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Tanshinone IIA inhibits LPS-induced NF-κB activation in RAW 264.7 cells: Possible involvement of the NIK–IKK, ERK1/2, p38 and JNK pathways

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Abstract

Nuclear factor κB (NF-κB) activation by NF-κB-inducing kinase (NIK)–IκBα kinase (IKK) pathway and mitogen-activated protein kinases (MAPKs) pathway are important in inflammation. We recently found that the tanshinone IIA, a diterpene isolated from *Salvia miltiorrhiza* (*S. miltiorrhiza*), reduced the production of pro-inflammatory mediators in RAW 264.7 cells stimulated with lipopolysaccharide (LPS). However, little is known about the inhibitory mechanisms of tanshinone IIA on the production of pro-inflammatory mediators. To investigate the inhibitory mechanism, we determined the inhibitory effects of tanshinone IIA on the activation of NF-κB and IκBα phosphorylation, and also examined phosphorylation of NIK and IKK as well as the activation of MAPKs such as p38 MAPK (p38), extracellular signal-regulated kinases 1/2 (ERK1/2), and c-Jun N-terminal kinase (JNK) in RAW 264.7 cells stimulated with LPS. Tanshinone IIA inhibited NF-κB-DNA complex, NF-κB binding activity, and the phosphorylation of IκBα in a dose dependent manner. Tanshinone IIA also inhibited the translocation of NF-κB from cytosol to nucleus. Moreover, the phosphorylation of NIK and IKK as well as the phosphorylation of p38, ERK1/2, and JNK in the LPS-stimulated RAW 264.7 cells were suppressed by the tanshinone IIA in a dose dependent manner. These results suggest that tanshinone IIA may inhibit LPS-induced IκBα degradation and NF-κB activation via suppression of the NIK–IKK pathway as well as the MAPKs (p38, ERK1/2, and JNK) pathway in RAW 264.7 cells and these properties may provide a potential mechanism that explains the anti-inflammatory activity of tanshinone IIA.

Keywords: Tanshinone IIA; Nuclear factor κB; NF-κB-inducing kinase; IκB kinase; Mitogen-activated protein kinase; Lipopolysaccharide

1. Introduction

Macrophages play significant roles in host defense mechanism. Although inflammatory mediators such as nitric oxide (NO) and pro-inflammatory cytokines are involved in host defense mechanisms, these overproduction contributes to the pathogenesis of several diseases such as otitis media, hearing loss, periodontitis, bacterial sepsis, rheumatoid arthritis, chronic inflammation, and autoimmune diseases (Watanabe et al., 2001; Hirose et al., 2001; Song et al., 2002). The nuclear factor κB (NF-κB) family is a key player in controlling inflammatory mediators. NF-κB is activated by phosphorylation of IκB via activation of the mitogen-activated protein kinases (MAPKs) such as p38 MAPK (p38), extracellular signal-regulated kinases 1/2 (ERK1/2), and c-Jun N-terminal kinase (JNK) as well as NF-κB-inducing kinase (NIK) and IκB kinase (IKK), and it is a pleiotropic transcription factor which regulates NO and pro-inflammatory cytokine production in the activated macrophages (Kwon et al., 2002; Guha and Mackman, 2001; Christman et al., 1998).

It would be valuable to develop potent inhibitors of pro-inflammatory mediators such as NF-κB inactivator for potential therapeutic use in inflammatory diseases. Some natural products are candidates of the potential source NF-κB inactivator.

Tanshinone IIA is one of the major diterpenes from *Salvia miltiorrhiza* Bunge (*S. miltiorrhiza*) (Jang et al., 2003). *S. miltiorrhiza* has been commonly used in traditional oriental herbal medicine to treat inflammatory diseases such as edema, arthritis,

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hepatitis and endangitis (Jang et al., 2003; Kim, 1989). In a previous study, we have shown that tanshinone IIA (Fig. 1) inhibited the productions of pro-inflammatory mediators such as NO, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) in RAW 264.7 cells stimulated with lipopolysaccharide (LPS) (Jang et al., 2003). However, little is known about the inhibitory mechanisms of tanshinone IIA on the production of pro-inflammatory mediators.

