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Immunomodulatory and anti-inflammatory effects of total flavonoids of *Astragalus* by regulating NF-κB and MAPK signalling pathways in RAW 264.7 macrophages

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Received July 5, 2018, accepted July 12, 2018

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Pharmazie 73: 589-593 (2008) *doi: 10.1691/ph.2018.8633*

Astragalus membranaceus Bunge has long been used to improve immune function in traditional Chinese medicine. The total flavonoids of *Astragalus* (TFA) are the main active components isolated from *Astragalus membranaceus* Bunge. Our recent study has shown that TFA has *in vivo* and *in vitro* immunomodulatory and anti-inflammatory effects; however, its potential mechanisms have not yet been elucidated. The present study aims to confirm the immunomodulatory and anti-inflammatory mechanisms of the action involved. Murine RAW 264.7 macrophages were treated with 10, 25 and 100 μg/ml of TFA. The mRNA expression levels of the tumour necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, IL-10, inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 were examined by RT-PCR in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. The protein expression levels of iNOS and COX-2, in addition to the phosphorylations of proteins in the mitogen activated protein kinase (MAPK) and nuclear factor (NF)-κB signalling pathways were measured by Western blot in LPS-stimulated RAW 264.7 macrophages. The results showed that TFA significantly inhibited TNF-α, IL-1β, IL-6, iNOS and COX-2 mRNA levels and increased IL-10 mRNA level in LPS-stimulated RAW 264.7 cells in a dose-dependent manner. Further studies revealed that TFA significantly inhibited iNOS and COX-2 protein levels, the phosphorylations of p38 and JNK in MAPKs pathway and IKKα/β, IκBα and the expression of nuclear NF-κB p65 in NF-κB pathway in LPS-stimulated RAW 264.7 cells. It suggests that TFA possesses immunomodulatory and anti-inflammatory effects by regulating MAPK and NF-κB signalling pathways in RAW 264.7 macrophages.

1. Introduction

Astragalus membranaceus Bunge is one of the oldest and most widely used crude drugs in traditional Chinese medicine. It has been listed in the Pharmacopoeia and Veterinary Pharmacopoeia of the People's Republic of China as a tonic and has multiple functions such as tonifying Qi and lifting yang, strengthening superficial resistance and reducing sweat, inducing diuresis to alleviate oedema, and promoting pus discharge and tissue regeneration (Pharmacopoeia Committee of PRC 2015; Veterinary Pharmacopoeia Committee of PRC 2011). Clinical practice and pharmacological studies have demonstrated that *Astragalus membranaceus* Bunge has a wide range of pharmacological activity including immunomodulation, cardiovascular protection, hepatoprotection, neuroprotection, anti-oxidation, anti-hyperglycaemia, anti-cancer, anti-virus, anti-inflammation and anti-allergic rhinitis (McKenna et al. 2002; Ryu et al. 2008; You et al. 2011; Cho and Leung 2007). The total flavonoids of *Astragalus* (TFA) are among the most beneficial active components isolated from the dried root of *Astragalus membranaceus* Bunge. A series of studies have shown that TFA have anti-oxidant, anti-tumour, anti-mutagenic, anti-atherosclerosis and other biological effects (Zhang et al. 2012; Zhang and Wang 2010; Wang et al. 2012). Accordingly, TFA have significant immunomodulatory and anti-inflammatory effects by regulating the cytokine and mediator tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, nitric oxide (NO) production in RAW 264.7 macrophages (Guo et al. 2016); however, its further potential mechanisms for these effects have not yet been elucidated.

As important effector cells of the immune system, macrophages play an essential role in immune response and host defence against a variety of infections (Linehan et al. 2000; Nazimek and Bryniarski 2012). Therefore, macrophages are recognized as target cells for a variety of immunomodulatory and anti-inflammatory drugs (Klein 2005). The nuclear factor (NF)-κB is essential for host defence and inflammatory responses (Li and Verma 2002), and the mitogen activated protein kinases (MAPKs) pathway is another major extracellular signal transduction pathway involved in inflammatory and immune responses (Takeda et al. 2003). NF-κB and MAPKs are therefore known as important targets for anti-inflammatory and immunomodulatory components. In this study, we investigated the effects of TFA on NF-κB and MAPKs pathways to confirm the immunomodulatory and anti-inflammatory mechanisms of TFA by using murine RAW 264.7 macrophages.

