



ORIGINAL ARTICLE

# Tizoxanide Inhibits Inflammation in LPS-Activated RAW264.7 Macrophages *via* the Suppression of NF- $\kappa$ B and MAPK Activation

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**Abstract**—Tizoxanide is the main active metabolite of nitazoxanide. Nitazoxanide and tizoxanide have a broad-spectrum anti-infective effect, including parasites, bacteria, and virus. In the present study, we investigated the anti-inflammatory effect of tizoxanide on lipopolysaccharide (LPS)-stimulated RAW264.7 cells and revealed underlying molecular mechanisms. The results showed that tizoxanide significantly suppressed production of NO as well as pro-inflammatory cytokines, such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in dose-dependent manner. Meanwhile, the levels of gene expression of these cytokines were inhibited significantly by tizoxanide that was discovered using RT-PCR. The increased protein levels of inducible nitric oxide synthase, heme oxygenase-1, and cyclooxygenase-2 by LPS in the cells were also reduced by tizoxanide. Moreover, we found that tizoxanide inhibited the phosphorylation of IKK- $\alpha$  and degradation of I $\kappa$ B by LPS in macrophage cells. The increased protein levels of p65 induced by LPS in the cytoplasm and nucleus were both decreased by tizoxanide, and the nuclear translocation of p65 was also restrained in cell imaging. In addition, tizoxanide considerably also inhibited LPS-activated JNK, p38, and ERK phosphorylation in RAW264.7 cells. Taken together, our results suggested that tizoxanide exerts anti-inflammatory effects, by inhibiting the production of pro-inflammatory cytokines and suppressing of the activation of the NF- $\kappa$ B and the MAPK signaling pathways in LPS-treated macrophage cells.

**KEY WORDS:** tizoxanide; RAW264.7 cells; lipopolysaccharide; anti-inflammation; NF- $\kappa$ B; MAPKs.

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## INTRODUCTION

Inflammation is a complex biological process of tissue damage or infection by recruiting regulatory host immune cells [1]. Despite its protective role in the host system against pathogens, uncontrolled inflammatory response leads to numerous health maladies, including acute and dangerous cardiovascular, pulmonary, and cerebrovascular conditions [1, 2]. Pharmacotherapy of inflammation is a globally important and challenging goal [1].

It is well known that macrophage is one of the most dominant and widely distributed inflammatory cells which

detect and can recognize the pathogen and initiate the inflammatory response by producing inflammatory mediators like nitric oxide (NO), and prostaglandins (PGs), and pro-inflammatory cytokines such as tumor necrosis interleukin (IL)-6, IL-1b, and factor  $\alpha$  (TNF- $\alpha$ ) [3–5]. Lipopolysaccharide (LPS), as a major lipidic components of the cell wall of most gram-negative bacteria, is often used to study experimentally induced activation of macrophage owing to the ability to trigger cascade in inflammatory processes [6, 7]. Toll-like receptor 4 (TLR4), a mammalian receptor for bacterial LPS, plays a beneficial role in immune responses to bacterial infections but is also a main driver of aberrant inflammation [8–10]. TLR4 can be activated by LPS and consequently promotes downstream signaling cascades, including the activation of nuclear factor-kappa B (NF- $\kappa$ B) and mitogen-activated protein kinases (MAPKs) family. MAPKs are classified into three components: p38 MAPK, extracellular signal-regulated kinases (ERKs), and c-Jun N-terminal kinase (JNK). Following phosphorylation and activation, NF- $\kappa$ B and MAPKs could upregulate gene expression of various inflammatory mediators [11, 12]. Inhibition of excessive inflammatory cytokines and mediators by suppressing the activation of NF- $\kappa$ B and MAPKs has been identified as a key step to inflammation treatment and therefore serves as an important target for drug development and discovery [13, 14].

Nitazoxanide (NTZ; Fig. 1b) is a synthetic thiazolide derivative (*N*-(5-nitrothiazol-2-gammal) salicylamide) that was developed as a cestocidal agent discovered by Rossignol [15]. In humans, NTZ undergoes rapid deacetylation in plasma (half-life of 6 min) to form its metabolite tizoxanide (TIZ; Fig. 1a) which is the only active form in the circulation unchanged even when plasma is diluted 10 folds [16]. Due to the remarkable efficacy in preclinical and clinical trials, NTZ received regulatory approval in the USA in 2002 for treatment of

*Cryptosporidium parvum* and *Giardia intestinalis* infections in non-immunodeficient children and adults [17, 18]. Indeed, the drug is a broad-spectrum agent which appears to be efficacious for the treatment of infections caused by luminal parasites, helminths, and anaerobic bacteria [17, 19, 20]. Serendipitously, in the results from clinical studies of AIDS, the antiviral activity of NTZ has aroused the attention on the scholars. Subsequently, the capacity of inhibiting rotavirus, influenza, norovirus, respiratory syncytial virus, and hepatitis B and C viruses was demonstrated by several studies *in vivo* or *in vitro* [17, 21–24]. These literatures indicate that the biological actions of NTZ are not means restricted to anti-parasitic activities, suggesting that NTZ has yet unknown pharmacological effects.

It is noteworthy that NTZ is the active pro-drug of TIZ, which is at least as active as NTZ against parasite, anaerobes and viruses [17, 24–26]. Although TIZ/NTZ susceptibility has been documented for different pathogens, the potential effect of these compounds on inflammatory response has nevertheless not been investigated so far. Nothing is known about TIZ/NTZ's mode of the anti-inflammatory activities and its molecular mechanisms, which is a limitation on its clinical use to treat inflammatory. Herein, we evaluate the anti-inflammatory activity of TIZ (NTZ active metabolite) by the mode of LPS-stimulated RAW264.7 cells and explore its potential mechanism.

## MATERIALS AND METHODS

### Chemical and Biological Materials

TIZ (purity  $\geq 99\%$ ) was synthesized by Key Laboratory of Veterinary Chemical Drugs and Pharmaceutics,

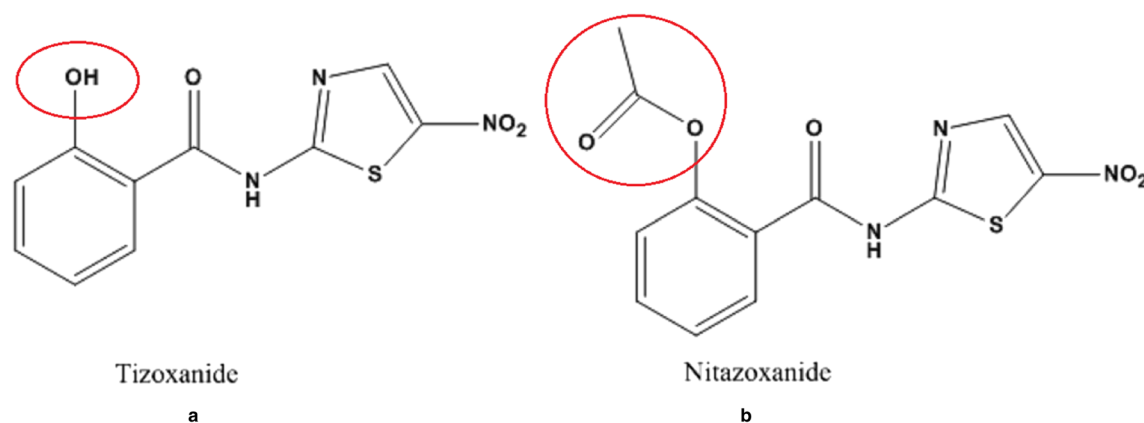


Fig. 1. Chemical structures of TIZ (a) and NTZ (b). The differences with a proton for TIZ and an acetyl moiety for NTZ show in circles.

