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Molecular Mechanisms of Inhibitory Activities of Tanshinones on Lipopolysaccharide-Induced Nitric Oxide Generation in RAW 264.7 Cells

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The effects of four tanshinones isolated from Tanshen (the root of *Salvia miltiorrhiza* Bunge, Labiatae) were tested for their inhibition of nitric oxide production in macrophage cells, and the underlying molecular mechanisms studied. Of the four tanshinones used, 15, 16-dihydrotanshinone-I, tanshinone-IIA and cryptotanshinone, but not tanshinone I, demonstrated significant inhibition of the LPS-induced nitric oxide production in RAW 264.7 cells, with calculated IC_{50} values of 5, 8, and 1.5 μM, respectively. Tanshinones exerted inhibitory activities on the LPS-induced nitric oxide production only when applied concurrently with LPS, and tanshinone-IIA and cryptotanshinone were found to inhibit LPS-induced NF-κB mobilization and extracellular-regulated kinase (ERK) activation, respectively. These results suggest that tanshinones inhibit LPS-induced nitric oxide generation by interfering with the initial stage of LPS-induced expression of certain genes. NF-κB and ERK could be the molecular targets for tanshinones for the inhibition of LPS-induced nitric oxide production in macrophage cells.

Key words: Tanshinones, Nitric oxide, Lipopolysaccharide, NF-*k*B, ERK

INTRODUCTION

Tanshinones are known to possess various biochemical and physiological activities, such as anti-inflammatory activity (Kim *et al.*, 2002) and the regulation of cytokine production in immune cells (Kang *et al.*, 2000). In addition to these immune-related activities, additional pharmacological activities have been reported; tanshinones induce apoptosis in Hepatic stellate cells, which play central roles in hepatic fibrosis (Kim *et al.*, 2003) and inhibit the osteoclast differentiation and bone resorption (Kim *et al.*, 2004).

Recently, four diterpenes were isolated from Tanshen (root of *Salvia miltiorrhiza* Bunge), by way of activity-guided fractionation, as the active principles responsible for anti-allergic properties; tanshinone-I, 15, 16-dihydrotanshinone-I, tanshinone-IIA and cryptotanshinone (Ryu *et al.*, 1999; Choi and Kim, 2004). Allergy and inflammation employ common mediators, which are difficult to discuss individually, even though the latter might occur in the process of the former. Therefore, any agent having both anti-allergic and anti-inflammatory activities would have greater potential for development as an effective anti-allergic drug.

Nitric oxide (NO) is synthesized *via* the oxidation of arginine, by a family of nitric oxide synthases (NOS), and plays a vital role in regulating physiological processes, e.g., blood vessel tone and neurotransmission, as well as in host defense and immunity (Furchgott and Zawadzki, 1980; Moncada and Higgs, 1993). However, increasing evidence indicates that the endogenous increase of NO through the induction of inducible NOS plays a complex role in modulating the inflammatory response (Wei *et al.*, 1995).

In this study, the effects of 4 tanshinones were tested on the LPS-induced NO production of RAW 264.7 macrophage cells, and the signaling events that mediate the inhibitory activities of tanshinones on the NO production was further determined.

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MATERIALS AND METHODS

Materials

The isolation and identification of the four tanshinones have been described in our previous report (Ryu *et al.*, 1997). The purity of tanshinones was higher than 95%. Antibodies for iNOS, p42/p48 ERK (ERK1/2), HRP-labeled anti-mouse and anti-rabbit, and agarose beads conjugated to phosphotyrosine antibodies were purchased from Sigma Chemical Co. (St. Louis, MI, USA) or Santa Cruz Biotechnologies (Santa Cruz, CA, USA) and the protein assay kits were from Promega (Madison, WI, USA).

Nitric oxide analysis

Nitric oxide was determined by measuring the amount of nitrite in cell culture supernatants using Griesss reagent (Promega), according to the manufacture's protocol (Seol *et al.*, 2004).

Viability assay

RAW 264.7 cells (5×10^4 cells/well) were treated with various concentrations of cryptotanshinone for 24 h, followed by the addition of 50 μL of the XTT labeling mixture, which was then incubated for 5 h at 37°C in 5% CO_2. The absorbance was measured at 540 nm.

Immunoprecipitation and immunoblotting

RAW264.7 cells were stimulated with either 1 μg/mL LPS alone or cryptotanshinone for 24 h. Cells were freeze-thawed 3 times in RIPA buffer and centrifuged at 45,000×g for 30 min. For iNOS immunoblotting, supernatants, containing 20 μg of protein, were analyzed by 8% SDS-PAGE gel and the immunoblots probed with antibodies for iNOS.

For the immunoprecipitation, the postnuclear supernatants were immunoprecipitated with beads conjugated to anti-phosphotyrosine antibodies. The beads were boiled for 5 min in Laemmli buffer and then analyzed by Western blotting with antibodies to ERK1/2.

