N-(*p*-Coumaryol)-Tryptamine Suppresses the Activation of JNK/c-Jun Signaling Pathway in LPS-Challenged RAW264.7 Cells

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Abstract

N-(*p*-Coumaryol) tryptamine (CT), a phenolic amide, has been reported to exhibit anti-oxidant and anti-inflammatory activities. However, the underlying mechanism by which CT exerts its pharmacological properties has not been clearly demonstrated. The objective of this study is to elucidate the anti-inflammatory mechanism of CT in lipopolysaccharide (LPS)-challenged RAW264.7 macrophage cells. CT significantly inhibited LPS-induced extracellular secretion of pro-inflammatory mediators such as nitric oxide (NO) and PGE$_2$, and protein expressions of iNOS and COX-2. In addition, CT significantly suppressed LPS-induced secretion of pro-inflammatory cytokines such as TNF-α and IL-1β. To elucidate the underlying anti-inflammatory mechanism of CT, involvement of MAPK and Akt signaling pathways was examined. CT significantly attenuated LPS-induced activation of JNK/c-Jun, but not ERK and p38, in a concentration-dependent manner. Interestingly, CT appeared to suppress LPS-induced Akt phosphorylation. However, JNK inhibition, but not Akt inhibition, resulted in the suppression of LPS-induced responses, suggesting that JNK/c-Jun signaling pathway significantly contributes to LPS-induced inflammatory responses and that LPS-induced Akt phosphorylation might be a compensatory response to a stress condition. Taken together, the present study clearly demonstrates CT exerts anti-inflammatory activity through the suppression of JNK/c-Jun signaling pathway in LPS-challenged RAW264.7 macrophage cells.

Key Words: *N*-(*p*-Coumaroyl) tryptamine, RAW 264.7 cells, Lipopolysaccharide, iNOS, COX-2, JNK, c-Jun

INTRODUCTION

The c-Jun N-terminal kinase (JNK) of mitogen-activated protein (MAP) kinases has been reported to be implicated in the pathogenesis of various inflammatory disorders including sepsis (Ip and Davis, 1998; Supinski *et al.*, 2009). JNK activation contributes to the development of inflammation-induced cell dysfunction and activation of caspases in several organs through the activation of transcription factor c-Jun (Cho and Choi, 2002; Wang *et al.*, 2004). Recently, we demonstrated that inhibition of JNK activation with aromadendrin significantly attenuates lipopolysaccharide (LPS)-induced inflammatory responses in RAW264.7 cells (Lee *et al.*, 2013).

Although macrophages play essential roles in the mobilization of the host defense against bacterial infection (Rehman *et al.*, 2012), aberrant activation of macrophages has been also reported to play pathogenic roles in various inflammatory disorders including sepsis (Kim *et al.*, 2012). In pathogenic conditions, abnormally activated macrophages produce excessive amount of a variety of pro-inflammatory mediators and cytokines that eventually aggravates the inflammatory conditions (Itharat and Hiransai, 2012). LPS, a component of the outer membrane of Gram-negative bacteria, is the most common cause of macrophage activation (Rietschel and Brade, 1992). LPS-induced activation of macrophages has been reported to cause a wide range of pro-inflammatory responses including secretion of pro-inflammatory mediators, expression of adhesion molecules and coagulation factors, phagocytosis, and cytoskeletal rearrangement (Sweet and Hume, 1996). Therefore, the suppression of aberrant macrophage activation might be a valuable therapeutic target for the treatment of inflammatory disorders.
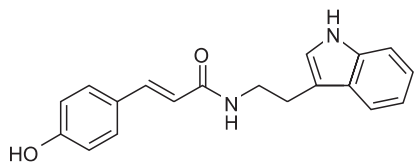
Fig. 1. Chemical structure of *N*-(*p*-coumaryol) tryptamine.

N-(*p*-Coumaryol) tryptamine (CT) and its derivatives such as *N*-(*p*-coumaroyl) serotonin have been reported to exhibit various pharmacological activities including anti-oxidant, anti-inflammatory, and growth-promoting properties (Takii *et al.*, 1999; Takii *et al.*, 2003). It has been reported that the antioxidant effect of CT and its derivatives was due to radical scavenging activity (Zhang *et al.*, 1997). However, the underlying mechanism by which CT exerts its pharmacological activity has not been clearly demonstrated. CT has been reported to be present in various medicinal herbs including Safflower oil cake (*Carthamus tinctorius L.*) (Takii *et al.*, 1999; Takii *et al.*, 2003) and *Ravensara anisata* (Andrianaivoravelona *et al.*, 1999). CT, used in the present study, was isolated from the stem of *Zea mays* (Sim *et al.*, 2014).

