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3,4,5-Trihydroxycinnamic Acid Inhibits Lipopolysaccharide-Induced Inflammatory Response through the Activation of Nrf2 Pathway in BV2 Microglial Cells

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Abstract

3,4,5-Trihydroxycinnamic acid (THC) is a derivative of hydroxycinnamic acids, which have been reported to possess a variety of biological properties such as anti-inflammatory, anti-tumor, and neuroprotective activities. However, biological activity of THC has not been extensively examined. Recently, we reported that THC possesses anti-inflammatory activity in LPS-stimulated BV2 microglial cells. However, its precise mechanism by which THC exerts anti-inflammatory action has not been clearly identified. Therefore, the present study was carried out to understand the anti-inflammatory mechanism of THC in BV2 microglial cells. THC effectively suppressed the LPS-induced induction of pro-inflammatory mediators such as NO, TNF-α, and IL-1β. THC also suppressed expression of MCP-1, which plays a key role in the migration of activated microglia. To understand the underlying mechanism by which THC exerts these anti-inflammatory properties, involvement of Nrf2, which is a cytoprotective transcription factor, was examined. THC resulted in increased phosphorylation of Nrf2 with consequent expression of HO-1 in a concentration-dependent manner. THC-induced phosphorylation of Nrf2 was blocked with SB203580, a p38 MAPK inhibitor, indicating that p38 MAPK is the responsible kinase for the phosphorylation of Nrf2. Taken together, the present study for the first time demonstrates that THC exerts anti-inflammatory properties through the activation of Nrf2 in BV2 microglial cells, suggesting that THC might be a valuable therapeutic adjuvant for the treatment of inflammation-related disorders in the CNS.

Key Words: Neuro-inflammation, 3,4,5-trihydroxycinnamic acid, Nrf2, Heme oxygenase-1

INTRODUCTION

Microglia are resident immune cells and play a critical role in host defense (Koh *et al.*, 2009) and tissue repair in the CNS. Upon activation, microglia release various cytokines and mediators, which include tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), monocyte chemoattractant protein-1 (MCP-1), and nitric oxide (NO) (Jung *et al.*, 2010). However, aberrant activation of microglia plays a pathogenic role in neuro-inflammatory conditions (Itagaki *et al.*, 1989; Matsumoto, 1992; McGeer and McGeer, 1998; Hailer, 2008) and results in the inflammation-mediated neuronal cell death (Ock *et al.*, 2009). Therefore, it may be important to control aberrant microglial activation for the treatment of many inflammatory diseases in the CNS.

The transcription factor NF-E2-related factor 2 (Nrf2) belongs to basic leucine-zipper (bZip) genes and is found in most cell types of the brain, including microglia. Under normal conditions, Nrf2 transcription is repressed by its negative regulator Keap1 (de Vries *et al.*, 2008). However, upon oxidant condition, Nrf2 dissociates from Keap1, translocates to the nucleus, and binds to antioxidant response element (ARE) and regulates the transcription of antioxidant proteins including heme oxygenase-1 (HO-1) (Lee *et al.*, 2011a). Activation of Nrf2 in microglia has been shown to exert neuroprotective influences by inhibiting pro-inflammatory cytokines (Koh *et al.*, 2009). In addition, it was reported that Nrf2 knockout mice were hypersensitive to the neuro-inflammation induced by lipopolysaccharide (LPS) (Innamorato *et al.*, 2008). HO-1 possesses antioxidative and anti-inflammatory properties against a variety of different stimuli (Ryter *et al.*, 2006). For example, LPS-stimulated macrophages can protect themselves from

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overproduction of NO by enhancing HO-1 expression (Lee *et al.*, 2005). It also was reported that HO-1 inhibits the production of TNF-α and IL-1β in response to LPS (Lee *et al.*, 2011a).

3,4,5-Trihydroxycinnamic acid (THC) is a derivative of hydroxycinnamic acids. It has been reported that hydroxycinnamic acids possess various biological properties such as anti-inflammatory, anti-tumor, and neuroprotective activities (Nagasaka *et al.*, 2007; Steinbrecher *et al.*, 2008; Kim, 2010). Ester derivatives of hydroxycinnamic acids have been also reported to exhibit a variety of biological activities (Lee *et al.*, 2011b). Caffeic acid phenethyl ester (CAPE) has been reported to activate HO-1 gene via regulation of Nrf2 (Lee *et al.*, 2010). However, biological activity of THC has not been extensively examined. Recently, anti-inflammatory activity of THC was identified in our study (Lee *et al.*, 2012). However, its anti-inflammatory action mechanism has not been clearly examined.

