INTRODUCTION

Inflammation, a complex biological response to harmful stimuli, is associated with various pathophysiological conditions. In response to inflammatory stimuli, macrophages release various pro-inflammatory molecules. Nitric oxide (NO) is a cellular signaling molecule that plays a key role in the pathogenesis of inflammation, while it is also required for normal physiological conditions (Gibaldi, 1993). NO is produced during oxidation of L-arginine to L-citrulline catalyzed by nitric oxide synthase (NOS) (Moncada and Higgs, 1993). Among three isoforms of NOSs, including endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS), inducible form of NOS is responsible for the excessive production of NO by macrophages during inflammatory processes (Nussler and Billiar, 1993). It is widely accepted that NO production is a critical event leading to inflammatory process and therefore, suppression of NO production and iNOS expression is considered as a useful strategy for the treatment of diseases associated with inflammatory conditions.

Heme oxygenase (HO), a member of phase II detoxifying enzymes, is the rate-limiting enzyme in catabolism of heme into free iron, biliverdin and carbon monoxide (CO). Among the three isoforms, HO-1 is a highly inducible protein sensitive to cellular stress and catalyzes a series of reactions required for cellular defense. Previous studies have shown that HO-1 induction plays an important role in a number of cytoprotection responses, including anti-oxidative and anti-apoptotic effects (Motterlini et al., 2000) and a growing body of evidence has indicated that HO-1 induction acts an adaptive defense mech-
anism to protect cells from various pathophysiological conditions (Maines, 1997). In addition, recent studies have also revealed that HO-1 induction modulates inflammatory process via suppressing the production of pro-inflammatory mediators, such as COX-2. TNF-α and iNOS (Wang et al., 2001). Furthermore, deficiency of HO-1 in mice exhibited a severe inflammation (True et al., 2007), while HO-1 over-expression prevented inflammatory responses in various experimental conditions (Otterbein et al., 2003; Onyiah et al., 2013). Therefore, based on previous reports, modulation of HO-1 activity may be a promising strategy for the treatment of the diseases associated with inflammatory conditions.

Chalcones, a group of polyphenolic and flavonoid family, are widely biosynthesized in plants. A number of previous studies have demonstrated that chalcones and its various derivatives regulate diverse biological functions and become the attractive molecule for the development of pharmacological agents in many diseases. In addition to the original observation showing the potent inhibitory effect on topoisomerase and subsequent on anti-cancer effects (Kim et al., 2013), chalcone and its derivatives also possess anti-bacterial (Avila et al., 2008), anti-fungal (Bato et al., 2007), anti-oxidant (Dinkova-Kostova et al., 2001) properties. In particular, recent studies have shown the potent anti-inflammatory activities of the chalcones in various experimental conditions (Kontogiorgis et al., 2008). For examples, treatment of immune cells with chalcones significantly suppressed production of many inflammatory mediators, including COX-2, TNF-α and NO (Wu et al., 2011). We have previously observed that introduction of hydroxyl group on phenyl moiety of chalcone skeleton increased biological activities (Karki et al., 2010; Karki et al., 2012). In the present study, as part of effort to develop optimal anti-inflammatory agents, we prepared synthetic chalcone derivatives containing hydroxyl group at different positions of the phenyl ring, and investigated their inhibitory effects on NO production in macrophages and further its potential mechanisms.

MATERIALS AND METHODS

Materials

All the cell culture reagents were purchased from Hyclone Laboratories (South Logan, UT, USA). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Griess reagent system and Luciferase assay kit and all related products were purchased from Promega (Madison, WI, USA). HO-1 antibody was obtained from Enzo Life Sciences (Farmingdale, NY, USA). Antibodies against phosphorylated and total forms of ERK1/2, JNK, p38 MAPK and Nrf2 were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). HRP-conjugated anti-mouse and anti-rabbit were purchased from Pierce (Rockford, IL, USA).

Cell culture

The RAW 264.7 macrophage cell line was purchased from the Korean cell line bank (Seoul, Korea) and routinely cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin at 37°C in an incubator with a humidified atmosphere of 5% CO₂.

