European Review for Medical and Pharmacological Sciences 2020; 24: 6446-6454

Tizoxanide mitigates inflammatory response in LPS-induced neuroinflammation in microglia *via* restraining p38/MAPK pathway

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Abstract. – OBJECTIVE: Traumatic brain injury (TBI) induced neuroinflammation is featured as excessive glial inflammatory activation and violent neurologic destruction and dysfunction. Massive microglia activation in situ and disrupt of blood-brain barrier contribute to severely collapsed nervous system. Tizoxanide (TIZ), a synthetic thiazolide derivative agent possessing a broad-spectrum anti-infective effect, currently shows a potential resistance against pathogens like bacteria, virus and parasites, while its underlying role in neuroinflammation is elusive. The study aimed to explore the effect of TIZ on neuroinflammation *in vitro* microglia.

MATERIALS AND METHODS: Primary microglia were accepted to neuroinflammatory activation via lipopolysaccharide (LPS) administration. TIZ was conducted to pretreatment of microglia. Cell viability, inflammatory cytokines, chemotaxis, nitric oxide release, inflammation-related enzymes, and mitogen-activated protein kinase (MAPK) pathway activation in microglia were investigated respectively.

RESULTS: We demonstrated that TIZ administration attenuates inflammatory cytokines and chemokines through quantitative real-time polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA) of medium supernatant. In addition, TIZ reduces pro-inflammatory mediators and nitric oxide release in microglia. Furtherly, TIZ inhibits the level of p38/MAPK pathway in LPS stimuli, indicating that TIZ negatively regulates neuroinflammation via inhibiting p38/MAPK pathway.

CONCLUSIONS: TIZ is verified to be an anti-inflammation effect on neuroinflammation in microglia *via* downregulation of p38/MAPK pathway, which restrains inflammation by reduced inflammatory cytokines, chemokines and mediators and decreased nitric oxide release. To summarize, TIZ is considered to be a promising reagent to alleviate neuroinflammation targeting microglia in nervous system injury.

Key Words:
Tizoxanide, Neuroinflammation, Microglia, p38/MAPK pathway, Traumatic brain injury.

Introduction

Traumatic brain injury (TBI) is identified as a category of neurological disease characterized by high disability and mortality in neurosurgery department[1,2]. Trauma-induced neuroinflammation accompanied with cerebral edema and intracranial hypertension leads to secondary injury and aggravates TBI pathological development[3,4]. Despite advanced treatment in the emergency treatments and intensive care of TBI, there is unavailable pharmacotherapy to mitigate TBI exacerbation. Given the multifactorial and complex TBI pathology, neurological deficits and massive functional neurons death are associated with excessive neuroinflammation featured by microglia and astrocyte activation *in situ*, which rapidly produces increased pro-inflammatory cytokines and accelerates destruction of blood–brain barrier (BBB)[5,6]. Increased permeability of BBB provokes peripheral leukocyte infiltrating to TBI lesion and deteriorates neuroinflammation progress, enlarging injured area in neural tissue studies[7-9].

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Acting as one of the inflammatory cells in nervous system, microglia in resting state play a regulated and monitored role in brain network[10,11]. Whereas, microglia activated by injury or infection initiate neuroinflammatory response, leading to nitric oxide (NO), and prostaglandins, and pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor α (TNF-α) generation[12,13]. Lipopolysaccharide (LPS), a major component of the cell wall in Gram-negative bacteria, is proved to trigger inflammatory cascade in microglia activation[14,15]. Meanwhile, LPS promotes downstream signaling pathways like mitogen-activated protein kinases (MAPK) pathways to induce microglial neuroinflammation[16,17]. Upregulated gene expression of various inflammatory mediators is observed following phosphorylation of MAPK family[18-20]. Hence, how to effectively restrain MAPK pathway activation in microglia may alleviate neuroinflammation after TBI. Nitazoxanide (NTZ), a novel thiazolide derivative discovered by Rossignol[21], deacetylates rapidly in human plasma to produce tizoxanide (TIZ). Due to the favorable results in preclinical and clinical trials, NTZ was approved to treat infection with non-immunodeficiency in USA. Notably, TIZ shows a prominent anti-infection effect in multiple diseases[22]. Besides, TIZ is found to exhibit pro-autophagy function in macrophage *via* regulating PI3K/AKT/mTOR pathway[23]. Hence, we hypothesize that TIZ may alleviate pathogenetic neuroinflammation in TBI *via* targeting microglia. In the study, we certify the anti-inflammatory property of TIZ in LPS activated microglia and verify the inhibited effect of TIZ on p38/MAPK pathway. Therefore, these findings suggest a potential pharmacotherapeutic effect of TIZ on TBI and other neuroinflammation-related diseases.