In the present study, in order to provide a novel pharmacological adjuvant that could suppress excessive activated microglia, the anti-inflammatory activity of THC and its underlying mechanism by which THC exerts cytoprotective action were examined in LPS-induced BV2 microglial cells.

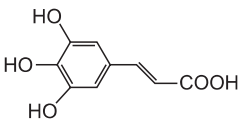

Fig. 1. Chemical structure of 3,4,5-trihydroxycinnamic acid (THC).

MATERIALS AND METHODS

Reagents and cell culture

The BV2 microglial cells (gift from Dr. T. H. Joh, Burke Institute, Cornell University, NY) was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) containing 5% heat-inactivated fetal bovine serum (FBS; Gibco BRL) and 50 μg/ml gentamicin (Sigma, St. Louis, MO, USA), and cultured at 37°C in a humidified atmosphere of 5% CO_2. Bacterial lipopolysaccharide (LPS) from *Escherichia coli* serotype 055:B5 was purchased from Sigma-Aldrich (St. Louis, MO, USA). 3,4,5-Trihydroxycinnamic aicd (THC, Fig. 1) was purchased from AApin Chemicals Limited (Abingdon, UK). The compound was solubilized in dimethyl sulfoxide (DMSO).

Nitrite quantification assay

The production of NO was estimated by measuring the amount of nitrite, a major stable metabolite of NO, using the Griess reagent (0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid). THC-pretreated BV2 microglial cells were stimulated with LPS in 12-well plates for 24 hr, and then 100 μl of each culture medium was mixed with an equal volume of Griess reagent. The absorbance at 540 nm was measured on a microplate reader. The results were expressed as percentages of released NO from LPS-stimulated BV2 cells. To prepare a standard curve, sodium nitrite was diluted in culture medium to concentrations.

Analysis of mRNA levels by semi-quantitative RT-PCR

The BV2 microglial cells were treated with THC in the absence or presence of LPS (200 ng/ml) for 6 hr. Total RNA was isolated using the Total RNA Extraction Kit (iNtRON Biotechnology, Inc, USA) according to the manufacturer's instruction. The total RNA (2 μg) obtained from cells was reverse-transcribed using oligo-(dT) 15 primers (Promega, Madison, WI, USA). PCR amplification conditions using primer sets specific for MCP-1, HO-1 and β-actin were optimized for each pair of primers. PCR primers were as follows: MCP-1 forward, 5'-CCTGCTGTTCACAGTTGCC-3'; MCP-1 reverse, 5'-TGAG GTGGTTGTGGAAA AGG-3'; HO-1 forward, 5′-TGAAGGAGGCCACCAAGGAGG-3′; HO-1 reverse, 5′-AGAGGTCACCCAGGTAGCGGG-3′; β-actin forward, 5'-ATCCTGA AAGACCTCTATGC-3'; β-actin reverse, 5'-AACG-CAGCTCAGTAACAGTC-3'. Parallel PCR analysis was run for the house keeping gene β-actin to normalize data for differences in mRNA quantity and integrity. PCR products were separated on agarose gel. For real-time PCR analysis, cDNA was synthesized as described before, using equal amounts of RNA. The cDNA products were used immediately for SYBR (Takara, JAPAN) real time RT-PCR using primers specific for TNF-α, IL-1β, and β-actin (BIO NEER, KOREA). Quantitative changes in mRNA levels were estimated by real time PCR using the following cycling conditions: 35 cycles of denaturation at 94°C for 10 sec; annealing at 61°C for 15 sec; and extension at 72°C for 20 sec; followed by 2 min at 72°C, in the presence of SYBR Green (1: 10,000 dilution of a stock solution from Molecular Probes) carried out in a 20 μl reaction volume.

Isolation of nuclear extract

BV2 microglial cells were cultured in 6-well plates and then treated 10, 50, and 100 μM concentrations of THC. Following 1 hr treatment of THC, cells were washed with ice-cold PBS, and harvested, and centrifuged at 5,000 rpm for 10 min at 4°C. The pellet was obtained and resuspended in hypotonic buffer containing 10 mM HEPES, 10 mM KCl, 1 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride. The lysates obtained were incubated for 10 min on ice with NP-40 and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant, consisting of the cytosolic fraction, was immediately frozen for further analysis. The pellet was resuspended in low salt buffer containing 20 mM HEPES, 0.4 M NaCl, 1 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride for 20 min on ice. Samples were gently mixed for 30 min at 4°C. After centrifugation at 13,000 rpm for 20 min, the supernatants containing nuclear extracts were obtained.

