Biochemical Pharmacology 81 (2011) 752–760

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

1,3,5-Trihydroxy-4-prenylxanthone represses lipopolysaccharide-induced iNOS expression via impeding posttranslational modification of IRAK-1

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ARTICLE INFO

Article history:
Received 3 November 2010
Accepted 21 December 2010
Available online 11 January 2011

Keywords:
1,3,5-Trihydroxy-4-prenylxanthone
Lipopolysaccharide
Inducible nitric oxide synthase
Interleukin (IL)-1 receptor-associated serine/threonine kinase 1
Transforming growth factor β -activated kinase-1

ABSTRACT

Both high level of nitric oxide (NO) and its generating enzyme, inducible NO synthase (iNOS), play important roles in pathophysiological conditions such as inflammatory processes. We previously found that 1,3,5-trihydroxy-4-prenylxanthone (TH-4-PX) isolated from *Cudrania cochinchinensis* repressed lipopolysaccharide (LPS)-induced NO production in RAW264.7 macrophages. Here we further examined the underlying mechanisms using RT-PCR and Western blot analyses. Consistent with NO inhibition, suppression of LPS-induced iNOS expression by TH-4-PX through abolishing IκB kinase (IKK) phosphorylation, IκB degradation and nuclear factor-κB (NF-κB) nuclear translocation was observed. After LPS stimulation, the increased nuclear level of c-Fos and c-Jun (major components of activator protein-1, AP-1) and the phosphorylated level of upstream signal molecules, such as c-Jun NH2-terminal kinase (JNK) and extracellular signal-regulated kinase, (ERK) were all significantly suppressed by TH-4-PX, while p38 remained unaffected. A further experiment revealed that TH-4-PX inhibited the phosphorylation of transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1), an upstream signaling molecule required for IKK and mitogen-activated protein kinases (MAPKs) activation. Stimulation with LPS also triggered the modification (phosphorylation and ubiquitination) and eventually the proteasomal degradation of membrane-associated interleukin (IL)-1 receptor-associated serine/threonine kinase 1 (IRAK-1), an essential signaling component to toll-like receptor (TLR)-mediated TAK-1 activation. Interestingly, the modified pattern of IRAK-1 in the presence LPS was significantly attenuated by TH-4-PX treatment. In conclusion, TH-4-PX inhibited LPS-induced NF-κB and AP-1 activations by interfering with the posttranslational modification (phosphorylation and/or ubiquitinylation) of IRAK-1 in the cell membrane to impede TAK1-mediated activation of IKK and MAPKs signal transduction.

1. Introduction

A regulated low level of nitric oxide (NO) production in the body is essential for maintaining homeostasis, though certain pathophysiological conditions associated with inflammation involve *de novo* synthesis of inducible NO synthase (iNOS) in immune cells, including macrophages [1]. A large body of evidence indicates many inflammatory diseases occurring through sustained and elevated activation of this particular enzyme. The biochemical process of iNOS protein expression is tightly regulated and complex, in which the endotoxin lipoolysaccharide (LPS) selectively binds to toll-like receptor 4 (TLR4), thus leading to signaling activation via downstream signaling factors, which include the adaptor MyD88, IL-1 receptor-associated protein kinases (IRAKs), tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6), and transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) [2]. Upon LPS stimulation, IRAK-1, a serine-threonine kinase, is phosphorylated within receptor complexes that include Tollip, MyD88, and TRAF6. Phosphorylation of IRAK-1 presumably triggers IRAK-1 to dissociate from the receptor complexes and translocate along with TRAF6 to the membrane-bound pre-associated TAK1-TAB1-TAB2 complex. Phosphorylated IRAK-1 is eventually ubiquitinated and degraded. TAK1-TAB1-TAB2-TRAF6 then dissociates from phosphorylated IRAK-1 and translocates to the cytosol, in which phosphorylated TAK1 is activated in a process that at least depends partially on TRAF6 self-ubiquitination [3–5]. TAK1 was later shown to be a pivotal factor for activation of IKK and mitogen-activating protein kinases (MAPKs) in response to TLR, IL-1R and TNFR stimulation [6].

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doi:10.1016/j.bcp.2010.12.022

These signaling pathways in turn activate a variety of transcription factors that include NF-κB (p50/p65) and AP-1 (c-Fos/c-Jun), which coordinate the induction of many gene-encoding inflammatory mediators. Thus, suppression and/or inhibition of the above-mentioned signaling molecules may have great potential for preventing and treating of inflammation-associated diseases.

In fact, many traditional herbs and spices along with their active constituents have been demonstrated to disrupt inflammatory signal transduction and prevent disease process [7]. Some phytochemicals, including polyphenols, terpenoids, and others [8–10], are found to be capable of targeting NO production by unique mechanisms. *Cudrania cochinchinensis* is a Chinese folk medicine with anti-inflammation, anti-microbial, antioxidant, and hepatoprotective properties [11–13]. Using endotoxin-stimulated macrophage as an assay platform, we found that 1,3,5-trihydroxy-4-prenylxanthone (TH-4-PX), a xanthone derivative isolated from *C. cochinchinensis*, inhibited significantly LPS-induced NO production in RAW264.7 macrophages. However, the mechanisms involved remain unclear. The present study aimed to explore the molecular mechanism underlying the repression effect of TH-4-PX on LPS-induced iNOS expression. We described for the first time that TH-4-PX is a novel anti-inflammatory natural compound that blocks LPS-induced NF-κB and AP-1 activations by interfering with the posttranslational modification (phosphorylation and/or ubiquitinylation) of IRAK-1 in the cell membrane to trim TAK1-mediated activation of IKK and MAPKs signal transduction.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, and antibiotics (penicillin/streptomycin) were purchased from Invitrogen (Carlsbad, CA). Anti-iNOS antibody was from Transduction Laboratories (Lexington, KY). Antibodies against total or phosphorylated IKK, ERK, JNK, p38, c-Jun, c-Fos and TAK1 were purchased from Cell Signaling Technology (Beverly, MA). Anti-NF-κB p65, anti-IκB, anti-IRAK-1, anti-β-actin, anti-histone, horseradish peroxidase-labeled goat anti-rabbit IgG and goat anti-mouse IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence (ECL) Western blotting detection reagent was from GE Healthcare (Little Chalfont, Buckinghamshire, UK). All other chemicals, including LPS (*Escherichia coli* serotype 0128:B12) and aminoguanidine, were from Sigma-Aldrich. (St. Louis, MO).