Synthesis of chalcone derivatives

Compounds used as starting materials and reagents were obtained from Aldrich Chemical Co., Junsei or other chemical companies, and utilized without further purification. HPLC grade acetonitrile (ACN) and methanol were purchased from Burdick and Jackson, USA. Thin-layer chromatography (TLC) and column chromatography (CC) were performed with Kieselgel 60 F254 (Merck) and silica gel (Kieselgel 60, 230-400 mesh; Merck) respectively. Since all the compounds prepared contain aromatic ring, they were visualized and detected on TLC plates with UV light (short wave, long wave or both). NMR spectra were recorded on a Bruker AMX 250 (250 MHz, FT) for 1H NMR and 62.5 MHz for 13C NMR, and chemical shifts were calibrated according to TMS. Chemical shifts (δ) were recorded in ppm and coupling constants (J) in hertz (Hz). Melting points were determined in open capillary tubes on electrothermal 1A 9100 digital melting point apparatus and were uncorrected.

ESI LC/MS analyses were performed with a Finnigan LC Advantage® LC/MS/MS spectrometry utilizing Xcalibur® program. For ESI LC/MS, LC was performed with 10 μL injection volume on a Waters X Terra® 3.5 μm reverse-phase C18 column (2.1×100 mm) with a gradient elution: from 65% to 95% of B in A for 5 min followed by 95% to 85% of B in A for 5 min and 65% of B in A for 5 min, where mobile phase A was 100% distilled water with 20 mM AF and mobile phase B was 100% ACN. MS ionization conditions were: Sheath gas flow rate: 70 arb, aux gas flow rate: 20 arb, 1 spray voltage: 4.5 KV, capillary temp.: 215°C, capillary voltage: 21 V, tube lens offset: 10 V. Retention time is given in minutes.

Compound 3

To an ice cold solution of 85% KOH (1.2 eq.) in methanol (10 mL) and H₂O (2 mL) methyl-3-thienylketone (1) (1.0 eq.) was added. After dissolution, aryl aldehyde (2 (1.0 eq.) was added dropwise. The reaction mixture was then stirred for 10 min to 3 h at 20°C. The mixture was neutralized with 4M aqueous HCl solution (pH adjusted to 2). The mixture was extracted with ethyl acetate (100 mL), washed with water (75 mL ×2) and saturated NaCl solution (50 mL). The organic layer was dried with magnesium sulfate and filtered. The filtrate was evaporated at reduced pressure, which was purified by silica gel column chromatography with a gradient elution of ethyl acetate/n-hexane to afford a solid compound 3 in 31.8 to 61.7% yield. Following the same procedure, four compounds were synthesized.

3-Phenyl-1-(thiophen-3-yl)prop-2-en-1-one (RK-I-105)

The same procedure described at general method was employed with 1 and 2 (R=a) to yield white solid 58.4%. Rf (EtOAc: n-Hexane=1:5, v/v): 0.37 Retention time: 16.98 min, [M]+: 215.1

1H NMR (250 MHz, CDCl₃) δ 8.16 (dd, J=2.7, 1.1 Hz, 1 H, 1-thiophene H-2), 7.83 (d, J=15.7 Hz, 1 H, -CO-C=CH-), 7.67 (dd, J=5.2, 1.0 Hz, 1 H, 1-thiophene H-5), 7.63-7.60 (m, 2 H, 3-phenyl H-2, H-6), 7.42-7.39 (m, 3 H, 3-phenyl H-4, H-3, H-5), 7.41 (d, J=15.7 Hz, 1 H, -CO-CH=C-), 7.37 (dd, J=4.3, 3.0 Hz, 1 H, 1-thiophene H-4). 13C NMR (62.5 MHz, CDCl₃) δ 183.96, 124.12, 143.04, 132.08, 128.94, 128.41, 127.43, 126.51, 122.65.
3-(2-Hydroxyphenyl)-1-(thiophen-3-yl)prop-2-en-1-one (TI-I-174)

The same procedure described at general method was employed with 1 and 2 (R=b) to yield dark green solid 34.9%. Rf (EtOAc : n-Hexane=1:2 v / v): 0.34
Retention time: 16.78 min, [M]+: 231.1