Western blot analysis

The BV2 microglial cells were pretreated with THC for 1 hr and stimulated with 200 ng/ml of LPS. Cells were washed with PBS and lysed with lysis buffer. Equal amounts of protein were separated on 10% SDS-polyacrylamide gel. Proteins were transferred to Hypond PVDF membrane and blocked in 5% skim milk in TBST for 1 hr at room temperature. Specific antibodies against extracellular signal-regulated kinase (ERK), phosphorylated (p)-ERK 1/2, p38, p-p38, c-Jun N-terminal kinase (JNK), p-JNK (1:1,000, Cell signaling), poly (ADP-ribose) polymerase (PARP) (1:1,000), Nrf2 (1:1,000, Santa Cruz), p-Nrf2 (1:1,000, Abcam), HO-1 (1:1,000; Abcam), MCP (1;1,000; Abcam) and β-actin (1; 2,500; Sigma) were diluted in 5% skim milk. After thorough washing with TBST, horseradish peroxidase (HRP)-conjugated secondary antibodies were ap-

plied. The blots were developed by the enhanced chemiluminescence detection (Amersham Biosciences).

Statistical analysis

All data are expressed as the mean ± standard deviation (SD). 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 double (**) marks represent statistical significance in p<0.05 and p<0.01, respectively.

RESULTS

THC suppresses production of NO and pro-inflammatory cytokines in LPS-stimulated BV2 microglial cells

To examine the anti-inflammatory potential of THC, the inhibitory effect on the production of NO, which is one of the pro-inflammatory mediators, was examined in LPS-activated BV2 microglial cells. As shown in Fig. 2A, THC significantly suppressed LPS-induced NO production in a concentration-dependent manner (Fig. 2A). In accordance, we previously demonstrated that THC significantly attenuates LPS-induced expression of iNOS, which is considered to be the source of excessive production of NO (Lee *et al.*, 2012). Given the fact that pro-inflammatory cytokines play a significant role in the progression of inflammation, the effect of THC on the mRNA expression of TNF-α and IL-1β was examined in LPS-stimulated BV2 microglial cells. THC suppressed the mRNA expression of TNF-α and IL-1β mRNA in a concentration-dependent manner in LPS-stimulated BV2 microglial cells (Fig. 2B, 2C).

THC inhibits LPS-induced mRNA and protein expression of MCP-1

MCP-1 has been reported to play a key role in the migration of activated microglia to sites of inflammation in the CNS (Jung *et al.*, 2010), effect of THC on the LPS-induced production of MCP-1 was examined by Western blot analysis and RT-PCR, respectively. Treatment of LPS resulted in the apparent up-regulation of MCP-1 protein expression and pretreatment of THC significantly suppressed LPS-induced MCP-1 protein expression in a concentration-dependent manner in BV2 microglial cells (Fig. 3A). Consistent with the decrease of MCP-1 protein, THC also significantly attenuated LPS-induced up-regulation of MCP-1 mRNA expression (Fig. 3B), suggesting a possibility that THC might attenuate the migration of activated microglia.

THC induces HO-1 expression via nuclear translocation of Nrf2

Nrf2 is a major transcription factor regulating a variety of cytoprotective enzymes including HO-1, which is involved in the regulation of acute inflammatory responses as well as cellular

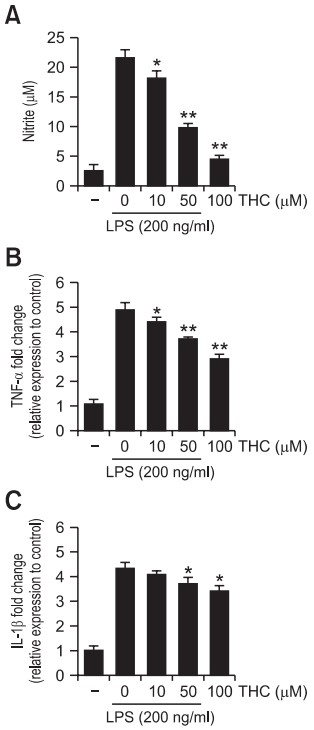

Fig. 2. Inhibitory effects of THC on LPS-induced inflammatory mediators in BV2 microglial cells. Cells were stimulated with LPS in presence or absence of THC 24 hr. (A) The amount of nitrite in culture medium was measured by using the Griess reagents. The level of TNF-α (B) and IL-1β (C) mRNA were determined by real-time PCR. THC significantly suppressed expression of TNF-α and IL-1β. Data from triplicate determination are shown (mean ± SD). **p*<0.05 and ***p*<0.01 indicate statistically significant differences from treatment with LPS alone.

