The anti-inflammatory effect of tussilagone, from *Tussilago farfara*, is mediated by the induction of heme oxygenase-1 in murine macrophages

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A R T I C L E   I N F O

Article history:
Received 30 July 2009
Received in revised form 12 September 2009
Accepted 23 September 2009

Keywords:
Tussilagone
Heme oxygenase-1
Anti-inflammatory effects
Macrophages

A B S T R A C T

Tussilagone (TSL), isolated from the flower of buds of *Tussilago farfara* (Compositae), is a sesquiterpenoid that is known to exert a variety of pharmacological activities. In the present study, we demonstrated that TSL exerts anti-inflammatory activities in murine macrophages by inducing heme oxygenase-1 (HO-1) expression. Treatment of RAW264.7 cells with TSL-induced HO-1 protein expression in a dose- and time-dependent manner without the induction of HO-1 mRNA expression. TSL-mediated HO-1 protein induction was not inhibited by treatment with actinomycin D, a transcriptional inhibitor, but by cycloheximide, a translational inhibitor. Moreover, mitogen-activated protein kinases (MAPKs) inhibitors such as SB203580, SP600125, and U0126 did not block TSL-mediated HO-1 protein expression, suggesting that the TSL-mediated HO induction may be regulated at the translational level. Consistent with the notion that HO-1 has anti-inflammatory properties, TSL inhibited the production of nitric oxide (NO), tumor necrosis factor (TNF)-α, and prostaglandin E2 (PGE2) as well as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression in lipopolysaccharide (LPS)-stimulated RAW264.7 cells and murine peritoneal macrophages. Inhibition of HO-1 activity by treatment with zinc protoporphyrin IX (ZnP), a specific HO-1 inhibitor, abrogated the inhibitory effects of TSL on the production of NO and PGE2 in LPS-stimulated RAW264.7 cells. Taken together, TSL may be an effective HO-1 inducer that has anti-inflammatory effects, and a valuable compound for modulating inflammatory conditions.

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1. Introduction

Heme oxygenases (HOs) catalyze the oxidation of heme to the biologically active products carbon monoxide (CO), biliverdin, and ferrous iron. Two distinct variants of HOs have been described in humans and rodents, each encoded by a different gene: HO-2, which is constitutively expressed, and HO-1, which is potently induced in many cell types by heme, inflammatory cytokines, and oxidative stress-related factors [1]. Several lines of evidence have shown that HO-1 participates in maintaining the cellular homeostasis and plays an important protective role in the tissues due to reducing oxidative injury and attenuating the inflammatory response [2]. The protective actions of HO-1 are thought to be mediated by the by-products of its enzymatic activity, bilirubin and carbon monoxide, which have antioxidant or anti-inflammatory properties [3,4]. It is also known that the HO-1/CO pathway suppresses LPS-induced toll-like receptor 4 (TLR4) signaling pathways by inhibiting the interaction of TLR4 with cavinolin-1 [5,6]. HO-1 expression or CO treatment inhibits the production of inflammatory cytokines and chemokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 induced by inflammatory mediators such as LPS in activated macrophages [7–10]. The upregulation of HO-1 expression or CO treatment suppresses LPS-induced expression of COX-2 and iNOS, and thereby inhibits the production of PGE2 and nitric oxide (NO), respectively [11,12]. An increasing number of therapeutic agents have been reported to induce HO-1 expression and exert their anti-inflammatory effects through HO-1 induction [13–17].

*Tussilago farfara* L. (Compositae) is a perennial herb that is widely spread in Korea, China, North Africa, Siberia, and Europe. The flower buds of *T. farfara* are known as an important folk medicine used in the treatment of cough and wheezing [18]. The extracts of *Farfarae flos* exhibit antioxidant effect, antimicrobial activity, and inhibitory effects on NO synthesis in LPS-activated macrophages and diacylglycerol acyltransferase activity [19–21]. Recently, it has been reported that tussilagone (TSL), the major component of *Farfarae flos*, suppresses iNOS and COX-2 expression in LPS-stimulated BV-2 microglial cells [22]. However, the molecular mechanism by which TSL exerts anti-inflammatory activity is not elucidated well. In the present study, as part of our continuing search to identify anti-inflammatory compounds from natural products, we identified TSL as a potent inducer of HO-1 expression. Our data showed that TSL inhibits the production of NO and PGE2 as well as the expression of iNOS and COX-2 in LPS-stimulated RAW264.7 cells.
RAW264.7 cells and murine peritoneal macrophages through the induction of HO-1 at translational level.

