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Inhibitory Effect of 3-(4-Hydroxyphenyl)-1-(thiophen-2-yl) prop-2-en-1-one, a Chalcone Derivative on MCP-1 Expression in Macrophages via Inhibition of ROS and Akt Signaling

Mi Jin Kim†, Taraman Kadayat†, Yeon Ji Um, Tae Cheon Jeong*, Eung-Seok Lee* and Pil-Hoon Park*

College of Pharmacy, Yeungnam University, Gyeongsan 712-749, Republic of Korea

Abstract

Chalcones (1,3-diaryl-2-propen-1-ones), a subfamily of flavonoid, are widely known to possess potent anti-inflammatory and anti-oxidant properties. In this study, we investigated the effect of 3-(4-Hydroxyphenyl)-1-(thio3-(4-Hydroxyphenyl phen-2-yl)prop-2-en-1-one (TI-I-175), a synthetic chalcone derivative, on endotoxin-induced expression of monocyte chemoattractant protein-1 (MCP-1), one of the key chemokines that regulates migration and infiltration of immune cells, and its potential mechanisms. TI-I-175 potently inhibited MCP-1 mRNA expression stimulated by lipopolysaccharide (LPS) in RAW 264.7 macrophages without significant effect on cell viability. Treatment of cells with TI-I-175 markedly prevented LPS-induced transcriptional activation of activator protein-1 (AP-1) as measured by luciferase reporter assay, while nuclear factor-κB (NF-κB) activity was not inhibited by TI-I-175, implying that TI-I-175 suppressed MCP-1 expression probably via regulation of AP-1. In addition, TI-I-175 treatment significantly inhibited LPS-induced Akt phosphorylation and led to a significant decrease in reactive oxygen species (ROS) production by LPS, which act as up-stream signaling events required for AP-1 activation in RAW 264.7 macrophages. Taken together, these results indicate that TI-I-175 suppresses MCP-1 gene expression in LPS-stimulated RAW 264.7 macrophages via suppression of ROS production and Akt activation.

Key Words: Chalcone, MCP-1, Reactive oxygen species, Inflammation, Lipopolysaccharide

INTRODUCTION

Inflammation is a complicated biological response in response to various harmful stimuli. While inflammation is initiated as a part of human body's immune response, excess and chronic inflammation is closely associated with development and progression of various pathophysiological conditions (Bedard and Krause, 2007). Inflammatory response is regulated by coordinated action of a number of pro- and anti-inflammatory mediators. Although inflammation is involved in the pathogenesis of diverse diseases, the mechanisms underlying and the proper clinical way for the management are still limited.

Monocyte chemoattractant protein-1 (MCP-1), also known as chemokine (C-C motif) ligand 2 (CCL2), is a member of the CC chemokine superfamily and plays an important role as an inflammatory chemokine that recruits a number of immune cells, including monocytes, T lymphocytes and dendritic cells, to the site of inflammation (Deshmane *et al*., 2009). Therefore, MCP-1 production is closely related with development of diseases associated with inflammation, including atherosclerosis, alcoholic liver injury and cancer (Deshmane *et al*., 2009; Mandrekar *et al*., 2011), while deficiency of MCP-1 gene caused decrease in recruitment of monocyte/macrophages resulting in diminishment of atherosclerosis (Gu *et al*., 1998). Based on previous reports, MCP-1 has been considered as a promising target for the treatment of chronic inflammation-associated diseases. Previous studies have revealed the signaling mechanisms implicated in MCP-1 gene expression

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***Corresponding Author**
E-mail: tcjeong@yu.ac.kr (Jeong TC), eslee@yu.ac.kr (Lee ES), parkp@yu.ac.kr (Park PH)
Tel: +82-53-810-2819 (Jeong TC), +82-53-810-2827 (Lee ES), +82-53-810-2826 (Park PH)
Fax: +82-53-810-4654 (Jeong TC), +82-53-810-4654 (Lee ES), +82-53-810-4654 (Park PH)
†The first two authors contributed equally to this work.

