) antibody at a 1:100 dilution in 0.2% BSA for 3 h at room temperature. Cells were washed and incubated with AlexaFlour 488 conjugated secondary antibody (anti-rabbit; Jackson Immuno Research Laboratories, Inc., West Grove, PA) at a 1:400 dilution at room temperature for 1 h. Negative control slides were incubated with 0.2% BSA in place of the primary antibody. Slides were imaged on Axiovert 200 microscope (Carl Zeiss MicroImaging, Thornwood, NY).

Membrane Permeability Assay BMVEC permeability was determined by measuring FITC-labeled dextran (Sigma-Aldrich) across the monolayer as described [24]. Briefly, cells were grown on gelatin-coated transwell membranes of 24-well plate (filter area, 0.33 cm^2, pore diameter size, 0.4 μm, Corning Costar, Cambridge, MA, USA). Monolayer confluent BMVEC media was replaced with glucose-free 1% serum-containing media and then placed in a hypoxic chamber (0.2% O_2, 5% CO_2) for 6 h, followed by 12 h of normoxia (21% O_2, 5% CO_2, and normal glucose) to mimic ischemia and reperfusion injury. TLR-4 inhibitor TAK242 (30 μM) and FITC-dextran 500,000-conjugate (0.5 mg/ml) was added to the luminal chamber at reoxygenation. Samples (100 μl media) were collected from abluminal chamber (at time 0, 1, 3, 6, 9, and 12 h) and transferred to 96-well plate for the fluorescence measurement on plate reader (Synergy HT; Bio-Tek) with an excitation wavelength of 490 nm and an emission wavelength of 520 nm. The readings were presented as relative fluorescence unit (FLU).

Cell Viability Assay Cell viability and proliferation were measured by MTT cell proliferation assay kit (Vybrant MTT cell proliferation assay kit, Thermo Fisher). BMVECs grown in 96-well plate were subjected to OGDR and respective treatments. Tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) was added in each well resulting intracellular purple formazan that was solubilized, and optical density was measured at 570 nm on a spectrophotometer.

Cell Migration Assay Cell migration assay (wound healing) was performed as described before [25]. Briefly, BMVECs were grown to confluence on a 12-well plate. Monolayer was wounded with a single sterile cell scraper of fixed diameter. Images of wounded areas were taken immediately before 6 h of OGD and after 30 h of reoxygenation. Cell migration was calculated by measuring migration distance normalized to initial distance of the wound using AxioObserver Zeiss Microscope software, and data are expressed as the percentage of untreated control cells.

Western Blot Analysis Briefly, equivalent amounts of cell lysates of brain tissue/microvessels/BMVECs (20 μg protein/lane) were loaded onto 10% SDS-PAGE, proteins separated, and proteins transferred to nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin followed by incubation for 12 h at 4 °C with primary antibody anti-claudin-5 (35–2500, Invitrogen), anti-occludin (71–1500, Invitrogen), anti-TLR-4 (ab22048, Abcam), anti-RIP3 (MABE28,EMD Millipore), anti-MyD88 (4283, Cell Signaling Tech.), or anti-pNFkB(S536, Cell signaling Tech.) at 1:1000 dilution or anti β-actin at 1:3000 dilution. After washing, membranes were incubated for 1 h at 20 °C with appropriate secondary antibodies (horseradish peroxidase [HRP]-conjugated; dilution 1:3000). Prestained molecular weight markers were run in parallel to identify the molecular weight of proteins of interest. For chemiluminescent detection, the membranes were treated with enhanced chemiluminescent reagent and the signals were monitored on Alpha Imager (Alpha Innotech; San Leandro, CA). Relative band intensity was determined by densitometry software (Alpha Innotech, ProteinSimple, San Jose, CA) and normalized with β-actin protein.

ELISA IL-6 BMVECs from diabetic animals subjected to OGDR secreted IL-6 in the media, and it was measured using commercially available ELISA kit (ab10072, Abcam) following the manufacturer's instruction. Results are expressed in pg/ml media.