1H NMR (250 MHz, DMSO-d6) δ 10.29 (s, 1 H, 3-phenyl 2-0H), 8.73 (br, 1 H, 1-thiophene H-2), 7.99 (d, J=15.7 Hz, 1 H, -CO-CH=C(CH3)−), 7.85 (dd, J=6.5, 1.2 Hz, 1 H, 3-phenyl H-6), 7.75 (d, J=15.7 Hz, 1 H, -CO-CH=C(CH3)−), 7.68-7.61 (m, 2 H, 1-thiophene H-5, 3-phenyl H-4), 7.26 (td, J=8.37, 1.4 Hz, 1 H, 1-thiophene H-4), 6.93-6.83 (m, 2 H, 3-phenyl H-3, H-5). 13C NMR (62.5 MHz, CDCl3) δ 183.52, 159.98, 143.48, 143.07, 137.13, 134.51, 130.06, 127.74, 127.06, 124.72, 124.46, 121.46, 119.64, 116.48.

3-(3-Hydroxyphenyl)-1-(thiophen-3-yl)prop-2-en-1-one (RK-III-277)

The same procedure described at general method was employed with 1 and 2 (R=c) to yield light yellow solid 61.7%. Rf (EtOAc : n-Hexane=1:2 v / v): 0.47
Retention time: 13.68 min, [M]+: 231.1

1H NMR (250 MHz, CDCl3) δ 8.17 (br, 1 H, 1-thiophene H-2), 7.78 (d, J=15.7 Hz, 1 H, -CO-CH=C(CH3)−), 7.67 (d, J=5.1, 1 Hz, 1 H, 1-thiophene H-5), 7.41-7.20 (m, 4 H, 1-thiophene H-4, -CO-CH=C(CH3)−, 3-phenyl H-4, H-6), 7.13 (s, 1 H, 3-phenyl H-2), 6.91 (d, J=7.6 Hz, 1 H, 3-phenyl H-4). 13C NMR (62.5 MHz, CDCl3) δ 183.22, 157.93, 143.48, 143.07, 137.13, 134.51, 130.06, 127.87, 127.30, 127.13, 120.04, 117.93, 115.44.

3-(4-Hydroxyphenyl)-1-(thiophen-3-yl)prop-2-en-1-one (TI-I-176)

The same procedure described at general method was employed with 1 and 2 (R=d) to yield light yellow solid 31.8%. Rf (EtOAc : n-Hexane=1:2 v / v): 0.54
Retention time: 14.58 min, [M]+: 231.1

1H NMR (250 MHz, DMSO-d6) δ 10.3 (s, 1 H, 3-phenyl 4-0H), 8.71 (br, 1 H, 1-thiophene H-2), 7.69 (d, J=8.5 Hz, 2 H, 3-phenyl H-2, H-6), 7.64-7.61 (m, 4 H, 1-thiophene H-1, H-5, -CO-CH=C(CH3)−, -CO-CH=C(CH3)−), 6.84 (d, J=8.56 Hz, 2 H, 3-phenyl H-3, H-5). 13C NMR (62.5 MHz, DMSO-d6) δ 182.92, 159.98, 143.35, 143.13, 133.37, 130.79, 127.74, 127.06, 125.71, 120.63, 115.71.

Measurement of cell viability

Cell viability was assessed using the CellTiter 96 Aqueous One kit (Promega, Madison, WI, USA). Briefly, RAW 264.7 cells were seeded in 96-well plates at 5×10⁴ cells/well. After overnight incubation, cells were treated with different concentrations of compounds. Then, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution was added and the cells were incubated for 2 h at 37°C. Cell viability was monitored via a SPECTROstar Nano microplate reader (BMG Labtech Inc., Ortenberg, Germany) by measuring absorbance at 490 nm.

Measurement of total Nitric Oxide production

Nitric oxide production was assessed spectrophotometrically as a formed nitrite (NO2). In brief, RAW 264.7 macrophages were seeded at a density of 5×10⁴ cells/well in 96-well plates. Cells were pretreated with indicated concentration of compounds for 1 h followed by treatment with 100 ng/ml LPS for additional 24 h. The 50 % of culture medium was reacted with 50 % of sulphanilamide and 50 % of N-1-naphthylethylenediamine dihydrochloride (NED). Then, the absorbance was measured at 540 nm using a SPECTROstar Nano microplate reader (BMG Labtech Inc., Ortenberg, Germany).

ELISA for TNF-α detection

RAW 264.7 macrophages were seeded at a density of 5×10⁴ cells/well in 96-well plates. Cells were pretreated with indicated concentration of TI-I-174 (10 μM) for 2 h followed by treatment with 100 ng/ml LPS for additional 4 h. The culture media were collected and measured using TNF-α ELISA kits (Biolegend, San Diego, CA, USA), according to the manufacturer’s instructions.