2. Materials and methods

2.1. Cell culture and peritoneal macrophage isolation

RAW264.7 cells were purchased from the American Type Culture Collection and maintained in Dulbecco’s Modified Essential Medium (DMEM) supplemented with penicillin (100 units/ml)–streptomycin (100 μg/ml) and 10% heat-inactivated fetal bovine serum (Cambrex, Charles City, IA, USA). Cells were maintained in a humidified 5% CO2 atmosphere at 37 °C. Peritoneal macrophages were isolated from Balb/c mouse. In brief, ten-week-old Balb/c females were purchased from Daehan Biolink Co. (Seoul, Korea). Mice were intraperitoneally injected with 1 ml of thioglycollate (Difco Laboratories, Detroit, MI). The peritoneal cavity was lavaged with 10 ml of ice-cold DMEM 4 days post-injection, followed by two times of washing. The washed cells were resuspended in HEPES-buffered DMEM and were seeded in culture plates for an overnight culture. The unbound cells were suctioned out after swirling the plate.

2.2. Reagents

SB203580, SP600125, and U0126 were purchased from Calbiochem (San Diego, CA). Zinc protoporphyrin IX (ZnPP) and copper protoporphyrin IX (CuPP) were from Porphyrin Products Inc. (Logan, UT, USA). Antibodies for HO-1, iNOS, and COX-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for p-p38 (Thr180/Tyr182), p-JNK (Thr183/Tyr185), p-Erk1/2 (Thr202/Tyr204) were from Cell Signaling Technology (Danvers, MA, USA). Anti-α-tubulin antibody was from Sigma (Saint Louis, MO).

2.3. Isolation of tussilagone

Tussilagone (TSL) was isolated from the dried flower buds of T. farfara as described previously [23]. TSL was obtained as white solid, showed a [M]+ peak at m/z 390 in the ESI-MS, corresponding to a molecular formula of C23H34O5. Its structure is shown in Fig. 1. The purity of TSL was checked by 1H and 13C NMR spectra, and its spectra showed highly pure signals without any other impurities. TSL was solubilized in 100% dimethyl sulfoxide and used at a final concentration of less than 0.05% dimethyl sulfoxide.

2.4. Measurement of NO, PGE2, and TNF-α and cell viability assay

RAW264.7 cells were seeded in 24-well plates at 5 × 105 cells/well. Peritoneal macrophages were seeded in 6-well plates at 4 × 106 cells/well. The plates were pretreated with various concentrations of TSL for 30 min and then incubated for another 24 h with or without 1 μg/ml of LPS. In some experiments, ZnPP or CuPP was added to the plates together with TSL. Nitrite concentration in the culture supernatant was measured by the Griess reaction. The nitrite level in each sample was calculated from a standard curve generated with sodium nitrite. The amount of PGE2 in the culture supernatant was measured using a Prostaglandin E2 Parameter Assay Kit (R&D systems, Minneapolis, MN, USA). The amount of TNF-α in the culture supernatant was measured with a TNF-α immunoassay Kit (R&D systems, Minneapolis, MN, USA). Cell viability was measured with MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide]-based colorimetric assay.

2.5. Western blot analysis

Proteins were extracted from cells in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5, 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 mM sodium vanadate, 150 mM NaCl). Fifty micrograms of protein per lane was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) and followed by transferring to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk, and then incubated with the corresponding primary antibody. After washing, the membranes were incubated with the appropriate secondary antibody conjugated to horseradish peroxidase. The signal was detected using the enhanced chemiluminescence system (Intron, Seongnam, Korea).

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

RAW264.7 cells were stimulated for 8 h with 1 μg/ml of LPS in the presence of various concentrations of TSL. The cells were harvested and...
total RNA was isolated using RNeasy Mini Kits according to the manufacturer's instructions (Qiagen, Santa Clarita, CA, USA). Two microgram of total RNA was used to synthesize 1st stranded cDNA using RT-PCR kit (Invitrogen, Carlsbad, CA, USA). For amplification of the iNOS, COX-2, and HO-1, the following primers were used: iNOS, 5′-CAG AAG CAG AAT GTG ACC ATC-3′ (sense) and 5′-CTT CTG GTC GAT GTG ATG AGC-3′ (antisense), COX-2, 5′-CCG AGG TGT ATG TAT GAG-3′ (sense) and 5′-ATCAGCCACAGGAGGAAG-3′ (antisense), HO-1, 5′-CGC AAC CAC ACA-3′ (sense) and 5′-TGA GCC CAT CTG TGA GGG-3′ (antisense). The cDNA for β-actin was also amplified as a control in a similar way using the following primers: 5′-GTG GGG CGC CCC AGG CAC CA-3′ (sense) and 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′ (antisense).

For PCR amplification, the following conditions were used: 94 °C for 5 min for 1 cycle, and then 94 °C for 1 min, 56 °C for 30 s and 72 °C for 1 min for 27 cycles. The amplified PCR products were separated with 1.5% agarose gel, and then stained with EtBr.

2.7. Statistical analysis

The data are expressed as the mean ± SD unless otherwise specified. Statistical significance was assessed by two-tailed unpaired student's t-test and p < 0.05 was considered statistically significant.