Ministry of Agriculture and Rural Affairs, Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, and characterized by LC-UV, LC-MS, and NMR methods (data were not shown). LPS and DMSO (dimethylsulfoxide) were purchased from Sigma-Aldrich (Saint Louis, MO). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (Auckland, NZ). RAW264.7 murine macrophage was from Shanghai Cell Bank, the Institute of Cell Biology, and Chinese Academy of Sciences (Shanghai, China). Anti-NF- $\kappa$ B mAbs, anti-p-NF- $\kappa$ B mAbs, anti-I $\kappa$ B mAbs, anti-p-I $\kappa$ B mAbs, anti-p-IKK $\alpha$  mAbs, anti-p-JNK mAbs, anti-JNK mAbs, anti-ERK mAbs, anti-p-ERK mAbs, anti-cyclooxygenase-2 (COX-2) mAbs, anti-p38 mAbs, anti-p-p38 mAbs, anti-Lamin B1 mAbs, and anti- $\beta$ -actin mAbs were acquired from Cell Signaling Technology (Beverly, MA, USA). The ELISA kits for IL-1 $\beta$ , IL-6, and TNF- $\alpha$  were obtained from R & D Systems Company (Minneapolis, MN, USA). In addition, IRDye® 800CW goat anti-rabbit or anti-mouse IgG was purchased from LI-COR corporate (Lincoln, NE, USA). Alexa Fluor 488-labeled secondary antibody, methylthiazolyldiphenyl-tetrazolium bromide (MTT), polyvinylidene difluoride (PVDF) membrane, and 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) were acquired from Beyotime Biotechnology (Shanghai, China). RIPA protein lysis buffer and nuclear-cytosol extraction Kit were also purchased from Beyotime Biotechnology (Shanghai, China).

### RAW264.7 Murine Macrophage Culture

RAW264.7 murine macrophage was obtained from the cell bank of the Chinese Academy of Science (Shanghai, China) and used for experiments not exceeding passages 10. RAW264.7 cells were maintained in DMEM containing 10% FBS at 37 °C in a moist atmosphere with 5% CO<sub>2</sub> and 95% air. When the cell confluency reached 80%, cells were stimulated by LPS (1.0  $\mu$ g/mL) in the presence or absence of TIZ. Additionally, 0.1% DMSO was added into TIZ prior at mixing in the complete medium to promote the dissolution of TIZ.

### Cell Viability Assay

MTT assay was used to detect the viability of RAW264.7 murine macrophage. When RAW264.7 cells were grown to 80–90% confluence, the cells were digested with 0.05% trypsin and were seeded on 96-well plates with a density of  $1 \times 10^5$  cells/mL in 100  $\mu$ L complete medium overnight. Subsequently, the cells were incubated with

various concentrations of TIZ in 37 °C and 5% CO<sub>2</sub> incubator for 24 h. Meanwhile, 1.0  $\mu$ g/mL LPS, co-treatment with 200  $\mu$ mol/L TIZ, and 1.0  $\mu$ g/mL LPS groups were set up. A volume of 20  $\mu$ L MTT was added into each well for 4 h incubation away from light, and then culture medium was removed with extra addition of 150  $\mu$ L DMSO to resolve the formazan. Finally, the optical density (OD) of the formazan of each well was measured with a microplate reader (BIO-RAD iMark, Hercules, CA, USA) at 490 nm.

### Enzyme-Linked Immunosorbent Assay

To detect the production of pro-inflammatory cytokines including IL-1 $\beta$ , IL-6, and TNF- $\alpha$  of RAW264.7 murine macrophage, the cells were seeded into 24-well plates. After cultivation of 12 h, RAW264.7 cells were simultaneously treated with LPS (1.0  $\mu$ g/mL) and TIZ (50, 100, 150  $\mu$ mol/L) in culture medium for 24 h. Cell culture media was centrifuged at 2000 $\times$ g for 10 min to remove debris and collected 50  $\mu$ L cell-free supernatants to assay immediately. According to the kits manufacturers' instructions, all materials and prepared reagents were equilibrated to room temperature prior to use. The biotinylated antibodies were detected by sequential incubation with streptavidin-horseradish peroxidase (HRP) conjugate and chromogenic substrates. Optical density measurements were taken at 450 nm in an Infinite M200 PRO plate reader (Tecan, Switzerland).

### NO Release Detection

Nitric oxide (NO) production of RAW264.7 cells was determined by measuring the concentration of nitrites and nitrates, and Griess reagent was used to detect the concentration of those chemical compounds [27, 28]. RAW264.7 cells were plated into 96-well plates at  $1 \times 10^5$  cells/mL with 12 h cultivation, and then the cells were pretreated with LPS (1.0  $\mu$ g/mL) for 1 h prior to the incubation of LPS (1.0  $\mu$ g/mL) and TIZ (50, 100, 150  $\mu$ mol/L). After incubation 6 h, the supernatant of cell culture medium was collected, and the Nitric Oxide Assay Kit (Beyotime, Shanghai, China) was applied to detect the levels of NO production of RAW264.7 cells. The OD of reaction solution was measured at 540 nm with Infinite M200 PRO (Tecan, Switzerland).

### Confocal Laser Scanning Fluorescence Microscopy

In order to detect the activity of TIZ inhibition the nuclear translocation of NF- $\kappa$ B protein, Alexa Fluor 488-

labeled secondary antibody and primary anti-NF- $\kappa$ B mAbs were used to analysis the levels and the position of NF- $\kappa$ B protein in RAW264.7 cells. Six-well plates with one glass slide was put in each well were cultivated overnight with  $2 \times 10^5$  cells/well. After the cells were pretreated with 1.0  $\mu$ g/mL LPS for 6 h, TIZ with various concentrations (50, 100, 150  $\mu$ mol/L) was added into each well and cultivated for 6 h. The cells were washed by PBS for 10 min and fixed with 4% paraformaldehyde at room temperature for 30 min. The fixed cells were permeabilized with 0.1% Triton X-100 for 5 min and blocked with 5% BSA at room temperature. The cells were then incubated with the primary anti-NF- $\kappa$ B mAbs at 4 °C overnight. Washed with PBS for 15 min, the cells were incubated with Alexa Fluor 488-labeled secondary antibody for 1 h. After washed, DAPI was used to dye the cells chromatin with blue. Thus, the nuclei of RAW264.7 cells were present in blue under the confocal laser scanning fluorescence microscopy (Zeiss, Germany) with an excitation wavelength of 350 nm for DAPI, while NF- $\kappa$ B of cytoplasm and nucleus was green with an excitation wavelength 540 nm. ZEN 2.1 software was applied to capture and edit the pictures.

### SDS-PAGE and Immunoblotting

Cells were seeded into six-well plates for further cultivation. After the confluence of RAW264.7 cells reached 80–90%, the cells were exposed to 1.0  $\mu$ g/mL LPS for 1 h prior to the extra addition of TIZ (50, 100, and 150  $\mu$ mol/L) for 6 h. Washed with pH 7.4 ice-cold PBS buffer three times, the cells were lysed with 200  $\mu$ L RIPA lysis buffer (Beyotime, Shanghai, China) for 5 min. Then, the lysate was collected into 1.5-mL tube and centrifuged at  $1000 \times g$  for 5 min at 4 °C. The concentration of protein was measured with BCA Protein Assay Kit (Beyotime, Shanghai, China). The protein solution was supplemented with 5 $\times$  SDS loading buffer and then denatured in boiling water at 100 °C for 10 min. Equal amounts of protein (30  $\mu$ g) were separated by subjecting to electrophoresis with a 6–12% SDS-Tris-glycine polyacrylamide gel and electrotransferred to the polyvinylidene difluoride (PVDF) membrane in western transfer buffer. The PVDF membrane was blocked in the 5% bull serum albumin (BSA) and then incubated in primary rabbit monoclonal antibodies at 4 °C overnight. Washed with Tris-buffered saline Tween-20 (TBST) 3 times for 15 min, the membrane was incubated with the IRDye® 800CW goat anti-rabbit secondary antibodies (1:5000) at room temperature for 40 min. Finally, the membrane was visualized by Odyssey Imager (Gene Company Limited).