Materials and Methods

Primary Microglia Extraction

Pregnant C57/B6J mice at 19 days post insemination were sacrificed *via* cervical dislocation, then fetal mice were removed *via* cesarean section and sacrificed in 75% alcohol for 5 minutes. All animal operations were approved by University of South China. The brains were placed into Dulbecco's Modified Eagle's Medium (DMEM, KeyGen, China) and the pia mater and vessel were subsequently removed from the brains. We used 2.5% trypsin to digest tissue for 15 minutes at 37°C. Then, the filtered cells were cultured at 37°C for 14 days. Cells were shaken on a shaking table at 37°C and we collected the super suspended microglia in new flasks. This investigation was approved by the Animal Ethics Committee of University of South China Animal Center.

Cell Culture and Treatment

Primary microglia were cultured using DMEM (Gibco, Rockville, MD, USA) appended 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% double antibiotics. TIZ purchased from APExBIO (Boston, MA, USA) was pretreated microglia for 24 hours. Then, LPS (100 ng/mL, Sigma-Aldrich, St. Louis, MO, USA) were administrated to stimulate microglia activation for 24 hours. The control group was added the same volume of phosphate-buffered saline (PBS). Cells were assigned to four different experimental groups. Control group (CON), LPS group (LPS), 50 μmol TIZ+LPS group (50 μmol TIZ) and 100 μmol TIZ+LPS group (100 μmol TIZ) were established in the study.

Western Blotting

Microglia were harvested using a Total Protein Extraction Kit (KeyGEN, Nanjing, China) with phosphatase and protease inhibitors. Following violent oscillation and low temperature centrifugation, protein was measured using a bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and balanced. Separated in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), the protein was blocked with a 5% skim milk and incubated overnight at 4°C with the primary antibodies (anti-p-p38 (1:1000; Abcam, Cambridge, MA, USA), anti- p38 (1:1000; Abcam, Cambridge, MA, USA), anti-β-actin (1:10000; Proteintech, Rosemont, IL, USA). Washed by Tris-Buffered Saline and Tween-20 (TBST) and incubated with the secondary antibody (Abcam, Cambridge, MA, USA, 1:2000) at room temperature, protein was visualized and using an enhanced chemiluminescence (ECL) system.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from microglia using a TRIzol reagent (Beyotime Biotechnology, Shanghai, China) following the manufacturer's protocol. Complementary deoxyribose nucleic

acid (cDNA) synthesis was performed using a qScript Flex cDNA Synthesis Kit (Quanta Biosciences, Beverly, MA, USA). RNAs RNA quantification was performed using a perfecta SYBR Green Supermix (Quanta Biosciences, Beverly, MA, USA). Melting curve was employed to analyze each RNA level. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization. The relative mRNA expression levels were quantified by the $2^{-\Delta\Delta Ct}$ methods. The primers of RNAs are described in Table I.

NO Measurement

The level of NO was measured spectrophotometrically at 480 nm using a NO Assay Kit (KeyGen, Nanjing, China) according to manufacturer's instruction. The level was examined in a spectrophotometer at 480 nm.

Cell Counting Kit-8 (CCK-8) Assay

Cell viability after LPS and different gradient of TIZ treatment was measured spectrophotometrically at 480 nm using a CCK-8 Assay Kit (KeyGen, Nanjing, China) according to manufacturer's instruction.