Total RNA isolation, reverse transcription and quantitative PCR (qPCR)

To measure the mRNA levels of the target genes, total RNAs were isolated using Qiagen lysis solution (Qiagen, Hilden, Germany) according to the manufacturer’s instructions and cDNAs were synthesized from 1 μg of total RNA of each sample using the GoScript Reverse Transcription system (Promega). Quantitative Real-time PCR was then performed with LightCycler 1.5 (Roche Diagnostics, Mannheim, Germany) using QPCR SYBR Green Capillary Mix (ABgene, Surrey, UK) at 95°C for 15 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. The primer sequences used for amplification of the target genes are listed in Table 1. The amount of target mRNA was measured via the comparative threshold (Ct) method after normalizing target mRNA C, values to those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, ΔCt) as a housekeeping gene. The sequences of the siRNA are listed in (Table 1).

Transient transfection and luciferase assay

Transcriptional activity was determined using luciferase reporter assay kit (Promega) according to the manufacturer’s instructions. Briefly, cells were initially seeded in 24-well plates at a density of 5×10⁵ cells/well. After overnight culture, the cells were co-transfected with control (pRL-TK) and expression vectors (pGL4/NF-κB or pTL/AP-1) using Fugene HD (Promega). After 6 h incubation, media were replaced with DMEM containing 10% FBS and cells were cultured for ad-

Table 1. Sequences of the primers used for quantitative RT-PCR

| Target gene | Primer | Sequence |
|-------------|--------|----------|
| IL-1β | Forward | 5'-GCCTCGTGCTGTCGGACCATAT-3' |
| Reverse | 5'-TCCTTTAGGCCCACAGCCACA-3' |
| COX-2 | Forward | 5'-GGGTCACGGACGACATT-3' |
| Reverse | 5'-GCACATTGGTGTGGTGGCT-3' |
| IL-6 | Forward | 5'-AACACAGGCGCTCTCTCATT-3' |
| Reverse | 5'-CAGGATCCCGGGAGAATCGT-3' |
| iNOS | Forward | 5'-GCTCGGTGTTGTGCCGACGAC-3' |
| Reverse | 5'-AAAGCAGGCGGACACATGCAA-3' |
| HO-1 | Forward | 5'-CGCATATAACCGCTACT-3' |
| Reverse | 5'-CCAGAGTGTCTACCGGACG-3' |
| GAPDH | Forward | 5'-ACACAGTCCATGCGCATAC-3' |
| Reverse | 5'-TCCACACCCTGTGGTCTGA-3' |
ditional 18 h. Thereafter, the cells were pretreated with indicated concentration of TI-I-174 for 1 h followed by 100 ng/ml LPS for additional 8 h and extracted with 0.1 ml of passive lysis buffer (Promega). Firefly and Renilla luciferase activities were measured by the Dual Luciferase Reporter Assay System (Promega). Statistical analyses for luciferase expression were carried out on the ratios of relative luciferase activity to Renilla luciferase.

**Transient transfection with small interfering RNA (siRNA)**

Cells were initially seeded on 35-mm dishes at a density of 7×10^5 cells/well. After overnight incubation, cells were transfected with scrambled control siRNA or siRNA targeting HO-1 (25 nM) or Nrf2 (50 nM) using Hiperfect transfection reagent (Qiagen) according to the manufacturer’s instructions. Gene silencing efficiency was assessed by qRT-PCR after 30 h of transfection. Murine specific siRNA targeting HO-1 and control siRNA were purchased from Bioneer (Daejeon, South Korea). The sequences of the siRNA used are listed in (Table 2).

**Preparation of cellular extracts and Western blot analysis**

RAW 264.7 macrophages were seeded in 35-mm dishes at a density of 1×10^6 cells per well. After overnight incubation, cells were treated with TI-I-174 and/or LPS as indicated in figure legends. Total proteins were then isolated using RIPA lysis buffer containing Halt Protease and Phosphatase Inhibitor Single-Use Cocktail (Thermo scientific, Rockford) as described previously. For immunoblot analysis, 20 μg of solubilized proteins were loaded by 10% SDS-PAGE. The proteins were then transferred to PVDF membranes, blocked with 5% skim milk in PBS/ Tween20 for 1 h and incubated with the designated primary antibodies overnight at 4°C. Subsequently, the membrane was incubated with secondary HRP-conjugated antibody. The images of the blots were captured using Fujiﬁlm LAS-4000 mini (Fujiﬁlm, Tokyo, Japan). The membranes were then stripped and re-probed with total form of MAPKs or β-actin antibody as the loading control.