### Real-Time Quantitative PCR Detecting

Total RNA of RAW264.7 murine macrophage was extracted with RNeasy Mini Kit (QIAGEN company), and real-time quantitative PCR was conducted according to the kit manufacturer's protocol (Invitrogen, Carlsbad, CA). The primer sequences of iNOS (inducible nitric oxide synthase), IL-1 $\beta$ , IL-6 and TNF- $\alpha$  were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and were as follows: iNOS: forward primer: GGAGTGACGGCAAA CATGACT, reverse primer: TCGATGCACAACCTG GGTGAAC; IL-1 $\beta$ : forward primer: ATGCCACC TTTTGACAGTGATG, reverse primer: GTTGATGT GCTGCTGCGAGATT; IL-6: forward primer: TAGTCCTTCCCTACCCCAATTTCC, reverse primer: TTGGTCCTTAGCCACTCCTTC; TNF- $\alpha$ : forward primer: CTCATTCCCTGCTTGTGGC, reverse primer: ACTTGGTGGTTTGCTACG; and  $\beta$ -actin: forward primer: GGCTGTATTCCCCTCCATCG, reverse primer: CCAGTTGGTAACAATGCCATGT. ABI 7500 real-time PCR thermocycler and SYBR green PCR Master Mix (Applied Biosystems) were applied to detect the relative quantities of target transcripts. The specificity of primers was assessed on the basis of dissociation curve of real-time quantitative PCR.  $2^{-\Delta\Delta C_t}$  method was used to calculate the relative expression of genes, while  $\beta$ -actin was regarded as reference gene to standardize the relative expression levels of target genes [29].

### Statistical Analysis

The assays were performed three times. All results were expressed as mean  $\pm$  standard deviation (SD). The criterion which based on the probability of  $P < 0.05$  and  $P < 0.01$  was used to identify significant differences between control and exposure groups in one-way analysis of variance (ANOVA) with LSD (least significant difference). All statistical analyses were performed using SPSS 17.0 (SPSS, Chicago, IL, USA).

## RESULTS

### Effects of TIZ on Cell Viability

MTT assay was performed to exclude the possibility that the inhibitory effects were due to the cytotoxicity of TIZ. As shown in the Fig. 2, RAW264.7 cells did not exhibit significant cytotoxicity after the cells were treated with TIZ at various doses on the cell viability assay. In addition, we did not find that LPS (1.0  $\mu$ g/mL) had a

significant effect on the RAW264.7 cells viability in a 24-h culture. These results indicated that the influences of TIZ on RAW264.7 cells were not due to the cytotoxicity of TIZ and LPS. Therefore, we mainly explored the effects of TIZ with below 150  $\mu\text{mol/L}$  on the RAW264.7 cells treated 1.0  $\mu\text{g/mL}$  LPS.

### Effects of TIZ on Release of Pro-inflammatory Cytokines

Release of cytokines is an important step in regulating host immune responses to inflammation [8, 10, 30]. To assess the effect of TIZ on release of pro-inflammatory cytokines including IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , ELISA was used to measure the production of these cytokines in LPS-stimulated RAW264.7 cells. The results revealed that the levels of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 were elevated at 9896, 158.8, and 4132 pg/mL after RAW264.7 cells were treated by LPS (Fig. 3). As expected, the levels of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 have been decreased at 1173, 69, and 21 pg/mL after 150  $\mu\text{mol/L}$  after TIZ treated. TIZ significantly suppressed the production of TNF- $\alpha$  (Fig. 3a), IL-1 $\beta$  (Fig. 3b), and IL-6 (Fig. 3c) in the LPS-treated macrophage for 24 h with a dose-dependent manner ( $P < 0.01$ ).

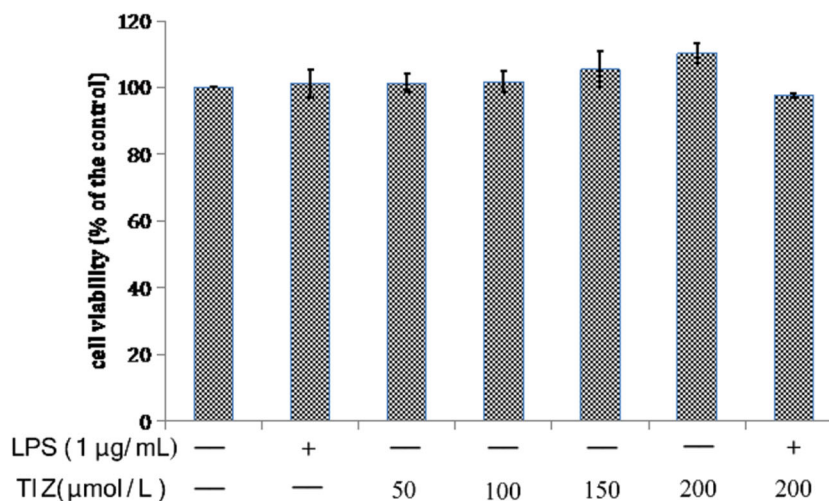
Furthermore, RAW264.7 cells were harvested to analyze cytokines mRNA by fluorescence real-time quantitative PCR to determine the inhibitory effects of TIZ on cytokine production related to the modulation of gene expression. As shown in Fig. 4, TIZ observably attenuated the gene transcription of the TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 respectively in a dose-dependent manner. These results indicated that the increased synthesis and release of pro-

inflammatory cytokines such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in LPS-stimulated RAW264.7 cells were inhibited by TIZ in dose-dependent manners.

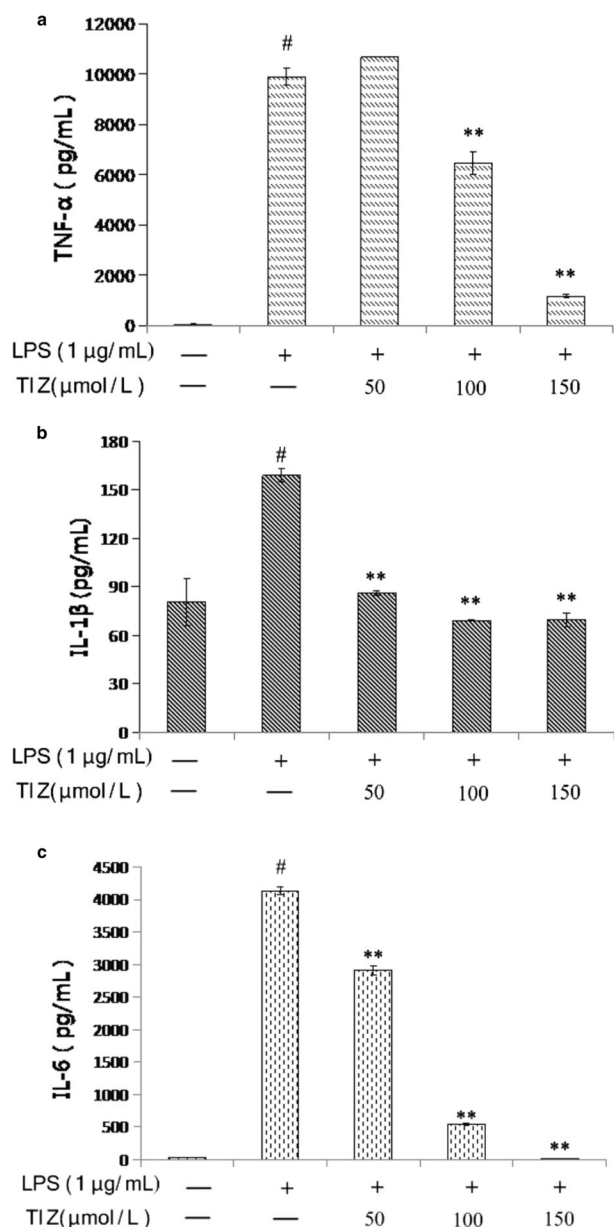
### Effects of TIZ on LPS-Stimulated NO Production