Statistical and Power Analyses Power analyses were conducted prior to in vivo treatment experiments and were based on the preliminary data for TLR-4 expression in control and diabetic animal brain samples (control 0.7 ± 0.2 and diabetic 1.1 ± 0.2) and predicted functional outcome composite score in control vs. diabetic animals. We hypothesized that TRL-4 inhibition will have no effect on control and will improve (increase the score) in diabetic rats to control levels. A sample size of six animals per group was predicted to provide 80% power to detect a significant difference. Two-way ANOVA (2 × 2 design) was used to assess disease and treatment effects in control and diabetic rats. For behavioral outcomes, repeated measures ANOVA (D0, D1, and D3) was performed across groups. A Bonferroni's post-test adjustment for multiple comparisons was

used for all post hoc mean comparisons for significant effects from all analyses. For TLR-4 expression in the brain homogenates, one-way ANOVA was used to compare the data followed by a Tukey's post hoc comparison. Effect of OGDR and TAK242 on permeability, claudin-5 and occludin expression was analyzed by two-way ANOVA. Data are expressed as mean ± SEM, and $p < 0.05$ was considered significant.

Results

Ischemic Stroke in Diabetes Increases TLR-4 Protein in Brain Parenchyma and Isolated Microvessels

TLR-4 protein in the brain was measured by two approaches. First set of experiments showed that TLR-4 protein is higher in total brain homogenates of both ischemic and non-ischemic hemispheres of diabetic animals compared to sham or control groups (Fig. 1a). In the second set of experiments, isolated microvessels were used which also showed a significant increase in TLR-4 expression in both hemispheres of diabetic animals (Fig.1b).

The Effect of In Vivo TLR-4 Inhibition on Neurovascular Injury

All measures of neurovascular injury (infarct size, edema, excess Hb, and HT) were greater in diabetic animals. There was a disease and treatment interaction such that TLR-4 inhibition reduced infarct size and excess hemoglobin only in diabetic animals (Fig. 2a, d). Treatment was effective in reducing edema in both control and diabetic groups.

The Effect of TLR-4 Inhibition on Neurobehavioral Outcomes

Ischemic stroke reduced the composite scores in both control and diabetic groups; however, it was more pronounced in diabetic animals (Fig. 3a). Inhibition of TLR-4 had no effect on adhesive removal ability of control animals. TLR-4 inhibition in diabetic animals improved the deficit in fine motor skills (Fig. 3a, b).

The Effect of TLR-4 Inhibition on BMVEC Membrane Permeability and Tight Junction Proteins

Immunofluorescence images showed increased TLR4 expression under OGDR conditions (Fig. 4a). Membrane permeability of BMVECs was significantly increased with OGDR (Fig. 4b). There was a trend for reduced permeability with the inhibition of TLR-4 signaling ($p = 0.0848$). After OGDR, expression of tight junction proteins occludin and claudin-5 was reduced in diabetic BMEVCs. Interestingly, inhibition of TLR-4 had differential effects under normoxic and hypoxic conditions: TAK242 lowered both protein levels in normoxia but prevented the hypoxia-induced decreases in OGDR (Fig. 4c).

The Effect of TLR-4 Inhibition on BMVEC Viability and Migration

To determine the effects of TLR-4 activation under hypoxic conditions, we measured cell viability and migration after OGDR and in combination with iron treatment in the presence and absence TLR-4 inhibition and antioxidant treatment. Hypoxia-mediated decrease in cell survival was prevented by