In this study, we clearly demonstrate that tanshinone IIA inhibited LPS-induced IκBα degradation and NF-κB activation. In addition, we report that tanshinone IIA suppressed the NIK–IKK pathway as well as MAPKs (p38, ERK1/2, and JNK) pathway in RAW 264.7 cells stimulated with LPS.

2. Methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and antibiotics were purchased from GIBCO BRL (Grand Islang, NY, USA). Anti-NF-κB p65, anti-IκBα, anti-phosphorylated IκBα (anti-p-IκBα), anti-NIK, anti-phosphorylated NIK (anti-p-NIK), anti-IKKα/β, anti-phosphorylated IKKα/β (anti-p-IKKα/β), anti-JNK, anti-phosphorylated JNK (anti-p-JNK), anti-ERK1/2, anti-phosphorylated ERK1/2 (anti-p-ERK1/2), anti-p38, and anti-phosphorylated p38 (anti-p-p38) mouse monoclonal or polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). NF-κB p65/NF-κB p50 transcription factor assay kit was purchased from Active Motif (Carlsbad, CA, USA). NF-κB specific oligonucleotide was purchased from Promega (Madison, WI, USA). PDTC (a specific inhibitor of NF-κB), SB203580 (a specific inhibitor of p38), PD98059 (a specific inhibitor of ERK1/2), and SP600125 (a specific inhibitor of JNK) were purchased from Calbiochem (San Diego, CA, USA). All other reagents were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA).

2.2. Isolation of tanshinone IIA

Tanshinone IIA was isolated from the roots of *S. miltiorrhiza*, which is based on the method described previously (Jang et al., 2003). The purity of tanshinone IIA was proven by nuclear magnetic resonance (NMR) and high performance liquid chromatography (LC)/mass spectrometry (MS) as >95%. The structure of tanshinone IIA was identified by comparing its spectral data ([^{1}H] NMR, [^{13}C] NMR and MS) with those reported in the literature (Ryu et al., 1997). Copies of the original spectra are obtainable from the author of correspondence.

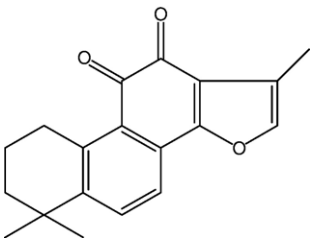

Fig. 1. Chemical structure of tanshinone IIA.

2.3. Cell culture

Murine macrophage RAW 264.7 cell line obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) was maintained at 1×10^6 cells/ml culture in DMEM supplemented with 10% heat-inactivated FBS, penicillin G (100 IU/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. On the following day, the medium was replaced with fresh DMEM, and the cells were then stimulated with LPS (1 μg/ml) in the presence or absence of tanshinone IIA isolated from *S. miltiorrhiza* for the indicated periods. The stock solutions of tanshinone IIA were prepared in dimethyl sulfoxide (DMSO), such that the final maximum concentration of DMSO in experimental media was 0.1%.

2.4. MTT assay

Cells were incubated for 24 h with 0–10 μg/ml of tanshinone IIA and some cells were activated for 24 h with LPS (1 μg/ml) after preincubation of tanshinone IIA for 2 h. Methylthiazoletetrazolium (MTT) (50 μg/ml) was add to 1 ml of cell suspension and incubated for 4 h, and the formazan formed was dissolved in acidic 2-propanol; optical density was measured using a microzplate reader (Spectra Max 250, Molecular Devices Co., Menlo Park, CA, USA) at 590 nm. The optical density of formazan formed in control (medium alone) cells was taken as 100% of viability.

2.5. NF-κB activation assay

To determine NF-κB-DNA complex formation, we conducted electrophoretic mobility shift assay (EMSA) essentially as previously described (Chaturvedi et al., 2000). Briefly, nuclear extracts (4 μg of protein) prepared from LPS-treated cells (2×10^6 cells/ml) were incubated with [^{32}P]-end-labeled 22-mer double-stranded NF-κB oligonucleotide from the human immunodeficiency virus (HIV) long terminal repeat, 5′-AGTT GAGGGGACTTTCCCAGGC-3′ for 15 min at 37 °C, and the DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5′-AGTTGAGGGGACTTTCCC AGGC-3′, was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. NF-κB binding activity was measured in nuclear extracts of treated cells using a commercially available sandwich ELISA (Active Mortif, Carlsbad, CA, USA). All procedures were performed according to the manufacturer's instructions.