Till date, numerous studies have demonstrated that NO proceed the inflammatory process, and therefore the production of NO reflects the development process of inflammatory [30–32]. The production of NO and the expression level of iNOS had significant upregulation after 1.0  $\mu\text{g/mL}$  LPS-treated macrophage [3]. In this study, the NO in the culture medium supernatants was collected and assessed. As shown in Fig. 5, the production of NO markedly upregulated in the 1.0  $\mu\text{g/mL}$  LPS-treated cells. After the LPS-stimulated RAW264.7 cells were incubated with TIZ (50, 100, 150  $\mu\text{mol/mL}$ ), the production of NO was significantly reduced 48–66% ( $P < 0.01$ ; Fig. 5a). Interestingly, the production of NO was elevated with the increasing concentration of TIZ (50, 100, 150  $\mu\text{mol/mL}$ ) though it was still suppressed on the whole. According to the molecular structure of TIZ, the active nitro group might affect the detection of NO production [33].

To validate the effects of TIZ suppressed the production of NO, the influence of mRNA transcriptions and protein expression of iNOS were evaluated by qRT-PCR and western blotting respectively. As shown in Fig. 5b, a lower iNOS protein expression appeared in normal cells and TIZ-treated cells, while higher expression appeared in LPS-stimulated RAW264.7 cells. Furthermore, the transcriptions of the iNOS genes were significantly decreased in normal cells and TIZ-treated cells ( $P < 0.01$ ; Fig. 5c).

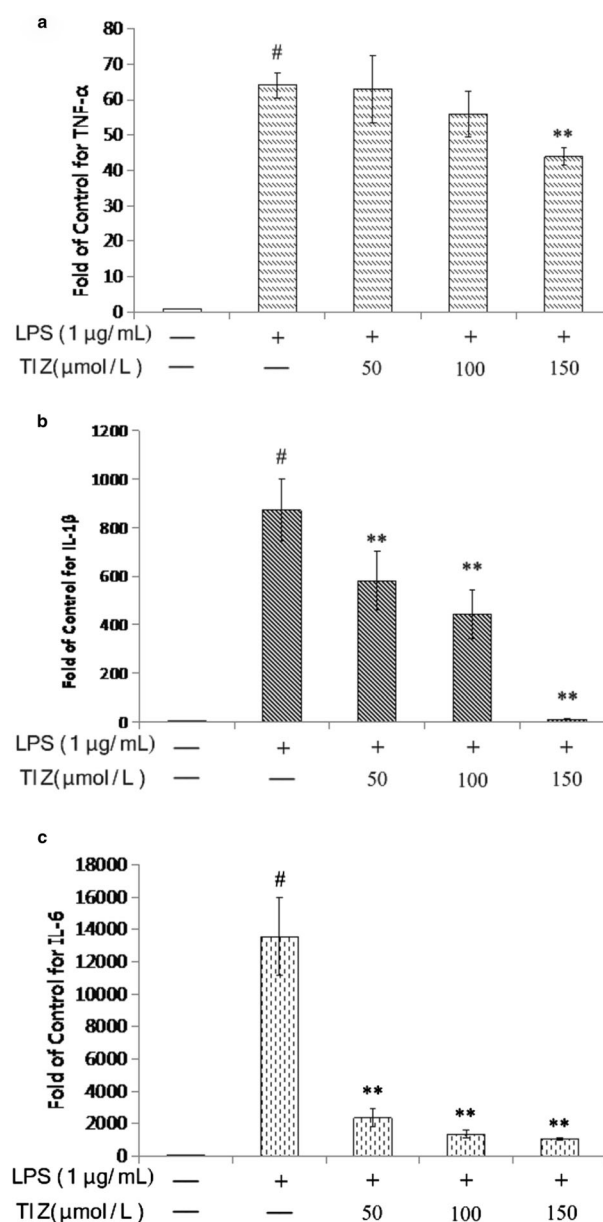


**Fig. 2.** Cytotoxicity of TIZ and LPS on RAW264.7 cells. Cells were treated with TIZ at 50, 100, 150, and 200  $\mu\text{mol/L}$  for 24 h, and cell viability was assayed by the MTT assay. Data are expressed as the means  $\pm$  S.D. of three independent experiments.



**Fig. 3.** Effect of TIZ on **a** TNF- $\alpha$ , **b** IL-1 $\beta$ , and **c** IL-6 production in LPS-stimulated RAW264.7 cells. Cells were incubated with or without LPS (1  $\mu$ g/mL) in the presence of various doses (50, 100, and 150  $\mu$ mol/L) of TIZ for 24 h. Values are expressed as mean  $\pm$  S.D. of three replicates. Number sign shows the significantly different ( $P < 0.01$ ) when single LPS-treated group compared with untreated group; double asterisks shows the significantly different ( $P < 0.01$ ) when TIZ groups compared with single LPS-treated group.

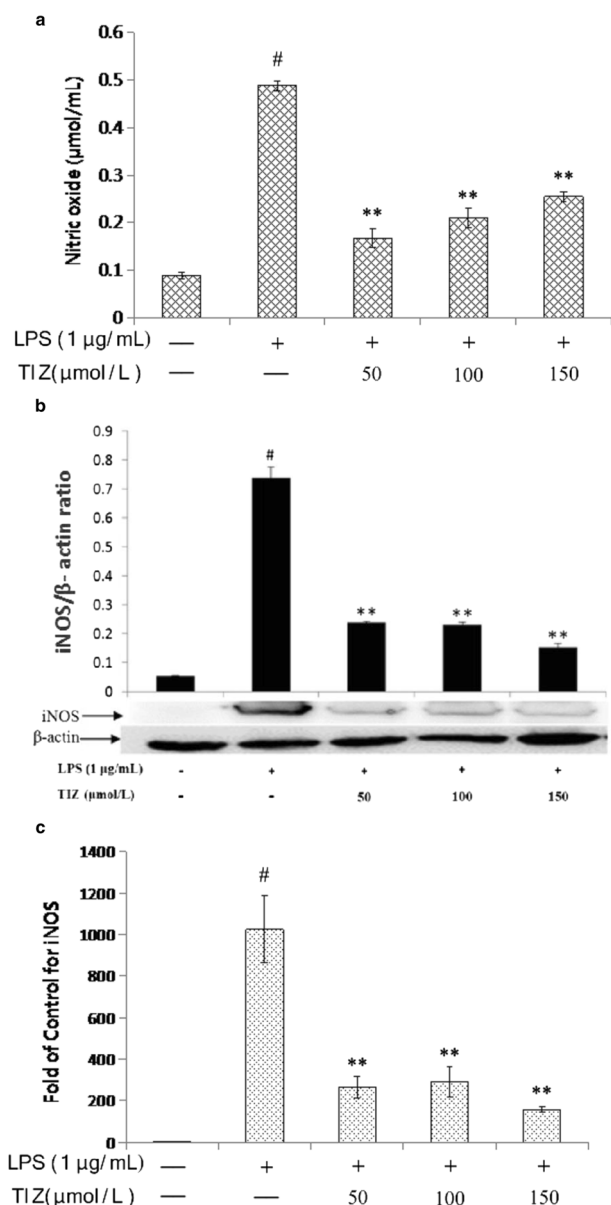
Together, these results indicated that the increased production of NO and expression of iNOS in LPS-stimulated RAW264.7 cells were inhibited significantly by TIZ.