2. Investigations and results

2.1. Effects of TFA on cytokine TNF-α, IL-6, IL-1β, IL-10 mRNA expression in LPS-stimulated RAW 264.7 cells

The effects of TFA on the mRNA expression of cytokine TNF-α, IL-6, IL-1β and IL-10 were determined by RT-PCR. As shown in Fig. 1, LPS treatment caused a marked increase in mRNA levels of TNF-α, IL-6, IL-1β and IL-10 compared with those of the control group ($P<0.01$). In contrast, co-treatment with TFA significantly attenuated the transcriptional levels of TNF-α, IL-6, IL-1β dependently and increased the transcriptional level of IL-10 compared with the LPS group ($P<0.05$ or $P<0.01$). It showed that

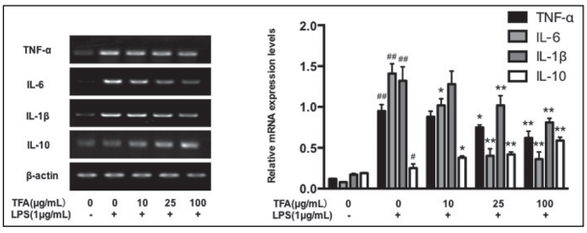

Fig. 1: Effects of TFA on cytokine TNF-α, IL-6, IL-1β, IL-10 mRNA expression in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with 10, 25, and 100 μg/ml of TFA and induced with 1 μg/ml of LPS for 12 h. The mRNA expression of TNF-α, IL-6, IL-1β and IL-10 was determined by RT-PCR. The relative mRNA expression levels of TNF-α, IL-1β, IL-6 and IL-10 were normalized using β-actin as references. The values are expressed as the means ± SEM of results obtained from at least three independent experiments. ##P < 0.01 vs. control group; *P < 0.05, **P < 0.01 vs. LPS group..

TFA modulated the LPS-stimulated RAW 264.7 macrophage function in the transcriptional levels of cytokines.

2.2. Effects of TFA on iNOS and COX-2 mRNA expression in LPS-stimulated RAW 264.7 cells

The effects of TFA on iNOS and COX-2 mRNA expression were examined by RT-PCT. As shown in Fig. 2, LPS treatment caused a

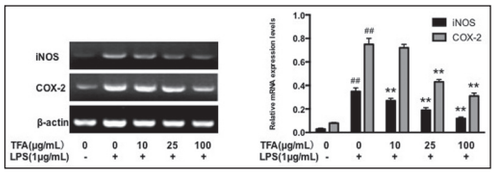

Fig. 2: Effects of TFA on iNOS and COX-2 mRNA in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with 10, 25, and 100 μg/ml of TFA and induced with 1 μg/ml of LPS for 12 h. The mRNA expression of iNOS and COX-2 was examined by RT-PCR. The relative mRNA expression levels of iNOS and COX-2 were normalized using β-actin as references. The values are expressed as the means ± SEM of results obtained from at least three independent experiments. ##$P < 0.01$ vs. control group; **$P < 0.01$ vs. LPS group.

marked increase in mRNA expression levels of iNOS and COX-2 compared with that of the control group (P<0.01). In contrast, co-treatment with TFA significantly decreased iNOS and COX-2 mRNA expression compared with the LPS group (P<0.05 or P<0.01). It showed that TFA attenuated the transcriptional levels of iNOS and COX-2.

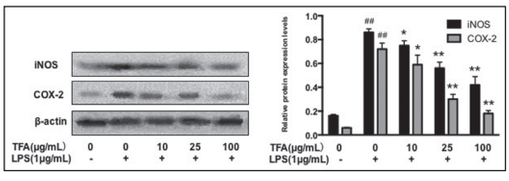

Fig. 3: Effects of TFA on iNOS and COX-2 protein expression in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with 10, 25, and 100 μg/ml of TFA and induced with 1 μg/ml of LPS for 24 h. The protein expression of iNOS and COX-2 was examined by Western blot. The relative protein expression levels of iNOS and COX-2 were normalized using β-actin as references. The values are expressed as the means ± SEM of results obtained from at least three independent experiments. ##$P < 0.01$ vs. control group; *$P < 0.05$, **$P < 0.01$ vs. LPS group.