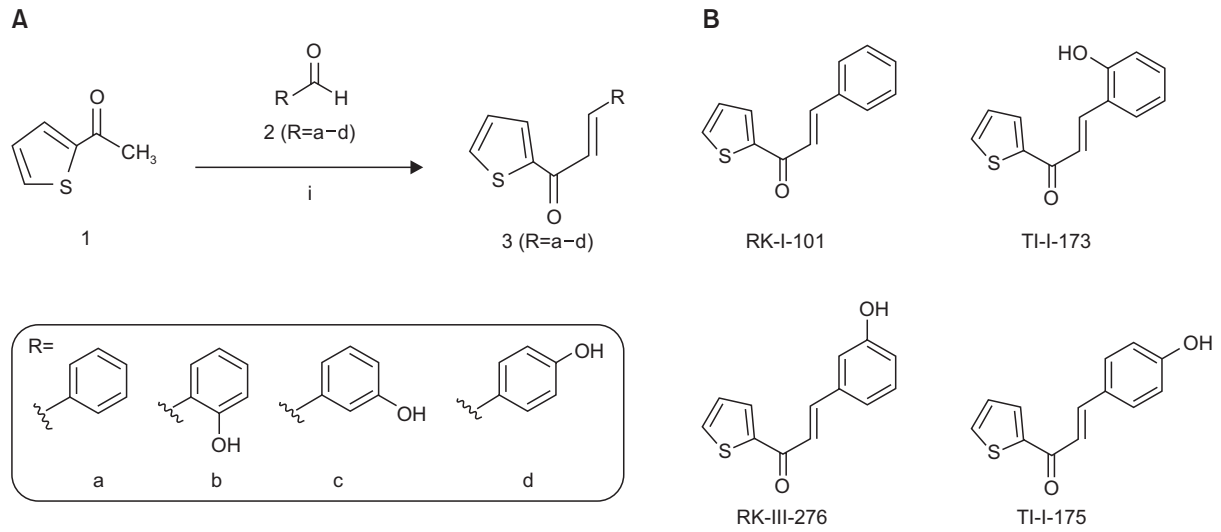

Fig. 1. Scheme for the preparation and chemical structures of the chalcone derivatives. (A) General synthetic scheme of phenyl/hydoxylphenyl-2-thienylpropenones. Reagents and conditions: (i) aryl aldehyde 2a-d (1.0 eq.)., KOH (1.2 eq.), MeOH / H2O (5:1), 10 min to 3 h, 0°C, 29.4-76.3%. (B) Chemical structures of the chalcone derivatives.

in response to inflammatory stimuli. ROS production derived from nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) initiates MCP-1 expression in endothelial cells (Chen *et al*., 2004) and NF-κB and AP-1 activation in response to ROS production has been directly shown to induce MCP-1 expression in macrophages and epithelial cells (Wang *et al*., 2000; Remppis *et al*., 2010). In addition, phosphatidylinositol 3-kinase (PI3K)/Akt signaling plays a crucial role in cytokine-induced MCP-1 gene expression via AP-1 activation (Bian *et al*., 2004; Park *et al*., 2009).

In response to inflammatory stimuli, macrophages release various pro-inflammatory molecules. In initial state, macrophages increase reactive oxygen species (ROS) production and and it is widely accepted that excessive ROS production is one of the key cellular events leading to the inflammatory process. In addition, ROS production is involved in production of various pro-inflammatory mediators, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and MCP-1 (Chen *et al*., 2004; Park *et al*., 2009; Peluso *et al*., 2010). Therefore, suppression of ROS production has been considered as a promising strategy for the prevention and/or treatment of diseases associated with chronic inflammatory conditions.

Chalcones (1,3-diaryl-2-propen-1-ones), a group of polyphenolic and flavonoid family, have been shown to possess diverse pharmacological activities. In addition to the original observation demonstrating the potent inhibitory effect on topoisomerase and subsequent on anti-cancer effects (Iwashita *et al*., 2000; Chowdhury *et al*., 2002), chalcone and its various derivatives have exhibited diverse biological properties, including anti-bacterial (Avila *et al*., 2008), anti-fungal (Batovska *et al*., 2007), anti-oxidant (Dinkova-Kostova *et al*., 2001), anti-tumorigenic (Zhang *et al*., 2013) and anti-inflammatory properties (Kim *et al*., 2014). In particular, with regards to anti-inflammatory effects, recent studies have shown that chalcones modulate activities of the transcriptional factors associated with inflammation, such as AP-1 and NF-κB (Ban *et al*., 2004; Yadav *et al*., 2011). Furthermore, anti-inflammatory activities of chalcones are linked with suppression of pro-inflammatory mediators such as cyclooxygenase-2 (COX-2), TNF-α and nitric oxide (NO) (Wu *et al*., 2011; Kim *et al*., 2014). Although MCP-1 plays an important role in the activation of inflammatory responses and is considered as a promising target for the modulation of inflammatory responses, effect of chalcones on the expression of MCP-1 and its potential mechanisms are not clearly understood. In the present study, as part of our continuing efforts to develop optimal anti-inflammatory and antioxidant agents, we prepared synthetic chalcone derivatives and investigated its inhibitory effect on MCP-1 expression in macrophages and further its potential mechanisms.

MATERIALS AND METHODS

Materials

All the cell culture reagents were obtained from Hyclone Laboratories (South Logan, UT, USA). Lipopolysaccharide (LPS), LY294002 and N-acetyl-L-cysteine (N-AC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Luciferase assay and MTS assay kits were procured from Promega (Madison, WI, USA). 5-Chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) was obtained from Molecular Probes (Eugene, OR, USA). Diphenyleneiodonium chloride (DPI) was obtained from Enzo Life Sciences (Farmingdale, NY, USA). Antibodies against phosphorylated and total forms of Akt were purchased 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% CO_2.

Chemical compounds

The synthetic chalcone compounds used for this study were provided by Prof. Eung-Seok Lee (Yeungnam University, S. Korea). The structures of the compounds and general synthetic scheme are shown in Fig. 1.

