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7-Methoxy-(9H-β-Carbolin-1-il)-(*E*)-1-Propenoic Acid, a β-Carboline Alkaloid From *Eurycoma longifolia*, Exhibits Anti-Inflammatory Effects by Activating the Nrf2/Heme Oxygenase-1 Pathway

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ABSTRACT

Eurycoma longifolia is an herbal medicinal plant popularly used in Southeast Asian countries. In the present study, we show that 7-methoxy-(9H-β-carbolin-1-il)-(*E*)-1-propenoic acid (7-MCPA), a β-carboline alkaloid isolated from *E. longifolia*, exerted anti-inflammatory effects by activating the nuclear factor-E2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) pathway. 7-MCPA inhibited lipopolysaccharide (LPS)-induced production of nitric oxide (NO), prostaglandin E_2 (PGE_2), and interleukin-6 (IL-6) in RAW264.7 cells and rescued C57BL/6 mice from LPS-induced lethality in vivo. LPS-induced expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and IL-6 was also significantly suppressed by treatment of 7-MCPA in RAW264.7 cells. 7-MCPA induced nuclear translocation of Nrf2 and increased transcription of its target genes, such as HO-1. Treating RAW264.7 cells with 7-MCPA increased the intracellular level of reactive oxygen species (ROS) and the phosphorylation level of p38 mitogen-activated protein kinase (MAPK); however, co-treatment with the antioxidant N-acetyl-cysteine (NAC) blocked 7-MCPA-induced p38 MAPK phosphorylation. Moreover, NAC or SB203580 (p38 MAPK inhibitor) blocked 7-MCPA-induced nuclear translocation of Nrf2, suggesting that 7-MCPA activated Nrf2 via a ROS-dependent p38 pathway. 7-MCPA induced HO-1 protein and mRNA expression and knockdown of Nrf2 with siRNA or SB203580 blocked 7-MCPA-mediated induction of HO-1 expression. Inhibiting Nrf2 or HO-1 abrogated the anti-inflammatory effects of 7-MCPA in LPS-stimulated RAW264.7 cells. We also demonstrated that 7-MCPA suppressed LPS-induced nuclear factor κB (NF-κB) activation. These results provide the first evidence that 7-MCPA exerts its anti-inflammatory effect by modulating the Nrf2 and NF-κB pathways and may be a potential Nrf2 activator to prevent or treat inflammatory diseases. J. Cell. Biochem. 117: 659–670, 2016.

KEY WORDS: *Eurycoma longifolia*; β-CARBOLINE ALKALOID; Nrf2; ANTI-INFLAMMATION; REACTIVE OXYGEN SPECIES; p38 MAPK

Inflammation is an adaptive response that is triggered by noxious stimuli and conditions, such as infection and tissue injury [Medzhitov, 2008]. Proinflammatory cells, mainly activated macrophages, mediate most of the cellular and molecular pathophysiology of inflammation by producing various kinds of inflammatory mediators, including tumor necrosis factor-α (TNF-α), nitric oxide (NO), interleukin-6 (IL-6), prostaglandin E_2 (PGE_2), and reactive oxygen species (ROS) [Laskin et al., 2011; Kang et al., 2012]. Excess or unregulated production of these mediators mediates or exacerbates a wide variety of diseases, such as sepsis, rheumatoid arthritis, atherosclerosis, and even cancer [Lawrence et al., 2002; Tabas and Glass, 2013].

One of the pathways implicated in the control of inflammation is the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2, a member of basic-leucine zipper transcription factor, regulates the expression of numerous detoxifying and antioxidant