2.6. Western blot for NF-κB, I-κB, NIK, IKKα/β, and MAPKs

The activation of NF-κB, p38, ERK1/2 or JNK was inhibited by pretreatment with positive controls, pyrolidine dithiocarbamate (PDTC, 10 μM), SB203580 (20 μM), PD98059 (30 μM), or SP600125 (20 μM), respectively (Birkenkamp et al., 2000; Bian

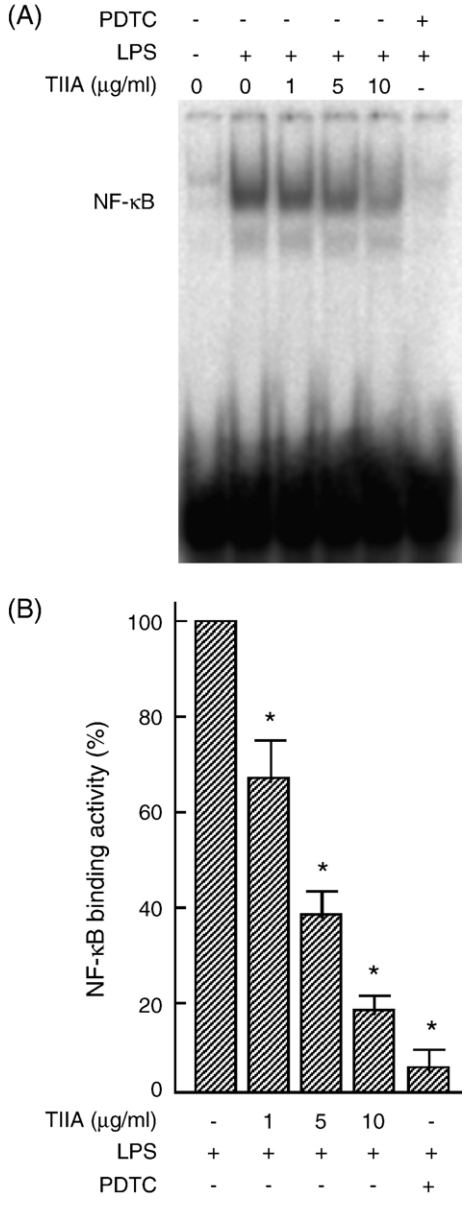

Fig. 2. Effects of tanshinone IIA (TIIA) on LPS-induced NF-κB-DNA complex formation in RAW 264.7 cells. Cells were preincubated for 2 h with or without PDTC (20 μM) or tanshinone IIA at indicated concentrations and then stimulated for 15 min with LPS (1 μg/ml). Nuclear extracts were prepared and protein (4 μg) was incubated with [^{32}P]-labeled NF-κB consensus oligonucleotide for 20 min. NF-κB-DNA complex formation was assayed by EMSA (A) and NF-κB binding activity was quantified by ELISA (B). Each column represents the mean±S.D. from three independent experiments. *$P<0.05$ indicates significant differences from the LPS-treated control group.

et al., 2003; Lee et al., 2002). Cells were treated in the presence or absence of these inhibitors or tanshinone IIA (1–10 μg/ml) for 2 h before LPS stimulation. RAW 264.7 cells were stimulated with 1 μg/ml LPS at 37 °C. LPS stimulation was stopped by the addition of ice-cold phosphate-buffered saline. The cells were then lysed for immunoblotting. To determine the levels of NF-κB, IκBα, and phosphorylated IκBα, cytoplasmic or nuclear extracts were prepared (Majumdar and Aggarwal, 2001) from LPS-treated cells, and to determine the phosphorylated levels of NIK, IKKα/β, p38, ERK1/2, and JNK, whole lysates were prepared from LPS-treated cells, and then resolved on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were electrotransferred to nitrocellulose filters, and probed with mouse monoclonal or polyclonal antibodies against NF-κB, IκBα, p-IκBα, NIK, p-NIK, IKKα/β, p38, p-p38, ERK1/2, p-ERK1/2, JNK, and p-JNK, and detected by chemiluminescence (ECL, Amersham, Arlington Heights, IL, USA).

2.7. Statistical analysis

All values are expressed as the mean±S.D. of three independent determinations. All experiments were done at least three times, each time with three or more independent observations. Statistical analysis was performed with analysis of variance (ANOVA) and Student's *t*-test.