The objective of the present study was to examine whether CT possesses the anti-inflammatory activity in LPS-challenged RAW264.7 cells and to understand its underlying mechanism by which CT exerts the anti-inflammatory property in order to provide a valuable pharmacological agent that could suppress aberrantly activated macrophages in inflammation-related conditions.

MATERIALS AND METHODS

Reagents and cell culture

Bacterial lipopolysaccharide (LPS) from *Escherichia coli* serotype 055:B5 was purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*-(*p*-Coumaryol) tryptamine (CT) (Fig. 1) was isolated and identified from *Zea mays* (Fig. 1) (Sim *et al.*, 2014). CT was dissolved in dimethyl sulfoxide (DMSO) and added to the cell culture at the desired concentrations. The macrophage RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) containing 5% heat-inactivated fetal bovine serum and penicillin-streptomycin (Gibco BRL) at 37°C, 5% CO_2. In all experiments, cells were incubated in the presence of the indicated concentrations of CT before the addition of LPS (200 ng/ml).

Cell viability

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RAW 264.7 macrophage cells were seeded at 5×10^5 cells per well and incubated with CT at various concentrations for 24 hr at 37°C. After incubation, MTT (0.5 mg/ml in PBS) was added to each well, and the cells were incubated for 3 hr at 37°C and 5% CO_2. The resulting formazan crystals were dissolved in dimethyl sulfoxide (DMSO). Absorbance was determined at 540 nm. The results were expressed as a percentage of surviving cells over control cells.
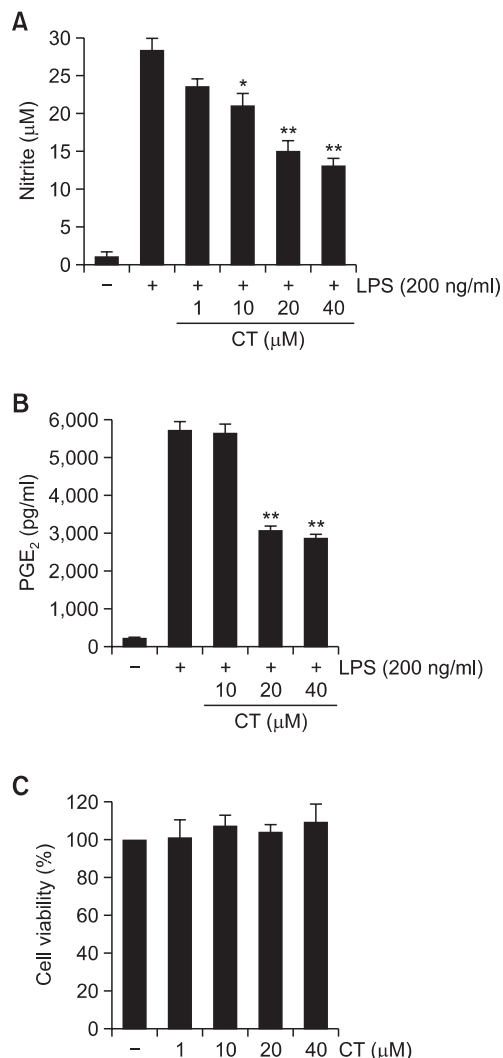

Fig. 2. Effects of N-(*p*-coumaryol) tryptamine on LPS-induced extracellular release of NO (A) and PGE_2 (B) in RAW264.7 macrophage cells. RAW264.7 cells were pretreated with indicated concentrations of N-(*p*-coumaryol) tryptamine for 1 hr before incubation with LPS (200 ng/ml) for 24 hr. The level of nitrite and PGE_2 were measured using Griess reagent and ELISA assay, respectively. N-(*p*-Coumaryol) tryptamine significantly suppressed LPS-induced extracellular release of NO and PGE_2. (C) Effect of N-(*p*-coumaryol) tryptamine on the viability of RAW264.7 cells. No significant cell death was observed with N-(*p*-coumaryol) tryptamine concentrations used in the present study. The data were obtained from three independent experiments and expressed as mean ± S.D. (n=3). *p<0.05 and **p<0.01 indicate statistically significant differences from treatment with LPS alone. CT stands for N-(*p*-coumaryol) tryptamine.