Immunofluorescence Staining

Microglia were fixated with 4% PFA for 10 minutes and blocked using an immuno-block reagent (Beyotime, Shanghai, China). Cells were incubated with iNOS (Abcam, Cambridge, MA, USA, 1:100), COX-2 (Abcam, Cambridge, MA, USA, 1:200), IBA1 (Abcam, Cambridge, MA, USA, 1:500), NF-κB (Abcam, Cambridge, MA, USA, 1:200) overnight at 4°C**,** washed with PBS, Alexa Fluor® 594 and 488 secondary antibodies (1:200, Abcam, Cambridge, MA, USA) was conducted to immunofluorescence staining at room temperature for 1 hour. Nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI) reagent (Sigma-Aldrich, St. Louis, MO, USA); then, the images were visualized and collected using a fluorescence microscope.

Enzyme-Linked Immunosorbent Assay (ELISA)

Medium were collected at 24 h post LPS stimuli. We centrifuged medium for 5 minutes and collected the supernatant. The supernatant was conserved at -80°C. ELISA was conducted using an ELISA Kit (MultiSciences, Hangzhou, China) according to manufacturer's instruction. The optical density (OD) value of each well was measured at 450 nm using a spectrophotometer.

Statistical Analysis

Data were described as means ± SD (standard deviations). Differences between two groups were analyzed using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Data were collected and analyzed using Statistical Product and Service Solutions (SPSS) 21.0 software (IBM, Armonk, NY, USA). $p<0.05$ is considered to be significant in statistics.

Results

Microglia Identification and Influence of TIZ on Cell Viability

To examine the purity of extracted microglia, we utilized immunofluorescence staining to reflect ion calcium splice protein antigen (IBA1), a biomarker of microglia, expression. The result

Table I. Primer sequences of quantitative reverse transcription-polymerase chain reaction.

Oligo Name	Sequence (5′ --------> 3′)	
TNF-α	Forward	CAGGCGGTGCCTATGTCTC
	Reverse	CGATCACCCCGAAGTTCAGTAG
IL-1β	Forward	GAAATGCCACCTTTTGACAGTG
	Reverse	TGGATGCTCTCATCAGGACAG
IL-6	Forward	CTGCAAGAGACTTCCATCCAG
	Reverse	AGTGGTATAGACAGGTCTGTTGG
CCL-2	Forward	TAAAAACCTGGATCGGAACCAAA
	Reverse	GCATTAGCTTCAGATTTACGGGT
CCL-3	Forward	TGTACCATGACACTCTGCAAC
	Reverse	CAACGATGAATTGGCGTGGAA
GAPDH	Forward	AGGTCGGTGTGAACGGATTTG
	Reverse	GGGGTCGTTGATGGCAACA