3. Results

3.1. Effects of tanshinone IIA on NF-κB activation of LPS-stimulated RAW 264.7 cells

To investigate the inhibitory effect of tanshinone IIA on the NF-κB activation, we observed the appearance of NF-κB-DNA complex and the binding activity of NF-κB in nuclear extracts of RAW 264.7 cells stimulated with LPS for 2 h. As shown in Fig. 2, treatment with LPS alone increased the levels of NF-κB-DNA complex and NF-κB binding activity. However, tanshinone IIA inhibited the increase of NF-κB-DNA complex and NF-κB binding activity in RAW 264.7 cells stimulated with LPS in a dose dependent manner (Fig. 2A and B). The LPS-induced NF-κB activation was also markedly suppressed by PDTC (positive control).

3.2. Effects of tanshinone IIA on cell viability

To investigate the effect of tanshinone IIA on cell viability, the cells were exposed to tanshinone IIA for 24 h. As shown in Fig. 3, the concentrations (1–10 μM) of tanshinone IIA used here had no effect on the viability of RAW 264.7 cells. The LPS at 1 μg/ml reduced the viability of RAW 264.7 cells, but tanshinone IIA concentration dependently reversed LPS-induced reduction of cell viability.

3.3. Effects of tanshinone IIA on NF-κB translocation and IκB degradation in activated RAW 264.7 cells

We also investigated the translocation of NF-κB from cytosol to nucleus and degradation of IκBα. As shown in Fig. 4A, treatment with LPS alone increased the translocation of NF-κB (p65) from cytosol to the nucleus, but the tanshinone IIA dose dependently inhibited the translocation of NF-κB (p65). Next, we observed whether inhibition of NF-κB translocation by tanshinone IIA was due to the degradation of IκBα. As shown Fig. 4B, treatment with LPS alone increased phosphorylation of IκBα, but the tanshinone IIA dose dependently inhibited the phosphorylation of IκBα, which resulted in the reduction of degradation of IκBα. This finding indicates that tanshinone IIA may inhibit a

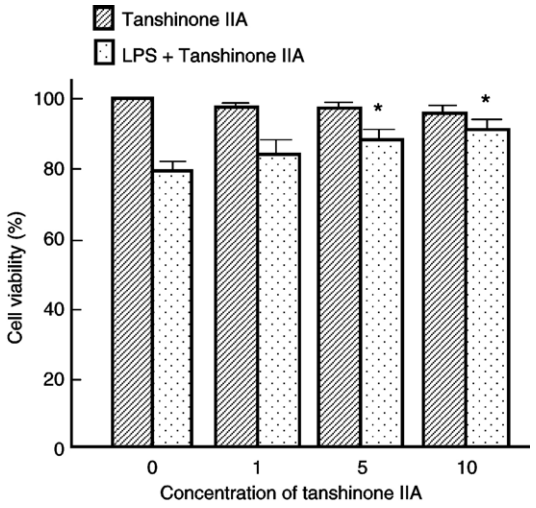

Fig. 3. Cells were incubated for 24 h with tanshinone IIA at indicated concentrations and some cells were activated for 24 h with LPS (1 μg/ml) after preincubation of tanshinone IIA for 2 h. Cell viability was measured by MTT assay. Data are mean ± S.D. in triplicate (*$P<0.05$; compared to the LPS-treated group).

potential NF-κB activation by IκBα degradation in RAW 264.7 cells stimulated with LPS.

3.4. Effects of tanshinone IIA on p38, ERK1/2, and JNK activation of LPS-stimulated RAW 264.7 cells

To investigate the molecular mechanism of NF-κB inhibition by tanshinone IIA in the LPS-stimulated RAW 264.7 cells, we observed the inhibitory effect of tanshinone IIA on p38, ERK1/2, and JNK activation. The maximal level of MAPK (ERK1/2, p38, and JNK) phosphoprotein expression has been known 10–30 min after LPS or phorbol 12-myristate 13-acetate (PMA) treatment in human and murine monocytes/macrophages (Birkenkamp et al., 2000; Bian et al., 2003; Lee et al., 2002). Therefore, we are carried out Western blot of MAPKs after LPS (1 μg/ml) treatment for 15 min. First, RAW 264.7 cells were pretreated with tanshinone IIA or SB203580 (positive control) in the indicated concentrations for 2 h and then stimulated with 1 μg/ml LPS for 15 min. Total cell lysates were then probed with phospho-specific antibody for p38. Phosphorylation of p38 was increased in cells treated with LPS alone. However, the tanshinone IIA inhibited the level of p38 phosphorylation in RAW 264.7 cells stimulated with LPS in a dose dependent manner (Fig. 5A). The LPS-induced p38 phosphorylation was also markedly suppressed by SB203580.