Nitrite quantification assay

The production of NO was estimated by measuring the amount of nitrite, a stable metabolite of NO, using the Griess reagent as described (Lee *et al.*, 2012). After CT-pretreated RAW264.7 macrophage cells were stimulated with LPS in 12-well plates for 24 hr, 100 μL of the cell supernatant was mixed with an equal volume of Griess reagent. Light absorbance was read at 540 nm. The results were expressed as a percentage of released NO from LPS-stimulated RAW 264.7 cells. To pre
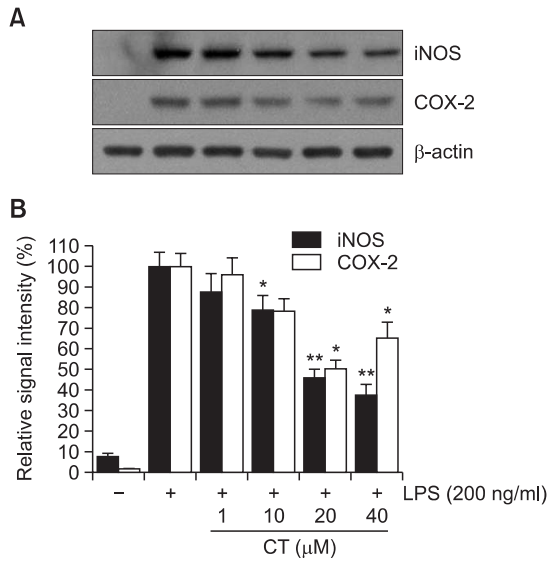
A

B

Fig. 3. Effects of N-(*p*-coumaryol) tryptamine on LPS-induced expression of iNOS and COX-2 in RAW264.7cells. (A) The cell lysates were subjected to SDS-PAGE, and then protein levels of iNOS and COX-2 were determined by Western blot analysis. N-(*p*-Coumaryol) tryptamine significantly attenuated LPS-induced over expression of iNOS and COX-2. Images are representative of three independent experiments that shows reproducible results. (B) Quantitative analysis of immunoblots of iNOS and COX-2. N-(*p*-Coumaryol) tryptamine significantly suppressed LPS-induced iNOS and COX-2 expression. The data were obtained from three independent experiments and expressed as mean ± S.D. (n=3). *$p<0.05$ and **$p<0.01$ indicate statistically significant differences from treatment with LPS alone. CT stands for N-(*p*-coumaryol) tryptamine.

A

B

Fig. 4. Effect of *N*-(*p*-coumaryol) tryptamine on LPS-induced extracellular secretion of IL-1β and in RAW264.7 macrophage cells. RAW264.7 cells were pretreated with indicated concentrations of *N*-(*p*-coumaryol) tryptamine for 1 hr, then incubated with LPS (200 ng/ml) for 24 hrs. The concentrations of IL-1β (A) andTNF-α (B) in collected cell culture media were measured by ELISA assay as described in the methods. *N*-(*p*-Coumaryol) tryptamine meaningfully reduced LPS-stimulated IL-1β and TNF-α cytokines in aconcentration-dependent manner. The values are expressed as mean ± SD for three independent experiments. *$p<0.05$ and **$p<0.01$ indicate statistically significant differences from treatments with LPSalone.

pare a standard curve, sodium nitrite was used to prepare a standard curve.

ELISA assay for cytokines
The RAW264.7 macrophage cells were treated with CT in the absence or presence of LPS. After 24 hr incubation, TNF-α and IL-1β levels in culture media were quantified using monoclonal anti-TNF-α or IL-1β antibodies according to the manufacturer's instruction (R&D Systems).