**Fig. 4.** Effect of TIZ on the genes transcription of **a** TNF- $\alpha$ , **b** IL-1 $\beta$ , and **c** IL-6 in LPS-stimulated RAW264.7 cells. Cells were incubated with or without LPS (1  $\mu$ g/mL) in the presence of various doses (50, 100, and 150  $\mu$ mol/L) of TIZ for 24 h. Values are expressed as mean  $\pm$  S.D. of three replicates. Number sign shows the significantly different ( $P < 0.01$ ) when single LPS-treated group compared with untreated group; double asterisks show the significantly different ( $P < 0.01$ ) when TIZ groups compared with single LPS-treated group.

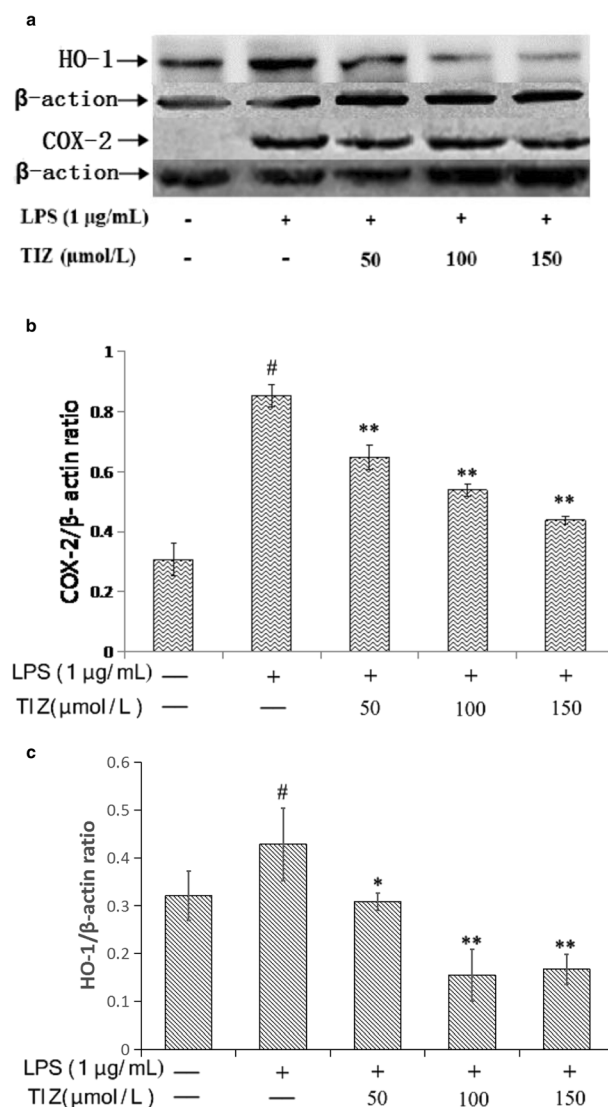
#### Effects of TIZ on COX-2 and HO-1

COX-2 and heme oxygenase-1 (HO-1) could be activated due to inflammation [12, 34]. To fully to understand



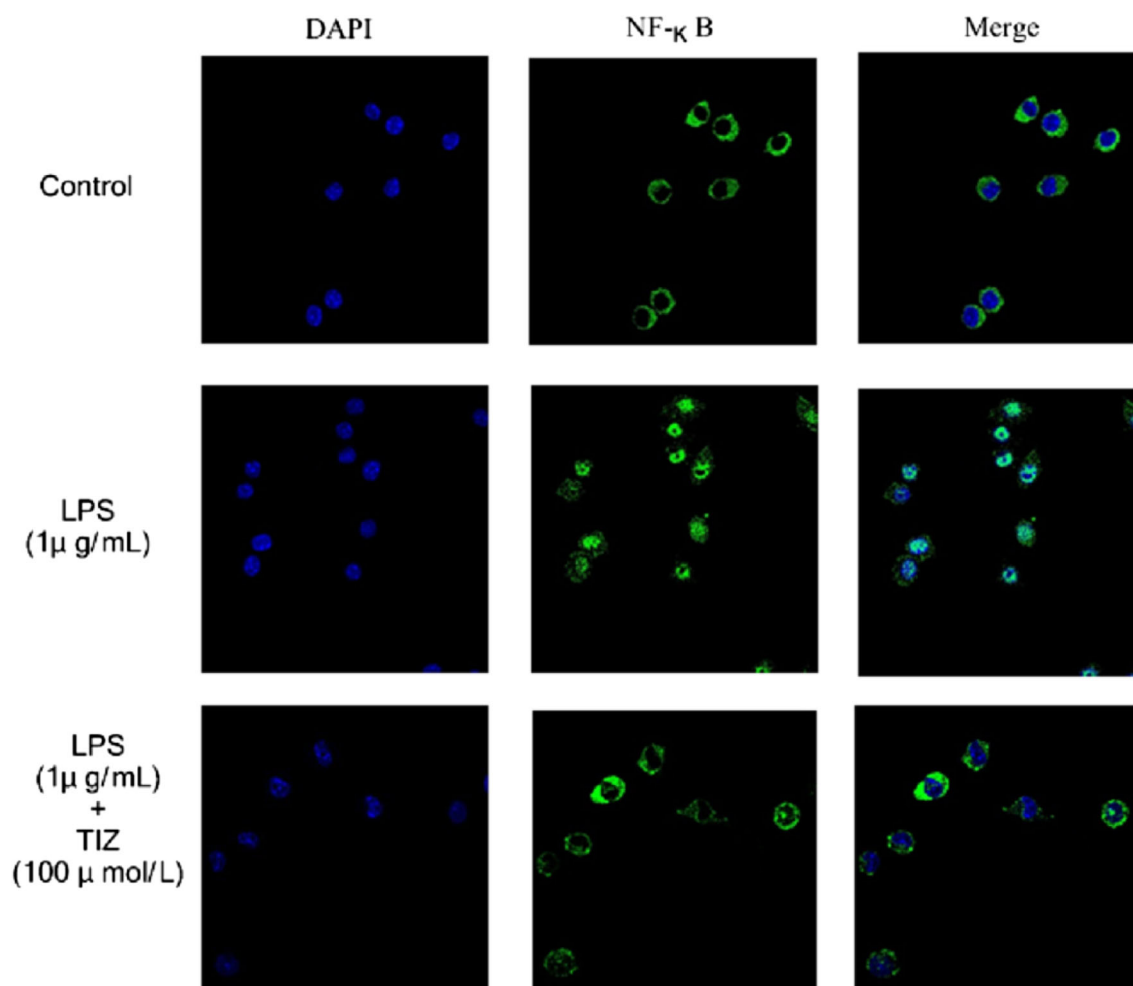
**Fig. 5.** Effects of TIZ on **a** NO production, **b** iNOS protein expression, and **c** iNOS gene transcription in LPS-stimulated RAW264.7 cells. Cells were incubated with or without LPS (1 µg/mL) in the presence of various doses (50, 100, and 150 µmol/L) of TIZ. Values are expressed as mean ± S.D. of three replicates. Number sign shows the significantly different ( $P < 0.01$ ) when single LPS-treated group compared with untreated group; double asterisks shows the significantly different ( $P < 0.01$ ) when TIZ groups compared with single LPS-treated group.

the anti-inflammatory effects of TIZ on LPS-stimulated RAW264.7 cells, western blotting was used to investigate the protein expression levels of COX-2 and HO-1. The result showed that the protein



**Fig. 6.** Effect of TIZ on relative protein expression levels of COX-2 and HO-1 in LPS-activated RAW264.7 cells. Cells were incubated with or without LPS (1 µg/mL) in the presence of various doses (50, 100, and 150 µmol/L) of TIZ. **a** The expressions of proteins were determined with western blotting. **b** The relative protein levels of COX-2. **c** The relative protein levels of HO-1. Values are expressed as mean ± S.D. of three replicates. Number sign shows the significantly different ( $P < 0.01$ ) when single LPS-treated group compared with untreated group; double asterisks show the significantly different ( $P < 0.01$ ) when TIZ groups compared with single LPS-treated group.

levels of COX-2 were notably decreased 24–49% and HO-1 were notably decreased 28–64% in the TIZ-treated macrophage cells, when compared with control groups (Fig. 6). The decreased production of COX-2 in the LPS-treated RAW264.7 cells with a dose-dependent manner suggested that TIZ exhibited anti-inflammatory activity.