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genes, such as heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase (NQO1), glutathione S-transferases, and γ-glutamyl cysteine synthetase catalytic subunit (GCLC) through consensus *cis*-elements called antioxidant-response elements [Jaramillo and Zhang, 2013]. Under basal conditions, the Kelch-like ECH-associated protein (Keap1) binds to Nrf2 and brings Nrf2 into the Keap1-Cul3-E3 ubiquitin ligase complex, leading to ubiquitination and subsequent degradation of Nrf2 [Jaramillo and Zhang, 2013]. Nrf2 is released from Keap1 in response to oxidative stress, translocates to the nucleus, forms a heterodimer with the small Maf protein, and binds to antioxidant-related elements in the promoter regions of antioxidant and cytoprotective genes [Jaramillo and Zhang, 2013]. In addition, phosphorylation of Nrf2 is an alternative mechanism for Nrf2 activation. A variety of kinases, including mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinases (ERK), c-jun N-terminal kinases (JNK) and p38 kinases, have been shown to involve in the phosphorylation of Nrf2 [Alfieri et al., 2008]. Activation of the Nrf2 defense response has been shown to protect against neurodegenerative diseases, aging, diabetes, photo-oxidative stress, cardiovascular disease, inflammation, pulmonary fibrosis, acute pulmonary injury, and cancer [Boutten et al., 2011; Jaramillo and Zhang, 2013].

HO-1 is the major anti-inflammatory and anti-oxidative enzyme that is regulated by activating Nrf2 [Kapturczak et al., 2004; Paine et al., 2010]. Heme oxygenase (HO) catalyzes the rate-limiting enzymatic step of heme degradation and produces carbon monoxide, ferrous iron, and biliverdin, which is converted into bilirubin via biliverdin reductase [Paine et al., 2010]. Two genetically distinct HO isozymes, HO-1 and HO-2, are known [Paine et al., 2010]. HO-2 represents the constitutive isoform [Paine et al., 2010]. By contrast, the inducible isoform HO-1 is highly up-regulated by various stimuli, including inflammatory mediators and oxidative stress-related factors [Maines and Panahian, 2001]. HO-1 maintains cellular homeostasis and plays an important protective role in tissues to reduce oxidative injury and attenuate the inflammatory response [Abraham and Kappas, 2008; Paine et al., 2010]. Expression of HO-1 or treatment with carbon monoxide suppresses production of the proinflammatory cytokines and chemokines in activated macrophages [Morse et al., 2003; Nakahira et al., 2006; Suh et al., 2006; Wang et al., 2009; Choo et al., 2015]. Thus, targeting the Nrf2/HO-1 pathway with natural phytochemicals could be a potential strategy for the prevention or treatment of inflammatory diseases [Motterlini and Foresti, 2014].

Eurycoma longifolia Jack. (Simaroubaceae) is an important medicinal plant in Southeast Asian countries [Bhat and Karim, 2010]. It is known locally as "Cay Ba Benh" in Vietnam, "pasak bumi" in Indonesia, and "tongkat ali" in Malaysia [Tran et al., 2014]. The roots of this plant have been widely used as a single ingredient or as a part of a mixture with other herbs for the treatment of sexual insufficiency, malaria, aches, dysentery, and fever [Bhat and Karim, 2010]. The effectiveness of *E. longifolia* is attributed to various bioactive constituents present in the plant, including quassinoids, canthin-6-one, and β-carboline alkaloids, etc. [Bhat and Karim, 2010]. A recent study showed that several quassinoids and β-carboline alkaloids from *E. longifolia* exert potent NF-κB inhibitory activities induced by TNF-α [Tran et al., 2014]. However, the anti-inflammatory activity of *E. longifolia* has not been studied well. In our continuing search for Nrf2 activators in herbal medicinal plants, we identified 7-MCPA, a β-carboline alkaloid, isolated from hairy-root cultures of *E. longifolia* as a potent Nrf2 activator. In the present study, we investigated the anti-inflammatory effects of 7-MCPA and its underlying mechanisms in RAW264.7 cells. We show here that 7-MCPA inhibited the lipopolysaccharide (LPS)-induced inflammatory responses in vitro by activating the Nrf2/HO-1 pathway via a ROS-dependent p38 MAPK pathway and protected mice from LPS-induced septic shock in vivo.

MATERIALS AND METHODS

CELL CULTURE AND CHEMICALS

RAW264.7 cells were cultured in Dulbecco's Modified Essential Medium (DMEM) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (Cambrex, Charles City, IA) under a humidified 5% CO_2 atmosphere at 37°C. Tin protoporphyrin IX (SnPP) and copper protoporphyrin IX (CuPP) were purchased from Porphyrin Products Inc. (Logan, UT). U0126, SB203580, and SP600125 were obtained from Calbiochem (San Diego, CA). N-acetyl-L-cysteine (NAC) and MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide were purchased from Sigma (St. Louis, MO). DAPI (4′,6-diamidino-2-phenylindole) and 2′,7′-dichlorofluorescin diacetate (DCF-DA) were from Invitrogen (Carlsbad, CA).

