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Original Article

TRB3 modulates microglial/macrophages polarization via down-regulating PPAR-gamma in rats suffering from diabetic cerebral ischemia

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Abstract: Objective: To investigate the role of TRB3 in the activation of microglia/macrophages during cerebral ischemia. Methods: Fifty-four SD rats were divided into sham group, CI (focal cerebral ischemia) group and CI+DM (diabetes mellitus) group. Type 2 diabetes was modeled by high fat diet and intraperitoneal injection of 60 mg/kg of streptozotocin before cerebral ischemia. Focal cerebral ischemia was achieved by 60 minutes of transient middle cerebral artery occlusion (MCAO). Twenty-four hours after ischemia, brain infarct area was measured after TTC staining. The ratio of cell apoptosis and M1/M2 microglia/macrophages of the 3 groups was also detected by flow cytometry within 24 hours after ischemia. Western blot was applied to detect the expression of TRB3 and PPAR-γ in ischemic portions of the brain tissue as well as the TRB3 knock-down or overexpressing CI+DM cell lines; RT-PCR was adopted for the examination of mRNA expression of IL-6, iNOS (markers for M1 type), IL-10, and arginase-1 (markers for M2 type) in the TRB3 knock-down CI+DM cell lines. In the meanwhile, the CI+DM rats also treated with PPAR-γ agonist (PGZ, 10 mg/kg) or inhibitor (GW9662, 0.3 mg/kg) after the modeling. Results: Compared with sham group and CI group, TTC staining revealed that diabetes mellitus combined with cerebral ischemic damage significantly induced the infarct volume and increased cell apoptosis. Compared with the CI group, the protein level of TRB3 and the ratio of M1/M2 microglia/macrophages in the brain tissue significantly increased in the CI+DM group. When knocking-down or overexpressing TRB3 in cells from the infarct tissues of CI+DM group, the ratio of apoptosis-positive cells and M1/M2 microglia/macrophages were significantly reduced while PPAR-γ protein expression level significantly increased. After treated with PGZ (10 mg/kg) and GW9662 (0.3 mg/kg), compared with the CI+DM group, the ratio of M1/M2 microglia/macrophages was significantly decreased in CI+DM+PGZ but obviously increased in CI+DM+GW9662 group. Conclusion: TRB3 could regulate microglial/macrophages polarization via down-regulated PPAR-gamma in rats with diabetic cerebral ischemia.

Keywords: TRB3, PPAR-gamma, microglial/macrophages polarization, diabetes mellitus, cerebral ischemic damage, apoptosis

Introduction

DM is a complex metabolic disorder characterized with chronic hyperglycemia, and the vascular complication is the main cause of disability [1]. Macrovascular complications in patients with diabetes mellitus refer to the aorta, coronary artery, brain basal arteries, renal artery and peripheral artery atherosclerosis [2]. The prevalence of atherosclerosis, coronary heart disease and cerebrovascular disease in patients with diabetes are 4-5 times higher than non-diabetic patients and 70-80% of patients with diabetes die of macrovascular complications [3, 4]. Studies also showed that 67% of patients with diabetes were complicated with acute stroke, suggesting that the risk of stroke in patients with diabetes is significantly increased [5]. Diabetes concurrent with ischemic injury will lead to increased neuronal damage and poor functional recovery [6]. Thus, it is of importance to explore the mechanisms of ischemic brain injury under diabetic conditions and develop more effective therapies for neuroprotection for this disease.

PPAR-γ (peroxisome proliferator-activated receptor gamma), is a nuclear receptor that plays a vital role in the regulation of macrophage polarization [7]. Microglia/macrophages can be activated into different phenotypes, for example, on the one hand microglia/macrophages can be activated into the M1 type after cerebral ischemia initiating an immune cascade reaction and producing the inflammatory cytokines and reactive oxygen species; on the other hand, microglia/macrophages can be activated into the M2 type, which is involved in damage repair in the inflammatory response [8-10].

Tribbles were first discovered in Drosophila embryogenesis regulation test [11], which contain three family members, TRB1, TRB2 and TRB3. Among them, TRB3 is the one that was studied most clearly. TRB3 could modify the activation of various intracellular signaling pathways, such as mitogen-activated protein kinases (MAPKs) controlling fundamental processes including mitosis, cell activation, apoptosis and modulation of gene expression [12].