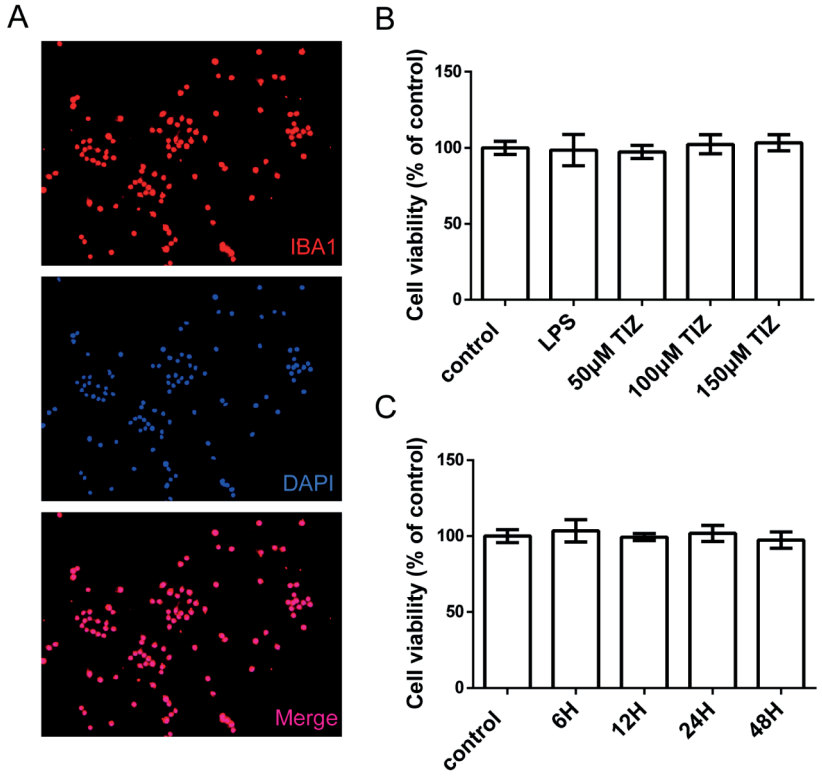

Figure 1. Microglia identification and influence of TIZ on cell viability. **A,** Representative IF staining of IBA1 (red) in extracted cells (magnification: 40×). **B,** Representative cell viability of microglia after LPS and TIZ (50, 100 and 150 μM) treatment. **C,** Representative cell viability of microglia within 48 hours after 100 μM TIZ treatment.

exhibited a remarkable expression of IBA1 in cells (Figure 1A), indicating that the purity of microglia is more than 99%. Next, the cells viability after LPS and different gradients of TIZ treatment for 24 hours was detected using CCK8 assay, displaying that 100 μg/mL LPS treatment and TIZ at various doses (50, 100 and 150 μmol/L) did not influence microglia viability (Figure 1B). Moreover, the cells viability was measured within 48 hours after 100 μmol/L TIZ treatment, showing no significant influence on microglia (Figure 1C). The results reveal that the effect of TIZ and LPS on microglia does not have cytotoxicity.

TIZ Decreases Expression of Pro-Inflammatory Cytokines and Chemokines in Microglia

We utilized qRT-PCR and ELISA to examine pro-inflammatory cytokines and chemokines in LPS and TIZ (50 and 100 μmol/L) treated microglia. At transcriptional level, we found that the RNA level of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 was markedly increased with LPS stimuli; however, the TIZ treatment significantly reduced the RNA expression of TNF-α, IL-1β, and IL-6 (Figure 2A-2C). Moreover, classic chemokines including CCL-2 and CCL-3 has exhibited a remarkable increase following LPS employment in microglia while the decreased level of CCL-2 and CCL-3 was observed in TIZ administration cells (Figure 2D and 2E). Besides, the release of cytokines and chemokines was measured in microglia medium. The results showed a marked increase of TNF-α, IL-1β, and IL-6 inconsistent with the expression of CCL-2 and CCL-3 whereas TIZ treatment reduced the expression both of the pro-inflammatory cytokines and chemokines (Figure 2F-2J). Thereof, the results suggest that TIZ inhibits the synthesis and release of pro-inflammatory cytokines and chemokines in activated microglia.

Administration of TIZ Reduces NO Release and Inflammation-Related Enzymes

We next evaluated the production of NO and the expression of inflammation-related enzymes such as inducible nitric oxide synthase (iNOS) and cy-

clooxygenase-2 (COX-2) in LPS activated microglia. The result of NO level showed a prominent elevation in the cell medium supernatants, while the production of NO reduced remarkably after TIZ conduct (Figure 3A). Immunofluorescence staining exhibited that high expression of iNOS and COX-2 in LPS treated microglia. After incubated with 100 μmol/L TIZ, we found that the iNOS and COX-2 expression decreased in microglia following LPS stimuli (Figure 3B). Hence, the results demonstrate that TIZ inhibits the release of NO level and reduces the expression of inflammation-related enzymes.