2.2. Extraction and isolation of TH-4-PX

The isolation of TH-4-PX was processed according to the method described by Kobayashi et al. [14]. Briefly, the air-dried roots of *C. cochinchinensis* were extracted four times with methanol (MeOH) under reflux to give the MeOH extract. The MeOH extract was partitioned with an ethyl acetate (EtOAc) and water mixture (1:1) to give the EtOAc extract and the water extract. The AcOEt extract was subjected to silica gel column chromatography eluting with *n*-hexane: EtOAc/MeOH to give eight fractions. Fraction 3 was further separated by HPLC (Cosmosil 5C18-AR, Nacalai Tesque, Tokyo, Janpan) to obtain TH-4-PX. The structure of TH-4-PX (Fig. 1) was elucidated with mass and NMR spectroscopy in comparison to published data [14,15]. The purity of TH-4-PX was >98% as judged by HPLC and ^{1}H NMR.

2.3. Cell culture and nitrite quantification

The macrophage cell line RAW 264.7 was obtained from ATCC (Rockville, MD) and cultured in DMEM supplemented with L-glutamine (2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% heat-inactivated FBS. Cells were plated at a density of 2×10^5 cells/well in 96-well culture plate and stimulated with LPS (1 μg/mL) in the presence or absence of different concentrations of TH-4-PX (1–30 μM) for 18 h simultaneously. TH-4-PX was dissolved in dimethyl sulfoxide (DMSO) and further diluted with sterile PBS and sterilized via a 0.2-μm filter. NO_2^- accumulation in the medium was used as an indicator of NO production. Briefly, the isolated supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 5% phosphoric acid) and incubated at room temperature for 10 min. $NaNO_2$ was used to generate a standard curve, and nitrite production was determined by measuring optical density at 550 nm [16]. All experiments were performed in triplicate. NO production by LPS stimulation was designated as 100% for each experiment.

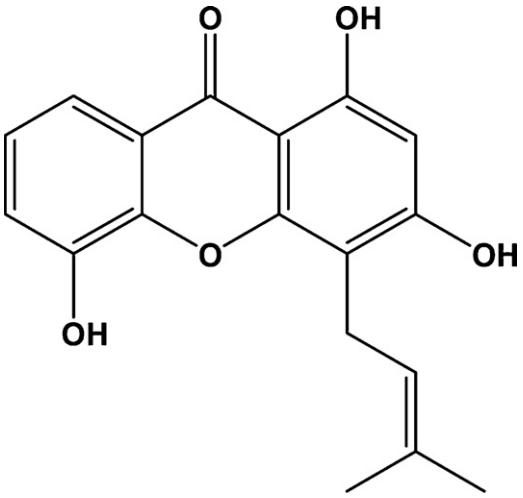

Fig. 1. Chemical structure of 1,3,5-trihydroxy-4-prenylxanthone (TH-4-PX).

2.4. Cell viability

Cell viability was determined colorimetrically using the MTT assay. After different treatments, 20 μl of 5 mg/mL MTT solution was added to each well and incubated for 4 h. The supernatants were aspirated, the formazan crystals in each well were dissolved in 200 μl of DMSO for 30 min at 37 °C, and optical density at 570 nm was read on a Microplate Reader (Bio-Rad, Hercules, CA). The mean absorbance for the duplicate cultures of each drug was calculated and the mean blank value was subtracted from these.

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized using the Superscript II (Invitrogen). PCR was performed with mouse specific primers for iNOS or GAPDH. The amplification conditions and primer sequences were the same as previously described [16,17].

2.6. Western blot analysis

Preparation of whole cell, nuclear or membrane protein extracts from control or drug treated cells was carried out as described previously [17]. The protein concentration of each sample was determined using a BCA protein assay kit (Pierce Chemical, Rockford, IL). Appropriate amount of total protein (20–30 μg) was used to detect iNOS, IKK, IκB, MAPKs or TAK1 and was electrophoresed on polyacrylamide gel. Nuclear protein was used to detect NF-κB, c-Fos and c-Jun. Membrane protein was used to