Measurement of cell viability

Cell viability was measured using the CellTiter 96 Aqueous One kit (Promega, Madison, WI, USA). Briefly, RAW 264.7 cells were seeded in 96-well plates at 5×10^4 cells/well. Cells were treated with indicated concentrations of compounds. After 24 h incubation, cells were further incubated with MTS solution (20 μl) for additional 2 h. Finally, cell viability was monitored using SPECTROstar Nano microplate reader (BMG Labtech Inc., Ortenberg, Germany) by measuring absorbance at 490 nm.

Measurement of Reactive oxygen species (ROS) production

Intracellular Reactive oxygen species (ROS) production was assessed by measurement of the changes in the fluorescence of 5-chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA). In brief, RAW 264.7 macrophages were seeded at a density of 5×10^4 cells/well in 96-well black plates. After overnight culture, cells were pretreated with compounds for 1 h and further stimulated with LPS for additional 24 h. Cells were then treated with 10 μM of CM-H_2DCFDA in dark for 30 min and washed with HBSS to remove excess dye. Finally, intracellular ROS production was determined using FLUOstar OPTIMA fluoremeter (BMG Labtech, Ortenberg, Germany) and analyzed by FLUOstar OPTIMA software from BMG. Excitation and emission wavelengths were set to 485 nm and 520 nm, respectively.

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. 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). The primer sequences used for amplification of the target genes are listed in Table 1.

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

Target gene	Primer	Sequence
MCP-1	Forward	5'-CCACTCACCTGCTGCTACTCAT-3'
	Reverse	5'-TGGTGATCCTCTTGTAGCTCTCC-3'
COX-2	Forward	5'-GGGCTCAGCCAGGCAGCAAT-3'
	Reverse	5'-GCACTGTGTTTGGGGTGGGCT-3'
IL-6	Forward	5'-ACAACCACGGCCTTCCCTACTT-3'
	Reverse	5'-CACGATTTCCCAGAGAACATGTG-3'
IL-8	Forward	5'-TTGCCTTGACCCTGAAGCCCCC-3'
	Reverse	5'-GGCACATCAGGTACGATCCAGGC-3'
GAPDH	Forward	5'-ACCACAGTCCATGCCATCAC-3'
	Reverse	5'-TCCACCACCCTGTTGCTGTA-3'

Transient transfection and luciferase assay

Transcriptional activities of AP-1 and NF-κB were 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 5×10^5 cells/well and were co-transfected with control (pRL-TK) and expression vectors (pGL4/NF-κB or pTL/AP-1) using Fugene HD (Promega). Cells were then treated with indicated concentration of TI-I-175 for 2 h followed by 100 ng/ml of LPS for additional 6 h and extracted with passive lysis buffer (promega). *Firefly* and *Renilla* luciferase activities were measured by the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Statistical analyses for luciferase expression were carried out on the ratios of relative luciferase activity to *Renilla* luciferase.

Preparation of cellular extracts and Western blot analysis

Raw264.7 macrophages were seeded in 35 mm dishes at a density of 1×10^6 cells/well. After overnight incubation, cells were pretreated with TI-I-175 and/or LPS as indicated in figure legend. Total proteins were extracted using RIPA lysis buffer containing Halt Protease and Phosphatase Inhibitor Single-Use Cocktail (Thermo scientific, Rockford, IL, USA) as described previously (Kim *et al*., 2014). For immunoblot analysis, 30 μg of solubilized proteins were separated in 10% SDS-PAGE and transferred to PVDF membranes. The membrane was incubated with the designated primary antibodies overnight at 4in. Subsequently, the membrane was incubated with secondary antibody conjugated with horse radish peroxidase (HRP). 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 Akt or β-actin antibody as a loading control.

Statistical analysis

Values were expressed 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 synthetic chalcone derivatives on cell viability and LPS-induced MCP-1 mRNA expression in RAW 264.7 macrophages

Chalcones have been shown to potently suppress production of inflammatory mediators in macrophages. In an effort to develop optimize agent for treatment of inflammation-associated diseases, we prepared series of chalcone derivatives, RK-I-101, TI-I-173, RK-III-276 and TI-I-175, containing hydroxyl groups at different positions of the phenyl ring (none, ortho, meta, or para), respectively (Fig. 1B) and examined inhibitory effects on production of inflammatory mediators. For this, we first examined if these synthetic chalcone derivatives affect cell viability. As shown in Fig. 2, TI-I-173 and TI-I-175 did not produce any cytotoxic effects up to 10 μM, while treatment with RK-I-101 and RK-III-276 generated significant cytotoxicity at 10 μM (Fig. 2). Therefore, these two compounds were not included for the continuing studies. In addition, we have

previously shown that *para*-hydroxyphenyl moiety exhibits the most effective structure activity for anti-inflammatory responses, including suppression of nitric oxide (NO) and ROS production (Kadayat *et al*., 2014). Thus, among the compounds prepared for this study, we decided to focus on TI-I-175 for continuing study.