**Statistical analysis**

Values were presented as mean ± SEM derived from at least three separate experiments. Data were assessed by one-way analysis of variance (ANOVA) and Tukey’s multiple comparison tests using GraphPad prism software version 5.01 (California, USA). Differences between groups were considered to be significant at \( p < 0.05 \).

**RESULTS**

**Effects of chalcone derivatives on cell viability and LPS-induced nitrite production in RAW 264.7 macrophages**

Previous studies have shown that chalcone derivatives potently suppress production of inflammatory mediators in macrophages (Kontogiorgis et al., 2008). In an effort to develop and optimize agent for treatment of inflammation-associated diseases, we prepared series of chalcone derivatives, RK-I-105, TI-I-174, RK-III-277 and TI-I-176, containing hydroxyl groups at different positions (none, ortho, meta, or para) of the phenyl ring, respectively (Fig. 1). Before investigating the inhibitory effects of these compounds on the production of inflammatory mediators, we first examined if these compounds affect cell viability. As shown in Fig. 2, RK-I-105 and TI-I-174 did not generate any toxic effects up to 10 μM, while treatment with RK-III-277 and TL-I-176 caused significant cytotoxicity at 10 μM. These compounds were not further included for con-

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**Table 2. Sequences of small interfering RNA used in transfection**

| Target gene | Primer | Nucleotide sequence |
|-------------|--------|---------------------|
| HO-1        | Forward| 5'-CAGAUCAGCACUACUGCAU-3' |
|             | Reverse| 5'-AUGACGUAGCGUAGUCUG-3' |
| Nrf2        | Forward| 5'-GCAUUAGUACAGCAGCAGA-3' |
|             | Reverse| 5'-UCUGUCCGUGCACUAAAGUC-3' |
| Scrambled   | Forward| 5'-UGGUUUACAUGUCGCUA-3' |
|            |        | 5'-UGGUUUACAUGUUGUGUGA-3' |
|            |        | 5'-UGGUUUACAUGUUUUCUGA-3' |
|            |        | 5'-UGGUUUACAUGUUUUCUGA-3' |
|            |        | 5'-UGGUUUACAUGUUUUCUGA-3' |

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**Fig. 1.** Scheme for the preparation and chemical structures of the chalcone derivatives. (A) General synthetic scheme of phenyl/hydroxyphenyl-3-thienylpropenones. Reagents and conditions: (i) aryl aldehyde 2a-d (1.0 eq.), KOH (1.2 eq.), MeOH/H2O (5:1), 10 min to 3 h, 0°C, 31.8-61.7%. (B) Chemical structures of the chalcone derivatives.
continuing study. For the investigation of the anti-inflammatory effects, we first examined the effects on LPS-stimulated nitric oxide (NO) production in RAW 264.7 macrophages. Pretreatment with TI-I-174 exhibited more potent inhibitory effect with the IC50 value of 5.75 μM roughly almost two-fold more potent than that of RK-I-105 (IC50 value of 9.63 μM) (Fig. 3). Based on these preliminary findings we focused subsequent efforts on TI-I-174.

**Effects of TI-I-174 on production of other inflammatory mediators in RAW 264.7 macrophages**

To further examine the anti-inflammatory properties of TI-I-174, we examined the effects of TI-I-174 on the expressions of other inflammatory mediators. As indicated in Fig. 4, treatment with TI-I-174 caused significant decrease in IL-1β expression (Fig. 4A). However, it did not generate significant effect on LPS-stimulated TNF-α secretion, IL-6 and COX-2 expression as determined by ELISA and quantitative RT-PCR, respectively (Fig. 4B-4D), implying that TI-I-174 regulates production of inflammatory mediators in a selective manner.