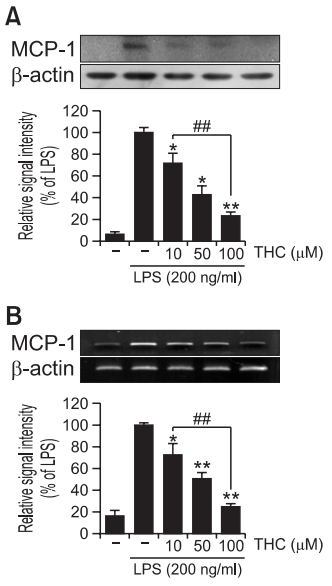

Fig. 3. Effect of THC on MCP-1 protein and mRNA expression in LPS-stimulated BV2 microglia cells. BV2 microglial cells were pretreated with THC (10, 50 and 100 μM) for 1 hr and then treated with LPS (200 ng/ml). (A) Cell lysates were subjected to SDS-PAGE, and then protein levels of MCP-1 were determined by Western blot analysis (top). Quantitative analysis was performed by densitometric analysis (bottom). (B) After 6 hr of LPS stimulation, representative image (top) and quantitative analysis (bottom) of MCP-1 mRNA expression were obtained using RT-PCR. THC significantly inhibited LPS-induced MCP-1 expression in both mRNA and protein levels. β-Actin was used as an internal control. Images are representative of three independent experiments that shows similar results. **p*<0.05 and ***p*<0.01 indicate statistically significant differences from treatment with LPS alone. ##*p*<0.01 indicates statistically significant differences between indicated groups.

antioxidant responses (Hsieh *et al.*, 2006; Innamorato *et al.*, 2008; Lee *et al.*, 2011a). In the present study, the effect of THC on the phosphorylation level of Nrf2 was examined in BV2 microglial cells. As shown in Fig. 4A, Nrf2 phosphorylation was significantly increased with THC treatment in a concentration-dependent manner (Fig. 4A). Furthermore, significant nuclear translocation of Nrf2 was observed with THC treatment in a concentration-dependent manner (Fig. 4B). Given that HO-1 is one of the downstream target genes of Nrf2, expression level of HO-1 was examined to determine whether increased phosphorylation of Nrf2 exerts its protective roles in the present study. Treatment of THC resulted in the up-regulation of HO-1 protein expression (Fig. 4C) as well as mRNA expression (Fig. 4D) in a concentration-dependent manner, suggesting that THC might provide cytoprotection through Nrf2-mediated HO-1 expression.

p38 mediates THC-induced phosphorylation of Nrf2

To understand the responsible signaling mechanism by which THC induces the phosphorylation and subsequent nuclear translocation of Nrf2, the involvement of multiple kinases that were previously reported as Nrf2 kinases was examined in the present BV2 microglial cell model. MAPK kinases have been reported to regulate NF-κB activation and synthesis of pro-inflammatory mediators in activated microglia (Zheng *et al.*, 2008), and to act as a specific target in inflammatory responses (Jung *et al.*, 2010). In the present study, THC markedly inhibited JNK and ERK 1/2 activation. However, phosphorylation of p38 kinase was significantly increased with THC treatment in a concentration-dependent manner (Fig. 5A). Blockade of p-38 activity with SB203058, a p-38 inhibitor, significantly abolished THC-induced Nrf2 phosphorylation and subsequent induction of HO-1 (Fig. 5B), suggesting that p-38 might be a key Nrf2 kinase in the present BV2 microglial cell model.