In addition, TRB3 targeted at human chromosome 20p13-p12.2, and gene of type 2 diabetes is also located in this area, so we had the hypothesis that TRB3 might have a natural link with diabetes [13, 14]. Studies showed that TRB3 could negatively regulate the PPAR-γ signal transduction pathway to promote insulin resistance [15-17]. Therefore, we suspected that TRB3/PPAR-γ might be a key signal pathway involving in the vicious circle between diabetes and ischemic brain injury by regulating the polarization of microglia/macrophages.

In this study, we explored the interaction between ischemic brain injury combined with diabetes and microglia/macrophages polarization. And we assessed the role of TRB3 in the activation of microglia/macrophages on cerebral ischemia under diabetes conditions.

Methods

Main reagents

DMEM culture medium, fetal bovine serum, trypsin, penicillin and streptomycin were purchased from Gibco (USA); Annexin V-FITC/PI kit was purchased from Yeasen (USA); Trizol and reagents for RT-qPCR analysis was purchased from Invitrogen (CA); antiTRB3, PPAR-γ, caspase-3 antibodies were from Shanghai Xuan Ling Biotech Company.

Animal model

All animal experiments were approved by local authorities and performed in accordance with international guidelines.

Focal cerebral ischemia was induced by 60 minutes of transient middle cerebral artery occlusion (MCAO). Rats were anticoagulated by oral administration of phenprocoumon. Type 2 diabetic rat model was established by given high fat diet and intraperitoneal injection of streptozotocin at the dose of 60 mg/kg, after 72 hours, if the tail blood glucose level was over 16.6 mmol/L, the diabetic rat model was built successfully.

Experimental design

First of all, we built CI (cerebral ischemia group) and CI+DM (cerebral ischemia + diabetes mellitus) rat models and set the sham operation group (n=18, respectively).

The brain tissue from the sham group (n=12), CI group (n=12) and CI+DM group (n=12) were taken out after modeling for histological analysis, cell culture and other molecular examinations.

After modeling, we performed TTC staining to observe and evaluate the infarct volume, adopted flow cytometry to detect and compare the ratio of apoptosis in the infarct tissues of sham group and other two modeling groups and to detect the primary M1 (iNOS) and M2 (CD206) polarization markers, in adherence-enriched microglia/macrophages from three groups [18]. Meanwhile, we also performed western blot to analyze the expression level of TRB3 in ischemic portions of the brain tissues.

After that, we transfected the TRB3-siRNA or TRB3-mRNA to cells from brain tissues in CI+DM group and performed western blot analysis to check the TRB3 expression level in the vehicle group (CI+DM cells), TRB3-siRNA group and TRB3-mRNA group. As the TBR3 was knocked down successfully, flow cytometry was performed again to see the ratio of cell apoptosis in the TRB3-siRNA group and TRB3-mRNA group and RT-PCR was adopted to analyzed

Table 1. The primer pairs in this study

Primer	Oligonucleotide sequences (5'-3')	Size (bp)
IL-6	CTGGTGACAACCACGGCCTTCCCTA	600
	Reverse: 5'-TCTGAGGTGCCCATGCTACAT -3'	
	ATG CTT AGG CAT AAC GCA CTA GGT T	
iNOS	ATGACCAGTATAAGGCAAGC	367
	GCTCTGGATGAGCCTATATTG	
IL-10	ACCTGGTAGAAGTGATGCCCCAGGCACTA	237
	TGCAGTTGATGAAGATGTCAAA	
Arginase-1	CAGAAGAATGGAAGAGTCAG	276
	CAGATATGCAGGGAGTCACC-	
GAPDH	TGAAGGTCGGAGTCAACGGATTTGGT	493
	CATGTGGGCCATGAGGTCCACCAC	

Note: IL-6 and iNOS are markers for M1 type macrophage, while IL-10 and arginase-1 are markers for M2 type macrophage; GAPDH was the internal reference.

mRNA expression of IL-6, iNOS (markers for M1 type) and IL-10, arginase-1 (markers for M2 type), to evaluate the macrophage polarization status. Meanwhile, we also detected the PPAR-γ and caspase protein levels in the vehicle group, TRB3-siRNA group and TRB3-mRNA group to explore the relationship between them.

As for other rat models, sham group (n=6), CI group (n=6) and CI+DM group(n=6), after the modeling, the rats were treated with the PPAR-γ agonist pioglitazone (PGZ, 10 mg/kg) or inhibitor GW9662 (0.3 mg/kg) every 24 hours until they were scarified. Then the brain tissues were taken for RT-PCR analysis of mRNA expression of IL-6, iNOS, IL-10, arginase-1 to evaluate the microglia/macrophages polarization status.

