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Tanshinone IIA from *Salvia miltiorrhiza* induces heme oxygenase-1 expression and inhibits lipopolysaccharide-induced nitric oxide expression in RAW 264.7 cells

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Abstract

Tanshinone IIA exerts anti-inflammatory effects and influences electron transfer reaction in mitochondria. In the present study, we demonstrated that tanshinone IIA increased intracellular production of reactive oxygen species (ROS), which in turn induces heme oxygenase-1 (HO-1) expression in RAW 264.7 macrophages. Tanshinone IIA inhibited COX-2 and iNOS expression in lipopolysaccharide-activated RAW 264.7 macrophages. Inhibition of HO-1 or scavenging of CO significantly reversed the inhibition of LPS-stimulated nitrite accumulation by tanshinone IIA, suggesting a novel role of HO-1 in the anti-inflammatory effect of tanshinone IIA.

Keywords: Tanshinone IIA; Cyclooxygenase-2; Heme oxygenase-1; RAW 264.7 macrophages

1. Introduction

The dried root of *Salvia miltiorhiza* is called 'Danshen' in China, which has been used as an anti-inflammatory agent for over one thousand years. However, mechanisms by which Danshen exerts these effects are poorly understood. Danshen extracts contain diterpene quinone and phenolic acid derivatives, including tanshinone (I, IIA and IIB), cryptotanshinone, isocryptotanshinone, miltirone, tanshinol (I and II) and salviol. These compounds exert antioxidant properties in vitro and in vivo (Wang et al., 2005). The anti-inflammatory effect may be explained, in part, by the inhibition of cytokine secretion, and iNOS expression (Jang et al., 2003; Dittmann et al., 2004) by tanshinones. Tanshinones have been shown to inhibit LPS-induced NF-kappaB mobilization and extracellular-regulated kinase (ERK) activation, which in turn inhibit LPS-induced nitric oxide generation in RAW 264.7 macrophages (Choi et al., 2004).

Heme oxygenases catalyze the rate-limiting step in heme degradation, resulting in the formation of iron, carbon monoxide, and biliverdin, which is subsequently converted to bilirubin by biliverdin reductase. Three isoforms of heme oxygenase (HO) have been described: an inducible isoform, HO-1, and two constitutively expressed isoforms, HO-2 and HO-3. HO-1 functions as a cytoprotective mechanism against inflammatory responses and ROS insults through the anti-inflammatory action of its

Abbreviations: CO, carbon monoxide; COX-2, cyclooxygenase-2; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; HO-1, heme oxygenase-1; iNOS, inducible nitric oxide; LPS, lipopolysaccharide; L-NAC, L-*N*-acetylcysteine; PBS, phosphate buffered saline; PI 3-K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; SnPP, tin protoporphyrin IX; TTFA, thenoyltrifluoroacetone.

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metabolite, CO, and the anti-oxidant activities of another metabolite, bilirubin (Otterbein and Choi, 2000; Lee et al., 2003). The induction of HO-1 expression is mediated through translocation of a redox-sensitive transcription factor Nrf2 (NF-E2 related factor 2) from cytosol to nuclei (Nguyen et al., 2003) following activation of the phosphatidylinositol 3-kinase (PI 3-K) survival signaling pathway (Martin et al., 2004).

In the present study, we investigated the effect of tanshinone IIA, one of the active ingredients in Danshen, on COX-2 and iNOS expression in LPS-activated RAW 264.7 macrophages. We found that tanshinone IIA inhibited LPS-induced COX-2 and iNOS protein expression and nitrite accumulation in RAW 264.7 macrophages. We demonstrated that suppression of LPS-induced iNOS expression by tanshinone IIA is mediated through HO-1 induction. We provide evidence that ROS generation, activation of the PI 3-K/Akt dependent pathway is involved in the induction of HO-1 and suppression of LPS-induced iNOS expression and nitrite accumulation.

2. Materials and methods

2.1. Materials

Affinity-purified mouse polyclonal antibodies to cyclooxygenase-2, p38 MAPK, phospho-Akt (Ser^{473}) and phospho-ERK were obtained from Transduction Laboratory (Lexington, KY). (2'-amino-3'-methoxyflavone) (PD 98059), and [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H- imidazole] (SB 203580) were purchased from Calbiochem (San Diego, CA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum, penicillin, and streptomycin were purchased from Life Technologies (Gaithersburg, MD). 5-Bromo-4-chloro-3-indolyl-phosphate/4-nitro blue tetrazolium (BCIP/NBT) substrate was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Protease inhibitor cocktail tablets were purchased from Boehringer Mannheim (Mannheim, Germany). Tanshinone IIA is a gift from Dr. Paul Chen at NanJing University of traditional Chinese medicine. All other chemicals were purchased from Sigma (St Louis, MO).