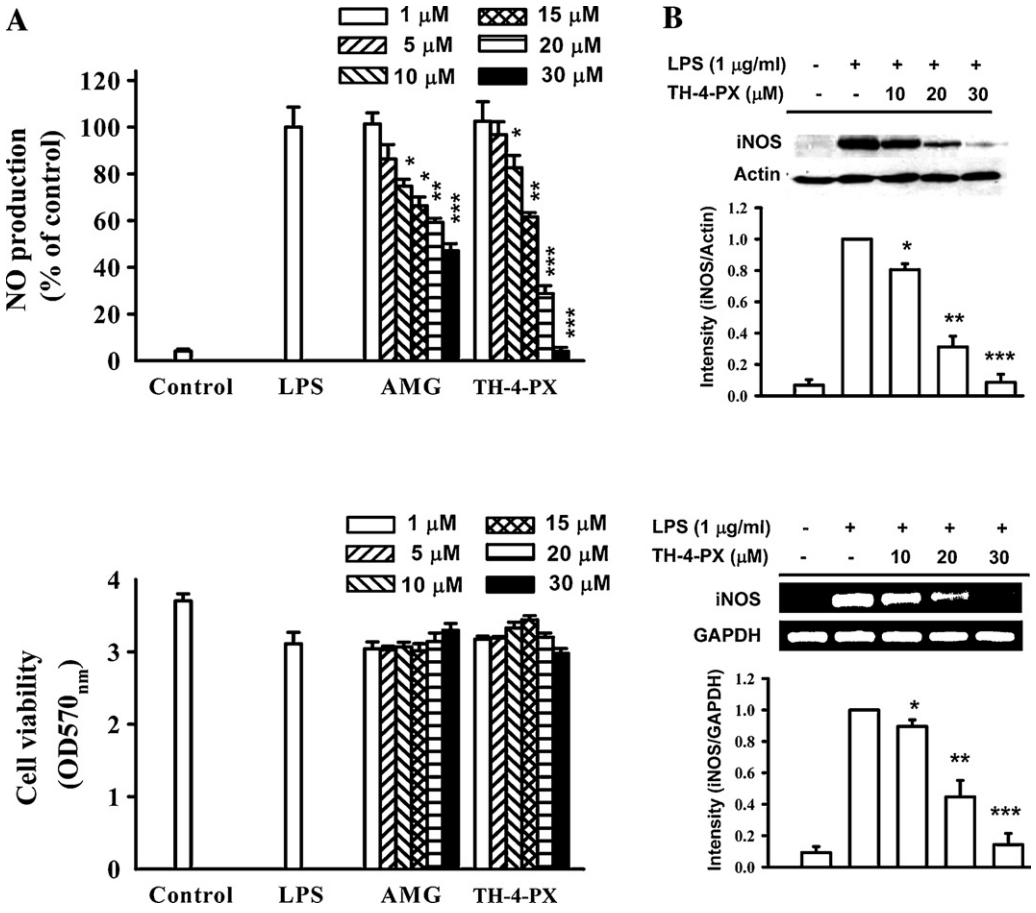

Fig. 2. Effect of 1,3,5-trihydroxy-4-prenylxanthone (TH-4-PX) on LPS-induced nitrite production, cell viability and iNOS expression in RAW264.7 macrophages. (A) Cells were treated with 1 μg/mL LPS in the absence or presence of TH-4-PX for 18 h. Results are expressed as the mean ± S.E.M. of five independent experiments each performed in triplicate. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, compared with LPS alone. (B) Cells were treated with LPS only or co-incubated with TH-4-PX. After incubation for 18 h or 6 h, total protein or total RNA was isolated for analyzing the expression of iNOS by Western blot analysis or RT-PCR, respectively. β-actin and GAPDH were used as loading controls. Bands were quantified with a densitometer. Results are expressed as the mean ± S.E.M. of three to four independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, compared with LPS alone.

detect the migration pattern of modified IRAK-1. The gels were transferred to polyvinylidene difluoride (PVDF, GE Healthcare) membranes by semidry electrophoretic transfer at 15 V for 60–75 min. The PVDF membranes were blocked overnight at 4 °C in 5% bovine serum albumin. The membrane was incubated with primary antibody and then with secondary antibody under appropriate condition. The signals were detected by ECL and evaluated by densitometry analysis.

2.7. Statistical analysis

Data were expressed as mean ± S.E.M. All experiments were performed at least three times independently to confirm our results. The data were analyzed by one-way analysis of variance (ANOVA) followed by *post-hoc* Dunnett's *t*-test for multiple comparisons. A p value < 0.05 was considered significant.

3. Results

3.1. TH-4-PX inhibits LPS-induced NO production, iNOS mRNA and protein expressions in RAW264.7 macrophages

The accumulated nitrite was 3.8 ± 1.1 μM of the control media, which increased to 59.5 ± 1.4 μM after LPS (1 μg/mL) stimulation for 18 h (represented as 100%). Co-treatment with TH-4-PX decreased the nitrite level concentration-dependently, but did not reduce the cell viability as compared with the LPS group without TH-4-PX treatment (Fig. 2A). More than 70% and almost 100% inhibition of NO production was obtained by 20 and 30 μM of TH-4-PX, respectively. Aminoguanidine (AMG), the selective iNOS inhibitor, was utilized as a positive control. Western blot analysis and RT-PCR were performed to determine whether the reduced production of NO by TH-4-PX was caused by the inhibition of iNOS protein or mRNA expression. As shown in Fig. 2B, iNOS protein expression was barely detectable in the un-stimulated cells, but markedly increased after 18 h of LPS stimulation. Cells co-treated with TH-4-PX showed a dose-dependent inhibition of iNOS protein expression. Similarly, the expression of iNOS mRNA was barely detectable in the un-stimulated cells. RAW 264.7 cells that were not treated with TH-4-PX expressed high levels of iNOS mRNA when stimulated with LPS for 6 h. Consistent with protein inhibition, TH-4-PX added also inhibited this LPS-stimulated iNOS mRNA production in a dose-dependent manner.

3.2. TH-4-PX inhibits IKK/NF-κB and MAPK/AP-1 pathways

The induction of iNOS depends mainly upon NF-κB. To examine whether the reduction of iNOS expression by TH-4-PX is attributed to the suppression of this signal pathway, the effects of TH-4-PX on LPS-induced nuclear translocation of NF-κB was studied. As expected, TH-4-PX inhibited LPS-induced NF-κB nuclear translocation in a concentration-dependent manner, when determined by Western blot analysis (Fig. 3). TH-4-PX also showed parallel inhibition of LPS-induced cytosolic degradation of IκB and phosphorylation of IκB kinase (IKK). Activator protein-1 (AP-1)