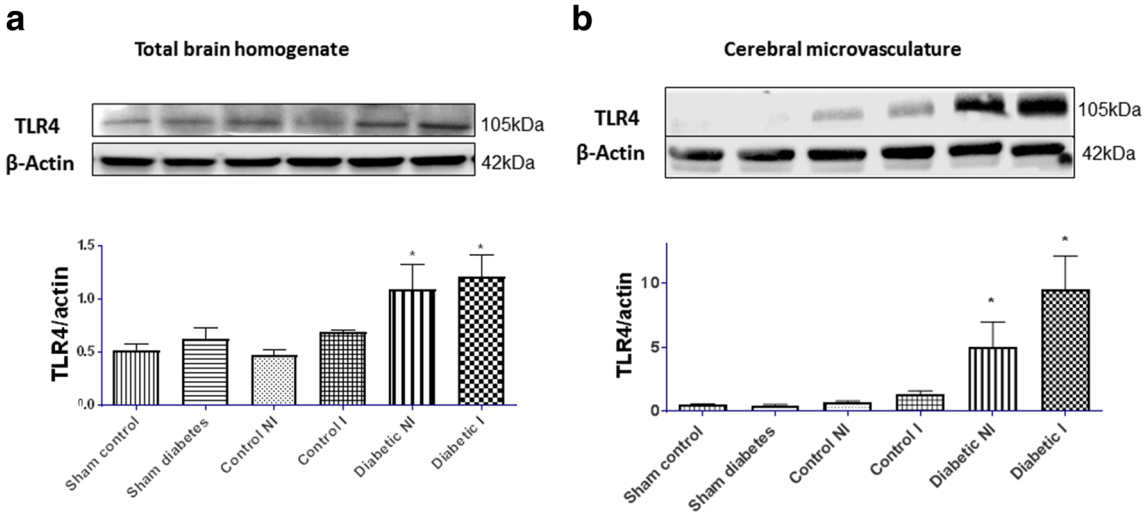

Fig. 1 Diabetes increases expression of TLR-4 in total brain homogenate and cerebral microvasculature after ischemic stroke. Control and diabetic rats were subjected to 60 min of MCAO or sham surgery and sacrificed after 72 h of reperfusion. **a**, **b** Representative Western blot images and measurement of TLR-4 protein expression in total brain homogenate and brain microvessels, respectively. Level of TLR-4 protein was significantly higher in both ischemic (I) and non-ischemic (NI) hemisphere of diabetic animals; *$p < 0.05$, compared with sham or control ($n = 4$)

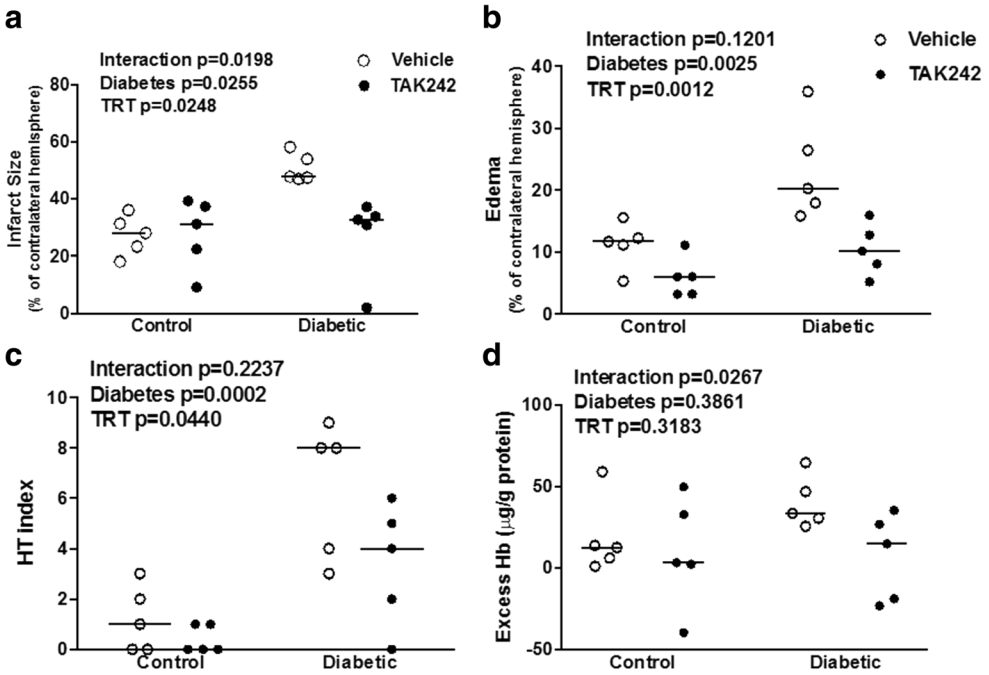

Fig. 2 Inhibition of TLR-4 reduces the neurovascular injury in diabetic animals subjected to ischemic stroke. Control and diabetic rats were subjected to 60 min of MCAO and treated with vehicle or TLR-4 inhibitor TAK-242 (3 mg/kg; i.p.) at reperfusion, 24 and 48 h after reperfusion ($n = 5$). After 72 h of reperfusion, animals were sacrificed to measure the neurovascular injury. **a** Diabetic animals had larger infarct size and treatment with TAK242 reduced infarct size only in the diabetic animals as indicated by the interaction. **b** Edema was greater in diabetic animals and treatment reduced edema in both control and diabetic animals. **c** Hemorrhagic transformation (HT) index was significantly higher compared to control animals. There was a treatment effect in both groups. **d** Diabetic animals showed excess Hb in ischemic hemisphere and inhibition of TLR-4 significantly reduced it only in diabetic animals