Histological analysis

The brains were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned (4 mm thickness), and stained with 2,3,5-triphenyl tetrazolium chloride (TTC) for the analysis of infarct condition. After brain tissues were stained with TTC, the infarct area was evaluated using a morphological image analysis system.

FACS analysis

A flow-based Annexin V assay kit was used to measure cell apoptosis according to the manufacturer's instructions. Briefly, the cells were digested from the infarct tissues and were washed with PBS three times, then resuspended by 400μl of ANX-V binding buffer and stained by 5μl of Annexin-V-fluorescein isothiocyanate (FITC) for 15 min at 37°C in the incubator. Subsequently, the above suspensions were transferred to the FACS tube and add 10μl of propidium iodide (PI) into the tube and incubate for 5 min on ice in the dark surroundings. Then flow cytometry was performed (Becton Dickinson, Bedford, MA, USA).

Western blot analysis

The extracted total protein was quantified by BCA method. After polyacrylamide gel electrophoresis and transferred to the PVDF membrane, the primary antibody for TRB3 (1:100), PPAP-γ (1:500) or caspase-3 (1:500) was add for the incubation for 12 hours. After washed with 5% TBST, the membrane was blot by skimmed milk and incubated with horseradish peroxidase-conjugated anti-mouse antibody. Finally, the bind was visualized by ECL (Amersham Biosciences) and quantified by Image Pro Plus software 6.0 version.

Cell culture and transfection

The infarct brain tissues from CI+DM rats were cut to small pieces and digested for the short-term culture and later transfection experiments [19]. According to the previous study, rat TRB3-siRNA was design as 5'-GGCACAGAGUACACCUGCATT-3' and 5'-UGCAGGUGUUACUCUGUGCCTT-3' and the TBR3-mRNA was designed as 5'-AGGACAAGATGCGAGCTAC-3', 5'-CTGTTCACAGCACCTAGAGC-3'. The randomly mixed sequences 5'-UUCUCCGAA CGUGUCACGUTT-3' and 5'ACGUGACACGUUCGGAGAATT-3' was also transfect to the vehicle group as control [20, 21]. The transfection was adopted PLKO.1 vector and lentivirus and lipo2000 were applied as the transfection agencies. After 24 h and 48 h transfection, cells were collected to exam the transfect rate and stabled with puro.

Quantitative RT–PCR

Total RNA was extracted from tissues or cells using Trizol method following the manufacturer's protocol. All the primers were synthesized by Invitrogen (Invitrogen, Shanghai, China) and the sequences used were shown in **Table 1** and the design referred from other related literatures [22, 23]. The RT-qPCR Kit (Invitrogen, USA) was applied for the reverse transcription and quantitative PCR (RT-qPCR) on ABI 7500