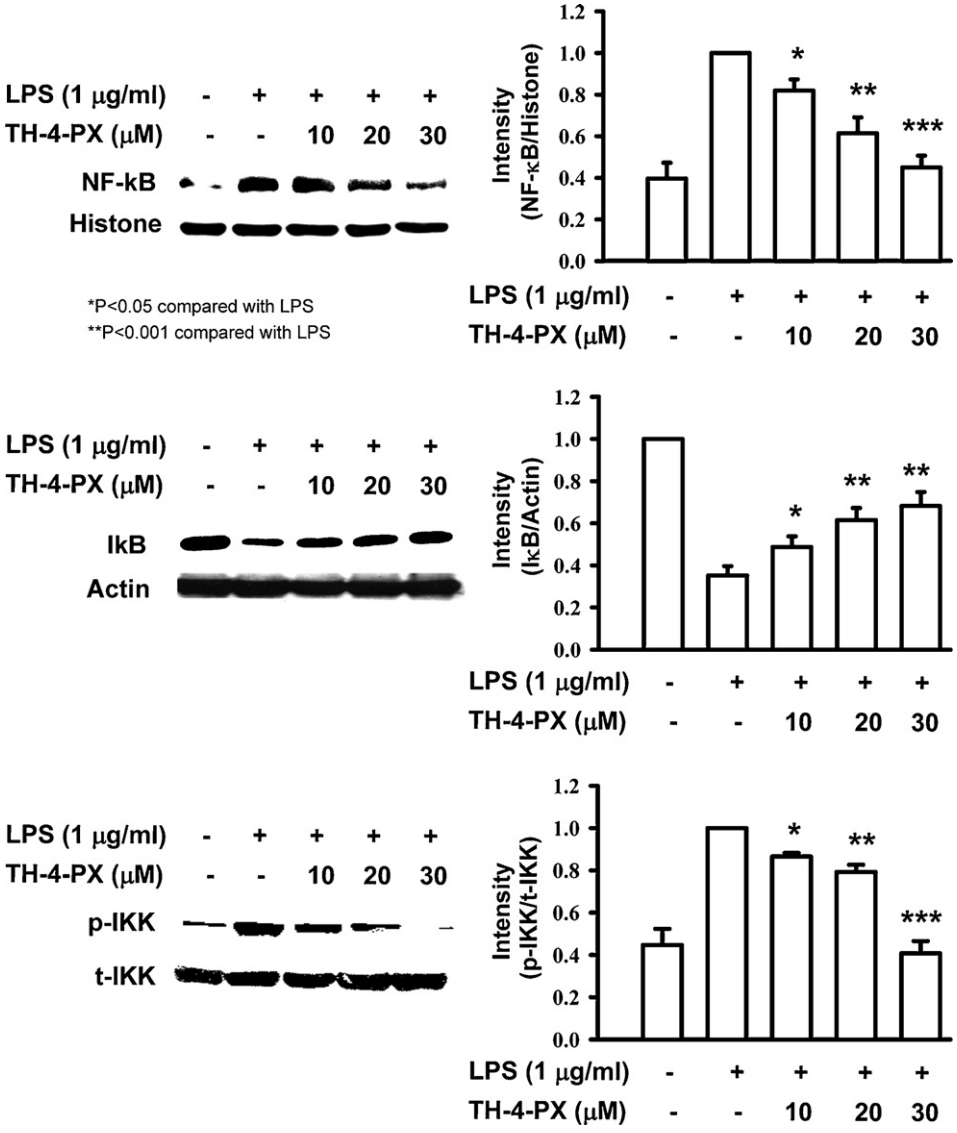

Fig. 3. 1,3,5-Trihydroxy-4-prenylxanthone (TH-4-PX) repressed LPS-induced NF-κB nuclear translocation, IκB degradation and IKK phosphorylation. TH-4-PX was added into cells 2 h before LPS stimulation and protein samples were prepared 1 h after LPS stimulation. Activations of signaling molecules were then evaluated by Western blot analysis as described Section 2. Bands were quantified with a densitometer. Results are expressed as the mean ± S.E.M. for each group from four to five independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as compared with LPS alone. The values under each lane indicate relative density of the band normalized to histone (for nuclear protein normalization), β-actin (for total protein normalization) or un-phosphorylated IKK, respectively.

is another critical transcription factor for iNOS expression induced by LPS, which is made up of a variety of dimers comprising members of the Jun, Fos, and ATF families of proteins [18,19]. We next tested the effect of TH-4-PX on LPS-induced AP-1 activation. RAW264.7 cells were exposed to LPS for 1 h, and the nuclear translocation of c-Jun and c-Fos were analyzed by Western blot. As shown in Fig. 4, the nuclear levels of c-Jun and c-Fos were markedly increased by LPS stimulation. In the presence of TH-4-PX, such phenomenon was repressed concentration-dependently.

Besides IKK, LPS can act through activation of MAPKs signal pathways to trigger NF-κB as well as AP-1 transcription [20,21]. Our previous study also indicated that MAPKs indeed participated in regulating LPS-induced iNOS expression [17]. Thus, we next examined whether TH-4-PX inhibited LPS-induced NO production by down-regulation of MAPKs activation. Following stimulation with 1 μg/mL LPS for 45 min, the phosphorylations of JNK, ERK and p38 MAPK significantly increased in RAW264.7 cells (Fig. 5). Pretreatment with various concentrations of TH-4-PX inhibited dose-dependently the phosphorylations of three MAPKs though in quite a different pattern. The most pronounced inhibition was shown against ERK phosphorylation. Phosphorylation of JNK was mildly repressed by TH-4-PX. In contrast, LPS-induced p38 phosphorylation was unaffected by TH-4-PX. These results suggest that regulation of ERK and JNK MAPK signaling cascade is another possible mechanism underlying the inhibitory effect of TH-4-PX in LPS-activated RAW264.7 cells.

3.3. TH-4-P suppresses TAK1 phosphorylation

Because TAK1 is the upstream signaling molecule of IKK, JNK and ERK [22,23], the effect of TH-4-PX on the LPS-induced phosphorylation of TAK1 was examined. First, we performed a time-course experiment to determine the time of phosphorylation of TAK1 induced by LPS. In parallel with the results of IKK phosphorylation, LPS also increased the phosphorylation of TAK1 after 15–60 min stimulation, but did not affect total TAK1 protein. As shown in Fig. 6A, significant phosphorylation was observed starting at 20 min, peaked at 45 min and declined at 60 min after LPS stimulation. Next, we pretreated the cells with various concentrations of TH-4-PX for 2 h and then exposed them to LPS