To examine the anti-inflammatory properties of TI-I-175, we investigated the effects of TI-I-175 on the mRNA expression of MCP-1, which is responsible for recruitment of immune cells to the site of inflammation, using quantitative real time PCR (qRT-PCR). As indicated in Fig. 3A, treatment with TI-I-175 generated significant decrease in LPS-induced MCP-1 mRNA expression. However, treatment of cells with TI-I-175 only slightly decreased expression of IL-8, a CC chemokine (Fig. 3B), and it did not cause any significant effect on LPS-induced COX-2 and IL-6 expression (Fig. 3C, D), implying that TI-I-175 regulates production of inflammatory mediators in a selective manner.

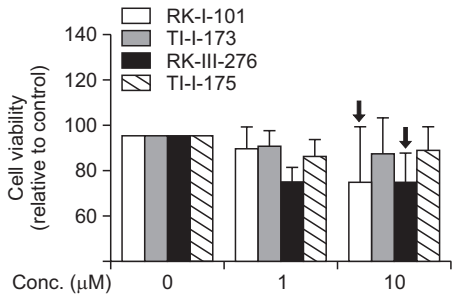

Fig. 2. Effects of chalcone derivatives on cell viability of RAW 264.7 macrophages. Cells were treated with the indicated concentrations of compounds for 24 h. Cell viability was assessed by MTS assay as described in material and methods. Values represent fold change compared with the control cells. Data are represented as mean ± SEM (n=3).

Effects of TI-I-175 on transcriptional activity of AP-1 and NF-κB in LPS-treated RAW 264.7 macrophages

MCP-1 gene contains biding sites for activator protein (AP-1), nuclear factor-κB (NF-κB) and sequence-specific protein 1 (Sp1) transcription factor (Wang *et al*., 1997). It has been reported that mutation of binding site of AP-1 or NF-κB in the promoter region of MCP-1 suppressed cytokine-induced MCP-1 expression (Martin *et al*., 1997), indicating a critical role of AP-1 and NF-κB in MCP-1 expression. To identify transcription factor(s) targeted by TI-I-175 for suppression of MCP-1 expression in RAW 264.7 macrophages, we investigated the effects of TI-I-175 on transcriptional activity of AP-1 and NF-κB via reporter gene assay. For this, cells 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-175. As depicted in Fig. 4, TI-I-175 treatment caused significant suppression of LPS-induced increase in transcriptional activity of AP-1 (Fig. 4A). In contrast, LPS-induced activation

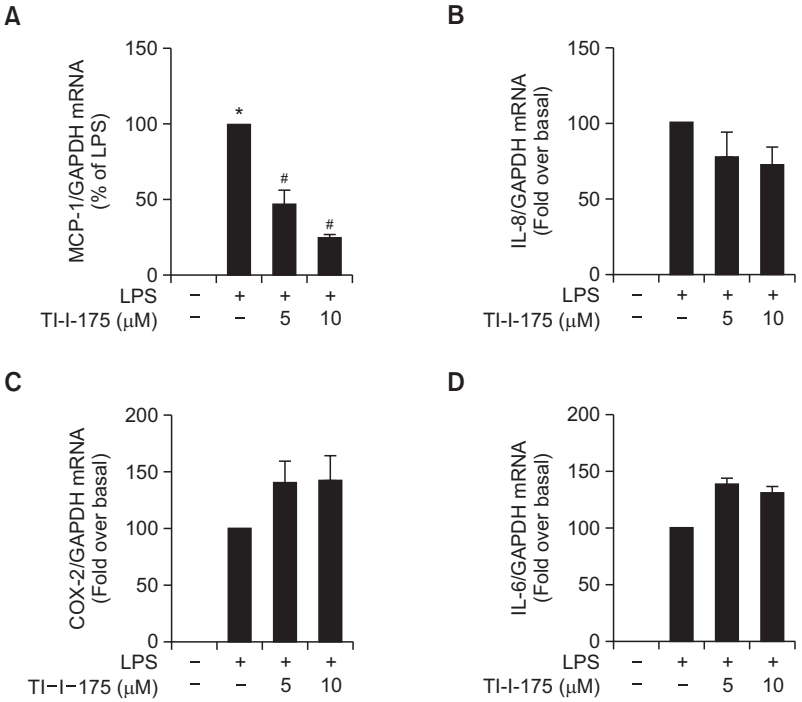

Fig. 3. Effect of TI-I-175 on MCP-1 expression in RAW 264.7 macrophages. (A) Cells were pretreated with the indicated concentrations of TI-I-175 for 2 h prior to stimulation of LPS (100 ng/ml) for 6 h. MCP-1 mRNA level was normalized to GAPDH mRNA. Values are presented as percentage (%) compared to the cells stimulated with LPS and are expressed as mean ± SEM (n=3). **p*<0.05 compared with control group;. #*p*<0.05 compared with cells treated with only LPS. (B-D) Cells were pretreated with TI-I-175 for 2 h followed by treatment with LPS (100 ng/ml) for additional for 6 h. Messenger RNA levels of IL-8 (B), COX-2 (C) and IL-6 (D) were assessed by qRT-PCR and the expression of target mRNA was normalized to GAPDH mRNA level as described previously. Values are presented as mean ± SEM (n=3).