Next, we observed the phosphorylation of ERK after LPS stimulation. The RAW 264.7 cells were pretreated with tanshinone IIA, and the total cell lysates were then probed with phospho-specific antibody for ERK1/2. Phosphorylation of ERK1/2 was increased in cells treated with LPS alone, but the tanshinone IIA inhibited the level of ERK1/2 phosphorylation in RAW 264.7 cells stimulated with LPS in a dose dependent manner (Fig. 5B). LPS-induced ERK1/2 phosphorylation was also suppressed by PD98059 (positive control).

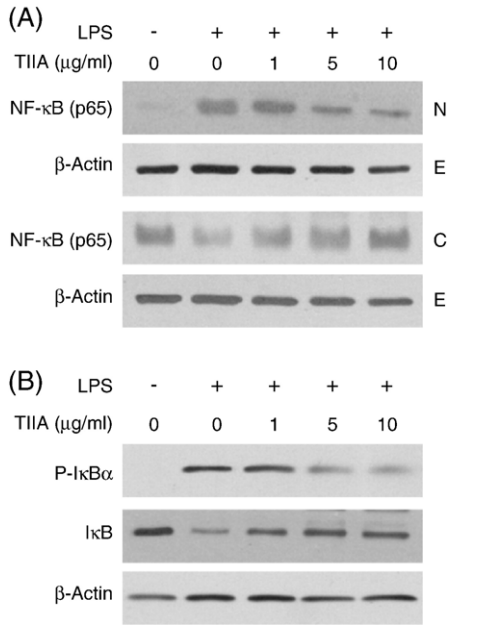

Fig. 4. Effects of tanshinone IIA (TIIA) on LPS-induced NF-κB activation in RAW 264.7 cells. Cells were preincubated for 2 h with or without tanshinone IIA at indicated concentrations and then stimulated for 15 min with LPS (10 μg/ml). Cytosolic extract (CE) and nuclear extract (NE) were isolated and investigated as to whether tanshinone IIA inhibited NF-κB translocation (A). The levels of phosphorylated IκBα (p-IκBα) and IκBα protein in CE were determined by Western blot (B).

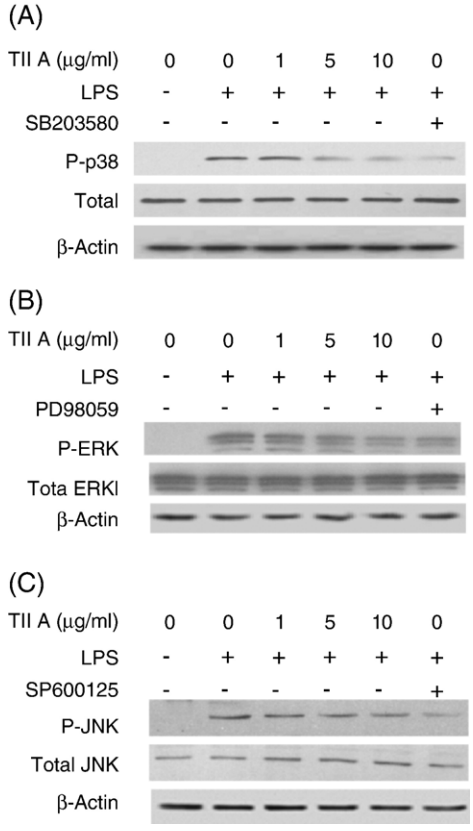

Fig. 5. Effects of tanshinone IIA (TIIA) on LPS-induced p38, ERK1/2, and JNK phosphorylation in RAW 264.7 cells. Cells were preincubated for 2 h with or without SB203580 (20 μM), PD98059 (30 μM), SP600125 (20 μM) or tanshinone IIA at indicated concentrations and then stimulated for 15 min with LPS (10 μg/ml). Total cell lysates were isolated and investigated as to whether tanshinone IIA inhibited p38 (A), ERK1/2 (B), and JNK (C) phosphorylation.