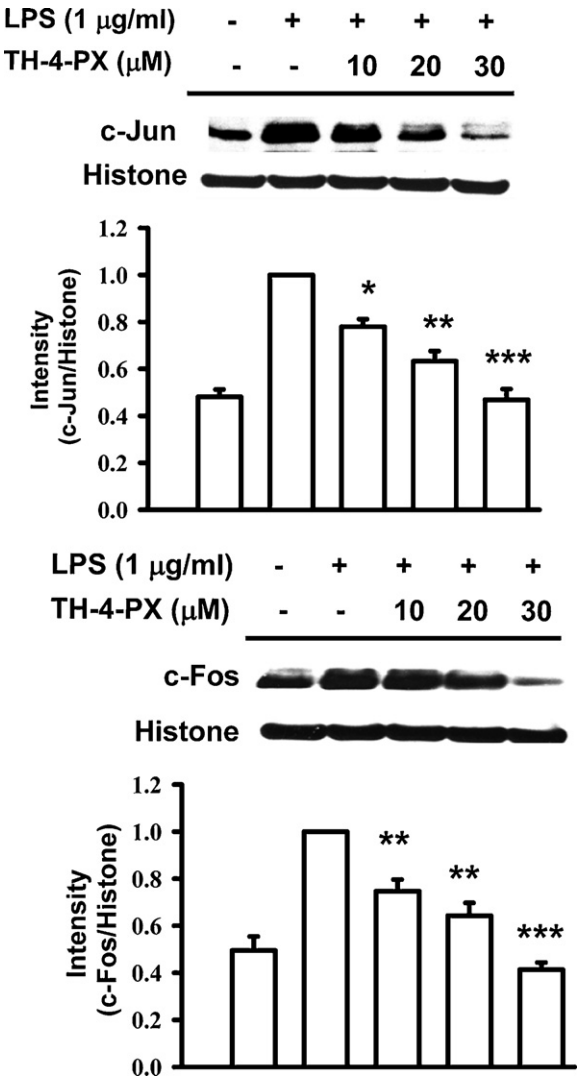

Fig. 4. Effect of 1,3,5-trihydroxy-4-prenylxanthone (TH-4-PX) on LPS-stimulated nuclear translocation of c-Jun and c-Fos in RAW264.7 cells. TH-4-PX was added into cells 2 h before LPS stimulation and protein samples were extracted 1 h after LPS stimulation. The nuclear levels of c-Jun and c-Fos were evaluated by Western blot analysis as described in Methods. Bands were quantified with a densitometer. Results are expressed as the mean ± S.E.M. for each group from four to five independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as compared with LPS alone. The values under each lane indicate relative density of the band normalized to histone.

for 45 min. As shown in Fig. 6B, treatment with TH-4-PX significantly inhibited the phosphorylation of TAK1 induced by LPS, but did not affect total TAK1 protein.

3.4. Effect of TH-4-PX on LPS-induced modification of IARK-1

Protein phosphorylation can have a profound effect on protein activity and stability as well as subcellular localization. IRAK-1 is posttranslationally modified (phosphorylation and/or ubiquitinylation) in cells treated with either LPS or IL-1, which leads to a slower migrating form of IRAK-1 in SDS-PAGE [24–26]. To confirm the kinetics of the IRAK-1 posttranslational modifications in RAW264.7 macrophages, cells were treated with LPS for varying durations. Membrane extracts from treated cells were subjected to Western blot analysis. In response to LPS, there is a time-dependent decrease in the unmodified IRAK-1 coordinated with a time-dependent increase in modified IRAK-1 in the membrane fraction (Fig. 7A). The results shown in the upper trace of Fig. 7A demonstrated that no modified IRAK-1 was significantly detected in the membrane pool within 5 min of LPS stimulation. An increase in modified IRAK1 was detected after 10 min and peaked around 30 min of LPS stimulation. Since phosphorylation often targets proteins for ubiquitin-dependent degradation, we examined whether LPS targets IRAK-1 for proteasomal degradation. In fact, the modified (phosphorylated and/or polyubiquitinated) IRAK-1 protein started to degrade after stimulation with LPS for 1 h (Fig. 7B and Fig. 8). In cultures pretreated with the proteasome inhibitor MG132, modified IRAK-1 accumulates in the membrane fraction in LPS-treated cultures, demonstrating that IRAK-1 degradation is proteasome-dependent (Fig. 7B). Interestingly, LPS-induced change in the apparent molecular mass of modified IRAK-1 and the decreased level of unmodified IRAK-1 were both significantly reversed by TH-4-PX treatment (Fig. 8).

4. Discussion

Macrophages play particularly important roles in inflammation by producing many pro-inflammatory molecules, including NO. The overproduction of NO can be harmful and cause various inflammatory diseases. Therefore, pharmacological interference with NO production is postulated to be useful in alleviating numerous disease states that are mediated by excessive and/or protracted activation of macrophages. In the present study, we demonstrated that TH-4-PH inhibits LPS-induced NO production in RAW 264.7 cells, a widely used macrophage-like cell line. This inhibitory effect is not mediated by the cytotoxic effect on macrophages. The parallel inhibition of iNOS protein and mRNA expression in LPS-stimulated cells implies that the blockade of transcription step could play a critical role. There are a number of binding sites for transcription factors located in the promoter of the iNOS gene, in which NF-κB and AP-1 are principally involved in LPS-mediated iNOS expression [17,27]. TH-4-PH at a similar concentration range indeed blocked the transcriptional activity of NF-κB and AP-1 as measured by repressing the increased nuclear translocation of p65, c-Fos and c-Jun in RAW264.7 cells by LPS stimulation. The critical step in NF-κB activation is IκB phosphorylation by IKK, which targets IκB for ubiquitin conjugation and degradation by the 26 S proteasome [28]. Moreover, the stimulation of macrophage TLR4 receptor rapidly activates not only the NF-κB pathway but also the MAPK pathways. MAPKs are a highly conserved family of protein serine/threonine kinases comprising p38, ERK, and JNK [29]. Using specific inhibitors of MAPKs, we proved that activation of MAPKs is essential in LPS-induced iNOS expression in RAW264.7 macrophages [17]. Many of the downstream targets of MAPK pathways are transcription factors including c-Jun, c-Fos, ATF2, and Elk-1 [18,30]. In line with this notion, the upstream signaling pathways for NF-κB activation such as IKK and those for AP-1 such as JNK and ERK were blocked by TH-4-PH.