Electrophoretic mobility shift assay

Nuclear extracts were prepared from the cells, as described previously (Im *et al.*, 1997). An oligonucleotide containing an NF-κB-binding site within the Ig κ-chain (5'-CCG GTT AAC AGA GGG GGC TTT CCG AG-3') was used as the probe. ^{32}P-labeled oligonucleotides (10,000 cpm) were incubated for 30 min at room temperature with 10 μg of nuclear extract in 20 μL binding buffer (10 mM Tris-HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng of poly (dI-dC) and 1 mM dithiothreitol). The reaction mixture was analyzed by electrophoresis on a 4% polyacrylamide gel in 0.5X Tris borate buffer.

RESULTS AND DISCUSSION

Effects of tanshinones on the LPS-induced nitric oxide production in RAW 264.7 cells

When the murine macrophage cell line RAW 264.7 cells were incubated with 1 μg/mL of lipopolysaccharide (LPS) for 24 h, the nitrite (NO_2^-) content in the medium increased up to four to five times. Of the 4 tanshinones (Fig. 1A), 15, 16-dihydrotanshinone-I, tanshinone-IIA and cryptotanshin-

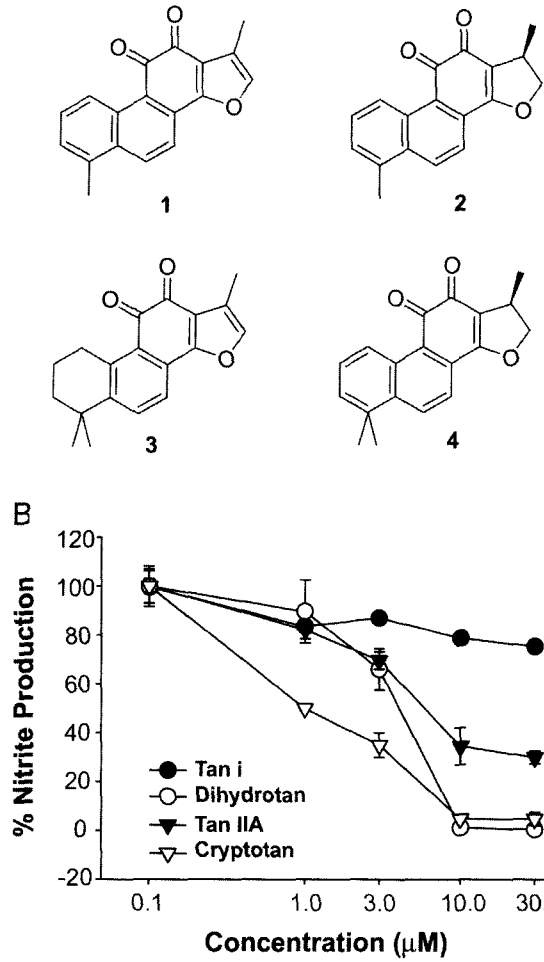

Fig. 1. Effects of tanshinones on the LPS-induced production of nitric oxide from RAW 264.7 cells. (A) Structures of tanshinones used. **1**: tanshinone-I (Tan I), **2**: 15, 16-dihydrotanshinone-I (Dihydrotan), **3**: tanshinone-IIA (Tan IIA) and **4**: cryptotanshinone (Cryptotan). (B) Drugs were dissolved in 100% DMSO, with the final DMSO concentration adjusted to 0.025%.

% NO production=(Treated-Blank-Spontaneous)/(Control-Blank-Spontaneous)

Control: cells were treated with LPS.
Treated: cells were treated with LPS and tanshinones.
Blank: only test material and Griess reagent were added.
Spontaneous: only Griess agent was added.

Each data point represents the mean±SEM. of triplicate determinations, with distinct triplicates measured for each determination (n=3).

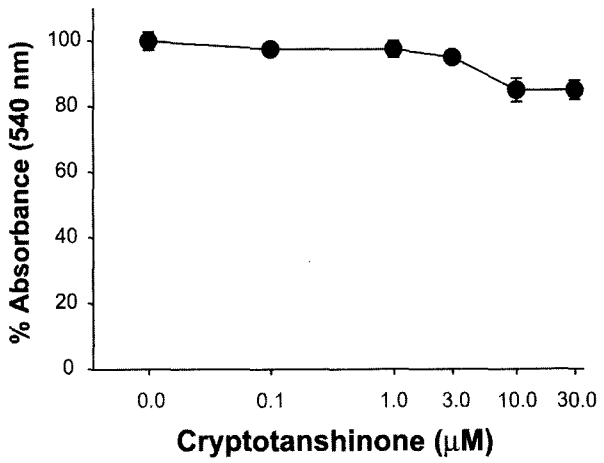

Fig. 2. Effects of cryptotanshinone on the viability of RAW 264.7 cells. Cells were treated with 0-30 μM of crytotanshinone for 24 h. XTT labeling mixture (50 μL) was added, followed by incubation for 5 hrs at 37°C in 5% CO_2. Each data point represents the mean±SEM of triplicate determinations.