**Fig. 7.** Immunofluorescence staining of NF- $\kappa$ B. Cells were incubated with or without LPS (1  $\mu$ g/mL) in the presence of TIZ (100  $\mu$ mol/L) for 6 h. NF- $\kappa$ B p65 was detected by Alexa Fluor 488-labeled immunostaining (green); nuclear was stained by DAPI (blue).

### Inhibit NF- $\kappa$ B Translocation to Nucleus

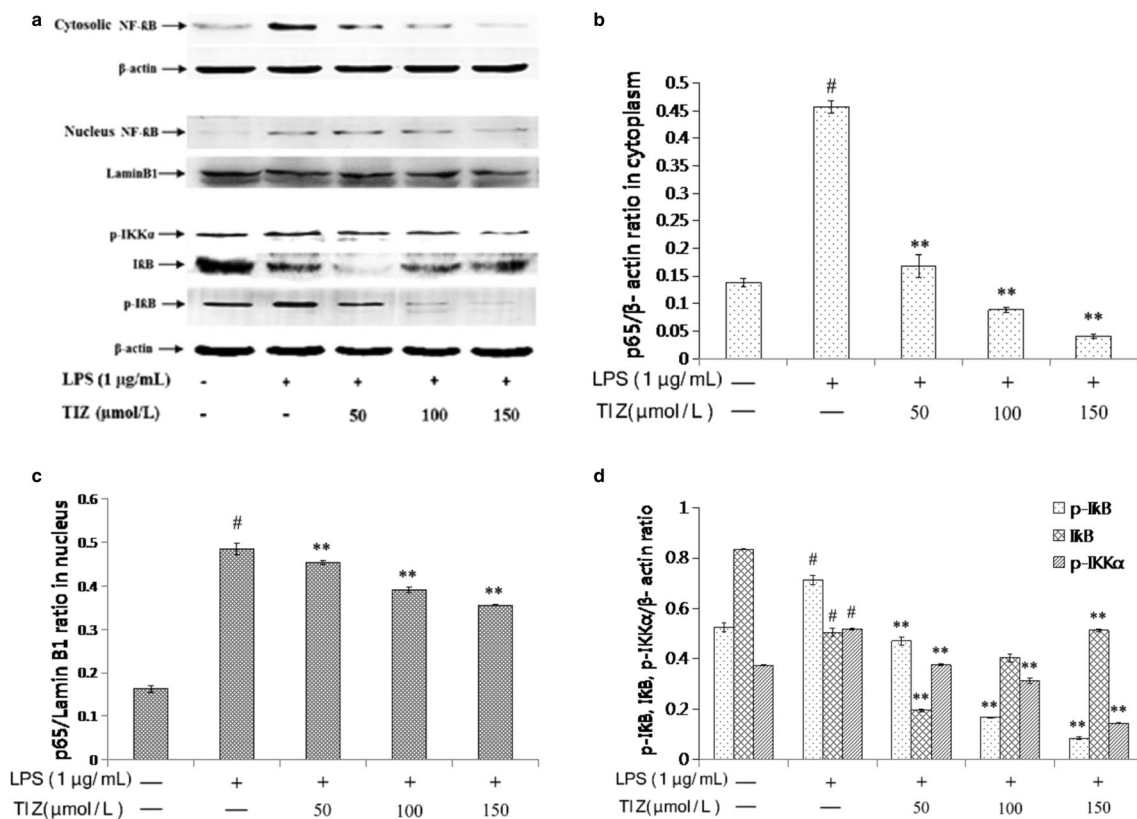
Regulating the expression of pro-inflammatory cytokines and inflammatory mediators in activated immune cells, NF- $\kappa$ B pathway plays a crucial role [3]. To investigate the effect of TIZ on LPS-activated NF- $\kappa$ B nuclear translocation, confocal laser scanning fluorescence microscopy was used to detect the NF- $\kappa$ B p65 translocation. As shown in Fig. 7, the expression of NF- $\kappa$ B in the nucleus of RAW264.7 cells stimulated with LPS alone significantly increased than normal cells without LPS and TIZ stimulation, suggesting that LPS incubation could significantly boost the NF- $\kappa$ B from cytoplasm translocation to nucleus. However, TIZ treatment for 6 h significantly attenuated the expression of NF- $\kappa$ B in the nucleus of LPS-stimulated

RAW264.7 cells. Therefore, the TIZ-treated cells showed an inhibition of this translocation.

### Inhibit the Proteins Expression of NF- $\kappa$ B Pathway

To better to interpret the mechanisms *in vitro*, the NF- $\kappa$ B signaling activation was assessed by western blotting in LPS-stimulated RAW264.7 cells. As shown in Fig. 8, NF- $\kappa$ B p65 expression was decreased 63–91% in cytoplasm and 5–26% in nucleus with various concentrations TIZ treatment (50, 100, 150  $\mu$ mol/mL) when compared with LPS-stimulated cells. As expected, LPS activated the NF- $\kappa$ B p65 subunit in cytoplasm and nucleus, while TIZ dramatically attenuated the activation with a dose-dependent manner. Furthermore, the data showed the





**Fig. 8.** Effect of TIZ on phosphorylation and degradation of NF- $\kappa$ B p65, p-I $\kappa$ B, and p-IKK $\alpha$ . Cells were treated with TIZ (50, 100, and 150  $\mu$ mol/L) and LPS (1  $\mu$ g/mL) for 6 h. **a** The expressions of proteins were determined with western blotting. **b** The activation of NF- $\kappa$ B p65 in the cytoplasm. **c** The activation of NF- $\kappa$ B p65 in the nucleus. **d** The degradation of p-I $\kappa$ B and p-IKK $\alpha$  with the increase of I $\kappa$ B. Values are expressed as mean  $\pm$  S.D. of three replicates. Number sign shows the significantly different ( $P < 0.01$ ) when single LPS-treated group compared with untreated group; double asterisks show the significantly different ( $P < 0.01$ ) when TIZ groups compared with single LPS-treated group.

phosphorylation of I $\kappa$ B and IKK $\alpha$  was activated by LPS and blocked by TIZ in RAW264.7 cells. The values of p-IKK $\alpha$  expression decreased 28–71% with various concentrations of TIZ treatment. Taken together, these results indicated that TIZ might participate in the inhibition of NF- $\kappa$ B pathway activated which mediates the inflammatory process of LPS-stimulated RAW264.7 cells.