Western blot analysis
The RAW 264.7 macrophage cells were incubated with CT for 1 hr prior to LPS treatment. Cells were washed with PBS and lysed in PRO-PREP lysis buffer (iNtRON Biotechnology, Seongnam, Korea). Equal amounts of protein were separated on 10% SDS-polyacrylamide gel. Proteins were transferred to Hypond PVDF membrane (Amersham Biosciences, Piscataway, NJ, USA) and blocked in 5% skim milk in TBST for 1 hr at room temperature. Specific antibodies against inducible NO synthase (iNOS), COX-2, extracellular signal-regulated kinase (ERK), phosphorylated (p)-ERK, p38, p-p38, c-Jun N-terminal kinase (1:1,000; Cell signaling Technology), Akt, p-Akt (1:1,000; Cell signaling Technology), and β-actin (1:2,500; Sigma) were diluted in 5% skim milk. After thoroughly washing with TBST, horseradish peroxidase-conjugated secondary antibodies were applied. The blots were developed by the enhanced chemiluminescence detection (Amersham Biosciences).
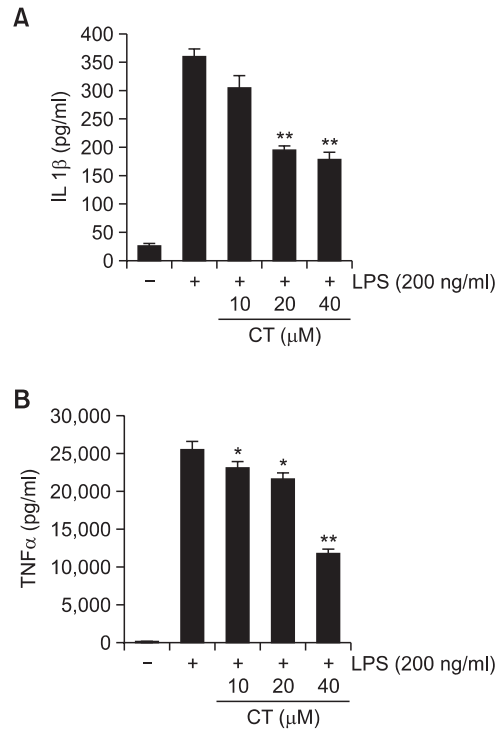
Statistical analysis
All values shown in the figures are expressed as the mean ± SD obtained from at least three independent experiments. Statistical significance was analyzed by two-tailed Student's *t*-test. Data with values of $p<0.05$ were considered as statistically significant. Single (* and #) and double (** and ##) marks represent statistical significance in $p<0.05$ and $p<0.01$, respectively.

RESULTS

N-(*p*-Coumaryol) tryptamine (CT) inhibits NO and PGE₂ secretion in LPS-stimulated RAW 264.7 macrophage cells
Given the previous reports that inflammatory mediators such as NO and PGE$_2$ play key roles in the progression of inflammation (Ock *et al.*, 2009; Lee *et al.*, 2012), the effects of CT on the extracellular release of NO and PGE$_2$ were examined in LPS-stimulated RAW264.7 macrophages. Cells were incubated with CT (1, 10, 20, or 40 μM) for 1 hr prior to LPS treatment (200 ng/ml). LPS showed markedly increased NO and PGE$_2$ production in RAW264.7 cells. However, CT significantly inhibited extracellular release of NO and PGE$_2$ in LPS-stimulated RAW264.7 cells in concentration dependent
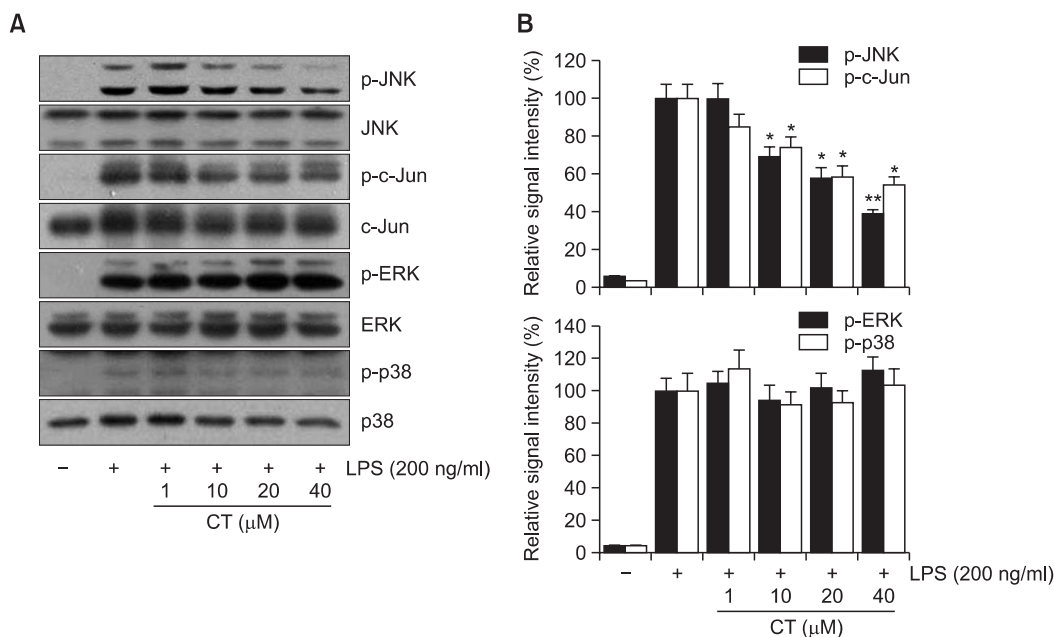
Fig. 5. Effect of N-(*p*-coumaryol) tryptamine on LPS-induced activation of MAPK signaling pathway in RAW264.7 macrophage cells. (A) representative immunoblots, (B) quantitative analysis of immunoblots. Cellswere challenged with 200 ng/ml LPS in the absence or presence of *N*-(*p*-coumaryol) tryptamine. LPS-induced increased phosphorylation of JNK and c-jun was significantly attenuated with *N*-(*p*-coumaryol) tryptamine treatment. However, phosphorylation of ERK and p38 was not affected with *N*-(*p*-coumaryol) tryptamine treatment, suggesting that JNK/c-jun signaling might play a key role in the LPS-induced activation of RAW264.7 cells. Images are representative of three independent experiments that shows reproducible results. The values are expressed as mean ± SD for three independent experiments. *p<0.05 and **p<0.01 indicate statistically significant differences from treatments with LPS alone. CT stands for *N*-(*p*-coumaryol) tryptamine.