TIZ Exerts Anti-Inflammatory Effect Via Inhibiting NF-κB and p38/MAPK Pathway

Regulating the expression of various pro-inflammatory cytokines and inflammatory mediators in microglia, NF-κB and MAPK pathways are a responsible mechanism. To explore the influence of TIZ on neuroinflammation in microglia, we first measured NF-κB expression using immunofluorescence staining, showing increased NF-κB expression in LPS stimulated microglia. However, 100 μmol/L TIZ administration alleviated the expression of NF-κB in cells (Figure 4A). Moreover, we detected the phosphorylation of p38/MAPK pathway using Western blotting, exhibiting that the level of phosphorylated p38 (p-p38) increased in microglia underwent LPS stimuli while the expression of p-p38 was down-regulated after TIZ treatment (Figure 4B and 4C). Hence, the TIZ-treated microglia show an inhibition of NF-κB and p38/MAPK pathway.

Discussion

Neuroinflammation associated with microglia and astrocytes *in situ* is a critical process aggravat-

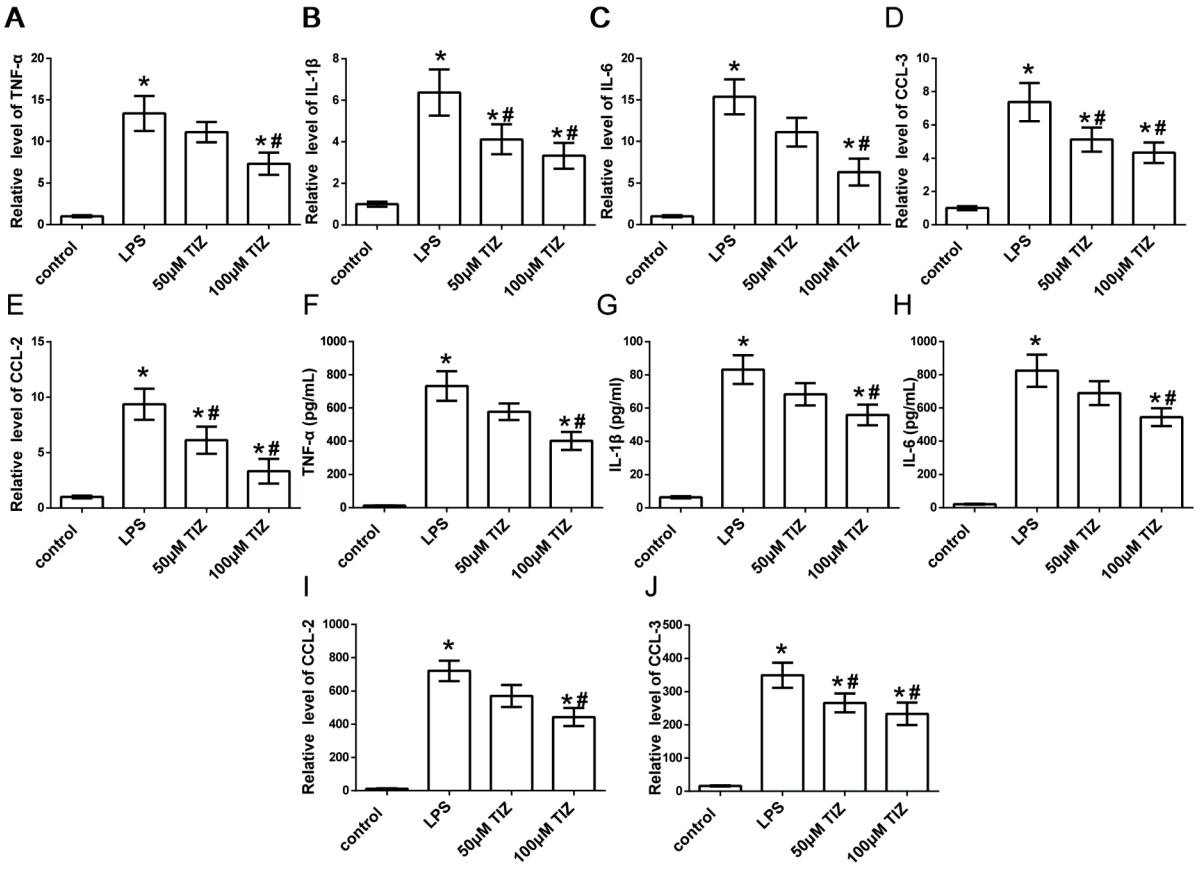

Figure 2. TIZ decreases expression of pro-inflammatory cytokines and chemokines in microglia. **A-E,** Representative RNA levels of TNF-α, IL-1β, IL-6, CCL-2 and CCL-3 in control, LPS, 50 μM TIZ+LPS (50 *μM* TIZ) and 100 μM TIZ+LPS (100 μM TIZ) group. **F-J,** Representative ELISA results of TNF-α, IL-1β, IL-6, CCL-2 and CCL-3 in control, LPS, 50 μM TIZ+LPS (50 μM TIZ) and 100 μM TIZ+LPS (100 μM TIZ) group. "*" means *vs.* control group and "#" means *vs.* LPS group with statistical significance.