3. Results

3.1. TSL induces HO-1 expression in RAW264.7 cells

To investigate the anti-inflammatory effect of TSL, we first determined whether TSL induces HO-1 expression in RAW264.7 cells (Fig. 2). RAW264.7 cells were treated with the increasing concentrations of TSL for 24 h and the expression level of HO-1 was determined by Western blot analysis (Fig. 2A). TSL increased the expression of HO-1 in a dose-dependent manner. At 30 μM TSL, the time course experiment of HO-1 induction revealed that HO-1 protein was increased 3 h after treatment and that its level continued to increase steadily even at 24 h (Fig. 2B). On the other hand, TSL did not affect cell viability as assessed by the MTT assay at concentrations that induced HO-1 expression (Fig. 2C), indicating that TSL induces HO-1 expression without affecting cell viability.

3.2. TSL does not induce the mRNA expression of HO-1

Since it has been known that HO-1 expression is regulated mainly at the transcriptional level through the signaling pathways involving mitogen-activated protein kinases (MAPKs) such as c-Jun NH2-
terminal kinase (JNK), extracellular signal regulated kinase-1/2 (Erk1/2) and p38 kinase [24], we investigated whether TSL-induced the mRNA expression of HO-1. Treatment of RAW264.7 cells with increasing concentrations of TSL for 24 h did not modulate the mRNA expression level of HO-1, as assessed by RT-PCR analysis (Fig. 3A). Moreover, treatment of the cells with TSL for various periods of time did not modulate the mRNA expression of HO-1 (Fig. 3B). To determine whether inhibition of MAPKs affected TSL-mediated HO-1 induction, RAW264.7 cells were exposed to the ERK1/2 inhibitor U0126, the JNK inhibitor SP600125, or the p38 inhibitor SB203580 (Fig. 3C). Cotreatment of these inhibitors failed to modulate both mRNA and protein expression of HO-1 induced by TSL. Furthermore, TSL did not induce the activation of ERK1/2, JNK, and p38 MAPKs (Fig. 3D). We then attempted to determine the effect of actinomycin D and cycloheximide on TSL-mediated HO-1 protein expression (Fig. 3E). Cotreatment of actinomycin D, a transcriptional inhibitor, did not significantly modulate TSL-mediated HO-1 protein expression, confirming that TSL-mediated induction of HO-1 may not occur at the transcriptional level. Cycloheximide, a translational inhibitor, however, completely blocked HO-1 protein expression induced by TSL. These results suggested that TSL does not induce the expression of HO-1 at the transcriptional level, but did at the translational level.

3.3. TSL suppresses production of NO, TNF-α and PGE2 in LPS-stimulated RAW264.7 cells

To investigate the anti-inflammatory effects of TSL, we determined the effect of TSL on the production of NO, TNF-α and PGE2 in LPS-stimulated RAW264.7 cells (Fig. 4). RAW264.7 cells were stimulated with 1 μg/ml of LPS for 24 h in the presence of increasing concentrations of TSL, and the amount of NO, TNF-α and PGE2 in the culture supernatant was measured (Fig. 4). TSL significantly attenuated the LPS-induced production of NO, TNF-α and PGE2 in a dose-dependent manner.

3.4. TSL down-regulates expression levels of COX-2 and iNOS in LPS-stimulated RAW264.7 cells

Next, we investigated the effect of TSL on the LPS-induced COX-2 and iNOS expression (Fig. 5). RAW264.7 cells were stimulated with...
1 µg/ml of LPS in the presence of increasing concentrations of TSL, and the expression levels of COX-2 and iNOS were determined (Fig. 5). Western blot analysis revealed that TSL suppressed LPS-induced iNOS and COX-2 expression in a dose-dependent manner (Fig. 5A). Consistently, LPS-induced mRNA expression levels of iNOS and COX-2 were also dose-dependently decreased in the presence of TSL, as assessed by RT-PCR analysis (Fig. 5B).

3.5. TSL suppresses production of NO, TNF-α and PGE2 in LPS-stimulated murine peritoneal macrophages

We further investigated the effect of TSL on the production of NO, TNF-α and PGE2 in LPS-stimulated peritoneal macrophages (Fig. 6). Macrophages were stimulated with 1 µg/ml of LPS for 24 h in the presence of increasing concentrations of TSL, and the amount of NO, TNF-α and PGE2 in the culture supernatant was measured. Similar with the results from RAW264.7 cells (Fig. 4), TSL significantly attenuated the LPS-induced production of NO, TNF-α and PGE2 in a dose-dependent manner. Consistently, the expression levels of COX-2 and iNOS were also effectively inhibited by pretreatment with TSL (Fig. 6D).