ANTIBODIES

Anti-poly ADP-ribose polymerase (PARP), anti-HO-1, anti-Nrf2 and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-IκBα, anti-phospho-IκBα, Anti-phospho-AKT, anti-AKT, anti-phospho-p38, anti-p38, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-JNK, and anti-JNK antibodies were from Cell Signaling Technology (Danvers, MA). Anti-α-tubulin antibody were purchased from Sigma. Alexa 546-conjugated goat anti-mouse secondary antibody was from Invitrogen.

ISOLATION OF 7-METHOXY-(9H-β-CARBOLIN-1-IL)-(*E*)-2-PROPENOIC ACID

7-methoxy-(9H-β-carbolin-1-il)-(*E*)-1-propenoic acid (7-MCPA) was isolated from the hairy root cultures of *E. longifolia* as previously described [Ngoc et al., 2015]. The chemical structure is shown in Figure 1. 7-MCPA was obtained as amorphous yellow powder, showed a $[M+H]^+$ peak at *m/z* 269, by electron spray ionization-mass spectrometry [Ngoc et al., 2015] and the purity of 7-MCPA was checked by ^1H and ^{13}C NMR spectra [Ngoc et al., 2015]. 7-MCPA was solubilized with dimethyl sulfoxide and used at a final concentration of less than 0.05% dimethyl sulfoxide.

MEASUREMENT OF NO, PGE_2, AND IL-6, AND THE CELL VIABILITY ASSAY

The amounts of NO, PGE_2, and IL-6 in the culture supernatants were measured as described previously [Kang et al., 2012; Choo et al., 2015]. Briefly, RAW264.7 cells were treated for 30 min with various

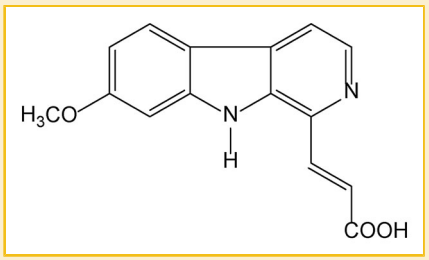

Fig. 1. Chemical structure of 7-MCPA.

concentrations of 7-MCPA and then stimulated 1 μg/ml LPS for 24 h. In some experiments, the cells were treated with 7-MCPA together with SnPP or CuPP. NO production was measured by the Griess reaction, and the amounts of IL-6 and PGE_2 were measured by ELISA according to the manufacturer's protocol (R&D systems, Minneapolis, MN). The MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] assay was used to determine the viability of RAW264.7 cells treated with 7-MCPA.

MEASUREMENT OF INTRACELLULAR REACTIVE OXYGEN SPECIES (ROS) LEVEL

2′,7′-dichlorofluorescin diacetate (DCF-DA) was used to measure the intracellular ROS levels as described elsewhere [Choo et al., 2015]. Briefly, RAW264.7 cells were pre-loaded for 30 min with 25 μM DCF-DA in PBS. Subsequently, the cells were washed with PBS twice, and then were incubated for 30 min with various concentrations of 7-MCPA at 37°C in the dark. The intensity of the fluorescence signal was detected with an excitation wave length of 480 nm and an emission wave length of 535 nm by Synergy MX Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT).

WESTERN BLOT ANALYSIS

Western blot analysis was performed as described elsewhere [Hwangbo et al., 2010; Choo et al., 2015]. Briefly, cells were lysed in lysis buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 5mM sodium orthovanadate, 1% NP-40 and protease inhibitors cocktail (BD Biosciences). In certain experiments, the cytoplasmic and nuclear fractions were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Thermo Fisher Scientific, Rockford, IL). Fifty micrograms of protein per lane were resolved by SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane and probed with the proper antibodies. The signal was detected using an enhanced chemiluminescent system (Intron, Seongnam, Korea).