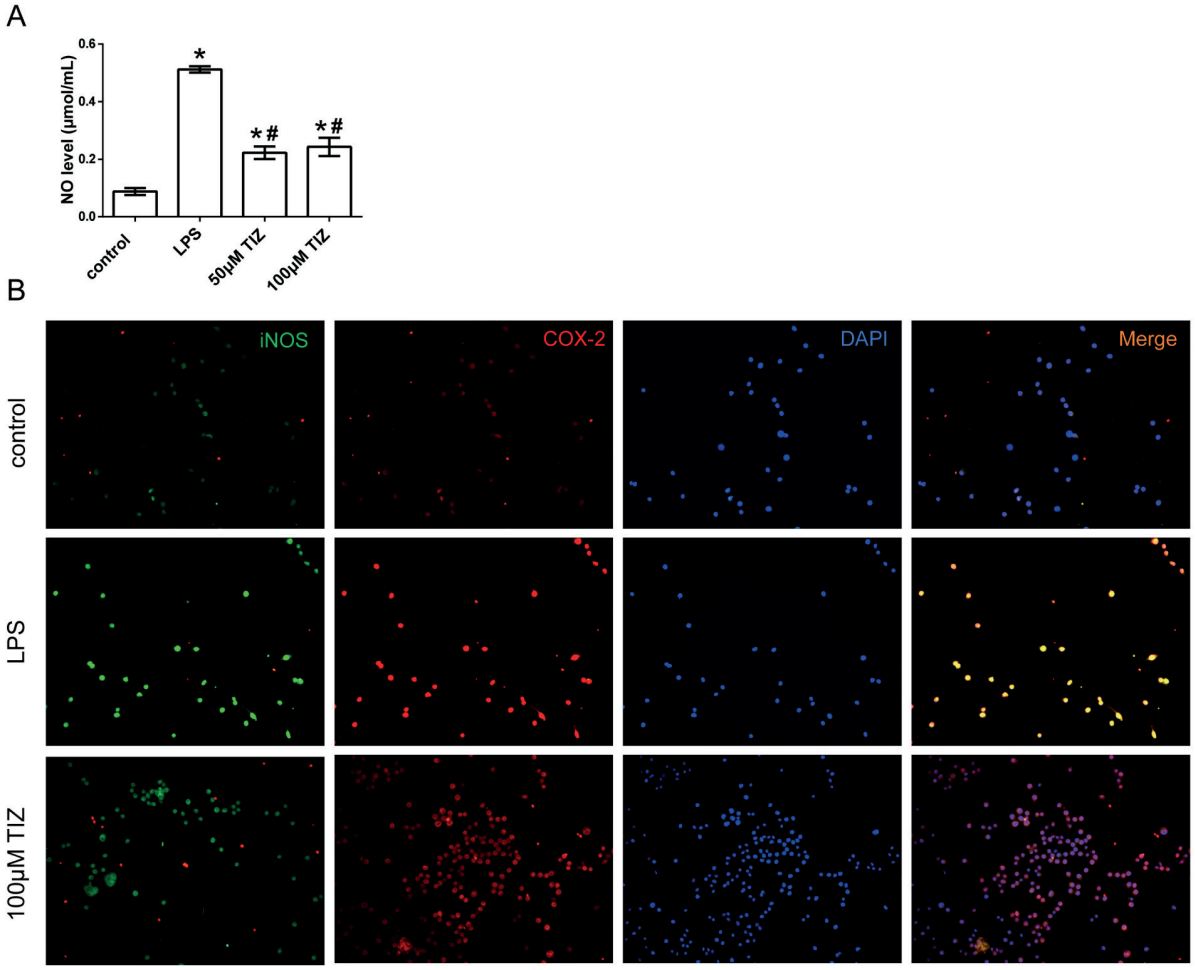

Figure 3. Administration of TIZ reduces NO release and inflammation-related enzymes. **A,** Representative NO level in control, LPS, 50 μM TIZ+LPS (50 μM TIZ) and 100 μM TIZ+LPS (100 μM TIZ) group. **B,** Representative IF staining of iNOS (green) and COX-2 (red) in control, LPS and 100 μM TIZ+LPS (100 μM TIZ) group (magnification: 40×). "*" means *vs.* control group and "#" means *vs.* LPS group with statistical significance.

ing neural tissue destruction after TBI. Inflammatory cascades trigger persistent secondary injury, contributing to neuron death, neurologic impairment and even threatening life. TIZ, a metabolite of NTZ, is showed as a promising polypharmacology function in various infective diseases[24-26]. Guo et al[27] analyzed quantification of TIZ in rat brain tissue and plasma by UHPLC-MS/MS. Notably, Shou et al[28] figured out that TIZ inhibited inflammation in RAW 267.4 macrophage *via* suppressing NF-κB and MAPK activation. This study explored the inhibitory function of TIZ on microglia induced neuroinflammation pathogenesis, and its regulated mechanism after LPS activation. Consistently, we found the effect of TIZ on inhibiting inflammation a in LPS activated primary microglia and further verified the underlying mechanism of NF-κB and MAPK pathway activation. The generation of pro-inflammatory cytokines is a marked biological phenomenon in acute inflammation and aggravates the damage of neural tissue and construction. Our finding that TIZ treatment decreased the generation and release of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 expression in LPS induced inflammatory microglia testified the inhibitory effect of TIZ on the negative regulation of neuroinflammation initiation. Importantly, Trabattoni et al[29] reported that TIZ regulated chemokines and elicits anti-viral innate immunity in HIV disease. In our study, we find that TIZ decreases the level of classical chemokines like CCL-2 and CCL-3 in LPS-induced microglia culture medium. Therefore, it is feasible that TIZ inhibits secondary neurologic injury *via* regulation of microglia-in-