Suppression of phosphorylation events for NF-κB and AP-1 translocation strongly suggested that the target of TH-4-PH could be activated at an earlier time in response to LPS. TAK1 kinase is an important signaling molecule, which mediates an activation signaling from the toll-like receptor to NF-κB [31]. TAK1, as an IKK kinase (IKKK), is thought to phosphorylate IKK in the TRAF6 pathway, resulting in further activation of NF-κB [22,23]. The phosphorylation of TAK1 is a common signal for activating downstream targets such as IKK and MAPK [32,33]. These kinases may represent novel sites for pharmacological intervention in a number of inflammatory conditions. Therefore, we examined the inhibitory effects of TH-4-PH on phosphorylation of TAK1. In parallel with the result of IKK phosphorylation, TH-4-PH also inhibited the phosphorylation of TAK1 (Fig. 6). The fact that TH-4-PX inhibited LPS-induced TAK-1 phosphorylation led to the

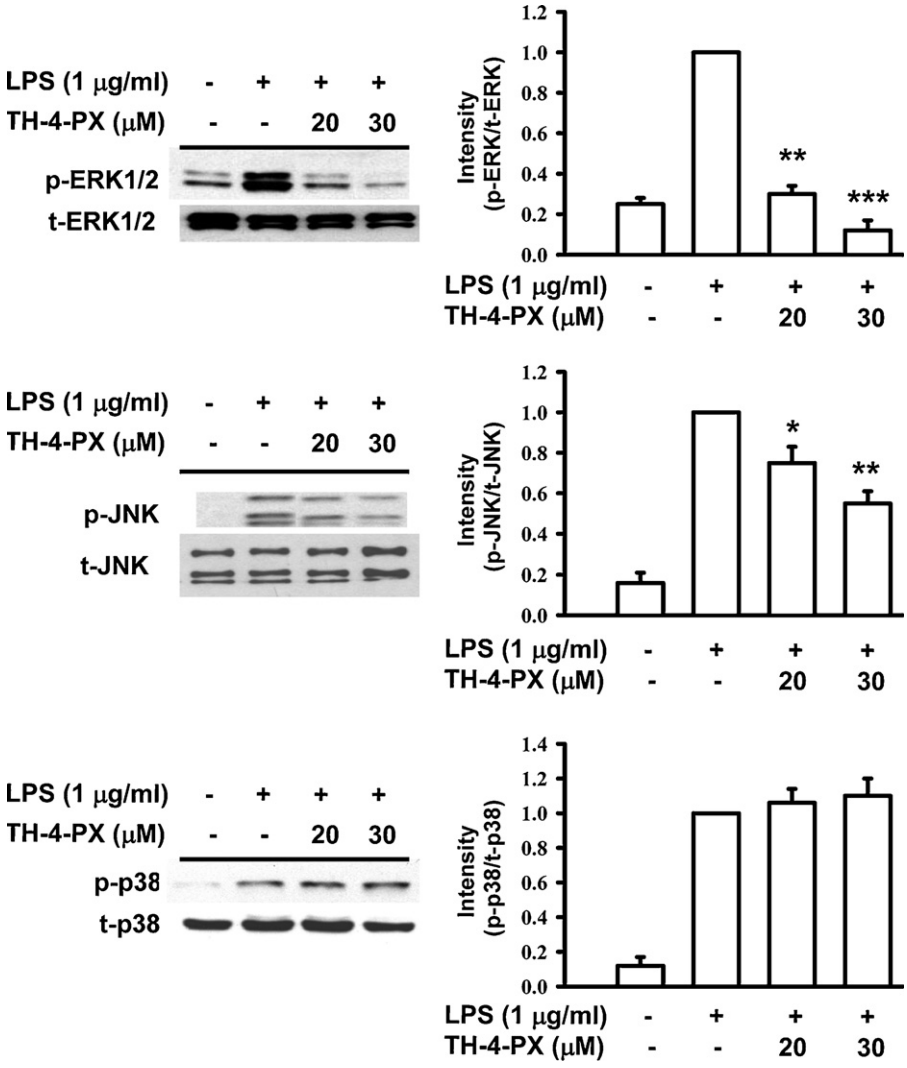

Fig. 5. Effect of 1,3,5-trihydroxy-4-prenylxanthone (TH-4-PX) on LPS-stimulated phosphorylation of MAPKs in RAW264.7 cells. TH-4-PX was added into cells 2 h before LPS stimulation and protein samples were extracted 45 min after LPS stimulation. The phosphorylated levels of p38, JNK and ERK were evaluated by Western blot analysis as described in Section 2. Individual non-phosphorylated protein was used as control. Bands were quantified with a densitometer. All analyses were representative of at least three independent experiments. Results are expressed as the mean ± S.E.M. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as compared with LPS alone.

conclusion that the LPS pathway upstream of TAK-1 was affected by TH-4-PX.

LPS signal initially leads to the assembly of the LPS recognition complex, which induces a cascade of association/phosphorylation events involving proteins such as IRAK-1. The critical role of IRAK-1 in TLR-mediated inflammatory responses has been previously reported in which IRAK-1 is linked to the induction of NF-κB activity by LPS [34]. The mammalian family of IRAK molecules has four members: IRAK-1, IRAK-2, IRAK-M and IRAK-4 [35]. Among these, only IRAK-1 and IRAK-4 possess protein kinase activity and play positive regulatory roles in IL-1 and TLR signaling. A stimulus-induced interaction between IRAK-1 and IRAK-4 is important in TLR signaling, in which MyD88 acts as a scaffold protein for the interaction [36]. IRAK-4 phosphorylates IRAK-1 leading to IRAK-1 activation [37]. Catalytically active IRAK-1 autophosphorylates, dissociates from the receptor, and forms a complex at the plasma membrane that contains the ubiquitin ligase TRAF-6, TAB2 (which binds lysine 63-linked ubiquitin chains) and the protein kinase TAK-1 [38,39]. Membrane-associated IRAK-1 is ubiquitinylated and eventually degraded by the proteasome [3,26]. The TRAF-6, TAK-1, and TAB1/2 complex phosphorylates and activates the IKK complex, resulting in the induction of NF-κB-regulated gene expression [40]. The results of the present study revealed that IRAK-1 activation regulated by LPS signaling pathways was also observed in RAW264.7 macrophages. Confronted by LPS stimulation, the expression of IRAK1 was present as the modified protein of species with much higher molecular mass (Fig. 7A). The modified forms of IRAK-1 appeared to have identical electrophoretic mobility, suggesting that the polyubiquitin was attached to IRAK1. We also confirmed that LPS-induced IRAK-1 protein turnover is mediated by the proteasome because MG132 restricted the accumulation of modified IRAK-1 in the membrane fraction of LPS-stimulated cells. Yamin and Miller [26] reported that LPS induces a time-dependent degradation of cytoplasmic pool of unmodified IRAK-1 protein. They further postulated a model in which the cytoplasmic pool of IRAK-1 is targeted to the plasma membrane when confronted by LPS stimulation, where it undergoes posttranslational modification (phosphorylation and ubiquitinylation) that eventually leads to its degradation. An exciting finding obtained from the present study is that TH-4-PH significantly impeded the LPS-triggered phosphorylation/ubiquitinylation of IRAK-1, which slowed down the subsequent proteasome-dependent degradation process. Therefore, unmodified IRAK-1 (75 kDa) protein of a low molecular mass was abundant in the cell membrane of TH-4-PH-treated cells but was less in non-treated ones. Results obtained here suggested that the target