NF-κB REPORTER ASSAY

NF-κB reporter assay was performed as described previously [Kang et al., 2012]. Briefly, RAW264.7 cells were transfected with pNF-κB-Luc (Stratagene, La Jolla, CA) together with pRL-CMV vector (Promega, Madison, WI) as an internal control using Fugene 6 (Promega). After 24 h, the cells were treated for 30 min with various concentrations of 7-MCPA and then stimulated with 1 μg LPS. Luciferase activity was determined with Synergy MX Multi-Mode Microplate Reader (BioTek, Instruments, Winooski, VT) by measuring light emission for 10 s. The results were normalized to the activity of *renilla*.

REAL-TIME QUANTITATIVE PCR

Total RNA was isolated using RNeasy Mini Kit from Qiagen (Santa Clarita, CA). One μg of total RNA was used to synthesis first stranded cDNA using Superscript RT kit (Invitrogen). Real-time quantitative PCR (qPCR) was performed to measure mRNA expression levels of iNOS, COX-2, IL-6, HO-1, NQO1 and GCLC as described previously [Choo et al., 2015]. The primers for iNOS, COX-1 IL-6 and β-actin were described elsewhere [Choo et al., 2015] and the primers for HO-1, NQO1 and GCLC were as follows; *HO-1*, 5′- CGC AAC AAG CAG AAC CCA-3′ (sense) and 5′- GCG TGC AAG GGA TGA TTT CC-3′ (antisense); *NQO1*, 5′-CGC CTG AGC CCA GAT ATT GT-3′ (sense) and 5′-GCA CTC TCT CAA ACC AGC CT-3′ (antisense); *GCLC*, 5′-GTC TGA CAC GTA GCC TCG GTA A-3′ (sense) and 5′-TGG CCA CTA TCT GCC CAA TT-3′ (antisense).

SMALL INTERFERING RNA AND TRANSFECTION

Nrf2 small interfering RNA (siRNA) and the scrambled control siRNA were obtained from OriGene Technologies (Rockville, MD). Briefly, RAW264.7 cells were plated in 60 mm culture dishes, and then transfected with Nrf2 siRNA or the control siRNA using Fugene HD, according to the instructions of the manufacturer (Promega). At 48 h after transfection, the cells were used for further experiments. Efficiency of Nrf2 knockdown was determined by Western blot analysis.

IMMNOCYTOCHEMISTRY AND CONFOCAL MICROSCOPY

Immunofluorescent confocal microscopy was performed with an OLYMPUS FV1000 as described previously [Hwanbo et al., 2010; Choo et al., 2015]. Briefly, cells were, fixed in fresh 4% paraformaldehyde, and permeabilized in 0.5% TritonX-100. After blocking with 1% goat serum, the cells were incubated with anti-Nfr2 antibody (1:200 dilution), washed, and followed by incubation with Alexa Fluor 546 goat anti-rabbit secondary antibody (1:250 dilution) for 3 h at room temperature. Subsequently, the cells were washed, stained with DAPI, and mounted. Confocal images were acquired as described previously [Hwangbo et al., 2010; Choo et al., 2015].

EFFECT OF 7-MCPA ON LPS-INDUCED MORTALITY IN C57BL/6 MICE

Male C57BL/6 mice (20–22 g) were housed in plastic cages and maintained at 22 ± 2°C and 50–60% relative humidity with 12-h light-dark cycles throughout the experiment and the animals were maintained in these facilities for at least 1 week before the experiment [Kim et al., 2014; Choo et al., 2015]. Animal experiments were approved by the Animal Research Committee of Kangwon National University. Mice (8 mice per group) were injected intraperitoneally with 7-MCPA (30 mg/kg and 10 mg/kg) dissolved in dimethyl sulfoxide:chremophore-EL:PBS (1:1:8 by volume) or control vehicle 1 h before LPS (Escherichia coli 0111:B4; Sigma-Aldrich, 40 mg/kg body weight) injection, as described previously [Kim et al., 2014; Choo et al., 2015]. Mortality was monitored for 5 days after injection of LPS, after which no further deaths of mice occurred.

STATISTICAL ANALYSIS

Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by the Fisher least significant difference test. $P < 0.05$ was considered statistically significant. The results are presented as the mean ± standard error of mean (SEM).