manners (Fig. 2A, B). In addition, CT showed negligible cytotoxicity in concentration ranges used in the study (Fig. 2C).

CT inhibits LPS-induced expressions of iNOS and COX-2

As CT inhibited LPS-induced extracellular release of NO and PGE$_2$ in RAW264.7 cells (Fig. 2), whether the attenuated production of NO and PGE$_2$ was attributable to downregulation of iNOS and COX-2 expression was examined in the absence or presence of CT. LPS treatment resulted in the significantly increased expression of iNOS and COX-2 proteins in RAW264.7 cells. Pretreatment of CT resulted in a significant suppression in LPS-induced iNOS and COX-2 overexpression levels in a concentration-dependent manner (Fig. 3), indicating that attenuated release of NO and PGE$_2$ is due to decreased expression of their responsible genes by CT.

CT attenuates LPS-induced release of pro-inflammatory cytokines such as IL-1β and TNF-α

To examine the effects of CT on the extracellular release of pro-inflammatory cytokines such as IL-1β and TNF-α, secretion of these cytokines was measured using ELISA assay in LPS-stimulated RAW264.7 cells. LPS treatment resulted in excessive extracellular release of IL-1β and TNF-α in RWA 264.7 cells. However, CT significantly attenuated LPS-induced extracellular release of IL-1β and TNF-α in a concentration-dependent manner (Fig. 4).

CT inhibits the phosphorylation of JNK/c-Jun in LPS-stimulated RAW264.7 cells

MAP kinase signaling pathways have been reported to be implicated in the LPS-induced inflammatory responses (Guha

and Mackman, 2001; Rushworth *et al.*, 2005). In the present study, to understand the underlying signaling mechanism by which CT exhibits its anti-inflammatory activity, the effect of CT on LPS-stimulated phosphorylation of JNK, ERK, and p38 kinase in RAW264.7 cells was examined. Cells were pretreated with CT at indicated concentrations (1, 10, 20, and 40 μM), and then were treated with LPS (200 ng/ml) for 30 min. CT significantly suppressed the LPS-induced phosphorylation of JNK and transcription factor c-Jun, a downstream target of JNK, in a concentration-dependent manner in RAW 264.7 cells (Fig. 5). However, no significant attenuation of LPS-induced ERK and p38 phosphorylation was observed (Fig. 5), suggesting that CT exerts its anti-inflammatory action through the suppression of JNK/c-Jun signaling pathway in RAW264.7 cells.