2.3. Effects of TFA on iNOS and COX-2 protein expression in LPS-stimulated RAW 264.7 cells

The effects of TFA on iNOS and COX-2 protein expression were examined by Western blot. As shown in Fig. 3, LPS treatment caused a marked increase in protein expression levels of iNOS and COX-2 compared with that of the control group (P<0.01). In contrast, co-treatment with TFA significantly attenuated the protein levels of iNOS and COX-2 compared with the LPS group (P<0.05 or P<0.01). The effects of TFA on iNOS and COX-2 protein expression are consistent with the effects of TFA on iNOS and COX-2 mRNA expression in LPS-stimulated RAW 264.7 cells.

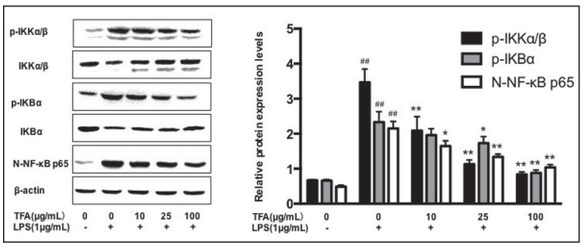

Fig. 4: Effects of TFA on NF-κB signalling pathway of LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with 10, 25, and 100 μg/ml of TFA and induced with 1 μg/ml of LPS for 30 min. The phosphorylations of IKKα/β, IKBα and the NF-κB p65 expression in the nucleus of RAW 264.7 cells were determined by Western blot. The relative protein expression levels of p-IKKα/β, p-IKBα and NF-κB p65 were normalized using IKKα/β, IκBα, and β-actin as references, respectively. The values are expressed as the means ± SEM of results obtained from at least three independent experiments. ##$P < 0.01$ vs. control group; *$P < 0.05$, **$P < 0.01$ vs. LPS group.

2.4. Effects of TFA on NF-κB signalling pathway of LPS-stimulated RAW 264.7 cells

To further confirm the effects of TFA on activation of the NF-κB pathway, the phosphorylation of IKKα/β, IKBα and the NF-κB p65 expression in the nucleus of RAW 264.7 cells were determined by Western blot analysis. As shown in Fig. 4, stimulation with LPS resulted in a marked phosphorylation of IKKα/β, IKBα and the accumulation of NF-κB p65 in the nucleus compared with those of the control group (P<0.01). Treatment with TFA, however, significantly inhibited the phosphorylation of IKKα/β, IKBα and

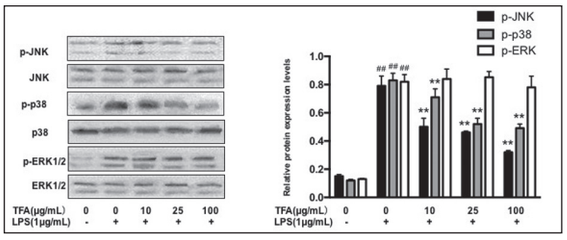

Fig. 5: Effects of TFA on MAPKs signalling pathway in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with 10, 25, and 100 μg/ml of TFA and induced with 1 μg/ml of LPS for 30 min. The phosphorylations of JNK, p38 and ERK1/2 were determined by Western blot. The relative protein expression levels of p-JNK, p-p38 and p-ERK1/2 were normalized using JNK, p38 and ERK1/2 as references, respectively. The values are expressed as the means ± SEM of results obtained from at least three independent experiments. ##$P < 0.01$ vs. control group; **$P < 0.01$ vs. LPS group.

the nuclear translocation of NF-κB p65 in LPS-stimulated RAW 264.7 cells compared with the LPS group (P<0.05 or P<0.01).

2.5. Effects of TFA on MAPKs signalling pathway in LPS-stimulated RAW 264.7 cells

We next evaluated the effects of TFA on MAPKs pathway activation including ERK1/2, p38 and JNK MAPKs phosphorylation levels in RAW 264.7 cells by Western blot analysis. As shown in Fig. 5, stimulation with LPS significantly increased the phosphorylation of JNK, p38 and ERK1/2 in RAW 264.7 cells compared with those of the control group (P<0.01). TFA significantly inhib-

ited the phosphorylation of JNK and p38 induced by LPS ($P<0.05$ or $P<0.01$), however, and had no significant effect on ERK1/2 phosphorylation induced by LPS ($P>0.05$).