RESULTS

7-MCPA INHIBITS LPS-INDUCED PRODUCTION OF NO, PGE_2, AND IL-6 IN RAW264.7 CELLS AND PROTECTS C57BL/6 MICE FROM LPS-INDUCED MORTALITY

To investigate the anti-inflammatory effect of 7-MCPA, we determined whether 7-MCPA inhibits production of PGE_2, IL-6, and NO in LPS-stimulated RAW264.7 cells. After RAW264.7 cells were stimulated with 1 μg/ml LPS for 24 h in the presence of various concentrations of 7-MCPA, the amounts of IL-6, PGE_2, and NO in the culture supernatant were measured (Fig. 2A, 2B, and 2C). Pretreating RAW264.7 cells with 7-MCPA attenuated LPS-induced production of IL-6, PGE_2, and NO in a concentration-dependent manner with IC_{50} values of 5.3 ± 0.9, 9.2 ± 1.5, and 4.9 ± 0.8 μM, respectively. In contrast, 7-MCPA did not affect cell viability, as measured by the MTT assay at concentrations that inhibited the LPS-induced inflammatory response (Fig. 2D). These results indicated that 7-MCPA inhibits the LPS-induced inflammatory response without affecting cell viability.

We next investigated whether 7-MCPA rescued C57BL/6 mice from LPS-induced mortality (Fig. 2E). Mice were intraperinoneally injected with 7-MCPA or vehicle 1 h before LPS challenge. 7-MCPA significantly increased mortality rate compared with vehicle-treated mice. In contrast to vehicle-treated mice that had a mortality rate of 62.5%, mice receiving 30 mg/kg 7-MCPA had a mortality rate of 12.5% after receiving LPS.

7-MCPA SUPPRESSES LPS-INDUCED EXPRESSION OF INOS, COX-2, AND IL-6 IN RAW264.7 CELLS

Next, we determined whether 7-MCPA suppresses the mRNA expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and IL-6 in LPS-stimulated RAW264.7 cells. RAW264.7 cells were stimulated for 8 h with LPS in the presence of various concentrations of 7-MCPA, and the mRNA expression levels of iNOS, COX-2, and IL-6 were determined by a real-time qPCR analysis. 7-MCPA treatment decreased mRNA of iNOS, COX-2 and IL-6 levels concentration-dependently (Fig. 3A–C). These results suggested that that 7-MCPA suppresses LPS-induced expression of iNOS, COX-2, and IL-6 at the transcriptional level.

7-MCPA INCREASES CELLULAR ROS LEVEL AND ACTIVATES NRF2 IN RAW264.7 CELLS

We next determined whether 7-MCPA activates Nrf2 in RAW264.7 cells. Treating RAW264.7 cells with 7-MCPA concentration-dependently increased Nrf2 nuclear translocation (Fig. 4A). To further confirm that 7-MCPA activates Nrf2, we determined the effect of 7-MCPA on the expression of Nrf2 target genes, such as HO-1, NQO1, and the γ-glutamyl cysteine synthetase catalytic subunit (GCLC), in RAW264.7 cells by real-time qPCR analysis. Treating RAW264.7 cells with 7-MCPA concentration-dependently increased HO-1, NQO1, and GCLC mRNA levels (Fig. 4B–D), suggesting that 7-MCPA activates Nrf2. Oxidative stress plays a critical role in activation of Nrf2 [Jaramillo and Zhang, 2013]. Therefore, we examined whether 7-MCPA increases ROS. Treating RAW264.7 cells with 7-MCPA increased the intracellular ROS level in a concentration-dependent manner. Co-treatment of RAW264.7 cells with 7-MCPA and the antioxidant NAC significantly suppressed both nuclear translocation of Nrf2 and intracellular ROS induced by 7-MCPA (Fig. 4A and 4E), suggesting that oxidative stress is involved in 7-MCPA-mediated activation of Nrf2.