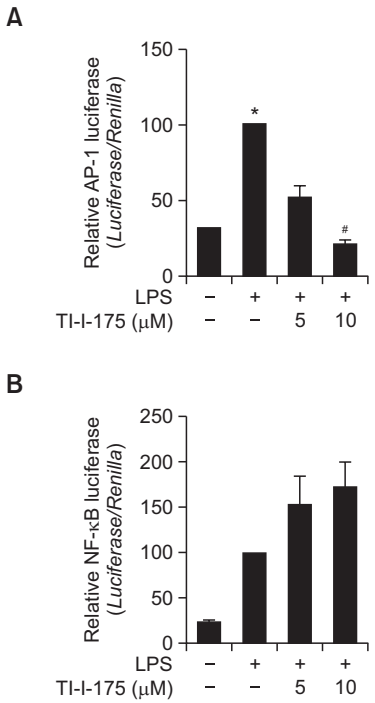

Fig. 4. Effects of TI-I-175 on transcriptional activation of AP-1 and NF-κB in RAW 264.7 macrophages. (A) Cells were transiently co-transfected with pAP1-*Luc* plasmid and *Renilla* reporter gene. After 24 h of culture, cells were pretreated with indicated concentration of TI-I-175 for 2 h followed by incubation with LPS (100 ng/ml) for additional 6 h. AP-1 dependent reporter gene expression was determined as described previously. Values are presented as percentage (%) compared to the cells stimulated with LPS and are expressed as mean ± SEM (n=3). **p*<0.05 compared with control cells; #*p*<0.05 compared with cells treated with LPS. (B) Cells were co-transfected with pGL4/NF-κB plasmid and *Renilla* reporter gene. After 24 h incubation, cells were pretreated with TI-I-175 for 2 h followed by stimulation with 100 ng/ml LPS for additional 6 h. Transcriptional activity of NF-κB was measured as described previously. Values are represented as percentage (%) compared to the cells stimulated with LPS and are expressed as mean ± SEM (n=3).

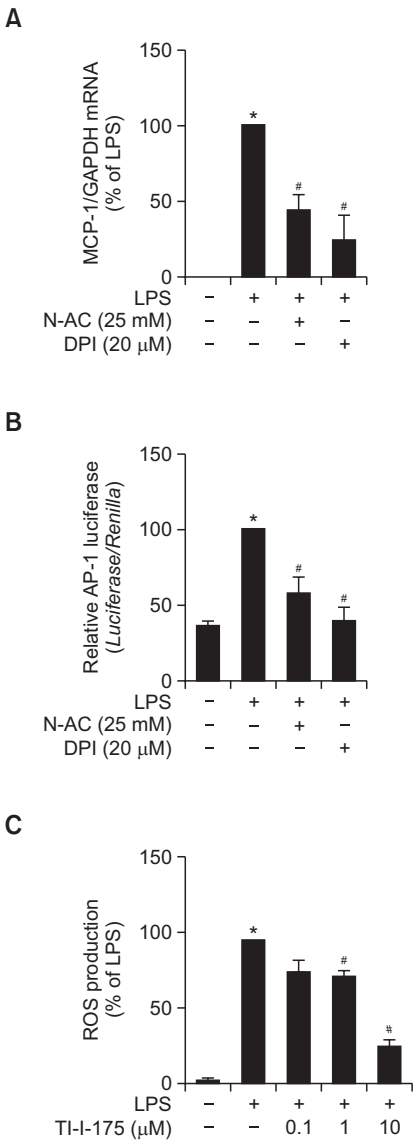

Fig. 5. ROS modulation is involved in the suppression of LPS-induced MCP-1 expression by TI-I-175 in RAW 264.7 macrophages. (A) Cells were pretreated for 2 h in the absence or presence of ROS inhibitors (N-AC (25 mM) and DPI (20 μM)) and then incubated with 100 ng/ml of LPS for 6 h. MCP-1 mRNA level was measured by qRT-PCR and the expression of target gene was normalized to GAPDH mRNA as described previously. Values are expressed as mean ± SEM (n=3). **p*<0.05 compared with control cells; #*p*<0.05 compared with cells treated with LPS. (B) Cells were treated with ROS inhibitor and LPS same as above and transcriptional activity of AP-1 was assessed by luciferase reporter assay as described previously. Values are presented as percentage (%) compared to the cells stimulated with LPS and are expressed as mean ± SEM (n=5). **p*<0.05 compared with control cells; #*p*<0.05 compared with cells treated with LPS. (C) Cells were pretreated with TI-I-175 for 2 h followed by stimulation with 100 ng/ml LPS for additional 24 h. The cells were then were stained with 10 μM CM-H_2DCFDA for 30 min in the dark conditions. ROS production was assessed by conversion of CM-H_2DCFDA to the fluorescent DCF, which was detected using fluorometer. Data are expressed as percentage (%) compared to the cells stimulated with LPS and are expressed as mean ± SEM (n=3). **p*<0.05 compared with the cells not treated with LPS; #*p*<0.05 compared with cells treated with LPS.

of NF-κB was not inhibited by treatment with TI-I-175 (Fig. 4B), suggesting that suppression of MCP-1 gene expression by TI-I-175 would be mediated via inhibition of AP-1 activation, rather than NF-κB.