2.2. Culture of RAW 264.7 cells and preparation of cell lysates

Cells of the murine macrophage cell line, RAW 264.7, were cultured as described previously (Lin et al., 2002). Cells were lysed in lysis buffer containing 50 mM Tris–Cl (pH 7.4), 150 mM NaCl, 100 μg/ml phenylmethylsulfonyl fluoride, 1% Nonindent P-40, and 4% protease inhibitor cocktails. Protein concentrations in the cell lysates were determined by a Bio-Rad protein assay following the manufacturer's recommendations. All cell lysates were stored at −70 °C until further measurements.

2.3. Polyacrylamide gel electrophoresis and Western blotting

Electrophoresis was ordinarily carried out on 10% sodium dodecyl sulphate polyacrylamide gels. Following electrophoresis, separated proteins on the gel were electrotransferred onto a polyvinyldifluoride membrane. Nonspecific bindings were blocked with blocking buffer containing 5% fat-free milk powder for 1 h at room temperature, followed by incubation with primary antibody in blocking buffer for 2 h. The polyvinyldifluoride membrane was then incubated with alkaline phosphatase-conjugated secondary antibody for 1 h. Subsequently, Western blots were developed with 5-bromo-4-chloro-3-indolyl-phosphate/4-nitro blue tetrazolium as substrate.

2.4. Statistical analysis

All data are expressed as the means ± SEM. Comparisons between groups were made by Students *t*-test. A difference between groups of ($P < 0.05$) was considered significant.

3. Results

3.1. Tanshinone IIA induces HO-1 expression via ROS generation in RAW 264.7 macrophages

Tanshinone IIA has been shown to mediate with the electron transfer reaction in mitochondria. We first examined whether tanshinone IIA may increase reactive oxygen species (ROS) production in RAW 264.7 macrophages. Treatment of RAW 264.7 macrophages with tanshinone IIA significantly increase intracellular ROS levels detected by a ROS-sensitive fluorescent probe, DCFDA (Fig. 1a). Because changes of intracellular redox status may lead to heme oxygenase-1 (HO-1) expression, we investigated whether tanshinone may induce HO-1 expression in RAW 264.7 macrophages. Tanshinone IIA elicited a dose-dependent induction of HO-1 protein (Fig. 1b). When cells were pretreated with *l*-NAC prior to the addition of tanshinone IIA, *l*-NAC inhibited tanshinone-induced HO-1 expression (Fig. 1c). Conversely, pretreatment of cells with BSO, an inhibitor of glutathione synthesis, significantly increased tanshinone IIA-induced HO-1 protein level. Tanshinone IIA could be converted to its semiquinone form by accepting electron from complex I (Zhou et al., 2003). We examined whether tanshinone-induced HO-1 expression is regulated by respiratory inhibitors. Blockage of electron flow from complex I to ubiquinone by rotenone did not alter the HO-1 protein levels. Similarly, inhibition of complex III by myxothiazol or inhibition of complex II by Thenoyltrifluoroacetone (TTFA) did not affect tanshinone-induced HO-1 protein expression (Fig. 1d), suggesting electron transport chain I, II, III were not involved.