7-MCPA ACTIVATES NRF2 VIA A ROS-DEPENDENT P38 MAPK PATHWAY

As ROS mediate activation of various kinases such as MAPKs [Ray et al., 2012], we investigated whether inhibiting MAPKs would affect 7-MCPA-mediated Nrf2 activation (Fig. 5A). Co-treatment with SP600125 (JNK inhibitor) or U0126 (MEK inhibitor) failed to modulate 7-MCPA-induced Nrf2 nuclear translocation. In contrast, SB203580 (p38 inhibitor) significantly blocked 7-MCPA-induced Nrf2 activation, as NAC did. We also confirmed this result with immunofluorescence, where SB203580 or NAC significantly blocked 7-MCPA-mediated Nrf2 nuclear translocation (Fig. 5B). We next determined whether 7-MCPA activates p38 MAPK (Fig. 5C). Treating RAW264.7 cells with 7-MCPA for 30 min increased the p38 MAPK phosphorylation level in a concentration-dependent manner. Moreover, NAC blocked 7-MCPA-mediated p38 MAPK phosphorylation. However, 7-MCPA did not induce ERK1/2, JNK, or AKT phosphorylation. A time course p38 MAPK phosphorylation experiment with 30 μM 7-MCPA revealed that the p38 MAPK phosphorylation level increased at 15 min and gradually returned to basal levels by 120 min after treatment (Fig. 5D). Taken together, these results suggest that 7-MCPA may mediate Nrf2 activation via a ROS-dependent p38 pathway.

7-MCPA INDUCES HO-1 EXPRESSION BY ACTIVATING NRF2 THROUGH A ROS-DEPENDENT P38 PATHWAY

Among Nrf2 target genes, HO-1 has pronounced anti-inflammatory activity [Kapturczak et al., 2004; Paine et al., 2010]. Thus, we further investigated 7-MCPA-mediated induction of HO-1. 7-MCPA concentration-dependently increased the expression level of HO-1 protein (Fig. 6A). In a time course experiment of HO-1 induction, the level of HO-1 protein began to increase at 3 h after 30 μM 7-MCPA treatment and reached a maximum at 6 h, before returning to basal levels at 24 h (Fig. 6B). A real-time PCR analysis revealed that the HO-1 mRNA expression level peaked at 3 h and decreased steadily for 12 h after the 7-MCPA treatment (Fig. 6C).

We next determined whether inhibiting p38 MAPK affected the 7-MCPA-mediated induction of HO-1. Similar with the effect of SB203580 on 7-MCPA-mediated activation of Nrf2, co-treatment with SB203580 significantly blocked 7-MCPA-induced HO-1 protein and mRNA expression as NAC did; however, JNK and MEK inhibitors failed to modulate 7-MCPA-induced HO-1 mRNA and protein expression (Fig. 6D and 6E), suggesting that p38 MAPK could be an essential role in 7-MCPA-induced expression of HO-1. Moreover, siRNA knockdown of Nrf2 blocked 7-MCPA-mediated HO-1