ROS modulation is involved in the suppression of LPS-induced MCP-1 expression by TI-I-175 in RAW 264.7 macrophages

Excessive ROS production is well-known to induce expression of a number of inflammatory genes in various experimental conditions. We next examined if TI-I-175 suppressed MCP-1 gene expression via modulation of ROS production. To test this, we first investigated whether ROS production is involved in LPS-induced MCP-1 expression. As shown in Fig. 5A, pretreatment with anti-oxidants, N-AC (ROS scavenger) and DPI (inhibitor of NADPH oxidase), significantly prevented LPS-induced MCP-1 mRNA expression. Pretreatment with ROS inhibitors also caused significant suppression in AP-1 activity stimulated with LPS (Fig. 5B), indicating that ROS generation plays an important role in LPS-induced MCP-1 expression

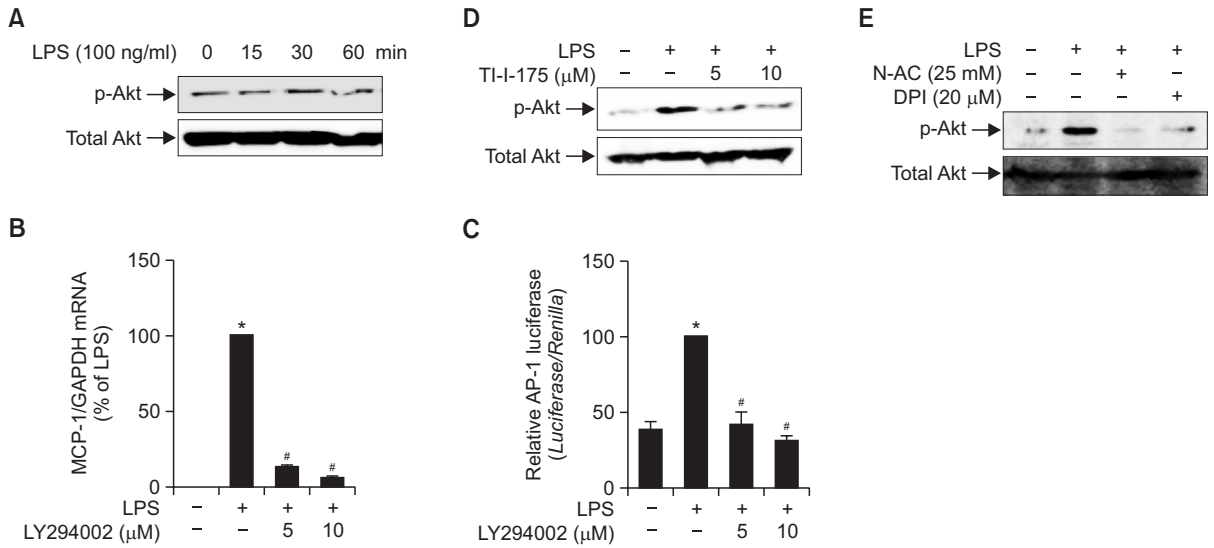

Fig. 6. TI-I-175 modulates Akt signaling for the suppression of LPS-induced MCP-1 expression in RAW 264.7 macrophages. (A) Cells were treated with LPS for the indicated time periods. Phosphorylated level of Akt was determined by Western blot analysis as described in material and methods. Representative images from three independent experiments are shown along with total form of Akt used for internal loading control. (B) Cells were pretreated with indicated concentration of LY294002 for 2 h followed by stimulation with 100 ng/ml LPS for additional 6 h. MCP-1 mRNA level was measured by qRT-PCR and normalized to GAPDH level as described previously. Data are expressed as mean ± SEM (n=3). **p*<0.05 compared with the cells not treated with LPS; #*p*<0.05 compared with cells treated with LPS. (C) Cells were transiently cotransfected with pAP1-*Luc* plasmid and *Renilla* reporter gene as described previously. After 24 h of incubation, cells were pretreated with indicated concentration of LY294002 for 2 h followed by incubation with LPS (100 ng/ml) for additional 6 h. AP-1 transcriptional activity was measured as described previously. Values are presented as percentage (%) compared to the cells stimulated with LPS and are expressed as mean ± SEM (n=4). **p*<0.05 compared with the cells not treated with LPS; #*p*<0.05 compared with cells treated with LPS. (D) Cells were pretreated with TI-I-175 for 2 h and followed by stimulation with 100 ng/ml LPS for additional 6 h. Phosphorylated level of Akt was measured by Western blot analysis as described previously. (E) Cells were pretreated with indicated concentration of ROS inhibitors (NAC or DPI) for 2 h and then incubated with 100 ng/ml LPS for additional 6 h. Phosphorylated level of Akt was determined by Western blot analysis as described previously. Representative images from three independent experiments are shown along with total form of Akt.

and AP-1 activation in RAW 26.7 macrophages. Finally, we next examined the effect of TI-I-175 on LPS-induced ROS production determined by CM-H_2DCF-DA assay using fluorometer. As expected, treatment of RAW 264.7 macrophages with TI-I-175 potently inhibited LPS-induced ROS production (Fig. 5C, IC_{50} value is 4.79 μM). All these results suggest that TI-I-175 suppresses LPS-induced MCP-1 mRNA expression via inhibition of ROS production and subsequent AP-1 activation.