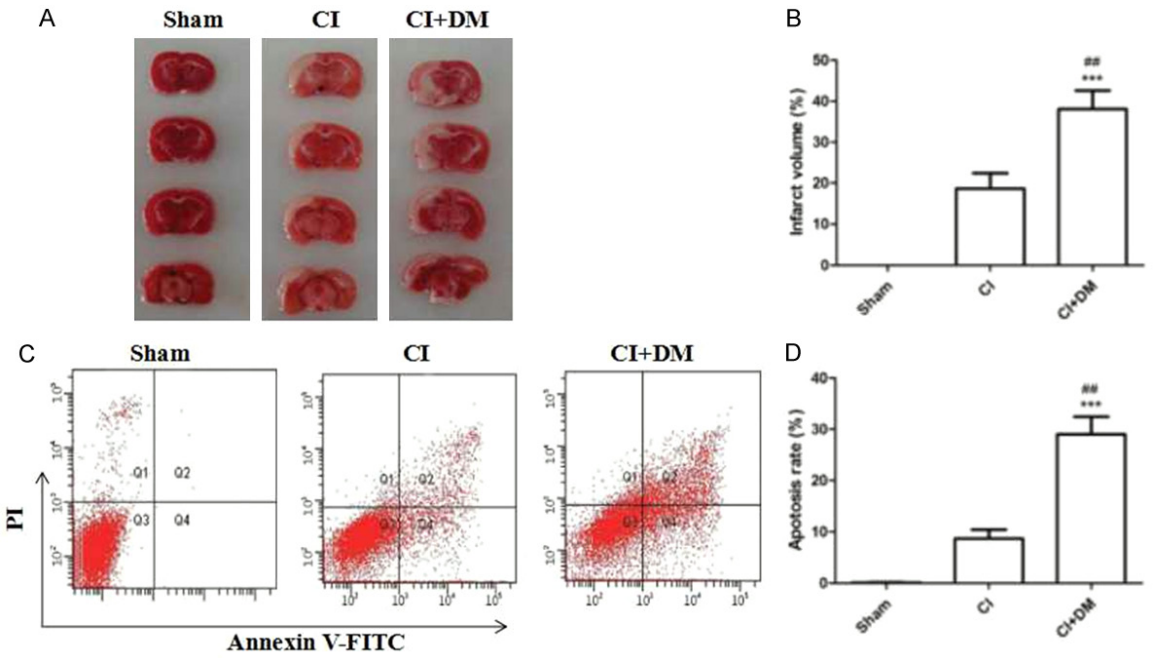

Figure 1. Hyperglycemia aggravated cerebral ischemic damage in rats. A. The coronal sections of each group were stained with TTC with red- and pale-colored regions indicating non-ischemic and ischemic portions of the brain, respectively. Evaluation of infarct area 24 h following reperfusion. B. After brain tissues were stained with TTC, the infarct area was evaluated using a morphological image analysis system; ***P<0.01, Vs Sham group; ##P>0.01, Vs CI group. C. The ratio of apoptosis in the cells of the 3 groups was detected by flow cytometry. D. Comparison of the ratio of apoptosis in the 3 groups; ***P<0.01 Vs Sham group; ##P<0.01 Vs CI group.

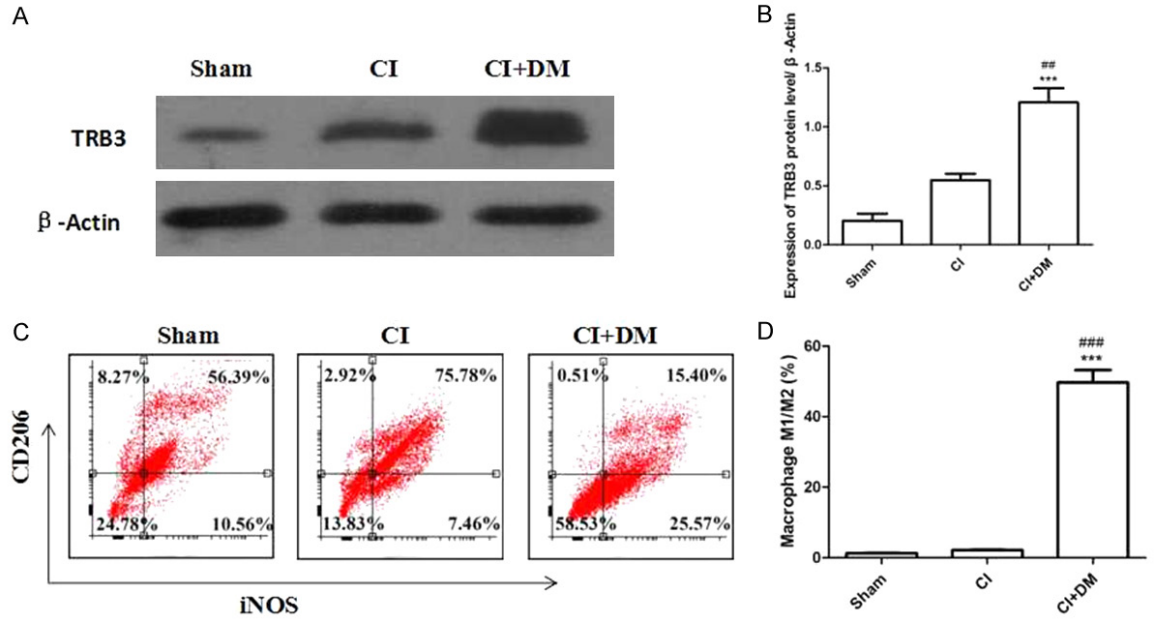

Figure 2. TRB3 expression was increased (A and B) and M1/M2 macrophage polarization (C and D) was changed in diabetic ischemic brain. (A) Western blot analysis of expression of TRB3 in ischemic portions of the brain tissue. (B) Analysis of TRB3 expression. Error bars represent standard deviations. ***P<0.01 Vs Sham group; ##P>0.01 Vs CI group. (C) The ratio of M1/M2 microglia/macrophages in the 3 groups was detected by flow cytometry as iNOS was the marker of M1 while CD206 was the marker of M2. (D) Comparison of the ratio of M1 and M2 microglia/macrophages between the 3 groups; ***P<0.01, Vs Sham group; ###P>0.001, Vs CI group.