one demonstrated significant inhibition of the LPS-induced NO production from RAW264.7 cells (Fig. 1B), with calculated IC_{50} values of 5, 8 and 1.5 μM, respectively. This was further confirmed by the immunoblotting of nitric oxide synthase after treatment of the cells with, for example, cryptotanshinone (Fig. 3A). Since the four tanshinones possess very similar structural characteristics, they are also likely to affect the LPS-induced nitric oxide expression in identical ways. Dexamethasone, a well-known anti-inflammatory agent that inhibits inducible NOS (Walker *et al.*, 1997; Ryu *et al.*, 2001), was used as a positive control and showed potent inhibitory activity toward LPS-induced NO production, with an observed IC_{50} value of about 1.0 μM (data not shown).

Since tanshinones possess cytotoxic activities in certain cell lines (Ryu *et al.*, 1997), firstly the inhibitory activities of tanshinones on the NO production from macrophage cells were tested to see if they were somehow related with their cellular toxicities. As shown in Fig. 2, cryptotanshinone, which is known to possess the strongest cytotoxic activities in tumor cells (Ryu *et al.*, 1997) and the most potent inhibitory activity for the LPS-induced nitric oxide production (Fig.1B), showed no significant cytotoxicity toward RAW 264.7 cells at concentrations up to 30 μM, the maximum concentration used to test the effects of tanshinones on NO production. These results suggest that other tanshinones would probably not have significant cytotoxicities, and these inhibitory activities of tanshinones on the NO production from macrophage cells are unlikely to be due to their detrimental effects on the physiological status of RAW 264.7 cells.

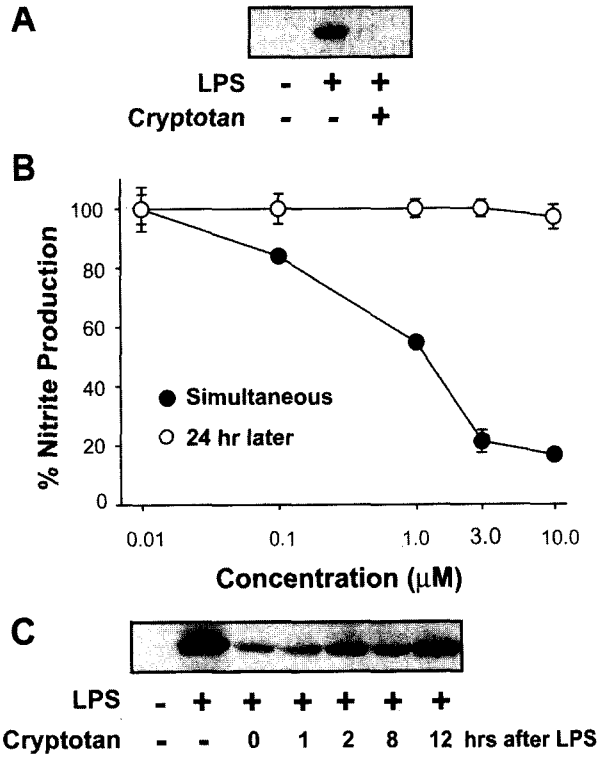

Fig. 3. Determination of the stage at which tanshinones inhibit the LPS-induced nitric oxide production from RAW264.7 cells. (A) RAW264.7 cells were stimulated with 1 μg/mL LPS alone or with 10 μM crytotanshinone. Samples were analyzed by SDS-PAGE gel and transferred to a nitrocellulose membrane. Immunoblots were probed with antibodies iNOS (1:1,000 dilution) and HRP-labeled anti-mouse (1:5,000 dilution). (B) Cells were simultaneously treated with cryptotanshinone (10 μM) and LPS (1 μg/mL) for 24 h following LPS stimulation. The nitrite concentrations in the media were determined at each concentration point, with each data point representing the mean±SEM. (C) Cells were treated with crytotanshinone (10 μM) for between 0 and 12 h following LPS stimulation (1 μg/mL). The expression of iNOS was determined in the same cells, as described in Fig. 3A.

Tanshinones inhibit the initial stage of LPS-induced gene expression

To determine the stage at which tanshinone affects the production of NO, cells were treated with cryptotanshinone at various time points following LPS stimulation. As shown in Fig. 3B, cryptotanshinone inhibited the production of NO by over 80% when the cells were simultaneously treated with LPS and cryptotanshinone. However, pre-stimulation of the cells with LPS markedly reduced the effects of cryptotanshinone, suggesting that it affected the initial stage of the LPS-induced expression of certain genes involved in the production of NO. The same results were essentially observed with the expression of iNOS. As shown in Fig. 3C, the inhibition of the LPS-induced iNOS expression decreased in proportion to the time interval between the LPS and cryptotanshinone treatments.