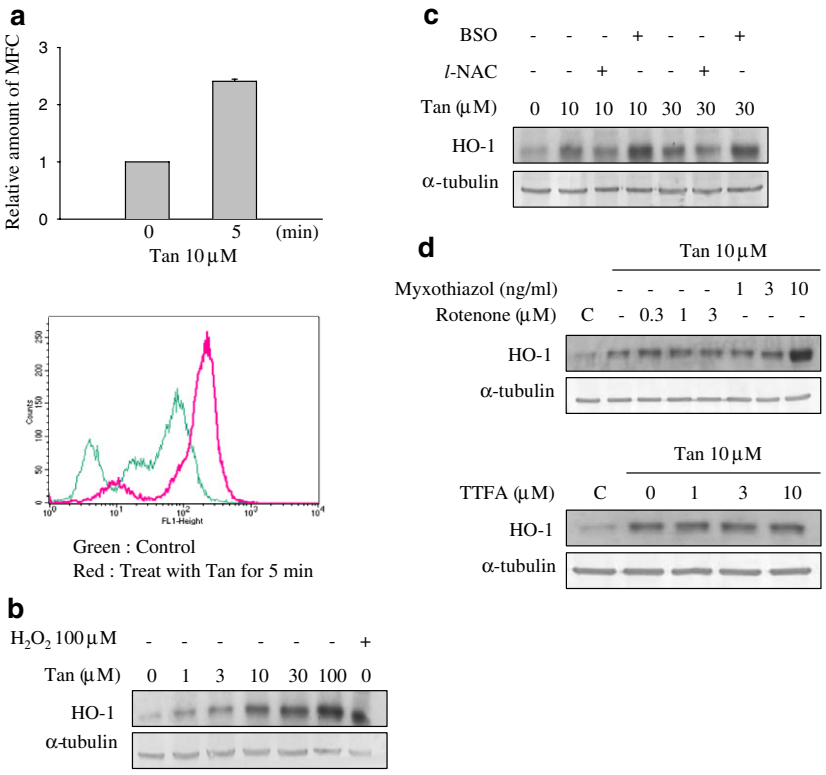

Fig. 1. Tanshinone IIA induces HO-1 expression via ROS generation in RAW 264.7 macrophages. In (a), RAW 264.7 macrophages were incubated with DCFH-DA (10 μM) for 6 h in the presence of tanshinone IIA (10 μM), and the fluorescence intensities were analyzed using FACS as described in Section 2. The upper panel shows means ± SEM of three independent experiments. In (b), cells were incubated with various concentrations of tanshinone IIA at 37 °C. In (c), cells were pretreated with L-NAC (3 mM) or BSO (10 μg/ml) before incubated with tanshinone IIA (10 μM) for 16 h and lysed. In (d), cells for pretreated with ETC inhibitors for 30 min before incubated with tanshinone IIA (10 μM) for 16 h. Cell lysates were electrophoresed and probed by Western blot with HO-1 specific antibodies. Equal loading in each lane was demonstrated by the similar intensities of α-tubulin.

3.2. Signaling pathways of tanshinone-induced HO-1 expression in RAW 264.7 macrophages

We next analyzed the signaling mechanisms by which tanshinone IIA induces HO-1 expression using specific pharmacological inhibitors. As shown in Fig. 2A, when cells were pretreated with LY 294002, an inhibitor of PI 3-K, and then challenge with tanshinone IIA for 24 h, LY 294002 significantly blocked tanshinone-induced HO-1 expression. Akt/PKB is a downstream Ser/Thr protein kinase of PI 3-K signaling pathway. To confirm tanshinone IIA activates PI 3-K pathway, the phosphorylation status of Akt/PKB was investigated. Treatment of RAW 264.7 macrophages with tanshinone IIA (10 μM) rapidly increased the level of phosphorylated Akt. The Akt/PKB phosphorylation increased at 15 min after tanshinone IIA was added and decline to basal level by 3 h (Fig. 2B). Subsequent experiments analyzed whether MAP kinase pathways were involved in the induction of HO-1 expression by tanshinone IIA. SB 203580 (a p38 MAPK inhibitor), PD 98059 (a MEK1/2 inhibitor) and SP 600125 (a JNK inhibitor) were used to treat the cells prior to addition of tanshinone IIA. As shown in Fig. 2A, PD 98059 significantly inhibited tanshinone-induced HO-1 protein expression, whereas SB 203580 (up to 10 μM) and SP 600125 (up to 10 μM) were without effect on HO-1 expression (Fig. 2A). Again, to confirm that tanshinone did activate ERK signaling pathway, we demonstrated that tanshinone IIA increased ERK phosphorylation (Fig. 2C). These data suggest that activation of PI 3-K and ERK signaling pathways are required for tanshinone-induction of HO-1 protein expression in RAW 264.7 macrophages.