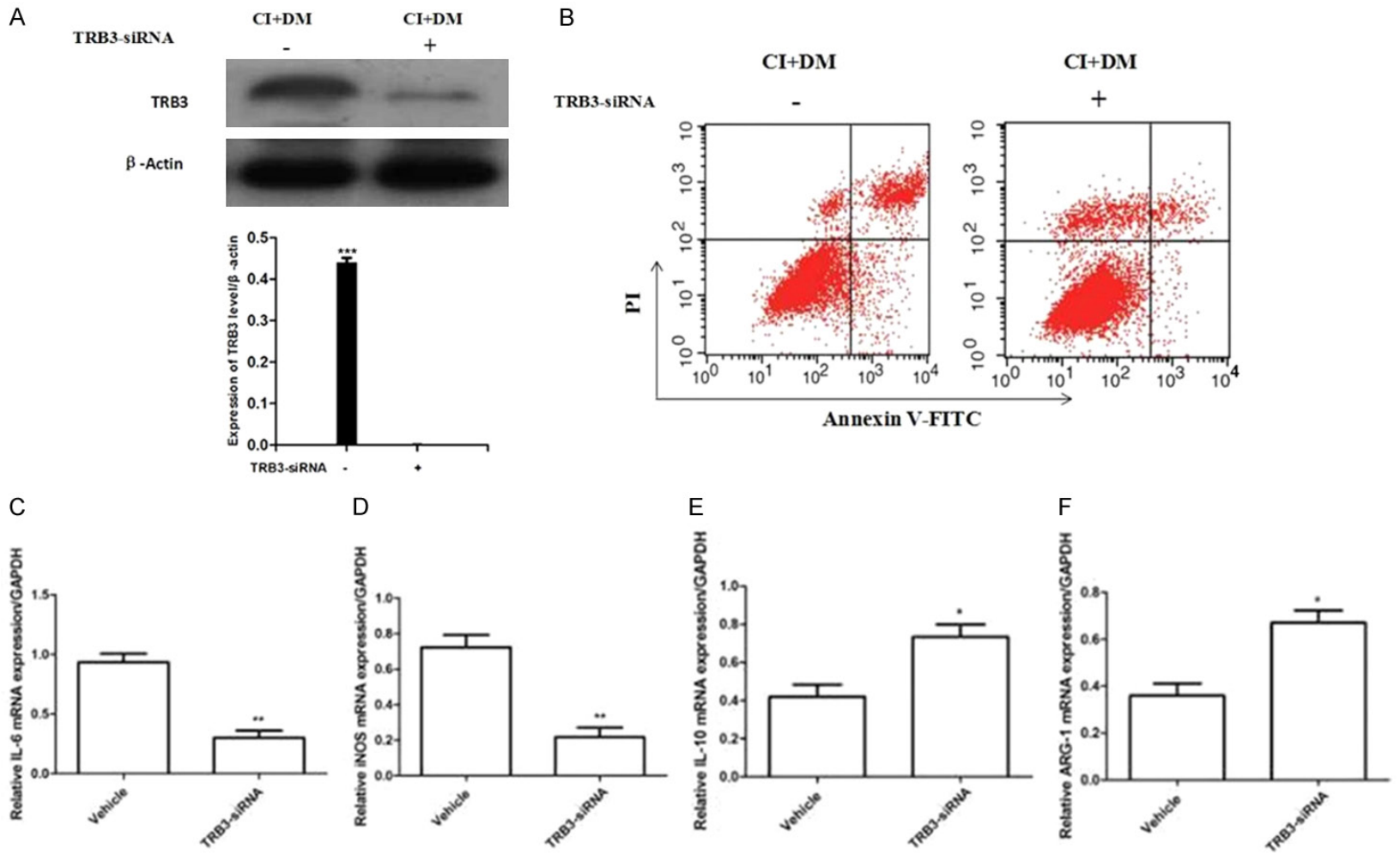

Figure 3. TRB3-siRNA reduced neuronal apoptosis and activity negates M1/M2 macrophage polarization. A. Western blot analysis of the expression of TRB3 in two group; error bars represent standard deviations, ***P<0.01. B. The ratio of apoptosis in the 2 groups was detected by flow cytometry. C-F. The expression level of IL-6, iNOS (these two are markers for M1 type macrophage), IL-10, arginase-1 (these two are markers for M2 type macrophage) mRNAs in the TRB3-siRNA transfected CI+DM cells via real-time PCR; *P<0.05 and **P<0.01, Vs vehicle group. IL-6, iNOS (markers for M1) and IL-10, arginase-1 (markers for M2).