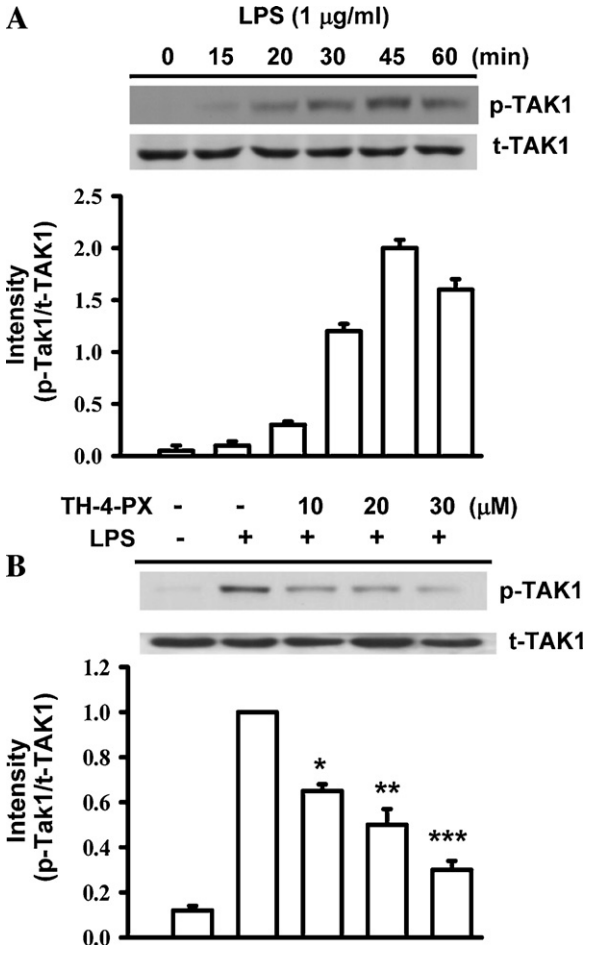

Fig. 6. Effect of 1,3,5-trihydroxy-4-prenylxanthone (TH-4-PX) on LPS-stimulated phosphorylation of TAK-1 in RAW264.7 cells. (A) RAW264.7 cells were treated with LPS (1 μg/mL) for 60 min, followed by extraction of the total protein. Protein fraction was resolved by 10% SDS-PAGE and probed for phosphorylated TAK-1 antibody. Non-phosphorylated TAK-1 was used as control. (B) TH-4-PX was added into cells 2 h before LPS stimulation and protein samples were extracted 45 min after LPS stimulation. Bands were quantified with a densitometer. All analyses were representative of at least three independent experiments. Results are expressed as the mean ± S.E.M. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as compared with LPS alone.

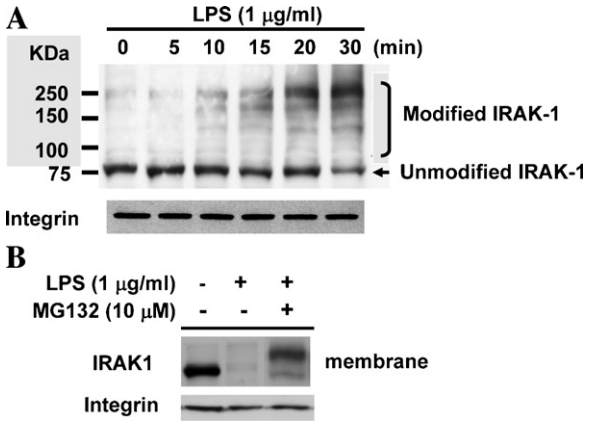

Fig. 7. LPS induced the modification (phosphorylation and polyubiquitination) of IRAK-1. (A) RAW264.7 cells were treated with LPS (1 μg/mL) for 30 min, followed by extraction of the membrane fraction. Membrane proteins were resolved by 10% SDS-PAGE and probed for IRAK-1 antibody. Integrin is the loading control for the membrane fraction. The molecular mass markers denote unmodified (75 kDa) and modified IRAK-1 (100–250 kDa) bands. (B) RAW264.7 cells were either untreated or pretreated with MG132 for 2 h. Cells were then stimulated with LPS for 1 h. Membrane lysates were prepared for IRAK-1 immunoblotting. Integrin is the loading control. MG132 is a proteasome inhibitor.

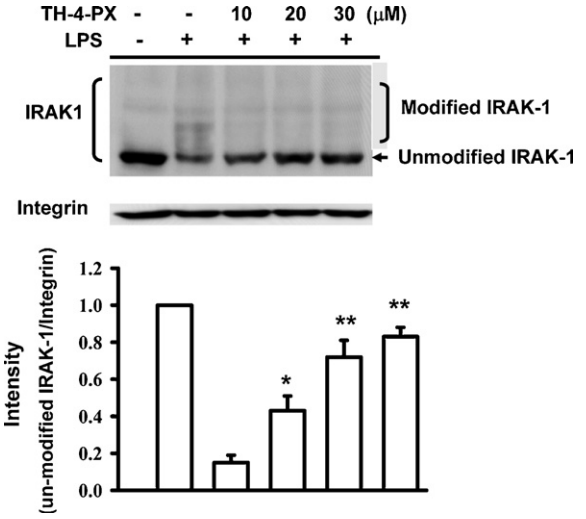

Fig. 8. LPS-induced change in the apparent molecular mass of modified IRAK-1 and the decreased level of unmodified IRAK-1 were attenuated by TH-4-PX treatment. RAW264.7 cells were either untreated or pretreated with TH-4-PX for 2 h. Cells were then stimulated with LPS for 1 h. Membrane lysates were prepared for IRAK-1 immunoblotting. Integrin is the loading control. Bands were quantified with a densitometer. Results are expressed as the mean ± S.E.M. from four independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as compared with LPS alone. The values under each lane indicate relative density of the band normalized to integrin.