TI-I-175 modulates Akt signaling for the suppression of LPS-induced MCP-1 expression in RAW 264.7 macrophages

PI3K/Akt signaling has been shown to induce MCP-1 expression in various experimental conditions (Murao *et al*., 2000; Bian *et al*., 2004). In addition, Akt is known to act as an upstream molecule leading to the transcriptional activation of AP-1 in endothelial cells (Bian *et al*., 2004). Based on these previous reports, we next investigated whether Akt signaling is involved in the suppression of LPS-induced MCP-1 expression by TI-I-175. As shown in Fig. 6, LPS treatment led to a rapid phosphorylation of Akt showing a maximal increase at 30 min treatment (Fig. 6A) and pretreatment with inhibitor of PI3K (LY294002) significantly inhibited LPS-induced MCP-1 gene expression (Fig. 6B). Furthermore, pretreatment with LY294002 also substantially decreased LPS-induced AP-1 activation (Fig. 6C), suggesting that PI3K/Akt signaling plays a critical role in LPS-mediated MCP-1gene expression via modulation of transcription factor AP-1. Importantly, pretreatment with TI-I-175 suppressed LPS-induced phosphorylation of Akt in RAW 264.7 macrophages (Fig. 6D), indicating that TI-I-175 suppressed LPS-induced MCP-1 expression via modulation of Akt signaling. In addition, consistent with the previous observation, ROS inhibitors (N-AC and DPI) completely blocked LPS-induced Akt phosphorylation (Fig. 6E). Collectively, these data imply that TI-I-175 suppressed LPS-induced MCP-1 expression in RAW 264.7 macrophages via suppression of ROS production and subsequent inhibition of Akt signaling and AP-1 activation.

DISCUSSION

Excessive production of reactive oxygen species (ROS) is considered as a critical event contributing to inflict diverse pathophysiological events (Apel and Hirt, 2004), although normal range of ROS is required for appropriate physiological processes as well. In particular, ROS is exceedingly produced in response to inflammatory stimuli (Kadayat *et al*., 2014) and considered as a critical mediator for inflammatory responses. Therefore, pharmacological modulation of excessive ROS production might be a promising strategy for the management of pathophysiological states associated with inflammation. In the present study, we synthesized chalcone derivatives in an attempt to develop optimal anti-oxidant and anti-inflammatory agents and demonstrated that TI-I-175 was

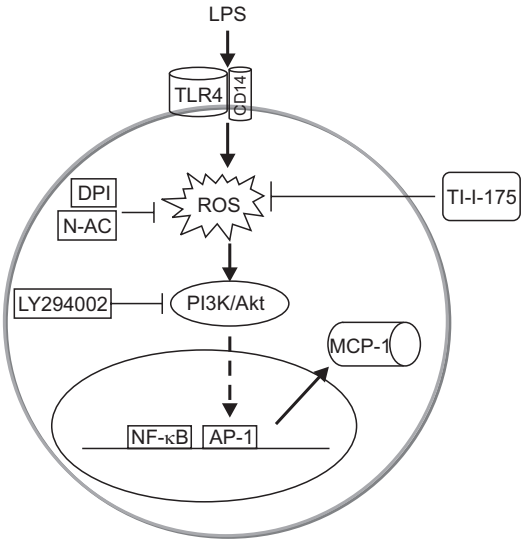

Fig. 7. Proposed model for the inhibitory effect of TI-I-175 on MCP-1 expression induced by LPS in RAW 264.7 macrophages. TI-I-175 treatment causes suppression of MCP-1 gene expression in RAW 264.7 macrophages stimulated with LPS. This inhibitory effect of TI-I-175 on LPS-induced MCP-1 gene expression would be mediated by modulation of AP-1 activation, not by NF-κB. Transcriptional activity of AP-1 in macrophages is regulated by a number of signaling mechanisms. Suppression of PI3K/Akt signaling would be a key signaling event causing the blockade of AP-1 activation and further suppression MCP-1 gene expression by TI-I-175. LPS-induced activation of Akt signaling (and AP-1) is mediated by enhanced ROS production. Interestingly, TI-I-175 treatment induces significant decrease in ROS production, which is a critical event leading to the suppression of Akt signaling and AP-1 activation. Detailed mechanisms underlying inhibiting transcriptional activation of AP-1 by Akt remain to be determined.

highly effective at inhibiting ROS production and expression of MCP-1, which plays a role in recruitment of immune cells and therefore involved in progression of inflammatory responses, in macrophages stimulated with LPS. In addition, we also demonstrated that its inhibitory effect on MCP-1 expression is mediated by suppression of Akt signaling and blockade of AP-1 activation.