TLR-4 neutralizing antibody or antioxidant NAC treatment (Fig. 5a). Measurement of cell migration is a measure of wound healing ability of cells. Inhibition of TLR-4 or NAC prevented the decrease mediated by OGDR+Fe treatment (Fig. 5b).

TLR-4 Signaling in BMVECs of Diabetic Animals

TLR-4 activation triggers downstream activation of RIP3 and MyD88. Thus, we measured the expression of RIP3 and MyD88 in OGDR in combination with iron. The notable increase in RIP3 expression was reduced with the inhibition of TLR-4 (Fig. 6a). MyD88 levels were increased in OGDR and OGDR+iron groups (Fig. 6b).

NFkB is a major transcription factor that regulates genes associated with innate and adaptive immune responses. OGDR resulted in an increase in the expression of phospho-NFkB and iron treatment did not further increase NFkB phosphorylation. TLR-4 inhibition reduced its expression to

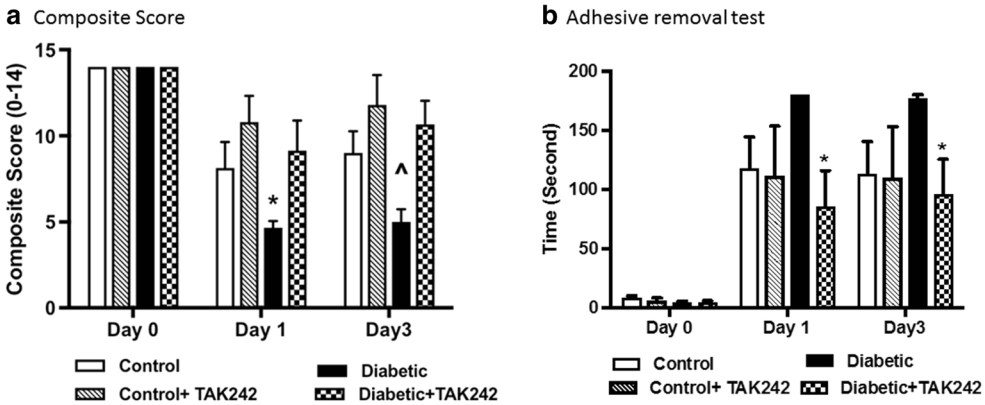

Fig. 3 Inhibition of TLR-4 improves the functional outcomes after ischemic stroke. **a** Composite score measured at day 1 after ischemic stroke injury was significantly lower in diabetic animals (*$p < 0.05$ vs. TAK242-treated control or diabetic animals; $n = 5$ control+TAK242, $n = 10$ control and diabetic, $n = 9$ diabetic+TAK242). On day 3, diabetic animals did not show any recovery (^$p < 0.05$ vs. all other groups). **b** Adhesive removal time was significantly reduced only in diabetic TAK242-treated animals (*$p < 0.05$ vs. diabetic)