mechanism by TH-4-PH was quite different from that of a proteasome inhibitor. TH-4-PH appears to affect the LPS pathway at the level and/or upstream of IRAK-1. The MyD88/IRAK-1 protein complex has been recently found to play critical roles in transferring LPS-TLR4 signaling into the cells and promoting the production of various inflammatory cytokines through activation of the NF-κB signal pathway [41,42]. The question of whether or not TH-4-PH represses IRAK-1 phosphorylation through interference with MyD88/IRAK-1 complex associations or the putative interaction between TH-4-PH and TLR4/CD14 complex requires further study. Nevertheless, our preliminary data indicated that TH-4-PX repressed LPS- and tumour necrosis factor-α-induced NO production equally (EC_{50}: 16.3 ± 1.6 vs. 15.2 ± 2.1 μM), suggesting a TLR4 antagonism property might be excluded.

Although xanthones in general are free radical scavengers that have been claimed to exert anti-inflammatory activity (ref [43]). Our preliminary study indicated that TH-4-PH failed to affect sodium nitroprusside-triggered NO production in a cell free condition (data not shown), precluding the possibility that TH-4-PH acts as a free radical scavenger to catch NO radical. Although many xanthone constituents (such as garcinone B, gambogic acid and cudratricusxanthone A) derived from natural source exert NO inhibition activity in RAW264.7 cells [44–46], most studies regarding their molecular mechanisms are limited to suppressing the IKK-IκB-NFκB or MAPKs signal pathways. With no exception, TH-4-PH also acts through the same pathway to inhibit LPS-induced iNOS expression. In fact, TH-4-PH was found to trim the phosphorylation of TAK-1, a common signal for the activation of downstream targets such as IKK and MAPK. Nevertheless, our data extend more understanding of the upstream molecular mechanisms underlying the iNOS inhibition activity of TH-4-PH. That is, blunting of TAK-1 phosphorylation by TH-4-PH might be due to the blocking of LPS-triggered phosphorylation/ubiquitinylation of IRAK-1. The present results suggested that TH-4-PX inhibited LPS-induced iNOS expression by interference with the posttranslational modification of IRAK-1, resulting in blocking TAK1-mediated activation of IKK and MAPKs signal transduction to down-regulate NF-(B and AP-1 activations in RAW264.7 macrophages. The possible regulatory mechanism of repression of LPS-

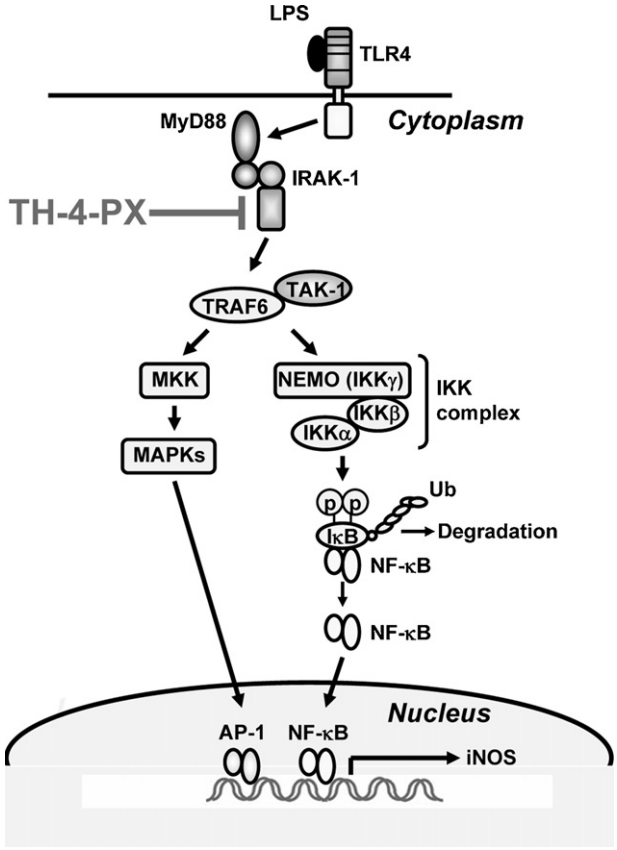

Fig. 9. Possible regulatory mechanism of 1, 3, 5-trihydroxy-4-prenylxanthone (TH-4-PX) involved in repressing LPS-induced iNOS expression in RAW264.7 macrophages. According to the findings obtained in the present study, we suggested that TH-4-PX is a novel anti-inflammatory natural compound that blocks LPS-induced NF-κB and AP-1 activations by interference with IRAK-1 activation to impede TAK1-mediated activation of IKK and MAPKs signal transduction. AP-1, activator protein-1; iNOS, inducible NO synthase; IκB, inhibitory IκB; IKK, IκB kinase; IRAK-1, interleukin (IL)-1 receptor-associated serine/threonine kinase 1; MAPKs, mitogen-activated protein kinases; MKK, MAPK kinase; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-κB; TAK-1, transforming growth factor β -activated kinase-1; TH-4-PX, 1, 3, 5-trihydroxy-4-prenylxanthone; TLR4, toll like receptor 4; TRAF6, tumour necrosis factor (TNF) receptor-associated factor 6.

induced iNOS expression by TH-4-PX is summarized in Fig. 9. In conclusion, the present data reveal that interference with IRAK-1 activation and the associated TAK-1 phosphorylation may partly account for the iNOS repression effect of TH-4-PH via blocking downstream IKK and MAPKs signaling transduction. To our knowledge, TH-4-PH is the first anti-inflammatory compound derived from plant source shown to exert its effect by blocking posttranslational modification of IRAK-1.

Acknowledgements

This work is supported by grant from National Research Institute of Chinese Medicine, Taipei, Taiwan (NRICM 99-DBCM-02) and by the National Science Council Grant funded of the Executive Yuan, Taiwan (NSC98-2320-B-241-003-MY3).

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