MCP-1 is the number of C-C chemokine family and has been characterized as a potent inflammatory chemokine which induces recruitment of monocytes and lymphocytes during inflammation and tissue injury (Tesch, 2008; Deshmane *et al*., 2009). MCP-1 has been shown to be involved in the development of diverse diseases related with inflammation as discussed earlier. During the inflammatory process, macrophages have been considered as one of major sources of MCP-1 production (Deshmane *et al*., 2009). Chalcones, a subfamily of flavonoid, are widely known as anti-inflammatory and anti-oxidant properties. Previous studies have shown that chalcones regulate expression of a number of inflammatory mediators, including macrophages adhesion molecules, IL-6, TNF-α, NO and MCP-1 (Hirai *et al*., 2007; Pan *et al*., 2013) and, therefore, they are considered as a promising molecule for the treatment of diseases associated with inflammation. In spite of these strong biological activities, high concentrations of chalcones have been shown to non-selectivity in terms of distinguishing between normal and tumorous cells (Zhang *et al*., 2013) and generate cytotoxicity in normal hepatocytes. In particular, chalcones potently suppress mitochondrial membrane potential and therefore causes excessive cell death in normal hepatocytes (Sabzevari *et al*., 2004), indicating that use of chalcones for the treatment of the diseases needs to be carefully monitored. In the present study, TI-I-175 did not produce any significant cytotoxic effects in the concentrations causing anti-inflammatory responses.

It is well known that intracellular ROS plays a key role in the expression of inflammatory mediators, including IL-8, TNF-α and ICAM-1 (Bedard and Krause, 2007; Leverence *et al*., 2011). In addition, previous studies have demonstrated that superoxide and H_2O_2 derived from Rac1-dependent NADPH oxidase activation causes up-regulation of MCP-1 gene expression in endothelial cells (Chen *et al*., 2004), and resveratrol inhibits MCP-1 production via modulation of Nox1 expression and subsequent ROS production in murine macrophages (Park *et al*., 2009), implying ROS would be critical mediator of MCP-1 gene expression. Growing evidences have demonstrated that chalcones efficiently regulate ROS production in various experimental conditions. For example, Isoliquiritigenin (ILTG) (4,2',4'-trihydoxylchalcone), a chalcone-derivative flavonoid, blocked TNF-α-induced intracellular ROS production in endothelial cells (Kumar *et al*., 2007). In this study, we also observed that TI-I-175 significantly inhibited LPS-induced MCP-1 gene expression and AP-1 activation via suppression of ROS production (Fig. 6), suggesting that anti-oxidant properties of TI-I-175 involves suppression of MCP-1 gene expression in macrophages.

PI3K/Akt signaling was originally known to be related with cancer development via induction of cell proliferation and suppression of apoptosis (Chen *et al*., 2001; Itoh *et al*., 2002). In addition, recent studies have suggested a possible role of Akt signaling in MCP-1 expression and mediation of endotoxin-induced inflammatory responses (Bian *et al*., 2004; Park *et al*., 2009). In this study, we have demonstrated Akt signaling plays an important role in LPS-induced MCP-1 expression in macrophages through AP-1 activation and TI-I-175 prevented LPS-induced phosphorylation of Akt (Fig. 6). Chalcones are well known for their potent anti-inflammatory and anti-oxidant properties. In addition, recent studies have demonstrated that chalcone regulates Akt signaling. For example, isobavachalcone (IBC), a natural chalcone derivative, potently diminished Akt signaling and revealed anti-proliferative effects in human cancer cells (Jing *et al*., 2010). DK-139, a novel chalcone derivative suppressed LPS-induced Akt phosphorylation in BV2 microglial cells (Lee *et al*., 2012), indicating that PI3K/Akt could be another promising signaling molecule mediating a number of biological responses by chalcones.

For the transcriptional activation of AP-1 by PI3K/Akt, we hypothesized that Akt signaling would activate another downstream signaling required for AP-1 activation, rather than directly activates AP-1. To further identify detailed signaling mechanisms underlying suppression of AP-1 by TI-I-175, we initially considered mitogen-activated protein kinases (MAPKs) as candidate molecules regulating AP-1 activation, since MAPKs are very well known as upstream signaling molecule required for AP-1 activation and Akt signaling has been also known to activate MAPKs signaling (Karin, 1995; Takeda *et al*., 2001). However, herein we found that TI-I-175 did not significantly affect LPS-induced activation of ERK1/2, p38MAPK and JNK (data not shown), indicating that MAPK

signaling pathways are not implicated in the modulation of AP-1 by TI-I-175 in RAW 264.7 macrophages. In the current study, we could not thoroughly address the mechanisms underlying regulation of AP-1 activation. Future studies will have to provide further insights into the mechanisms for AP-1 regulation and MCP-1 expression by chalcone compounds.

In conclusion, we demonstrated that TI-I-175, a newly synthesized chalcone derivative, effectively suppressed LPS-stimulated MCP-1 expression in macrophages. The suppressive effect was mediated via modulation of ROS production, suppression of Akt signaling and finally blockade of AP-1 activation (Fig. 7). In addition to the previous reports demonstrating the potent anti-inflammatory effects of chalcone, the present study provide the evidence for the inhibitory effect of a synthetic chalcone derivative on the expression of MCP-1 required for recruitment of immune cells to the sites of inflammation. Future studies validating therapeutic effect in *in vivo* model are now required.

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