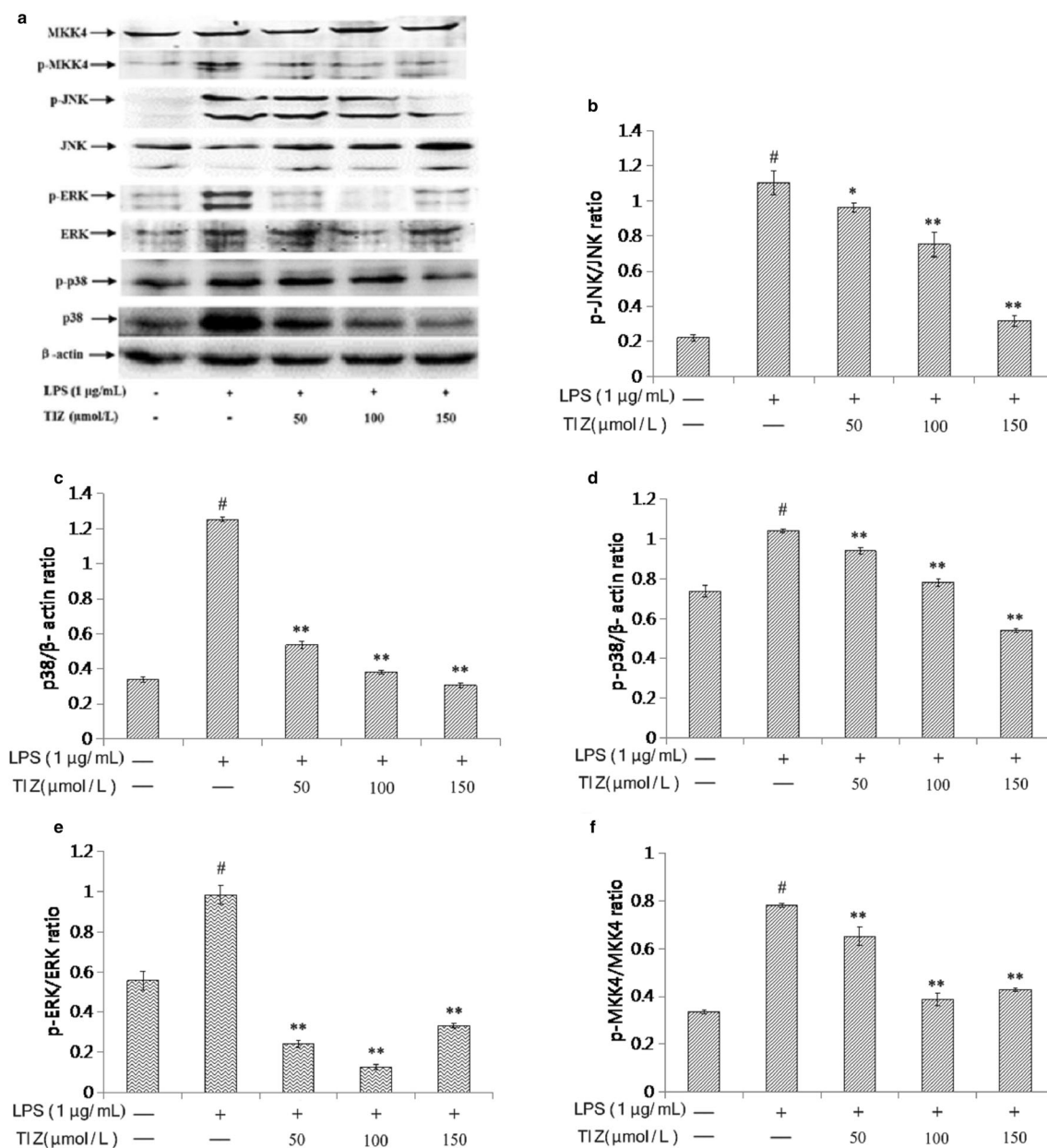
### Inhibit the Proteins Expression of MAPK Pathway

In the regulation of inflammatory process, the members of MAPKs signaling pathway play an important role [12]. Thus, the levels of MKK4, p-MKK4, JNK, p-JNK, ERK, and p-ERK protein expression were investigated in LPS-stimulated RAW264.7 cells. As shown in Fig. 9, LPS stimulation significantly increased the levels of phosphorylations and/or expression of JNK, p38, ERK, and MKK4 protein; however, the situation was suppressed effectively

with TIZ incubation 6 h. For example, compared with LPS-stimulated cells the values of p-ERK/ERK and p-JNK/JNK decreased 64–85% and 13–71% with various concentrations of TIZ treatment. The data indicated that TIZ could participate in the inhibition of the MAPK members activated in LPS-stimulated RAW264.7 cells.

### DISCUSSION

This study investigated the inhibitory effects of TIZ on inflammatory pathogenesis, and its molecular mechanism by LPS-activated RAW264.7 murine macrophages. The current results were the first time to figure out that TIZ repressed the inflammation in LPS-stimulated RAW264.7 cells based on an underlying mechanism involved in the activation of NF- $\kappa$ B and MAPK family molecule. In addition, the current study revealed the thiazolide compounds

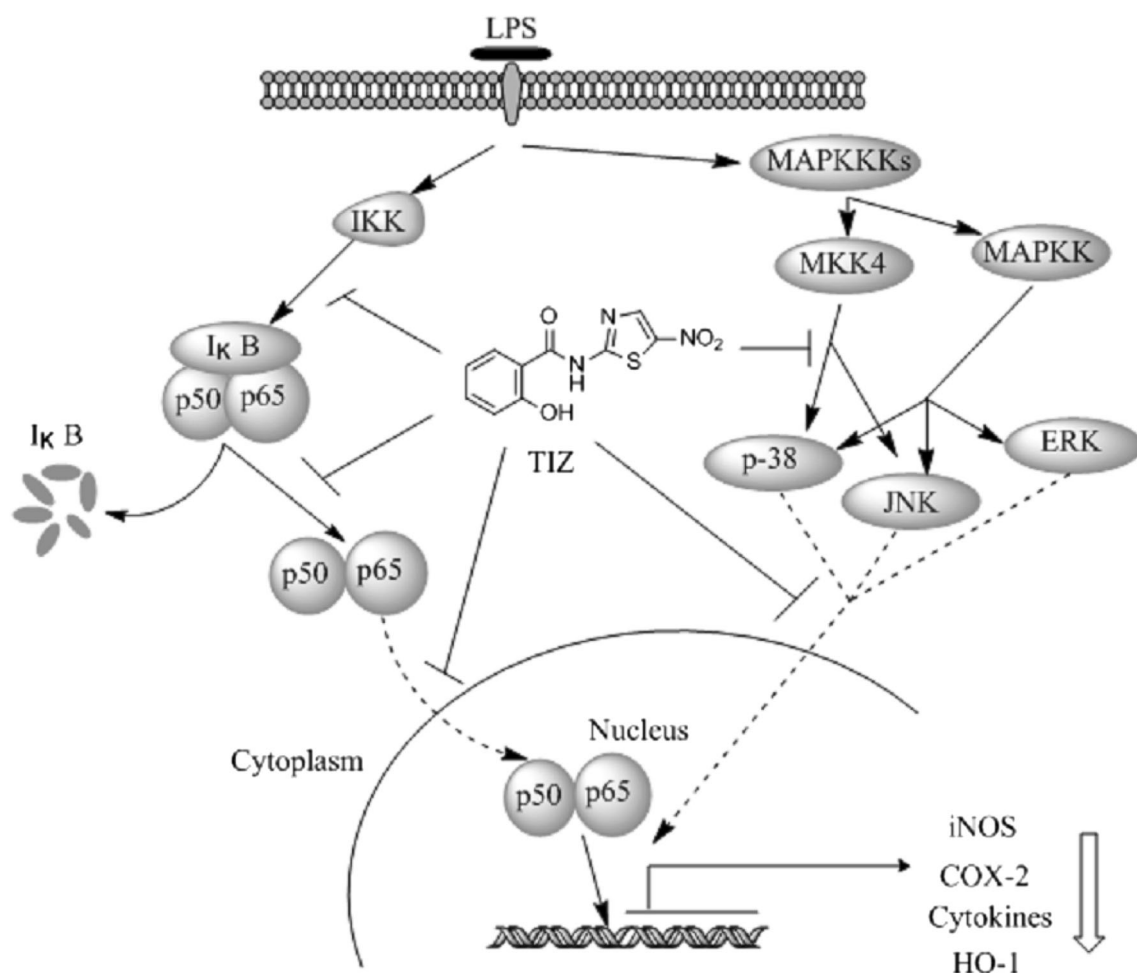


**Fig. 9.** Effects of TIZ on phosphorylation and degradation of MKK4, JNK, ERK, and p38 in LPS-activated RAW264.7 cells. Cells were treated with TIZ (50, 100, and 150 μmol/L) and LPS (1 μg/mL) for 6 h. **a** The expression of proteins was detected by western blot analysis. **b** The degradation and phosphorylation of JNK. **c** The relative protein levels of p38. **d** The relative protein levels of p-p38. **e** The degradation and phosphorylation of ERK. **f** The degradation and phosphorylation of MKK4. Values are expressed as mean ± S.D. of three replicates. Number sign shows the significantly different ( $P < 0.01$ ) when single LPS-treated group compared with untreated group; single asterisk or double asterisks shows the significantly different ( $P < 0.05$  or  $P < 0.01$ ) when TIZ groups compared with single LPS-treated group.

could be a new anti-inflammatory agent and inhibitor of NF-κB and MAPK pathway.