CT appears to inhibit LPS-induced Akt phosphorylation in LPS-stimulated RAW 264.7 cells

It has been previously reported that suppression of PI3K/ Akt signaling plays a key role in the attenuation of LPS-induced NF-κB activation (Dilshara *et al.*, 2013; Liu *et al.*, 2013). To understand the possible involvement of Akt signaling pathway in the present study, the effect of CT on LPS-induced Akt phosphorylation was examined. LPS showed markedly increased phosphorylation of Akt and CT significantly attenuated LPS-induced Akt phosphorylation in a concentration-dependent manner (Fig. 6).

Inhibition of JNK but not Akt exhibits the suppression of LPS-induced pro-inflammatory responses

The present data demonstrated that CT significantly suppresses LPS-induced activation of JNK/c-jun (Fig. 5) and Akt

(Fig. 6) signaling pathways. In order to delineate the exact signaling pathway by which CT exerts its anti-inflammatory responses, blockade of each signaling pathway was achieved
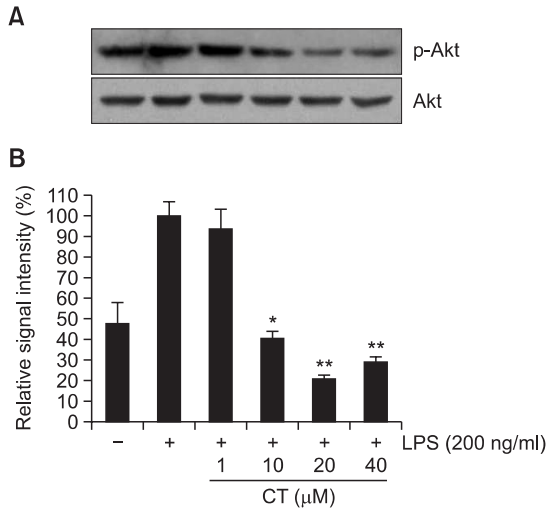

Fig. 6. Effect of N-(*p*-coumaryol) tryptamine on LPS-induced activation of Akt signaling pathway in RAW264.7 macrophage cells. (A) representative immunoblots, (B) quantitative analysis of immunoblots. *N*-(*p*-coumaryol) tryptamine appeared to significantly attenuate Akt phosphorylation in RAW264.7 macrophage cells. The images shown are representative of three independent experiments and the values are expressed as mean ± SD for three experiments. *p<0.05 and **p<0.01 indicate statistically significant differences from treatments with control alone. CT stands for *N*-(*p*-coumaryol) tryptamine.

with specific inhibitors and involvement of each signaling pathway in LPS-induced inflammatory response was examined. Inhibition of JNK/c-Jun signaling pathway with SP600125 showed significant suppression of c-jun and Akt phosphorylation, which is quite similar with CT (Fig. 7A). However, inhibition of Akt signaling pathway with wortmannin, a PI3K inhibitor, exhibited only Akt phosphorylation but not c-jun phosphorylation (Fig. 7A). Furthermore, inhibition of JNK/c-Jun signaling pathway significantly attenuated LPS-induced iNOS and COX-2 expression (Fig. 7B). However, inhibition of Akt signaling pathway with wortmannin could not inhibit LSP-induced expression of iNOS and COX-2 (Fig. 7B), strongly suggesting that CT exerts its anti-inflammatory action through the suppression JNK/c-Jun signaling pathway and that apparent suppression of Akt phosphorylation by CT might be due to the lessened necessity of compensatory action as CT attenuates the LPS-induced damage, rather than direct inhibition of Akt phosphorylation by CT.

DISCUSSION

The present study clearly demonstrated that *N*-(*p*-coumaryol) tryptamine (CT) suppresses LPS-induced inflammatory responses through the suppression of JNK/c-Jun signaling pathway in LPS-challenged RAW264.7 macrophage cells. CT significantly inhibited LPS-induced extracellular release of pro-inflammatory mediators and cytokines. In addition, CT significantly attenuated LPS-induced expression of iNOS and COX-2 proteins.