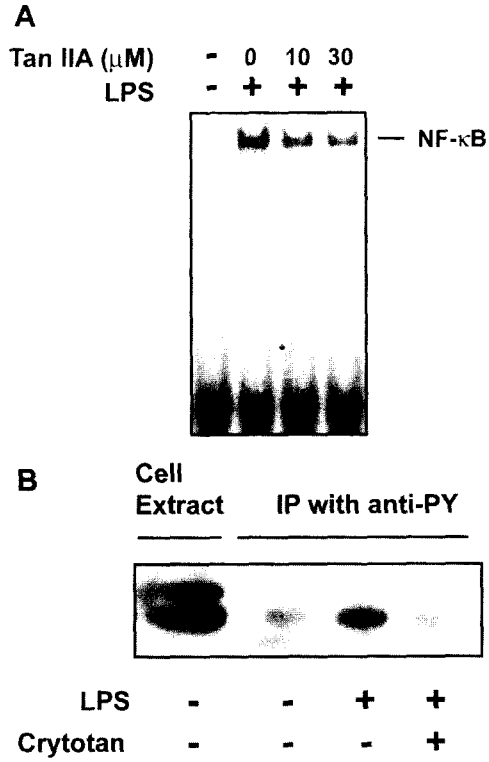

Fig. 4. Effects of tanshinones on the LPS-induced NF-κB mobilization and ERK activation. (A) Cells (2×10^6) were treated with 10 or 30 μM tanshinone IIA (30 min), followed by LPS (100 ng/mL, 30 min) (Cho et al., 2002). Nuclear extracts from RAW264.7 cells were incubated with ^{32}P-labeled κB oligonucleotide, and analyzed by electrophoresis on a 4% polyacrylamide gel. B. Cells were treated with 10 μM cryototanshinone for 20 min, followed by 1 μg/mL LPS for 5 min. Immunoprecipitation and immunoblotting were conducted as described in the Methods section. Rabbit polyclonal antibodies for ERK1/2 (1:2,000 dilutions) and HRP-labeled anti-rabbit antibodies (1:5,000) were used.

NF-*k*B and ERK1/2 are involved in the inhibitory activities of tanshinones on LPS-induced nitric oxide production

NF-κB belongs to the Rel family of transcription factors (Bethea *et al.*, 1997), and plays a central role in the regulation of iNOS expression (Baeuerle and Henkel, 1994). Since the expression of iNOS was dose dependently inhibited by tanshinone, its influence on the nuclear localization of NF-κB was determined. Since NF-κB mediates a variety of cellular functions; cell growth or death, the tanshinone with the weakest cellular toxicity, tanshinone IIA, was selected. As shown in Fig. 4A, the LPS-induced nuclear translocation of NF-κB was moderately inhibited by tanshinone IIA. Even though other tanshinones were not tested for their LPS-induced NF-κB translocation, similarities in their structural and functional aspects suggest that other tanshinones are likely to have stronger inhibitory activities on NF-κB translocation than thanshinone IIA.

It is well established that the p38 MAP kinase is critical for the LPS-induction of inducible NO synthase expression, NO production and activation of NF-κB DNA-binding activity in macrophages (Chen *et al.*, 1999; Chen and Wang, 1999). Interestingly, recent studies have shown that ERK plays important roles in mediating the NO production involving NF-κB mobilization (Jaramillo *et al.*, 2003; Aga *et al.*, 2004). In addition, tanshinone has shown inhibitory activities on the ERK activation induced by RANKL in osteoclast precursors (Kim *et al.*, 2004) and FcεRI-mediated ERK activation in mast cells (Choi and Kim, 2004). Based on this information, the effects of cryptotanshinone on the LPS-induced ERK activation were tested. As expected, LPS provoked the tyrosine phosphorylation of ERK1/2, and crytotanshinone at 10 μM completely inhibited this LPS-induced ERK activation (Fig. 4B).

The inhibitory activities of tanshinones on NO production have previously been reported (Jang *et al.*, 2003). However, their molecular details remain to be clearly understood. In this study, 15, 16-dihydrotanshinone-I, tanshinone-IIA and cryptotanshinone, but not tanshinone I, have been shown to possess significant inhibitory activities toward LPS-induced NO production in macrophage cells. Our data also show that tanshinones inhibit the initial stage of LPS-induced gene expressions involved in NO production, and NF-κB and ERK could be part of the signaling components that mediate the inhibitory activities of tanshinones toward LPS-induced NO production in macrophage cells.

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