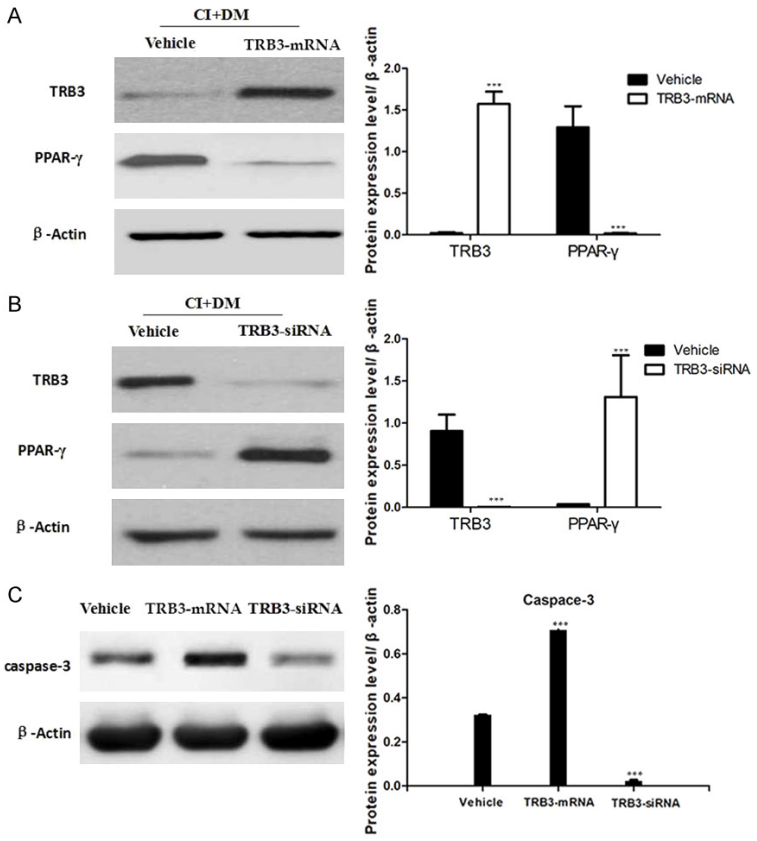

Figure 4. TRB3 decrease the expression level of PPAR-γ. (A&B) western blot analysis of expression of TRB3 and PPAR-γ in cells with overexpressed TRB3 (A) Or siRNA knockdown of TRB3 (B) Expression; ***P<0.01 Vs Vehicle group. (C) Western blot analysis of caspase-3 expression level in 2 transfected groups and the vehicle group; ***P<0.01 Vs Vehicle group.

Real Time PCR System (Applied Biosystems, USA). The internal reference was GAPDH and the relative expression level of gene was calculated using $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data in the figures are presented as means ± SEM and were analyzed by Student's t-test between two groups or by one-way ANOVA with post hoc Bonferroni test when three or more groups were compared; the correlation between protein expression levels was examined by Pearson's linear regression analysis. In all statistical comparisons, a *P* value <0.05 was considered statistically significant.

Result

Hyperglycemia aggravated cerebral ischemic damage in rats

At 24 h after reperfusion, 2,3,5-Triphenyltetrazolium chloride (TTC) staining revealed an extensive pale area of infarct in brain sections of the rats subjected to MCAO. Conversely, no infarct area was detected in the sham-operated animals, suggesting that the increased infarct volume was a result of the occlusion. And diabetes mellitus combined with cerebral ischemic damage significantly induced the infarct volume, compared to that in the rats only with CI damage (**Figure 1A**, **1B**).

In the FACS assay, a large number of apoptosis-positive cells was observed in the brain tissues from CI+DM rats. The number of apoptosis-positive cells was significantly reduced in the CI group, compared with the CI+DM group (**Figure 1C**, **1D**).

TRB3 increased brain damage and mediated M1/M2 macrophage alteration

The protein levels of TRB3 significantly increased in the CI+DM group compared with the CI group (**Figure 2A**, **2B**).