3.3. Induction of HO-1 by tanshinone IIA leads to inhibition of LPS-induced iNOS and COX-2 expression

To examine the potential anti-inflammatory action, we assess the effect of tanshinone IIA on LPS-induced iNOS and COX-2 expression in RAW 264.7 macrophages. Treatment with tanshinone IIA significantly inhibited LPS-induced iNOS and COX-2 expression (Fig. 3A) in RAW 264.7 macrophages. Tin protoporphyrin IX (SnPP) (20 μM), a HO competitive inhibitor, reverse the inhibition of tanshinone IIA on LPS-induced nitrite accumulation (Fig. 3B). To assess the role of CO in the inhibition of LPS-induced iNOS expression by tanshinone IIA, we examine whether treatment of cells with CO scavenger, hemoglobin, would reverse the inhibition of LPS-stimulated nitrite accumulation in RAW 264.7 macrophages by tanshinone. As shown in Fig. 3C, hemoglobin reversed

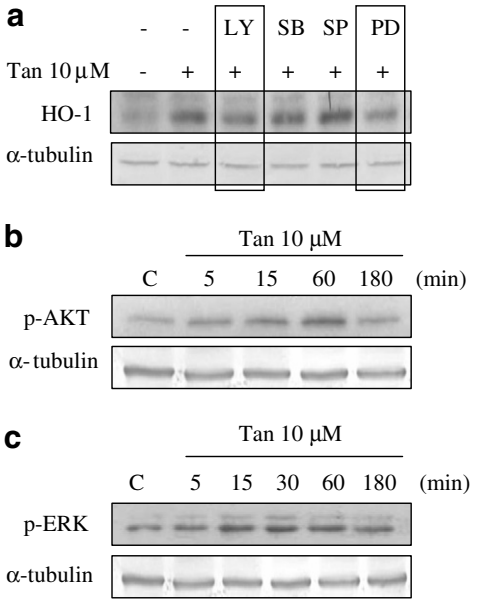

Fig. 2. Signaling pathways of tanshinone-induced HO-1 expression in RAW 264.7 macrophages. In (a), cell were pretreated with LY 294002 (20 μM) or with various MAPK inhibitors, including SB 203580 (10 μM), PD 98059 (5 μM) and SP 600125 (10 μM) for 30 min before incubated with tanshinone IIA (10 μM) for 16 h. Cell lysates were electrophoresed and probed by Western blot with HO-1 and α-tubulin specific antibodies. In (b) and (c), cells were treated tanshinone IIA (10 μM) for various time periods and lysed. Cell lysates were electrophoresed and probed by Western blot with phospho-Akt (Ser^{473}) specific antibodies (b) or with phospho-ERK specific antibodies (c). Equal loading in each lane was demonstrated by the similar intensities of α-tubulin.

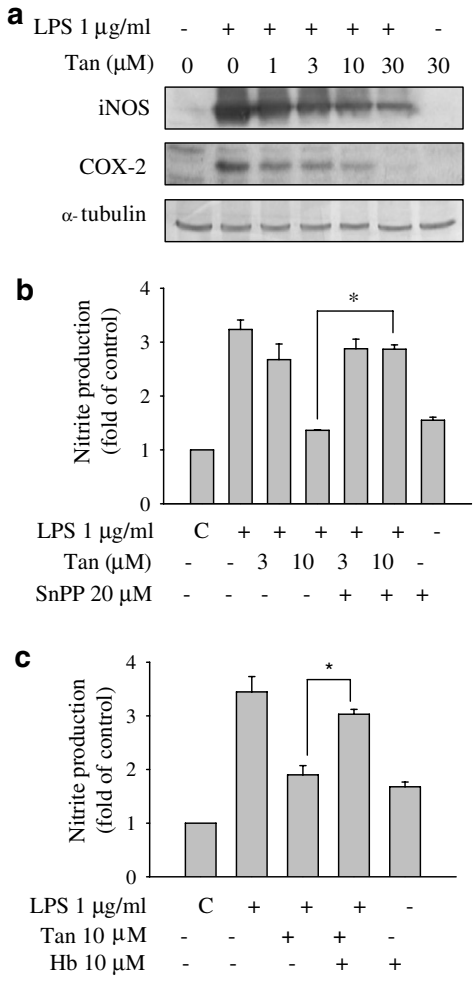

Fig. 3. Induction of HO-1 by Tanshinone IIA leads to inhibition of LPS-induced iNOS and nitrite production: Cells were pretreated with tanshinone IIA (1-30 μM) for 30 min before the addition of 1 μg/ml of LPS and incubated for 24 h. Cells were lysed and cell lysate subjected to Western blot analysis using anti-iNOS, or anti-COX-2 antibodies (panel a). In (b), cells were pretreated with tin protoporphyrin (SnPP) (20 μM) for 30 min before the addition of 1 μg/ml LPS in the presence or absence of tanshinone IIA (10 μM) and incubated for 24 h. In (c), cells were pretreated with hemoglobin (Hb) (10 μM) in the presence or absence of tanshinone IIA (10 μM) for 30 min before the addition of 1 μg/ml LPS and incubated for 24 h. The medium was removed and analyzed for nitrite accumulation from RAW 264.7 macrophages. Data represent means ± SEM of three independent experiments done in triplicate (panels b and c).

the inhibition of LPS-induced nitrite accumulation by tanshinone. Collectively, these data suggest that tanshinone IIA can inhibit iNOS induction through a mechanism that involves the action of CO.