**Effect of TI-I-174 on LPS-induced iNOS expression in RAW 264.7 macrophages**

In continuing studies, we examined the molecular mechanisms underlying suppression of NO production. Nitric oxide is produced during the conversion of arginine into citrulline by nitric oxide synthase (NOS). Among the three isoforms of NOS, inducible form of NOS (iNOS) is responsible for NO production in response to LPS treatment. We therefore further examined the effect of TI-I-174 on iNOS expression. To elucidate the mechanisms underlying suppression of LPS-induced NO production, we investigate the effect of TI-I-174 on LPS-induced iNOS expression. As shown in Fig. 5A, TI-I-174 treatment significantly attenuated LPS-induced iNOS protein expression in a dose-dependent manner. Quantitative RT-PCR (qRT-PCR) analysis further indicated that LPS-induced increase in iNOS mRNA level was significantly inhibited by treatment with TI-I-174 (Fig. 5B) as expected, indicating that TI-I-174 suppresses NO production through inhibition of iNOS expression following LPS stimulation.

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**Fig. 2.** Effects of chalcone derivatives on viability of RAW 264.7 macrophages. Cells were treated with the indicated concentrations of compounds for 24 h. Cell viability was measured by MTS assay as described in material and methods. Data represent the mean ± SEM (n=3).

**Fig. 3.** Effect of TI-I-174 on LPS-induced nitrite production in RAW 264.7 macrophages. Cells were pretreated with the indicated concentrations of TI-I-174 for 1 h followed by stimulation of LPS (100 ng/ml) for additional 24 h. Values are presented as percentage (%) compared to the cells treated with LPS and are expressed as mean ± SEM (n=3). *p<0.05 compared with the corresponding control values (2nd column group).

**Fig. 4.** Effects of TI-I-174 on production of pro-inflammatory mediators in RAW 264.7 macrophages. (A) and (B) Cells were pretreated with TI-I-174 for 2 h followed by treatment of 100 ng/ml LPS additional for 6 h. IL-1β (A) and COX-2 (B) mRNA level was measured by qRT-PCR and the expression of target mRNA was normalized to GAPDH mRNA as described previously. Values are presented as mean ± SEM (n=3-4). *p<0.05 compared with control cells. (C) Cells were treated with TI-I-174 for 2 h followed by stimulation of 100 ng/ml LPS additional for 6 h. IL-6 mRNA level was normalized to GAPDH mRNA. Values are presented as mean ± SEM (n=3). *p<0.05 compared with control; *p<0.05 compared with cells treated with LPS. (D) Cells were pretreated with TI-I-174 for 2 h followed by LPS (100 ng/ml) additional for 4 h, and the production of TNF-α protein accumulated in the media was measured by ELISA. Values are presented as mean ± SEM (n=3). *p<0.05 compared with control.
Fig. 5. Effect of TI-I-174 on LPS-induced iNOS expression in RAW 264.7 macrophages. (A) Cells were pretreated with TI-I-174 for 2 h followed by incubation with 100 ng/ml LPS for additional 24 h. Expression of iNOS protein level was measured by Western blot analysis as described previously. Representative image from three independent experiments that showed similar results is shown along with β-actin for internal loading control. (B) Cells were treated with TI-I-174 for 2 h followed by stimulation of 100 ng/ml LPS for additional 6 h. iNOS mRNA level was normalized to GAPDH mRNA. Values are presented as mean ± SEM (n=3). *p<0.05 compared with control; #p<0.05 compared with cells treated with LPS.

Effect of TI-I-174 on transcriptional activity of AP-1 and NF-κB in LPS-treated RAW 264.7 macrophages

Gene expression of iNOS is regulated by various transcription factors. In particular, AP-1 and NF-κB have shown to play a critical role in LPS-induced iNOS expression in macrophages. To further elucidate the mechanisms underlying down-regulation of iNOS expression, we therefore examined the effect of TI-I-174 on transcriptional activity of AP-1 and NF-κB using reporter gene assay. RAW 264.7 macrophages were transfected with the pAP-1-Luc plasmid (containing direct repeats of the AP-1 recognition sequences) or with the pNF-κB-Luc plasmid (containing repeats of NF-κB recognition sequences), and then stimulated with LPS in the absence or presence of TI-I-174. Reporter gene assay revealed that TI-I-174 treatment significantly inhibited transcriptional activation of AP-1 following LPS stimulation (Fig. 6A). However, TI-I-174 had no significant effect on the transcriptional activation of NF-κB by LPS stimulation (Fig. 6B), indicating that TI-I-174 regulates iNOS gene expression probably by inhibiting the activation of AP-1, but not NF-κB.