The primary M1 (iNOS) and M2 (CD206) polarization markers were evaluated in brain tissues from three groups via flow cytometry, showing that almost all the microglia/macrophages shifted to M1 type as iNOS expression increased and CD206 expression decreased comparing with the sham group and CI group (**Figure 2C**, **2D**).

TRB3-siRNA reduced neuronal apoptosis and activity negates M1/M2 macrophage polarization

As shown in **Figure 3A**, **3B**, compared with vehicle group, the protein level of TRB3 significantly decreased in the TRB3-siRNA group (P<0.01). In the flow cytometry, the number of apoptosis-positive cells was significantly reduced in the TRB3-siRNA group compared with the vehicle group (**Figure 3B**), meanwhile, the protein level of caspase-3 significantly decreased in TRB3-siRNA group but increased in the TRB3-Mrna

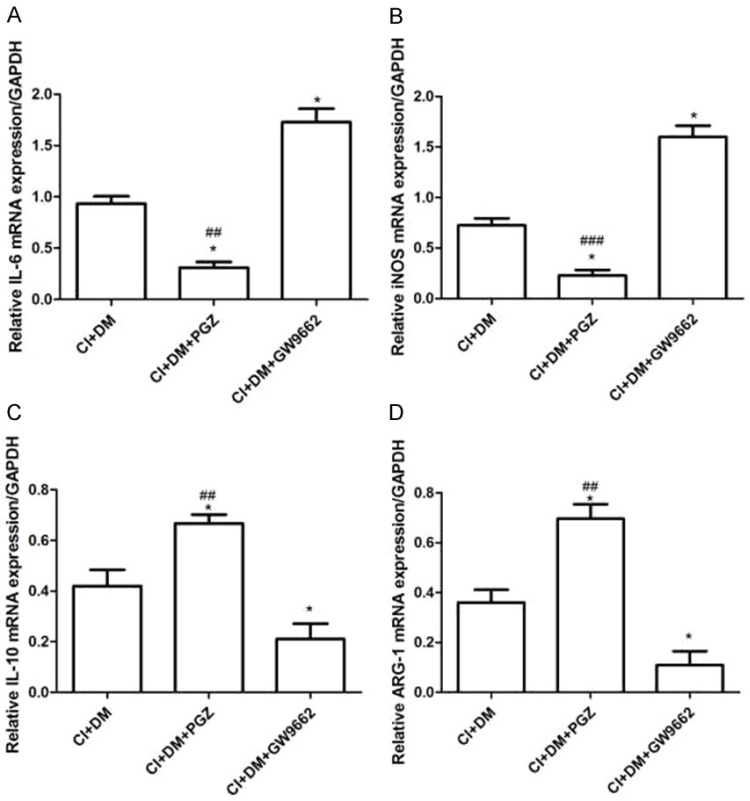

Figure 5. PPAR-γ mediated M1/M2 macrophage alteration. A-D. Real-time PCR analysis of the expression level of IL-6, iNOS, IL-10, arginase-1 mRNA in CI+DM rats treated with 10 mg/kg PGZ or 0.3 mg/kg GW9662; *P<0.01, Vs CI+DM group; ##P>0.01 and ###P<0.001, Vs CI+DM+GW9662 group.

group (**Figure 4C**), which was consist with the result of flow cytometry.

We also analyzed mRNA expression of IL-6, iNOS (markers for M1 type) and IL-10, arginase-1 (markers for M2 type) in the TRB3-siRNA group, respectively. As shown in **Figure 3C-F**, most of microglia/macrophages shifted to M2 type as IL-6 and iNOS expression decreased and IL-10, arginase-1 expression increased in the TRB3-siRNA group.

TRB3 decrease the expression level of PPAR-γ

As shown in **Figure 4A**, **4B**, TRB3 protein expression level was significantly increased in the TRB3-mRNA group and obviously decreased in the TRB3-siRNA group, compared with the vehicle group. Meanwhile, the relationship between the TRB3 and PPAR-γ expression level was also analyzed by Pearson's linear regression analysis, of which the result indicated that TRB3 protein expression level was conversely related with PPAR-γ expression (Pearson r=-0.8434, P=0.0084).