Finally, to determine the JNK phosphorylation, RAW 264.7 cells were pretreated with tanshinone IIA in the indicated concentrations for 2 h and then stimulated with 1 μg/ml LPS for 15 min. Total cell lysates were probed with phospho-specific antibody for JNK. LPS stimulation increased JNK phosphorylation. However tanshinone IIA inhibited the level of JNK phosphorylation in RAW 264.7 cells stimulated with LPS in a dose dependent manner (Fig. 5C). The LPS-induced JNK phosphorylation was markedly suppressed by SP600125 (positive control).

These data suggest that tanshinone IIA may have the inhibitory effect for LPS-induced phosphorylation of p38, ERK1/2, and JNK in RAW 264.7 cells.

3.5. Effects of tanshinone IIA on NIK and IKK activation of LPS-stimulated RAW 264.7 cells

Since the NIK–IKK pathway is also involved in the activation of NF-κB, we observed the inhibitory effect of tanshinone IIA on NIK and IKKα/β activation. We carried out Western blot of NIK and IKKα/β after LPS (1 μg/ml) treatment for 5 min. First, RAW 264.7 cells were pretreated with tanshinone IIA in the indicated concentrations for 2 h and then stimulated with 1 μg/ml LPS for 15 min. Total cell lysates were then probed with phospho-specific antibody for NIK and IKKα/β. Phosphorylation of NIK and IKKα/β was increased in cells treated with LPS alone. However, the tanshinone IIA inhibited the levels of NIK and IKKα/β phosphorylation in RAW 264.7 cells stimulated with LPS in a dose dependent manner (Fig. 6A and B). These data suggest that tanshinone IIA may have the inhibitory effect for LPS-induced phosphorylation of NIK and IKKα/β in RAW 264.7 cells stimulated with LPS.

4. Discussion

In the present study, we show that the inhibitory mechanisms of tanshinone IIA on the production of pro-inflammatory mediators in RAW 264.7 cells are stimulated with LPS. LPS has been shown to initiate multiple intracellular signaling events, including the stimulation of pathways that lead to the activation of NF-κB, NIK, IKK and MAPKs such as p38, ERK1/2, and JNK (Li and Verma, 2002; Majumdar and Aggarwal, 2001; Cavaillon and Haeffner-Cavaillon, 1990; Garrington and Johnson, 1999). NF-κB is a transcriptional factor of several genes involved in immune responses and plays a central role in a number of signaling pathways in monocytes/macrophages (Guha and Mackman, 2001). The activation of NF-κB is essential for the triggering and coordination of innate and adaptive immune responses including the production of pro-inflammatory mediators (Mancuso et al., 2002; Sha et al., 1995; Li and Verma, 2002). The data presented show that tanshinone IIA inhibited the increase of NF-κB-promoter DNA complex and NF-κB binding activity in RAW 264.7 cells stimulated with LPS in a dose dependent manner. Many terpenes, including diterpene, triterpene, and sesquiterpenes isolated from phytomedicinal plant have proved to possess anti-inflammatory activity both in vitro and in vivo, and most of them inhibited NF-κB activity (Zhou et al., 2003, 1999; Niu et al., 2000; Wang et al., 1999), although the precise mechanisms of action have not been fully characterized. NF-κB proteins exist as dimmers composed of various combination of structurally related proteins, including p65 (RelA), p52 (NF-κB_2), and p50 (NF-κB_1) (Li and Verma, 2002). In resting state, the NF-κB dimmers are retained in the cytoplasm by associating with the inhibitory proteins, IκBs (IκBα, IκBβ, and IκBε) (Sha et al., 1995; Li and Verma, 2002; Wang et al., 1999; Ruland and Mak, 2003). Upon stimulation with LPS, IκB proteins are phosphorylated and degraded, following NF-κB to translocate to the nucleus where it can activate certain genes through binding to transcription-regulatory elements in a nucleotide sequence-specific manner (Guha and Mackman, 2001). Our data show that the tanshinone IIA dose dependently inhibited the nuclear translocation of NF-κB and the phosphorylation of IκBα. The activation of NF-κB is regulated by cellular kinases such as MAPKs (Guha and Mackman, 2001). MAPKs are a highly conserved family of protein serine/threonine kinases and include the p38, ERK1/2, and JNK subgroups (Garrington and Johnson, 1999; Ruland and Mak, 2003), and p38 is activated by LPS stimulation and has been postulated to be important in the control of these end points (Bhat et al., 1998). It is important to establish the relative contribution of these MAPKs to LPS-induced signaling in macrophages (Chen and Wang, 1999). The ERK pathway is activated by the dual-specific MAPK kinase (MAPKK) known as MEK1/2 (Birkenkamp et al., 2000). MAPKs have been shown to be involved in the regulation of cytokine response (Guha and Mackman, 2001). The activation of ERK is thought to be involved in LPS-induced macrophage responses, such as the increased production of pro-inflammatory cytokines and inducible nitric oxide synthase (iNOS) (Bhat et al., 1998; Ajizian et al., 1999). LPS stimulation of RAW 264.7 cells rapidly activates the JNK pathway (Hambleton et al., 1996). The JNK is also known as the stress-activated protein kinase (SAPK1) (Dumitru et al., 2000). Another important issue to be solved is the molecular mechanisms by which MAPKs are activated in LPS-induced signaling (Chakravortty et al., 2001). Thus, p38, ERK1/2, and JNK activation is used a hallmark of LPS-induced signal transduction in RAW 264.7 cells. Therefore, to