Effects of TI-I-174 on LPS-induced activation of MAPKs in RAW 264.7 macrophages.

Mitogen-activated protein kinases (MAPKs) signaling plays a key role in the activation of AP-1. To further identify the upstream signaling molecule involved in the modulation of transcriptional activity of AP-1 by TI-I-174, we further examined the effect of TI-I-174 on LPS-induced activation of MAPKs. As shown in Fig. 7, TI-I-174 significantly reduced phosphorylated level of JNK, while it did not significantly affect activation of p38MAPK and ERK1/2, suggesting that JNK would be an upstream signaling molecule to modulate activity of AP-1.

Role of HO-1 in TI-I-174-mediated suppression of NO production in LPS-stimulated RAW264.7 macrophages

HO-1 is widely known to modulate inflammatory response in various experimental conditions. We next examined whether HO-1 induction is involved in suppression of NO production by TI-I-174. To test this hypothesis, we first examined if TI-I-174 induced HO-1 expression. As shown in Fig. 8, treatment of RAW 264.7 macrophages with TI-I-174 led to a marked increase in HO-1 mRNA expression in a dose (Fig. 8A) and time dependent manner (Fig. 8B), showing a maximal increase after 3 h treatment with TI-I-174 and returned to the baseline level after 24 h of treatment (Fig. 8B). TI-I-174 treatment also caused significant increase in HO-1 protein expression level in a dose (Fig. 8C) and time dependent manner (Fig. 8D). TI-I-174 treatment also transiently increased HO-1 protein expression. It caused a maximal increase at 8 h treatment and returned to the almost normal level at 24 h, showing a pattern similar to that of mRNA expression.

Nuclear factor-erythroid 2 (Nrf2) is well known as a key transcription factor responsible for various anti-oxidative proteins, including HO-1 (Na and Surh, 2014). To further elucidate
the mechanisms underlying HO-1 expression by TI-I-174, we examined whether Nrf2 signaling plays a role in the induction of HO-1 by TI-I-174. For this, we investigated the effect of TI-I-174 on Nrf2 expression. As shown in Fig. 8E, treatment with TI-I-174 rapidly increased Nrf2 protein expression.

Finally, to verify that HO-1 induction is involved in TI-I-174-mediated suppression of nitrite production, we investigated whether gene silencing of HO-1 is sufficient to rescue nitrite production. As shown in Fig. 8F, transfection with siRNA targeting HO-1 efficiently suppressed HO-1 expression (Upper panel). In addition, inhibitory effect of TI-I-174 on LPS-induced nitrite production was partially restored by transfection with siRNA targeting HO-1 (compare 3rd and 4th column), while scramble siRNA transfection did not significantly affect LPS-induced nitrite production (5th column), suggesting that suppression of LPS-induced nitrite production by TI-I-174 is mediated, at least in part, by HO-1 induction.

**DISCUSSION**

Nitric oxide (NO), a gaseous cellular signaling molecule, plays an important role in a number of normal physiological processes, such as vasodilation and neurotransmission. However, NO is also considered as a pro-inflammatory mediator and overproduction of NO is involved in the pathogenesis of inflammatory disorders in many tissues (Ruan, 2002). Therefore, development of pharmacological agent inhibiting NO production would be a promising strategy for the management of inflammatory diseases. Chalcones, a group of phenolic compounds, exhibited potent anti-oxidant and anti-inflammatory properties in diverse in vitro and in vivo conditions (Berger et al., 2006; Kontogiorgis et al., 2008). In the present study, we prepared synthetic chalcone derivatives in an attempt to develop optimal anti-inflammatory agents. Among these chalcone derivatives, TI-I-174 was highly effective at...
inhibiting NO production in macrophages stimulated with LPS, and we further demonstrated that TI-I-174 mediated its effects by inducing HO-1 expression and blockade of AP-1 activation.