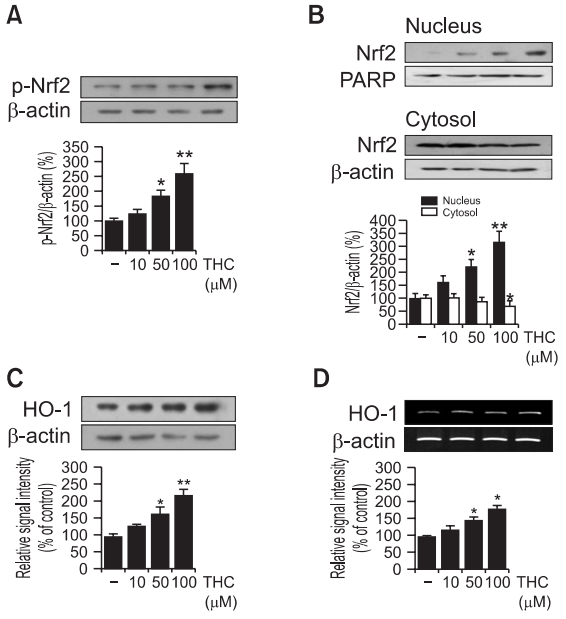

Fig. 4. Effects of THC on Nrf2 activation and HO-1 expression. (A&B) Phosphorylation and nuclear translocation of Nrf2. BV2 microglia cells were treated with THC (10-100 μM), and then Western blot analysis was performed. Total level of phosphorylated Nrf2 was determined (A) and nuclear translocation of Nrf2 was examined in nuclear and cytosol fractions (B). (C, D) HO-1 protein and mRNA expression. HO-1 protein level (C) was determined by immunoblotting after THC treatment. After 6 hr of THC treatment, the total RNA was isolated, HO-1 mRNA level (D) was determined by RT-PCR. β-Actin was used as an internal control. Quantitative analysis was carried out using densitometric analysis. Images are representative of three independent experiments that shows similar results. **p*<0.05 and ***p*<0.01 indicate statistically significant differences from treatment with LPS alone.

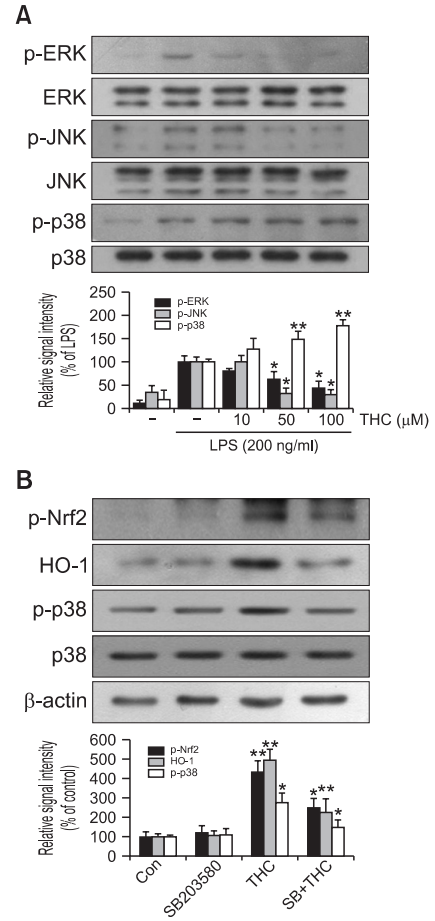

Fig. 5. THC-induced Nrf2 phosphorylation was mediated by p-38 MAPK. BV2 microglial cells were stimulated with 200 ng/ml LPS in the absence or presence of THC. (A) Western blot analysis was then performed to evaluate the activation of MAP kinases signaling pathways (top: representative image, bottom: quantitative analysis). Phosphorylation of p38 was increased in a concentration-dependent manner, but phosphorylation of ERK and JNK was decreased with THC treatment, suggesting that p-38 might play an important role in the THC-induced Nrf2 phosphorylation. (B) BV2 microglia cells were pretreated with the p38 inhibitor, SB203580 (5 μM), for 1 hr prior to THC treatment. THC-induced Nrf2 phosphorylation was blocked via p38 inhibition (top: representative image, bottom: quantitative analysis), indicating that p38 is responsible for the THC-induced phosphorylation of Nrf2. β-Actin was used as an internal control. Images are representative of three independent experiments that shows similar results. Quantitative analysis was carried out using densitometric analysis. **p*<0.05 and ***p*<0.01 indicate statistically significant differences from treatment with LPS alone.