In accordance with previous reports that CT and its derivatives have pharmacological properties such as anti-inflammatory and anti-oxidant actions (Takii *et al.*, 1999; Takii *et al.*,
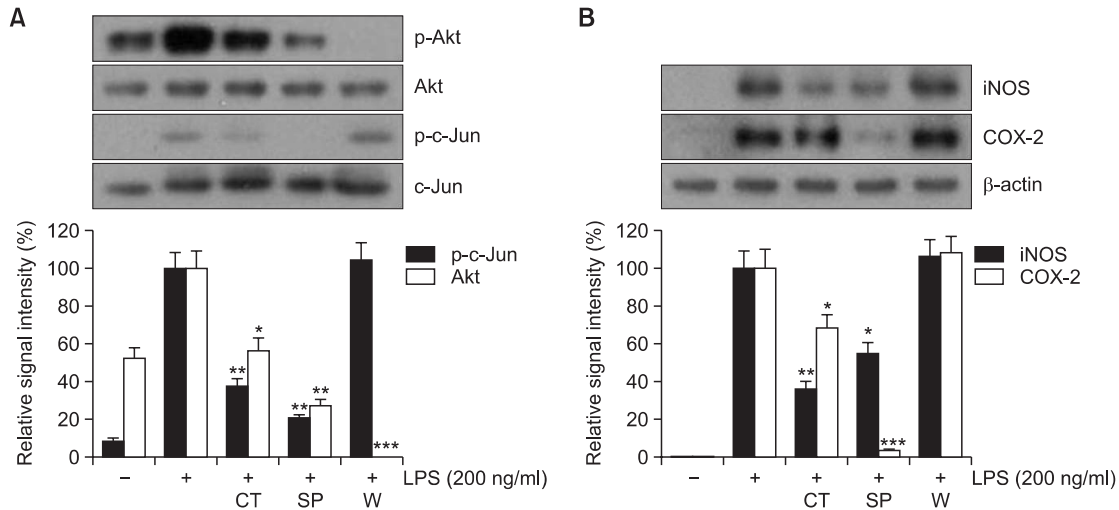

Fig. 7. Role of JNK and Akt signaling pathways in *N*-(*p*-coumaryol) tryptamine-mediated suppression of LPS-induced RAW264.7 cell activation. RAW264.7 cells were pretreated with CT (*N*-(*p*-coumaryol)tryptamine), SP (SP600125, JNK inhibitor), or W (wortmannin, Akt inhibitor), and then exposed to LPS (200 ng/ml) for 1 hr. The cell lysates were prepared and subjected to Western blotting analysis. (A) Phosphorylation levels of Akt and c-jun were examined in the presence of CT, SP, or W. CT and SP significantly suppressed LPS-induced phosphorylation of Akt and c-jun. However, W did not inhibit LPS-induced c-jun phosphorylation. (B) The protein levels of iNOS and COX-2 were examined. Suppression of LPS-induced iNOS and COX-2 expressions were observed with N-(*p*-coumaryol) tryptamine (CT) and alsowith JNK inhibitor (SP) but not with Akt inhibitor (W). The images on top are representatives of threeindependent experiments. The data for quantitative analyses on bottom were obtained from threeindependent experiments and expressed as mean ± SD (n=3). *p<0.05 and **p<0.01 indicate statisticallysignificant differences from treatments with control alone.

2003), the present study demonstrated that CT possesses anti-inflammatory properties in LPS-induced RAW 264.7 macrophage cells. However, the underlying mechanism by which CT and its derivatives exert the anti-inflammatory activity has not been clearly demonstrated. Therefore, the present study elucidated that CT exerts the anti-inflammatory action through the suppression of LPS-induced activation JNK/c-Jun signaling pathway.

Macrophages play essential roles in the host defense against bacterial infection (Rehman *et al.*, 2012). However, aberrant activation of macrophages plays detrimental roles in multiple inflammation-related disorders including sepsis by producing a wide range of pro-inflammatory mediators and cytokines (Rietschel and Brade, 1992). Macrophages initiate LPS-induced pro-inflammatory gene transcription when LPS binds to its membrane receptor, TLR4, which leads to the phosphorylation of multiple kinases, which subsequently activates various pro-inflammatory transcription factors including NF-κB and AP-1 (O'Connell *et al.*, 1998; Guha and Mackman, 2001). We previously reported that LPS causes increased expression of pro-inflammatory mediators and the increased degradation IκB (Vo *et al.*, 2012). CT significantly attenuated LPS-induced extracellular release of pro-inflammatory cytokines such as IL-1β and TNF-α. Although pro-inflammatory cytokines such as IL-1β and TNF-α have been reported to play key roles in the development of inflammatory responses, other cytokines and transcription factors might also play a certain role. Therefore, further studies are necessary to clearly explain the effect of CT other cytokines and transcription factors in LPS-mediated inflammatory conditions.