3.6. Inhibition of HO-1 activity blocks anti-inflammatory activity of TSL

Since TSL was able to induce HO-1 expression, and suppress iNOS and COX-2 expression as well as NO and PGE2 production in LPS-stimulated RAW264.7 cells and peritoneal macrophages, we examined whether TSL-mediated HO-1 induction could be responsible for the anti-inflammatory effects of TSL. To test this, we utilized a specific HO-1 inhibitor, zinc protoporphyrin IX (ZnPP), and an inactive compound, copper protoporphyrin IX (CuPP). ZnPP dramatically reversed TSL-mediated suppression of production of NO and PGE2 in LPS-stimulated RAW264.7 cells (Fig. 7). In contrast, CuPP showed no effect. Taken together, these observations suggest that HO-1 induction mediates the inhibitory effects of TSL on LPS-induced inflammatory responses in macrophages.

4. Discussion

Tussilagone (TSL) is the major constituent of the flower buds of T. farfara. In the present study, we demonstrated the potential involvement of HO-1 induction in the anti-inflammatory activity of TSL. We found that TSL-induced HO-1 expression in RAW264.7 cells, and that this induction was correlated with the suppression of iNOS and COX-2 expression as well as NO and PGE2 production in LPS-stimulated RAW264.7 cells and murine peritoneal macrophages. Inhibition of HO-1 activity by ZnPP abolished these effects, suggesting that HO-1 expression is involved in the inhibition of LPS-induced inflammatory responses by TSL.

TSL has been evaluated for its various pharmacological activities including antagonistic activity of platelet activating factor [25] and inhibition of diacylglycerol acyltransferase [23]. Recently, the extract of the flower buds of T. farfara or TSL has been reported to exert anti-inflammatory activities including inhibition of iNOS and COX-2 expression in microglial cells [22]. However, the underlying mechanisms that could explain the anti-inflammatory effect of TSL remain to be elucidated. In the present study, we showed that TSL induces HO-1 expression in RAW264.7 cells in a time- and dose-dependent manner. The induction of HO-1 is widely recognized as an effective cellular strategy to counteract a variety of cellular damage and inflammation [1,2,4]. A growing body of evidence has also shown that HO-1 efficiently represses inflammatory responses by inhibiting the production of NO and PGE2.

Fig. 6. TSL inhibits LPS-induced production of NO, PGE2, and TNF-α in murine peritoneal macrophages. Macrophages were pretreated with indicated concentrations of TSL for 30 min, and then stimulated with LPS (1 µg/ml) for 24 h. The amounts of NO (A), PGE2 (B), and TNF-α (C) in culture supernatants were determined. Mean values from two independent experiments performed in triplicate are shown; bar, the mean±S.D. Asterisks indicate a significant difference (*, p<0.01) compared with the control. (D) Macrophages were pretreated for 30 min with indicated concentrations of TSL, followed by the stimulation with LPS (1 µg/ml) for 24 h. Subsequently, total lysates were prepared and the expression levels of iNOS and COX-2 were determined by Western blot analysis and compared with those of α-tubulin.
various inflammatory cytokines [1,2]. Several plant-derived components, including curcumin, resveratrol, and 2'-hydroxychalcone, have been reported to induce HO-1 and exert anti-inflammatory activities in different types of cells [26–29]. Similar with these findings, TSL induced HO-1 expression and suppressed LPS-induced iNOS and COX-2 expression as well as NO and PGE2 production.

It has been known that HO-1 expression is regulated mainly at the transcriptional level [24]. The most crucial transcription factor in HO-1 expression seems to be nuclear factor-erythroid 2-related factor (Nrf2). Most of the HO-1 inducers derived from plants activate Nrf2 expression at the transcriptional level [24]. The most crucial transcription factor in HO-1 expression as well as NO and PGE2 production.

Inhibition of HO-1 activity reverses the anti-inflammatory effects of TSL in RAW264.7 cells. RAW264.7 cells were pretreated with 30 μM of TSL in the presence of ZnPP or CuPP for 30 min and then stimulated with LPS (1 μg/ml) for 24 h. The amounts of NO (A) and PGE2 (B) in culture supernatants were determined. Mean values from two independent experiments performed in triplicate are shown. bar, the mean ± S.D. Asterisks indicate a significant difference (*, p < 0.01) compared with the control.

Fig. 7. Inhibition of HO-1 activity reverses the anti-inflammatory effects of TSL in RAW264.7 cells. RAW264.7 cells were pretreated with 30 μM of TSL in the presence of ZnPP or CuPP for 30 min and then stimulated with LPS (1 μg/ml) for 24 h. The amounts of NO (A) and PGE2 (B) in culture supernatants were determined. Mean values from two independent experiments performed in triplicate are shown. bar, the mean ± S.D. Asterisks indicate a significant difference (*, p < 0.01) compared with the control.

inflammatory activity of TSL. Further-