3. Discussion

It is well known that macrophages play a fundamental role in regulating inflammation and immune responses. Therefore, macrophages are often used in studying anti-inflammatory and immune drugs (Cheng et al. 2014; Morimoto et al. 2014). Our recent study has demonstrated that TFA have significant immunomodulatory and anti-inflammatory effects by regulating cytokine and mediator TNF-α, IL-1β, IL-6, NO production in RAW 264.7 macrophages (Guo et al. 2016). In the present study, we further found that TFA mediated these cytokines' mRNA, inducible enzymes' mRNA and related protein expression by regulating NF-κB and MAPKs signalling pathways.

The cytokines play a crucial role in various kinds of diseases (Popa et al. 2007; Dinarello 2005; Gabay 2006; Nishimoto and Kishimoto 2004). In particular, they are considered to be important initiators of the inflammatory response and mediators of the development of various inflammatory diseases (Glauser 1996; Männel and Echtenacher 2000). Pro-inflammatory cytokines may be produced by macrophages in inflammatory response, for example, when stimulated by LPS: the TLR4 signalling is activated, and then the expression of several cytokines TNF-α, IL-6, and IL-1β increases in macrophages (Fujiwara and Kobayashi 2005; Reddy and Reddanna 2009; Rossol et al. 2011; Ferwerda et al. 2008; Guha and Mackman 2001). In contrast, the anti-inflammatory factor IL-10 can inhibit the development of inflammation (Mosser and Zhang 2008). Therefore, a good anti-inflammatory drug should inhibit the release and expression of pro-inflammatory cytokines and increase the release and expression of anti-inflammatory cytokines. We have reported that TFA markedly inhibited the over-secretion of cytokines TNF-α, IL-6 and IL-1β in LPS-stimulated macrophages (Guo et al. 2016). It also inhibited the over-production serum TNF-α and IL-1β in adjuvant-induced arthritic rats (Liu et al. 2017). In this study, our data showed that TFA inhibited the over-expression of TNF-α, IL-6 and IL-1β mRNA in LPS-stimulated RAW 264.7 cells, and it was consistent with our previous results. Further, TFA increased IL-10 expression in RAW 264.7 cells. This suggested that TFA exerted modulatory effects on the secretion of cytokines through modulating these cytokines' mRNA expression.

INOS and COX-2 are two critical inducible enzymes responsible for the production of the mediators NO and PGE_2, respectively. The pro-inflammatory mediator NO is strongly connected with inflammation and immune regulation, which is produced by iNOS when acting on L-arginine (Xie and Nathan 1994; Zamora et al. 2000). The modulation of iNOS-mediated NO release is one of the major contributing factors during the inflammatory process (Kim et al. 2003). The PGE_2 generated by COX-2 can induce many inflammatory diseases, including cancer, rheumatoid arthritis and cardiovascular disease (Turini and DuBois 2000). The level of iNOS and COX-2 expression is well correlated with the degree of inflammation (Kimura et al.1998). Our report has demonstrated that TFA modulated the over-production of NO in LPS-stimulated RAW 264.7 macrophages (Guo et al. 2016). We have also shown that TFA inhibited serum PGE_2 production in adjuvant-induced arthritic rats (Liu et al. 2017). In the present study, our results showed that TFA inhibited the over-expression of iNOS and COX-2 mRNA and proteins in LPS-stimulated RAW264.7 cells. These results are consistent with our previous reports and imply that TFA might suppress NO and PGE_2 production through inhibiting iNOS and COX-2 mRNA and protein expression, respectively.

The NF-kB signalling pathway is regarded as the most commonly involved pro-inflammation pathway (Gilmore 2006; Chung et al. 2010). Many studies have shown that NF-κB plays a pivotal role in multiple immune and inflammatory responses by regulating genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes such as COX-2 and iNOS (Surh et al. 2001; Tsatsanis et al. 2006). The expression of many inflammation and immune-related genes is regulated through the NF-κB signalling pathway. In unstimulated cells, NF-κB resides in the cytoplasm as an inactive complex bound to the inhibitory protein IκB. In response to various stimuli such as LPS, the inhibitor of the IκB kinases (IKKs) is activated, the IκB protein is phosphorylated and subsequently degraded, and then dissociates to release free NF-κB dimers. The free NF-κB is rapidly transferred into the nucleus interacting with the NF-κB binding sequences, and triggers iNOS, COX-2, IL-1β, and TNF-α expression (Cho et al. 2009; Zhu et al. 2013, 2015; Hong et al. 2015). In this study, the experimental results showed that treatment with LPS alone significantly increased the phosphorylation of IKKα/β and IKBα, and the expression of NF-κB p65 in the nucleus. Phosphorylation was markedly inhibited by co-treatment with TFA in LPS-induced RAW264.7 macrophages, however, suggesting that the activation of NF-κB pathway is blocked by the inhibition of the phosphorylation of IKKα/β and IKBα, as well as NF-κB p65 expression in the nucleus by TFA.