4. Discussion

'Danshen' has long been used as an anti-inflammatory agent in China. In the present study, we provided evidence that tanshinones may induce HO-1 expression. The induction of HO-1 expression was mediated through reactive oxygen species, which lead to activation of PI 3-K, and ERK signaling pathways in RAW 264.7 macrophages. In addition, we demonstrated that CO is the key molecule mediating the anti-inflammatory effect of HO-1. We showed that scavenging of CO by hemoglobin attenuated the inhibition of tanshinones on LPS-stimulated nitrite accumulation in RAW 264.7 macrophages. The schematic illustration of the signaling pathway is shown in Fig. 4.

Tanshinone IIA has been shown to mediate electron transfer reaction in rat heart mitochondria (Zhou et al., 2003). Incubation of RAW 264.7 macrophages with tanshinone dramatically increased intracellular ROS production measured by DCFDA, a ROS-sensitive fluorescent probe. However, inhibition of ETC complexes did not affect the tanshinone-induced HO-1 expression. Instead, increasing glutathione concentrations by the glutathione precursor, L-*N*-acetyl-cysteine, inhibited HO-1 induction; and decreasing glutathione by the γ-glutamylcysteine synthetase inhibitor, L-*N*-buthionine-[S,R]-sulfoximine, potentiates the tanshinone-induced HO-1 expression. These data support the notion that tanshinones may increase HO-1 gene expression through ROS production. It has been shown that tanshinone IIA may accept electron from NADH dehydrogenase of complex I. Although it is not clear of whether the electron reenters the respiratory chain, oxygen radicals were formed during tanshinone-mediated electron transport (Zhou et al., 2003).

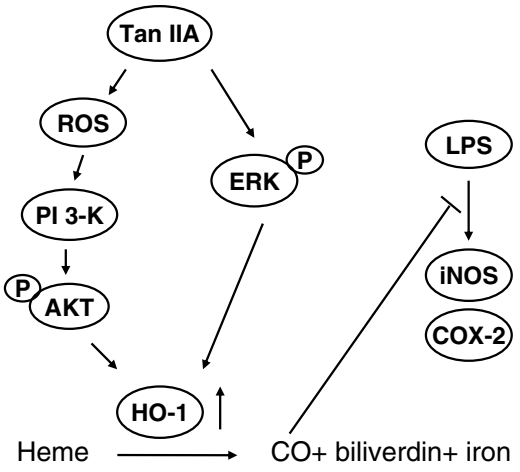

Fig. 4. Schematic diagram illustrating of the signaling pathways by which tanshinone IIA induces HO-1 expression and inhibits LPS-induced iNOS and COX-2 expression in RAW 264.7 cells. Tanshinone IIA induces HO-1 expression through PI 3-K/Akt and ERK pathway. HO-1 catalyzes heme to biliverdin, iron, and CO. CO plays an important role in the inhibition of LPS-induced iNOS expression by tanshinone IIA.

HO-1 gene is a prototypical phase II enzyme. Transcriptional activation of HO-1 gene requires binding of transcription factor to the antioxidant responsive elements (AREs) in the promoter proximal region of HO-1 gene. The AREs can be regulated, at least in part, by the Nrf-2 protein. Given activation of PI 3-K may increase the Nrf2 protein level in nuclear, activation of PI 3-K /Akt signaling pathway may mediate HO-1 induction (Nguyen et al., 2003). Consistent with this finding, we found that tanshinone increased Akt phosphorylation in RAW 264.7 macrophages, and inhibition of PI 3-K pathway by specific inhibitor blocked the tanshinone induced HO-1 expression. We also showed that the ERK pathway may be involved. It is not unusual that multiple signaling pathways may converge on HO-1 transcription to mediate their antioxidant activities.

In conclusion, this study provides evidence for a novel role of tanshinone in the regulation of HO-1 expression. Our results raise the possibility that the anti-inflammatory effects of tanshinone are mediated by tanshinone-induced HO-1 expression.

Acknowledgments

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