duced neuroinflammatory response and cell migration-induced glial aggregation. Acting as a specific anti-infective reagent, TIZ confers negative effect on glutamate receptor in the treatment of neuropathic pain[30]. We here display that TIZ treatment in microglia dramatically inhibits iNOS and COX-2 expression after LPS induction, which is potentially associated with the reduction of pain mediators. Hence, the release of NO also decreased in microglia induced neuroinflammation. Previous studies show that microglia activation by LPS attributes to the involvement of NF-κB and MAPK pathway. Likewise, we certify that LPS activates inflammation response in microglia *via* up-regulation of NF-κB and p38/MAPK, and further confirm the repression of NF-κB and MAPK pathway resulted from TIZ treatment, indicating that the role of TIZ against neuroinflammation is due to suppression of NF-κB and p38/MAPK pathway. Therefore, TIZ is a promising therapeutic reagent that alleviates secondary neuroinflammation in TBI and other neurological diseases. TIZ induces autophagy in mammalian cells and inhibits mycobacterium tuberculosis *via* stimulating autophagy[31,32]. Importantly, TIZ is reported to induce autophagy by inhibiting PI3K/Akt/mTOR pathway in RAW264.7 macrophage cells[23]. We also speculate that TIZ may regulate autophagy in microglia and neuron after TBI and the molecular mechanism need to be investigated in future vitro study. Ruiz-Olmedo et al[33] suggest that TIZ exhibits low permeation into BBB, indicating no TIZ in cerebrospinal fluid in normal rats. However, more researches concerning the dose and pharmacokinetics of TIZ in brain tissue of vivo TBI model are needed after the disruption of BBB. Importantly, the novelties of the work are that we first demonstrated the potentiality that TIZ plays an anti-neuroinflammatory role in microglia via inhibition of p38 phosphorylation, which takes a new view on the exploration of TBI therapy.