PPAR-γ mediated M1/M2 macrophage alteration

CI+DM rats treated with PGZ (10 mg/kg, an agonist of PPAR-γ), and GW9662 (0.3 mg/kg, an inhibitor of PPAR-γ). As **Figure 5A-D** showed, most of microglia/macrophages shifted to M2 type as IL-6 and iNOS expression decreased and IL-10, arginase-1 expression increased in the CI+DM+PGZ compared with the CI+DM group and CI+DM+GW9662 group.

Discussion

In this study, we found that in the CI+DM rat modeling group, the volume of ischemic portion was obviously larger and the ratio of apoptosis cells was elevated significantly compared with the sham group and the group with CI only, indicating that the DM will aggravated the cerebral ischemic damage in rats by increasing the apoptosis in never cells. Li et al. compared the apoptosis development in STZ induced DM rats and STZ induced DM rats with occlusion in the middle cerebral artery, and the results showed that the fragmented DNA and Bax protein expression level was evidently higher than the normal rats and the rats with DM only [24]. In addition, Zhao et al. also reported that DM had an enhancement effects on the apoptosis inducement in the rats with global cerebral ischemia/reperfusion [25]. Both of their findings were consisted with ours.

Currently, there were a lot of studies have reported that association between the TRB3 expression level and cell apoptosis. For example, Zhou et al. knocked down the expression of TRB3 in lung cancer cells and found that the apoptosis was induced, which might regulate by the down-regulation of Notch protein; Wang et al. found that proteinuria could notably induce renal tubule apoptosis, which would be alleviate after genetically inhibition of TRB3 [26, 27]. Therefore, we further explored the

expression of TRB3 in the brain tissues of rats and the results indicated that the brain damage degree was positively correlated with the TRB3 expression level, especially, the latter might play an important role in the procedure of ischemic brain damage. Additionally, the microglia/macrophages alternation was also close related with the cerebral ischemia, which may be another key factor to reveal the mechanisms of how the DM expands the ischemia brain damage [28]. Thus, we also detected the microglia/macrophages polarization of which the result indicated that almost all the microglia/macrophages showed in M1 type.

In order to further explore the inner relationship among microglia/macrophages polarization, TRB3 and diabetic cerebral ischemia, we transfected mRNA and siRNA to overexpress and knockdown the TRB3 expression level, and then we found that the ratio of apoptosis cells decreased and the microglia/macrophages polarization reversed to M2 type when the expression of TRB3 decreased, additionally, there was an opposite result when the expression of TRB3 increased. All of these finding suggested that the high expressed TRB3 might induce the apoptosis and M1-like microglia/macrophages increasing so as to enhance the brain damage after the rats cerebral ischemic injury.

The receptor and ligand of PPAR-γ can be activated for the combination with specific DNA response elements to regulate the transcription and translation of various genes, so as to get involved with many physiological and pathological processes [29]. In this study, the expression of PPAR-γ was conversely associated with TRB3 as PPAR-γ was notably decrease in the TRB3 overexpression group compared with the control group, and its expression was elevated significantly in the TRB3 knockdown group, which was consistent with other researchers' studies [30-32]. Additionally, when we applied PPAR-γ agonist or inhibitor to CI+DM rat models respectively, the results showed that compare with the rat model did not treat with medicine, the microglia/macrophages polarization showed more in M2 type in the CI+DM+PGZ group and more in M1 type in CI+DM+GW9662 group, indicating that activating the expression of PPAR-γ would decrease the ratio of M1-like microglia/macrophages. We suspected that the mechanism might be that on the one hand, PGZ, an insulin sensitizer, can improve the sensitivity of insulin directly to alleviate the insulin resistance of patients via inhibiting the excessive endoplasmic reticulum stress (ERS) by regulating the expression of JNK, caspase, CHOP, etc., which were pro-apoptotic factors activated by the persistent and severe ERS, to inhibit the apoptosis so as to alleviate the brain damage [33, 34]; on another hand, GW9662, a PPAR-γ inhibitor, can negatively control the differentiation of M2 type microglia/macrophages, therefore the ratio of M1 type microglia/macrophages is promoted and the inflammation of brain damaged tissue was worsened [35].

In summary, TRB3 could regulate microglial/macrophages polarization via down-regulating PPAR-γ in rats with diabetic cerebral ischemia, therefore, inhibiting the expression of TRB3 or M1 type microglial/macrophages polarization may be a new preventing and treating target for the patients with diabetic cerebral ischemia. However, as there are limitations of this study, such the unstable cell line, the research with enough clinical samples should be taken to verify the results.

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Disclosure of conflict of interest

None.

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