Chalcone (1,3-diaryl-2-propan-1-ones) has been shown to exhibit a variety of pharmacological activities. In addition to pharmacological responses by natural chalcone, its structural modifications on propenone moiety by introduction and/or substitution of aromatic rings exhibited a wide range of pharmacological activities, including anti-oxidant and anti-inflammatory activities. In this study, we incorporated the hydroxyl group on phenyl moiety in phenyl-3-thienylpropenones at different positions (none, ortho, meta, or para) (Fig. 1). We demonstrated that introduction of hydroxyl group at ortho position produced more potent suppressive effect on nitrite production, whereas introducing at meta and para positions are toxic to the macrophages (Fig. 2). It seems ortho-hydroxy would a beneficial moiety to suppress NO production without producing cytotoxic effects in macrophages. Although it is not sufficient to demonstrate structure activity relationship (SAR) at this stage, it would be interesting to further investigate SAR of the chalcone derivatives on suppressing NO production for future study.

Chalcones are known as potent inducers of phase II detoxifying enzymes, and a number of previous studies suggest that beneficial actions of chalcones are derived from induction of endogenous cytoprotective pathways, such as HO-1 (Foresti et al., 2005; Abuarqoub et al., 2009). In this present study, we have demonstrated that TI-I-174 mediated its effects by inducing HO-1 expression and blockade of AP-1 activation (Fig. 8A), while it did not affect transcriptional activity of NF-κB (Fig. 6B). While it is widely accepted that HO-1 induction suppresses NF-κB signaling, modulation of NF-κB activity by HO-1 signaling would be depending on experimental conditions. It has been shown that HO-1 induction or CO production negatively regulates NF-κB signaling in macrophages (Ashino et al., 2008). However, many other studies have also reported that HO-1 induction does not affect NF-κB activity, even if it suppresses inflammatory response, indicating that HO-1 can inhibit inflammatory response through the mechanisms independent of NF-κB regulation. For example, it has been shown that a synthetic chalcone compound induces HO-1 expression and suppresses NO production, but HO-1 induction does not mediate suppression of NF-κB signaling (Park et al., 2009). In addition, HO-1 induction by a synthetic chalcone derivative (3', 4', 5', 3, 4, 5-hexamethoxy-chalcone, CH) did not mediate inhibition of iκB-α phosphorylation and IKK activity (Alcaraz et al., 2004). Furthermore, HO-1 induction by statins further significantly increased phosphorylation of iκB-α and translocation of p65 into the nucleus in the presence of LPS (Hsieh et al., 2008). These reports further indicate that HO-1 induction modulates NF-κB signaling in a context-dependent manner. At this stage, even though we cannot identify the detailed mechanisms underlying no significant effect on NF-κB transcriptional activity by HO-1 induction, data demonstrated in the present study imply that AP-1, rather than NF-κB, would be the key target by which TI-I-174 inhibits NO production.

In the present study, we demonstrated that TI-I-174 treatment caused increase in HO-1 expression and blockade of AP-1 activation. Since previous studies have also shown that HO-1 induction negatively regulates AP-1 activation (Dijkstra et al., 2004; Sasaki et al., 2006; Yasui et al., 2007), we further investigated whether HO-1 induction is required for blockade of AP-1 activation by TI-I-174. However, gene silencing of HO-1 by siRNA transfection did not restore TI-I-174-mediated suppression of AP-1 activation and iNOS mRNA accumulation (data not shown). These results are not supportive to the notion that HO-1 induction inhibits AP-1 activation and iNOS transcription, but in accordance with the previous observations showing that CO, a by-product of heme catabolism by HO-1, inhibited LPS-stimulated nitrite production without affecting iNOS expression (Sawle et al., 2005) and HO-1 induction by a synthetic chalcone derivative (3', 4', 5', 3, 4, 5-hexamethoxy-chalcone, CH) did not mediate suppression of transcriptional activity by CH (Alcaraz et al., 2004). Taken together, data presented here imply that TI-I-174 suppress NO production via two individual pathways (HO-1 induction and AP-1 inhibition), and also suggest that inhibition of NO production by TI-I-174 is a product of multiple pathways acting in concert.

In conclusion, we demonstrated that TI-I-174, a synthetic chalcone derivative, potently suppressed LPS-stimulated NO production in macrophages. This effect was mediated via induction of HO-1 and inhibition of AP-1 activation. Based on these findings, we propose that TI-I-174 would be a promising agent for the treatment of diseases associated with inflamma-
tion. Further studies are now required to validate the therapeutic effects of TI-I-174 in an in vivo model.

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