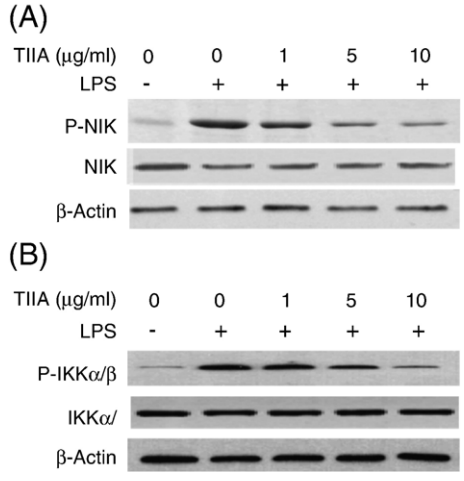

Fig. 6. Effects of tanshinone IIA (TIIA) on LPS-induced NIK and IKKα/β phosphorylation in RAW 264.7 cells. Cells were preincubated for 2 h with or without tanshinone IIA at indicated concentrations and then stimulated for 15 min with LPS (10 μg/ml). Total cell lysates were isolated and investigated as to whether tanshinone IIA inhibited NIK (A) and IKKα/β (B) phosphorylation.

further confirm the inhibitory mechanism of NF-κB activation by tanshinone IIA, we investigated the effect of tanshinone IIA on the p38, ERK1/2, and JNK phosphorylation in RAW 264.7 cells stimulated with LPS for 15 min. Surprisingly, the levels of p38, ERK1/2, and JNK phosphorylation were suppressed by the treatment of the cells with tanshinone IIA in a concentration dependent manner.

Recent studies have shown that phosphorylation of IκB is also regulated by both α and β isoforms of IKK. These kinases and additional upstream intermediates such as NIK may represent a novel site for pharmacological intervention in a number of inflammatory conditions (Karin and Delhase, 1998; Muzio et al., 1998; Irie et al., 2000). Therefore, we observed the inhibitory effect of tanshinone IIA on NIK and IKKα/β activation in RAW 264.7 cells stimulated with LPS. In this study, phosphorylation of NIK and IKKα/β was increased in cells treated with LPS alone, but the tanshinone IIA inhibited the levels of NIK and IKKα/β phosphorylation in a dose dependent manner. A more recent report has shown that transcriptional factors such as AP-1 or STAT-1 play an important role in the intracellular signal transduction pathway in inflammatory process (Chen et al., 2005). Therefore, further studies are required to understand fully the cross-talks between tanshinone IIA and AP-1 or STAT-1 activation.

In conclusion, these results suggest that tanshinone IIA may inhibit LPS-induced NF-κB activation by IκBα degradation via suppression of the NIK–IKK pathway as well as the MAPKs (p38, ERK1/2, and JNK) pathway in RAW 264.7 cells and these properties may provide a potential mechanism that explains the anti-inflammatory activity of tanshinone IIA.

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