As another major signal transduction pathway, the MAPKs pathway also plays a critical role in the control of cellular responses to cytokines and stress, as well as in the regulation of cell growth differentiation (Imajo et al. 2006). MAPKs are a family of protein kinases including JNK, p38 and ERK1/2. It has been reported that MAPK phosphorylation can induce NO and PGE_2 production in stimulated macrophages (Chang et al. 2006; Chen et al. 1999). P38, JNK and ERK can be activated through LPS stimulation, p38 being involved in regulating iNOS and TNF-α gene expression, JNK being involved in regulating iNOS expression and ERK being associated with TNF-α production (Uto et al. 2005; Dumitru et al. 2000; Bak et al. 2012). Our present results indicated TFA could suppress the phosphorylation of JNK and p38 in LPS-stimulated RAW264.7 macrophages but had no significant effect on ERK phosphorylation. This suggests that TFA exerted immunomodulatory and anti-inflammatory effects by regulating JNK and p38 MAPKs signalling pathways.

In conclusion, TFA can decrease TNF-α, IL-1β, IL-6, iNOS, and COX-2 expression and alleviate the inflammatory reaction in RAW264.7 macrophages stimulated by LPS. The NF-κB and MAPK signalling pathways are involved in the protection of TFA during inflammation. Therefore, TFA may be promising candidate drug for interventions in inflammatory related diseases.

4. Experimental

4.1. Reagents

LPS (*Escherichia coli* 055:B5) and dimethyl sulphoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Foetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin and other reagents for cell culture were purchased from Invitrogen-Gibco (Grand Island, NY, USA). Mouse anti-COX-2 monoclonal antibody, mouse anti-iNOS monoclonal antibody, mouse anti-JNK monoclonal antibody, mouse anti-ERK monoclonal antibody, mouse anti-p38 monoclonal antibody, mouse anti-phospho-JNK monoclonal antibody, mouse anti-phospho-ERK monoclonal antibody, mouse anti-phospho-p38 monoclonal antibody, horseradish peroxidase (HRP)-linked goat anti-mouse IgG, and β-actin monoclonal antibody were purchased from Santa Cruz (Santa Cruz, CA, USA). Rabbit anti-IκBα monoclonal antibody, rabbit anti-p-IκBα monoclonal antibody, rabbit anti-IKKα/β monoclonal antibody, rabbit anti-p-IKKα/β monoclonal antibody, rabbit anti-NF-κB p65 monoclonal antibody, HRP-linked goat anti-rabbit IgG were purchased from Cell Signaling Technology, Inc. (Chicago, IL, USA). All the other chemical reagents used in this study were of analytical grade.

4.2. Cell culture

The murine RAW 264.7 macrophage cell line was obtained from the China Cell Line Bank (Beijing, China). The cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate at 37 °C in a humidified atmosphere with 5 % CO_2 and 95 % air.

4.3. Extraction and purification of TFA

TFA were isolated from the dried roots of *Astragalus membranaceus* Bunge, which was identified and authenticated macroscopically and microscopically according to the Pharmacopoeia of the People's Republic of China. The voucher specimen (No. 151022) was deposited at the Herbarium of Yanbian University (Yanji, Jilin, China). TFA (Batch number: DST160804-093) were prepared and provided by Shanghai Yuanye Biotech Co., Ltd. (Shanghai, China). Briefly, the dried roots of *Astragalus*