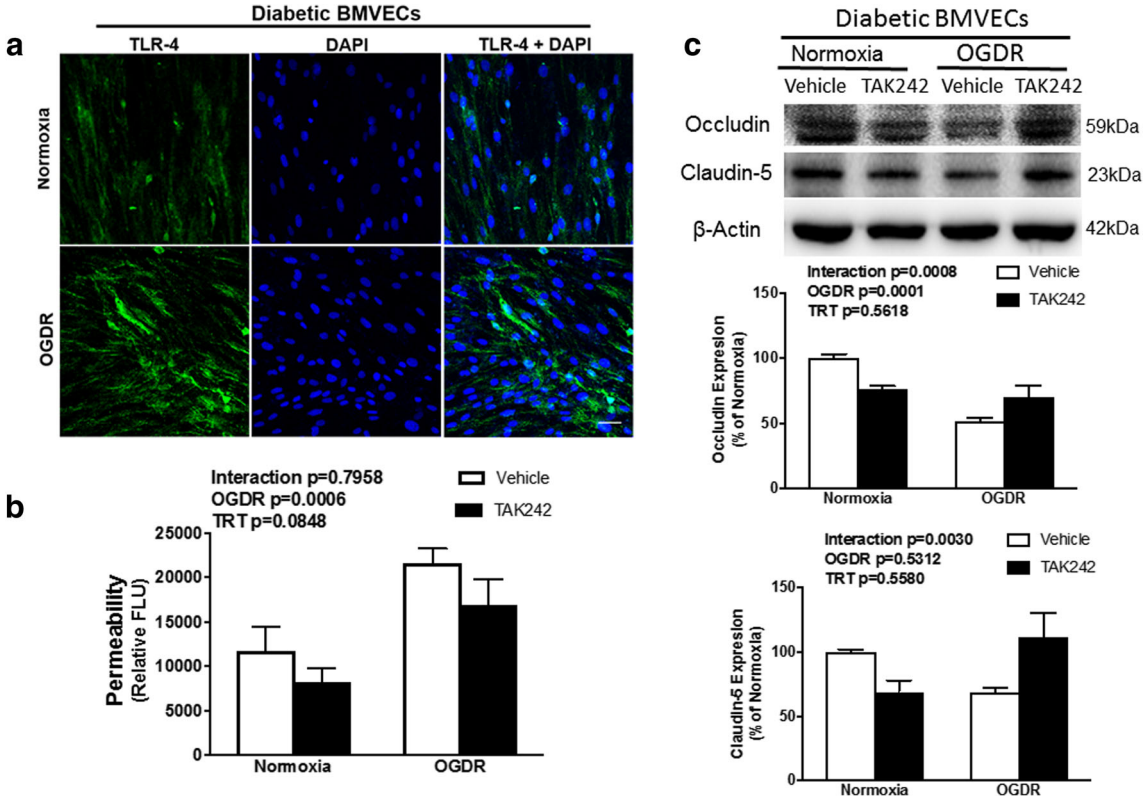

Fig. 4 OGDR increased the expression of TLR-4 in BMVECs of diabetic animals. **a** Representative immunofluorescent images of TLR-4 (green), nuclei stained with DAPI (blue) and merged images in BMVECs exposed to 6 h OGD and 12 h of reoxygenation. There was no positive staining when primary antibody was omitted (not shown). Scale bar is 50 μm. **b** OGDR increased the membrane permeability and reduced the expression of tight junction proteins in BMVECs. **c** Representative images of immunoblots for occludin and claudin-5 after 6 h OGD and 12 h of reoxygenation in BMVECs isolated from diabetic GK rats. Treatment with TLR-4 inhibitor (TAK242; 30 μM) reduced tight junction proteins under normoxic conditions but increased expression of both occludin and claudin-5 under OGDR conditions indicated by the interaction ($n = 4–5$)

control normoxia levels (Fig. 7a). IL-6 levels, a major pro-inflammatory cytokine, were increased by OGDR compared to normoxia. Addition of iron in OGDR condition did not potentiate the secretion of IL-6; however, IL-6 levels remained