NF-κB is a major transcription factor responsible for the expression of a variety of pro-inflammatory genes such as mediators such as iNOS, COX-2, and cytokines (Siebenlist *et al.*, 1994). Therefore, the aberrant activation of NF-κB has been associated with various pathological conditions including cancers and autoimmune diseases (Li and Verma, 2002). We previously reported that suppression of NF-κB transcription mediated anti-inflammatory properties of natural products against LPS-induced inflammation in RAW264.7 cells (Vo *et al.*, 2014a; Vo *et al.*, 2014b). The present data showed that CT significantly suppressed the expression of iNOS and COX-2, presumably through the suppression of NF-κB transcription. However, further studies are necessary to clearly delineate the exact mechanism by which CT leads to the suppression of iNOS and COX-2 expression.

Many studies have shown that LPS activates all three MAP kinases such as ERK, JNK, and p38 in macrophages (Sweet and Hume, 1996) and that many of the downstream targets of MAPK pathways are transcription factors, which regulate various genes encoding inflammatory mediators (Zhang *et al.*, 2006; Lee *et al.*, 2009; Zhang *et al.*, 2011). In the present study, LPS treatment exhibited increased phosphorylation of all three MAPKs. However, CT selectively attenuated LPS-induced JNK/c-Jun activation in a concentration-dependent manner with concurrent significant attenuation of LPS-induced pro-inflammatory responses. The JNK/c-Jun signaling pathway has been implicated in the development of inflammatory responses in various conditions leading to cellular dysfunction and organ failures (Ip and Davis, 1998; Supinski *et al.*, 2009). It has been also reported that inhibition of JNK activation significantly attenuates lipopolysaccharide (LPS)-induced inflammatory responses in RAW264.7 cells (Lee *et al.*, 2013).

No noticeable changes were observed in phosphorylation of ERK and p38 with CT. Furthermore, inhibition of JNK signaling pathway with specific JNK inhibitor mimicked the action of CT, suggesting that selective suppression of JNK/c-Jun signaling pathway might be sufficient to exert anti-inflammatory effects of CT in RAW 264.7 cells and that JNK/c-Jun pathway rather than ERK and p38 plays a key role in pro-inflammatory responses in RAW264.7 cells.

It has been reported that LPS-induced activation of PI3K/Akt signaling pathway contributes to the development of inflammatory responses including the activation of NF-κB transcription (Madrid *et al.*, 2001; Joh and Kim, 2011). Many reports have shown that suppression of Akt signaling attenuates LPS-induced inflammatory responses in various models (Shao and Lin, 2008; Dilshara *et al.*, 2013; Liu *et al.*, 2013). In the present study, LSP treatment resulted in the phosphorylation of Akt and CT appeared to significantly suppress Akt phosphorylation in a concentration-dependent manner in RAW264.7 cells. To the contrary, it has been also reported that activation of PI3K/Akt signaling pathway reduces LPS-induced inflammation in various models (Kidd *et al.*, 2008; Xu *et al.*, 2010; Zong *et al.*, 2012). Activation of Akt/PI3K signaling pathway has been reported to exert significant anti-inflammatory effects through the suppression of NF-κB-mediated transcription (Ha *et al.*, 2011; Vo *et al.*, 2014a). Therefore, the role of PI3K/Akt signaling cascades in LPS-induced inflammatory response still remains controversial (Takeshima *et al.*, 2009). In the present study, inhibition of PI3K/Akt signaling pathway failed to suppress LPS-induced expression of iNOS and COX-2, which was shown with JNK inhibition and CT treatment. The data strongly suggest that the PI3K/Akt pathway might not be responsible for the LPS-induced inflammatory responses. Apparent attenuation of Akt phosphorylation with CT might be considered due to the lessened mobilization of compensatory intracellular components as CT attenuates the LPS-induced inflammation, rather than direct inhibition of Akt phosphorylation by CT. However, further studies are necessary to clearly delineate the phenomenon.

In conclusion, the present data clearly demonstrates that CT exerts anti-inflammatory property through the inhibition of JNK/c-Jun signaling pathway in LPS-challenged RAW264.7 macrophage cells. The present study strongly suggests that CT might be a valuable therapeutic agent for the treatment of inflammation-related pathogenic conditions.

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