DISCUSSION

The present study clearly demonstrates that THC significantly inhibits LPS-induced inflammatory responses through the activation of Nrf2 transcription factor in BV2 microglial cells. THC significantly suppressed LPS-induced production of NO, up-regulation of MCP-1, and pro-inflammatory cytokines such as TNF-α and IL-1β. THC induced the phosphorylation of Nrf2 and phosphorylated Nrf2 translocated to the nucleus, consequently resulting in the expression of cytoprotective genes including HO-1. THC-induced phosphorylation of Nrf2 was mediated by p38 MAPK.

Hydroxycinnamic acids and their ester derivatives have been reported to possess a variety of pharmacological activities including antitumor, antiviral, anti-inflammatory, immunosuppressive, and neuroprotective activities (Nagasaka *et al.*, 2007; Kim, 2010; Lee *et al.*, 2011b). Although it has been reported that THC exerts inhibitory activity against human neutrophil elastase, which is involved in the regulation of inflammatory processes (Steinbrecher *et al.*, 2008), the biological activity of THC has not been extensively determined. Recently, we reported that THC exhibits greater anti-inflammatory activity compared to caffeic acid, a prototype of hydroxycinnamic acids, in a BV2 microglial cell model (Lee *et al.*, 2012). However, the exact mechanism by which THC exerts anti-inflammatory activity needs to be determined.

Nrf2 is found in most cell types of the brain, including microglia, where it could modulate brain redox homeostasis and regulate inflammatory conditions (Lee *et al.*, 2011a). It has been known that activation of Nrf2 in microglia following brain injury plays a role in inhibiting microglia hyperactivation and preventing neuronal death caused by microgliosis (Koh *et al.*, 2009). It has been reported that Nrf2 exerts cytoprotective effects in animal models and CNS cell types for neurodegeneration (Innamorato *et al.*, 2008). Recent evidences have indicated that sulforaphane-treated animals displayed Nrf2 activation and increased in HO-1 (Lin *et al.*, 2008). Furthermore, CAPE-mediated activation of Nrf2 signaling seems to repress NF-κB signaling (Lee *et al.*, 2010). Therefore, Nrf2 signaling could be an attractive therapeutic target for brain inflammation. In the present study, THC resulted in the increased Nrf2 activation as well as up-regulation of HO-1, one of its downstream target genes. Under normal conditions, HO-1 levels are low, but can be up-regulated by a variety of stimuli, including oxidative stress and cytokines, and play a cytoprotective role in oxidative and inflammatory damage (Lu *et al.*, 2010). Actually, LPS-stimulated macrophages can protect themselves from overproduction of superoxide anion and NO by enhancing HO-1 expression (Lee *et al.*, 2005). It has been also reported that HO-1 inhibits the production of TNF-βa and IL-1β in response to LPS (Lee *et al.*, 2011a).

Many kinases and signaling pathways have been reported to be involved in the phosphorylation of Nrf2. MAPKs have been reported to exert a significant effect on the regulation of pro-inflammatory mediators and cytokines by controlling the activation of NF-κB in microglia (Jung *et al.*, 2010). The present results showed that p38 MAPK, but not ERK and JNK, was significantly activated with THC. Furthermore, blockade of p38 significantly attenuated THC-mediated Nrf2 phosphorylation and HO-1 expression, suggesting that p38 MAPK signaling pathway plays a key role in THC-induced Nrf2 phosphorylation. PI3K-Akt signaling pathway has been reported to play a role in Nrf2 activation in RAW 264.7 cells (Ha *et al.*, 2011). Contrary to the previous report, the decrease of Akt phosphorylation was observed with THC in the present study (data not shown), suggesting that Akt signaling pathway might not be responsible for the phosphorylation of Nrf2 in the present BV2 microglial cell model. Based on a previous report that PKC mediates nuclear translocation of Nrf2 in human monocytic cells (Rushworth *et al.*, 2005), the role of PKC in the THC-induced phosphorylation of Nrf2 was examined using Ro-31-8220, a pan-PKC inhibitor. The results indicated that PKC signaling does not play a noticeable role in THC-induced Nrf2 phosphorylation because Nrf2 phosphorylation and HO-1 expression levels were not changed with Ro-31-8220 (data not shown).

In conclusion, the present study clearly demonstrates that THC exerts anti-inflammatory activity through the activation of Nrf2 transcription, which is achieved by p38 MAPK signaling pathway in LPS-stimulated BV2 microglial cells, suggesting that THC might be a valuable therapeutic agent in the treatment of inflammation-related brain disorders.

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