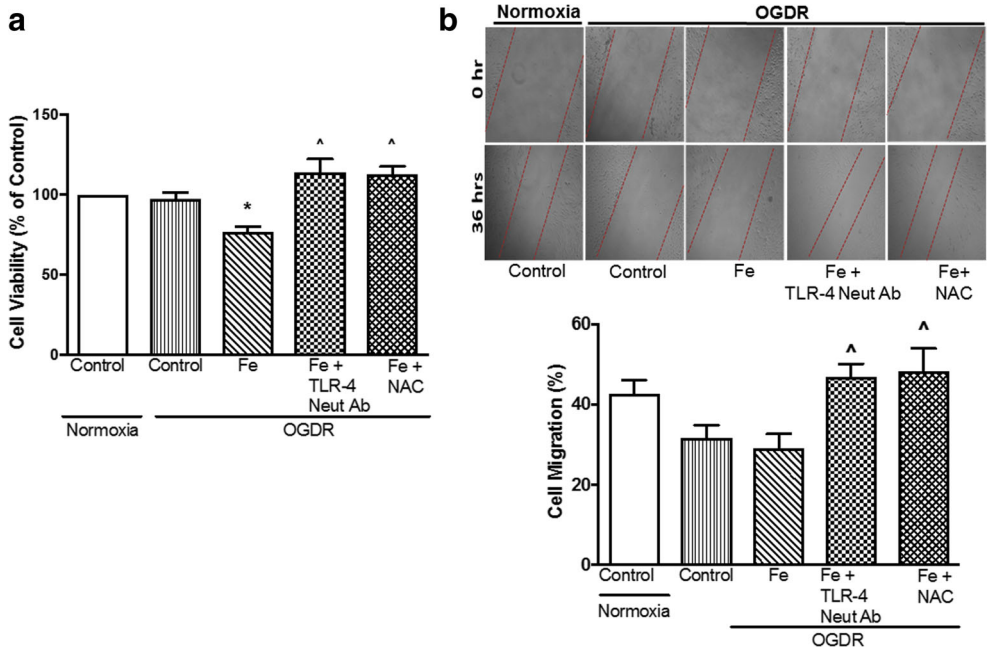

Fig. 5 Inhibition of TLR-4 improved cell viability and cell migration of BMVECs of diabetic rats upon OGDR and iron treatment. **a** Cell viability was measured after 6 h OGD and 12 h of reoxygenation and iron treatment in BMVECs isolated from diabetic GK rats. Cells were treated with iron(III)sulfate (Fe; 0.1 mM) and TLR-4 neutralizing antibody (TLR4 Neu Ab;10 μg/ml) or *N*-acetyl cysteine (NAC; 1 mM). **b** Representative image and measurement of cell migration after 6 h OGD and 30 h of reoxygenation and iron treatment in BMVECs. Inhibition of TLR-4 or oxidative stress with NAC increased the viability and cell migration on OGDR and addition of iron. (*$p < 0.05$ vs. control, ^$p < 0.05$ vs. OGRD+Fe; $n = 4$)

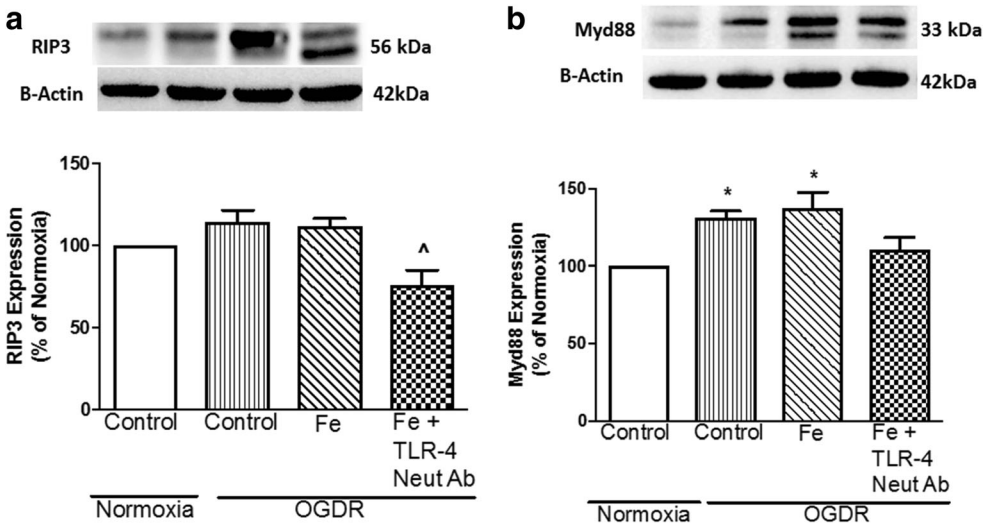

Fig. 6 Inhibition of TLR-4 reduces expression of RIP3 and Myd88 proteins of upon OGDR and iron treatment in BMVECs of diabetic rats. Representative image and measurement of RIP3 (**a**) and Myd88 (**b**) expression after 6 h of OGD followed by 12 h of reoxygenation in BMVECs. Cells were treated with iron(III)sulfate (Fe; 0.1 mM) and TLR-4 neutralizing antibody (TLR4 Neu Ab;10 μg/ml). Addition of TLR-4 neutralizing antibody significantly reduced the expression of downstream proteins RIP3 and Myd88 after OGDR and addition of iron (^$p < 0.05$ vs. OGRD+Fe; *$p < 0.05$ vs. control; $n = 4$)

significantly higher compared to normoxia. TLR-4 inhibition reduced IL-6 levels, but it was significantly different only with NAC treatment (Fig. 7b).