Under excessive and prolonged inflammation, the host may be threatened with serious diseases such as myocardial infarction, acute lung injury, and even cancer [35].

As a canonical model on inflammation research, LPS can directly activate RAW264.7 macrophages and lead to the over-expression of inflammatory cytokines and mediators [36]. Increased release of inflammatory cytokines (e.g. IL-1β, IL-6 and TNF-α), as well as excess production of NO



**Fig. 10.** In the proposed model of the molecular mechanism, TIZ exerts its anti-inflammatory effects in LPS-stimulated RAW264.7 cells. The anti-inflammatory functions have been shown through inactivating phosphorylation of I $\kappa$ B, p-38, JNK, and ERK and inhibiting translocation of NF- $\kappa$ B from cytoplasm to nucleus. Ultimately, NF- $\kappa$ B and MAPK pathway are blocked to produce pro-inflammatory enzymes, iNOS, COX-2, HO-1, and pro-inflammatory cytokines.  $\leftarrow$  means activation;  $\perp$  means inhibition.

and prostaglandins under inflammatory conditions, all contribute to intracellular cascades of inflammation related molecular degeneration. Therefore, a good strategy in fighting inflammation is to search for new agents with multifactorial mode of anti-inflammatory action [37].

NTZ is a synthetic thiazolide derivative and the active prodrug of TIZ [17, 26, 38]. NTZ exhibits polypharmacology actions *via* its active metabolite TIZ [24, 38], but less is known about its potential role in anti-inflammatory. When LPS is bound with TLR4, it will initiate the inflammatory response in macrophage, which additionally activates NF- $\kappa$ B and MAPK pathway and responds to release of pro-inflammatory cytokines [39, 40]. Inhibition of these cytokines and their upstream signaling molecules is an effective therapy for inflammatory

diseases [39]. Upon LPS stimulation, macrophages could subsequently secrete excessive inflammatory cytokines including IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in the early stage of inflammatory diseases. IL-1 $\beta$  arranges the following immune responses to both the local and systemic levels, which stimulate macrophages to secrete other inflammatory cytokines [3, 41]. IL-6 could expand the inflammatory cascade and lead to the inflammatory process which plays an important role in the innate and adaptive immune [29]. As an acknowledged key cytokine, TNF- $\alpha$  stimulates other cytokines producing and promotes immune response process in the pathogenesis of inflammation [2]. Hong et al. reported the production of IL-6 was suppressed by NTZ in stimulated macrophages [42]. In the present study, the efficacy of TIZ on IL-1 $\beta$ , IL-6, and TNF- $\alpha$  was further

assessed *in vitro* for the first time. As expected, the three cytokine production of LPS-stimulated macrophages all downregulated with concentration-dependent by TIZ. Furthermore, the qPCR data revealed that TIZ significantly blocked their genes transcription.

Blocking over-production of NO was applied as a promising strategy targeting to anti-inflammatory diseases due to NO acts as a critical intracellular messenger in the development of inflammatory pathogenesis [29, 43]. Up-regulated iNOS in cells consequently triggers the burst of NO generation and physiological dysfunction [29]. COX-2 is responsible for catalyzing arachidonic acid (AA) conversion to prostaglandin E2 (PGE2) and should maintain the low level of expression in normal cells because excessive PGE2 could induce inflammation and seriously threaten host health [12]. HO-1 is an essential enzyme to cleave heme which could be a sensitive indicator of cellular stresses such as LPS-activated inflammation [34]. In this study, the data demonstrated that COX-2, HO-1, and iNOS expressions were downregulated and NO production was decreased by TIZ in LPS-stimulated RAW264.7 cells.

Peak plasma TIZ concentrations in healthy human volunteers after 7 days of treatment with 1 g NTZ every 12 h reached 22  $\mu\text{g/ml}$  [16]. Single doses of 4 g yielded C<sub>max</sub> values of 17.5  $\mu\text{g/ml}$ , with an upper range of 26.5  $\mu\text{g/ml}$  [16]. Above 3  $\mu\text{M}$  NTZ and/or TIZ caused an increase in autophagosomes in MCF-7 cells [26]. This study indicated that TIZ begin to have activity against inflammation at 50  $\mu\text{M}$  (about 15  $\mu\text{g/mL}$ ). Undoubtedly, the above results suggested that attributed to attenuate the inflammatory cytokines, TIZ has a protective effect on LPS-stimulated inflammation. Therefore, we supposed that TIZ might participate in regulation of TLR4 signaling pathway.

It has been illustrated that NF- $\kappa$ B and MAPKs are the main downstream of TLR4 pathway which participate in the LPS-stimulated inflammatory response [11, 12]. Bound with I $\kappa$ B, NF- $\kappa$ B complex cannot translocate from cytoplasm to the nucleus and its activity is restricted [2]. NF- $\kappa$ B could be released *via* the phosphorylation and degradation of the inhibitory protein I $\kappa$ B during LPS stimulation [29]. Subsequently, p65 subunit separated from NF- $\kappa$ B complex translocates to nucleus and triggers the target gene transcription such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$  through the canonical pathway [3, 44, 45]. MAPK is a family containing parallel kinase modules JNK, p38, and ERKs which phosphorylate both other protein kinases and involved in inflammation, gene transcription, apoptosis, and differentiation [12, 29, 36, 46]. *Via* investigation the effects of TIZ on LPS-stimulated NF- $\kappa$ B and MAPKs in RAW264.7 cells, we found that TIZ regulated the NF- $\kappa$ B pathway by suppressing LPS induction

to IKK $\alpha$ , p-I $\kappa$ B, and NF- $\kappa$ B-p65 activity. Noteworthy, TIZ blocked LPS-stimulated degradation of I $\kappa$ B, which held back the nuclear translocation of NF- $\kappa$ B. Meanwhile, our studies demonstrated that the increased phosphorylation of JNK, p38, and ERKs of MAPK pathways which LPS stimulated was inhibited by treatment with TIZ in RAW264.7 cells. The above signaling pathways were taken together as illustrated in Fig. 10.

In summary, we explored the effect of TIZ on the inhibition of inflammation in RAW264.7 macrophage for the first time. The findings revealed the potential mechanism of TIZ on ameliorating inflammatory responses stimulated by LPS in RAW264.7 cells, which is involved in the attenuation of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and NO production *via* the blockade of NF- $\kappa$ B and MAPK pathways. Further studies are warranted to develop thiazolidine analogues of therapeutic applications for inflammatory disorders on account of NTZ/TIZ holding great promises for the treatment of inflammatory.

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## COMPLIANCE WITH ETHICAL STANDARDS

**Conflict of Interest.** The authors declare that they have no competing interests.

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