Table: Primer sequences used for RT-PCR

Genes	Forward (5′→3′)	Reverse (5′→3′)	Expected size
TNF-α	AGAATGAGGCTGGATAAGA	AGAGGTTCAGTGATGTAGCG	384 bp
IL-1β	TCATTGTGGCTGTGGAGAAG	GCTTGTGAGGTGCTGATGTA	535 bp
IL-6	GCCTTCTTGGGACTGATG	CTGGCTTTGTCTTTCTTGTT	383 bp
IL-10	ACCTGGTAGAAGTGATGCC	CAAGGAGTTGTTTCCGTTA	367 bp
iNOS	CTCCTTTGAGCCCTTTGT	GAGCGAGTTGTGGATTGTC	658 bp
COX-2	ATGGTCAGTAGACTTTTACA	GGAGAGACTATCAAGATAGT	856 bp
β-actin	CTGTCCCTGTATGCCTCTG	ATGTCACGCACGATTTCC	218 bp

membranaceus Bunge were sliced and macerated in 95% ethanol and then extracted. The ethanol extraction was repeated 3-4 times and the pooled extract was then concentrated to a specific gravity of 1.35 by evaporation. The concentrated solution was extracted with ethyl acetate. The concentrated ethyl acetate extraction was further separated by silica gel columns.

4.4. RT-PCR analysis

RAW 264.7 cells (4×10^5 cells/well) were seeded in a 6-well plate, treated with or without 10, 25 and 100 μg/ml of TFA for 1 h, and then challenged with 1 μg/ml of LPS for 12 h. The total RNA from RAW 264.7 cells was extracted using TRI REAGENT™ (Invitrogen) according to the manufacturer's instructions. Sense and antisense primers for RAW 264.7 cells mRNA expression are shown in the Table. All mRNA primers were synthesized by SBS Genetech Co., Ltd. (Beijing, China). The parameters of PCR reactions were 94 ˚C for 3 min for one cycle, then 94 ˚C for 1 min, 55 ˚C for 30 s, and 72 ˚C for 1 min for 30 cycles. The amplified PCR products were separated on 1% agarose gels and visualized with ethidium bromide staining and UV irradiation. Band intensities were quantified with Quantity One software (Bio-Rad), and the bands of TNF-α, IL-1β, IL-6, IL-10, iNOS and COX-2 mRNA were normalized using β-actin as references.

4.5. Western blot analysis

RAW 264.7 cells (4×10^5 cells/well) were seeded overnight in a 6-well plate. The cells were treated with or without 10, 25 and 100 μg/ml of TFA for 1 h, and then challenged with 1 μg/ml of LPS for 12 h (for iNOS and COX-2) and 30 min (for NF-κB and MAPKs). The cells were harvested by centrifugation and washed once with phosphate buffer saline (PBS). The washed cells were treated with ice-cold lysis buffer and the supernatant was obtained by centrifugation. The protein concentrations were determined using the Bio-Rad protein assay reagent (Bio-Rad, Munich, Germany) according to the manufacturer's instructions. Protein samples were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.2 % Tween 20 (TBST) at room temperature for 1 h, followed by incubation with primary antibodies (iNOS, COX-2, IκBα, p-IκBα, IKKα/β, p-IKKα/β, NF-κB p65, JNK, p-JNK, p38, p-p38, ERK1/2, p-ERK1/2) overnight at 4 ˚C. Blots were washed with TTBS and incubated with secondary antibodies for 1 h at room temperature. Blots were washed with TTBS again and then developed by the enhanced chemiluminescence (ECL). The densitometry analysis of all bands was performed using ImageJ software (GeneGnome, Syngene, Frederick, MD, USA). The same size square was drawn around each band to measure the proteins' density. The protein levels of iNOS and COX-2, and NF-κB p65 in the nucleus were expressed as a relative value to that of β-actin. The p-IKKα/β, p-IκBα, p-JNK, p-p38, p-ERK1/2 phosphorylation levels were expressed as a relative value to that of IKKα/β, IκBα, JNK, p38 and ERK1/2, respectively.

4.6. Statistical analysis

All values are expressed as the means±SEM of results obtained from at least three independent experiments. One-way analysis of variance (ANOVA) and Student's *t*-test were used to assess differences between the treatment groups. A P-value <0.05 was considered significant.

Acknowledgements: This work was supported by a grant from the National Natural Science Foundation of China (Nos. 31660727, 31260622 and 31060349) and the Scientific and Technological Development Planning Funding Project of Jilin Province (No. 20160204016NY).

Conflicts of interest: None declared.

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