Discussion

Acute ischemic stroke is a debilitating disease. Diabetes is one of the most commonly found comorbidities associated with poor stroke outcomes, but underlying reasons why recovery is further compromised in diabetes is not known. Number of studies have shown TLR-4 signaling could mediate inflammatory responses in cerebral ischemia and plays important role in cell proliferation, differentiation, and apoptosis [12, 16, 26].The current study was designed to explore the role of TLR-4 in worsened vascular injury after stroke in diabetes [5] and focused on endothelial cell survival in conditions that mimic neurovascular injury in diabetic stroke. Our novel findings show that (1) ischemic stroke increases the expression of TLR-4 not only in the brain but also in the microvasculature of diabetic animals; (2) TLR-4 inhibition

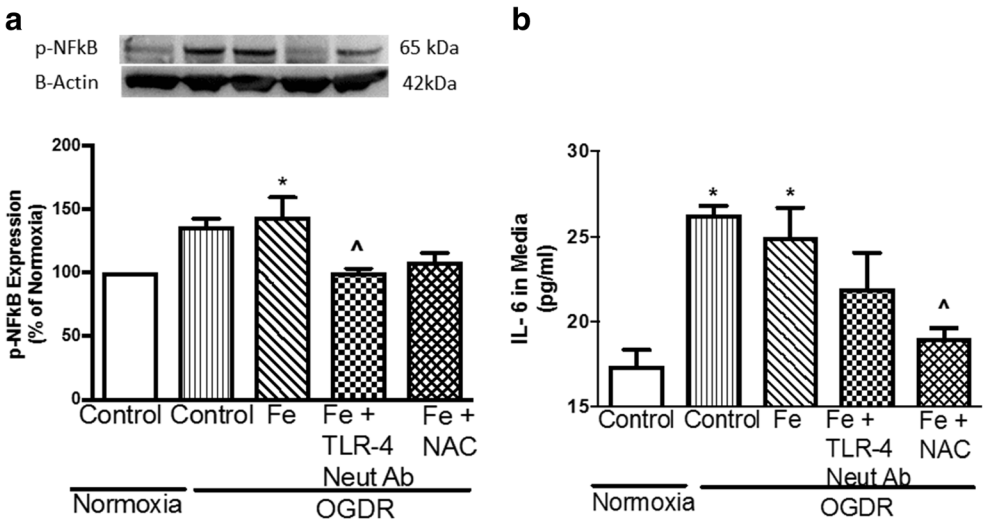

Fig. 7 Inhibition of TLR-4 reduced the expression of NF-κB in cell lysate and secretion of IL-6 in media upon OGDR and iron treatment in BMVECs of diabetic rats. **a** Representative image and measurement of NF-κB and **b** measurement of IL-6 in collected media after 6 h of OGD and 12 h of reoxygenation and iron treatment in BMVECs isolated from diabetic GK rats. Cells were treated with iron(III)sulfate (Fe; 0.1 mM) and TLR-4 neutralizing antibody (TLR4 Neu Ab;10 μg/ml) or *N*-acetyl cysteine (NAC; 1 mM). Inhibition of TLR-4 (TLR-4 neut Ab) and oxidative stress (NAC treatment) both reduced the NF-κB expression (*$p < 0.05$ vs. control, ^$p < 0.05$ vs. OGDR+Fe; $n = 4$)

after reperfusion prevents amplified neurovascular injury (greater infarct size, HT, and excess Hb) and improves functional outcomes in diabetes, and (3) inhibition of TLR-4 restores membrane permeability while increasing expression of tight junction proteins, cell survival, and angiogenic potential of BMVECs isolated from diabetic animals.