Conclusions

Taken together, we proved that TIZ treatment reduces neuroinflammatory level through inhibiting p38/MAPK pathway in microglia. Thus, TIZ may be a novel protective agent targetinganti-inflammation in TBI.

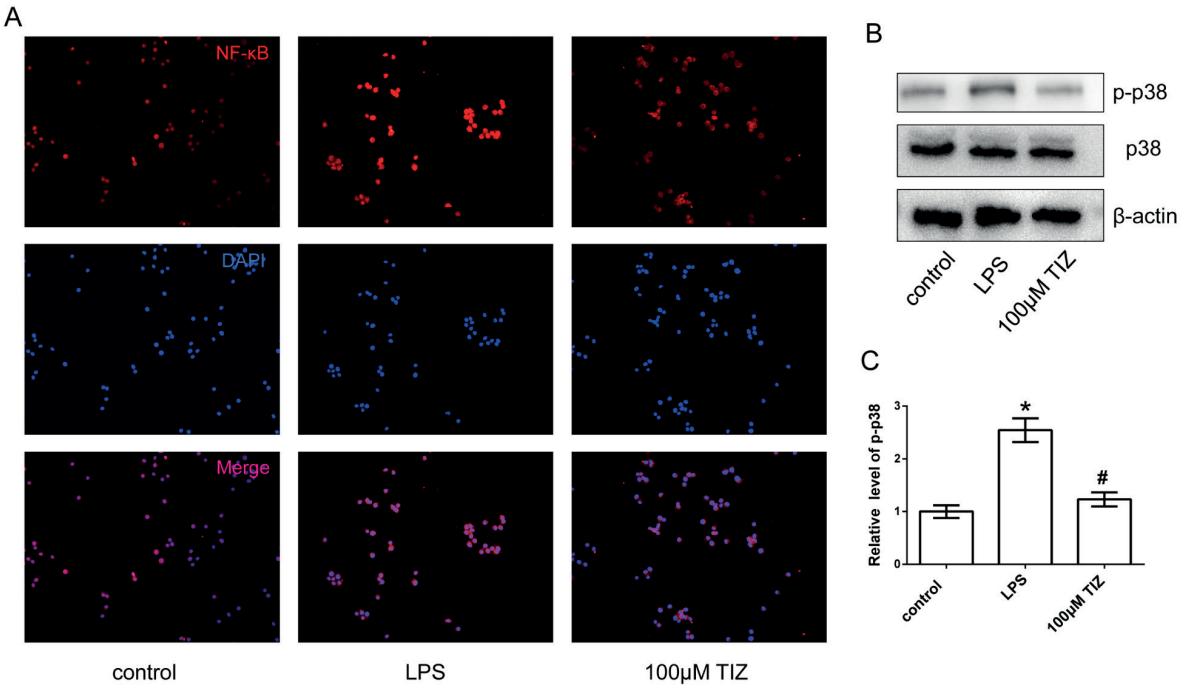

Figure 4. TIZ exerts anti-inflammatory effect *via* inhibiting NF-κB and p38/MAPK pathway. **A,** Representative IF staining of NF-*κB* (red) in control, LPS and 100 μM TIZ+LPS (100 μM TIZ) group (magnification: 40×). **B,** Representative Western blotting of p-p38 and p38 in control, LPS and 100 μM TIZ+LPS (100 μM TIZ) group. **C,** Quantitative analysis of p-p38 level in control, LPS and 100 μM TIZ+LPS (100 μM TIZ) group. "*" means *vs.* control group and "#" means *vs.* LPS group with statistical significance.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding Acknowledgements

Hunan Provincial Commission of Health (20200497).

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