Clinical and experimental studies have demonstrated that ischemia reperfusion injury in diabetes results in greater bleeding into the brain and worsened outcomes compared with non-diabetics [17, 27–29]. Functional recovery after stroke is also impeded in diabetes but underlying reasons are not fully understood. As recently highlighted by American Heart Association [30], cerebrovascular health is crucial for brain health. In addition to providing constant blood flow to the brain, small vessels of the brain forms BBB and provide neurotrophic functions by secreting growth factors necessary for survival within the neurovascular unit [31]. Thus, in the current study, we focus on the microvascular injury and integrity in diabetes. Our studies showed that TLR-4 protein is not only increased in total brain homogenates but also in the microvessels isolated from diabetic animals after stroke. While microvessel preparation includes multiple cell types including endothelial cells, vascular smooth muscle cells, and possibly embedded pericytes, in vitro BMVEC studies provide additional information on critical role of endothelial cells. A recent study reported that inhibition of TLR-4 signaling by TAK242 prevents neuronal apoptosis and improves outcomes in diabetic rats [16]. Our results at the brain, vascular tissue, and endothelial cell levels enhance the significance of this past study and show that TLR-4 inhibition also provides acute vascular protection by reducing edema and bleeding while improving short-term functional outcome after stroke in diabetes.

Amplified HT in diabetes may also influence the restorative and regenerative processes within the neurovascular networks. Toxic effects of extravasated blood components including red blood cells (RBCs), coagulation factors, complement components, and immune globulins can increase the severity of stroke injury [8, 32, 33]. Hemin, the oxidative form of heme released from RBCs exerts its toxic effects via release of excessive iron, depletion of glutathione and production of free radicals in ICH models [9]. TLR-4 is activated by many endogenous ligands such as heme and fibrinogen [34], which are produced in brain after ischemic injury. However, there is paucity of information about the effect of free iron in vascular injury, particularly endothelial cell death after ischemic stroke in diabetes. Microvascular endothelial cells have stronger inflammatory responses and show greater levels of TLR-4 and CD14 expression than macrovascular endothelial cells [15]. Our group has previously reported that there is a greater loss of angiogenic properties in BMVECs isolated from diabetic animals after OGDR [7]. Thus, in an effort to address the role of TLR-4 in vascular repair after an ischemic event, we used BMVECs isolated from diabetic animals. To mimic the ischemia-reperfusion injury and release of free iron, cells were exposed to OGD and iron was added during reoxygenation. We observed that OGDR increased TLR-4 expression and membrane permeability and this was accompanied with decreased expression of tight junction proteins (occludin and claudin-5), whereas inhibition of TLR-4 prevented this effect. Thus, it can be speculated that the vasoprotection achieved with TLR-4 inhibition in vivo could be due to improved BBB integrity. In the current study, we used in vivo TLR-4 inhibition starting immediately after reperfusion. While additional in vivo studies are needed to better understand the role of iron-induced TLR-4 signaling in compromised neurovascular repair and functional recovery in diabetes, improvement of viability and migration ability of BMVECs with the inhibition of TLR-4 may have long-term effects as well. TLR-4 activation triggers the RIP3 kinase-dependent programmed necrosis that occurs through either TIR domain-containing adapter-inducing interferon-β (TRIF) or MyD88 signal transduction. It has been demonstrated in murine sickle cell disease model that heme triggers TLR-4 signaling leading to endothelial cell activation and vaso-occlusion, and it requires redox-active iron to mediate these processes [35, 36]. Heme released during hemolysis activates TLR-4 signaling in endothelial cells leading to release of Weibel-Palade body degranulation and activation of NF-κB. This can further activate NADPH oxidase-mediated production of reactive oxygen species, interleukins, and cytokines [36]. We also observed the activation of NF-κB with hypoxia and iron treatment, while inhibition of TLR-4 and oxidative stress with NAC blocked its activation and prevented cytokine release.

Transmembrane pattern recognition receptors, such as TLRs, play an important role in the induction and regulation of immune/inflammatory responses [37, 38]. TLR-4 is expressed by variety of cell types including immune cells and vascular cells [39], but most TLR-4 studies focused on immune cells such as monocytes, macrophages, and lymphocytes. Indeed, information related to the role of TLR-4 in the inflammatory response by vascular cells, in particular BMVECs, is relatively scarce. The current study provides novel information that ischemic stroke in diabetes increases the expression of TLR-4 in both neuronal and vascular tissue and its inhibition can improve the neurobehavioral outcomes. Our results also suggest that endothelial TLR-4 inhibition has therapeutic potential by providing vascular protection and restoration in diabetes.

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