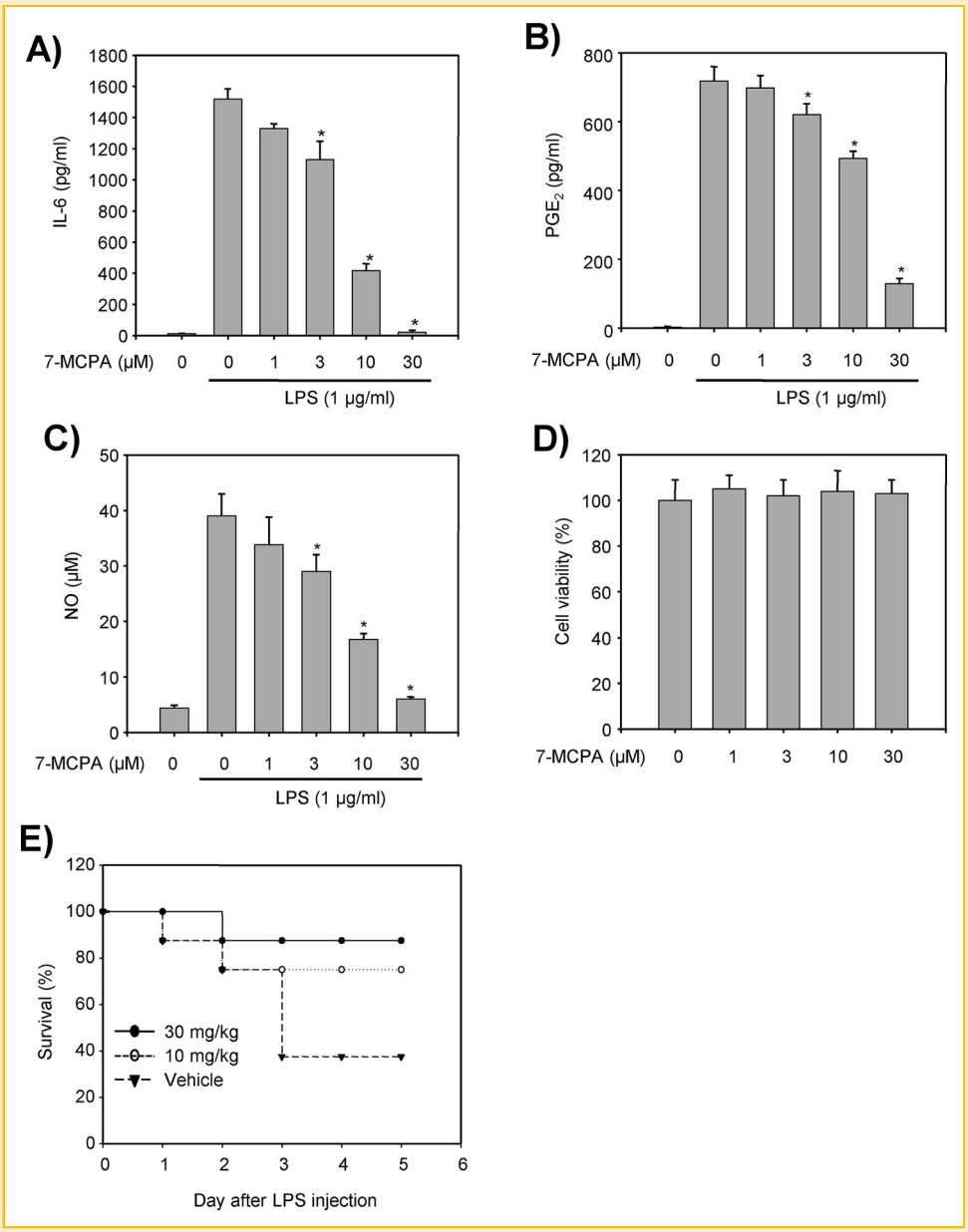

Fig. 2. 7-MCPA inhibits LPS-induced inflammatory responses. A–C: RAW264.7 cells were incubated for 30 min with the indicated concentrations of 7-MCPA prior to stimulation with LPS (1 μg/ml) for 24 h. The amounts of IL-6 (A), PGE_2 (B), and NO (C) in the culture supernatants were determined. Data are presented as mean ± SEM (* $P < 0.01$ compared with LPS only, n = 6). D: RAW264.7 cells were incubated for 24 h with the indicated concentrations of 7-MCPA, and cell viability was determined by MTT assay. Data are presented as mean ± SEM (n = 6). E: The protective effect of 7-MCPA on LPS-induced mortality in C57BL/6 mice. C57BL/6 mice (8 mice/group) were challenged with LPS after being injected with the indicated 7-MCPA doses. Survival was determined during 5 days after LPS injection.

expression (Fig. 6F). These results suggest that 7-MCPA induces HO-1 expression by activating Nrf2 via a ROS-dependent p38 pathway.

INHIBITION OF THE NRF2/HO-1 PATHWAY BLOCKS 7-MCPA ANTI-INFLAMMATORY ACTIVITY

We examined whether 7-MCPA-mediated induction of HO-1 plays the major role in the anti-inflammatory effect of 7-MCPA. SnPP, a specific HO-1 inhibitor, attenuated 7-MCPA-mediated inhibition of the production of NO, PGE_2, and IL-6 in LPS-stimulated RAW264.7 cells; however, CuPP, a negative control of SnPP, had no effect (Fig. 7A–C). Furthermore, Nrf2 knockdown by siRNA significantly reversed 7-MCPA-mediated suppression of iNOS and COX-2 expression in LPS-stimulated RAW264.7 cells (Fig. 7D). These results suggest that inducing HO-1 by activating Nrf2 could play the major role in the anti-inflammatory effect of 7-MCPA in RAW264.7 cells.

7-MCPA INHIBITS LPS-INDUCED ACTIVATION OF NF-κB

Activating Nrf2 attenuates activation of NF-κB [Li et al., 2008; Cuadrado et al., 2014]; thus, we examined whether 7-MCPA

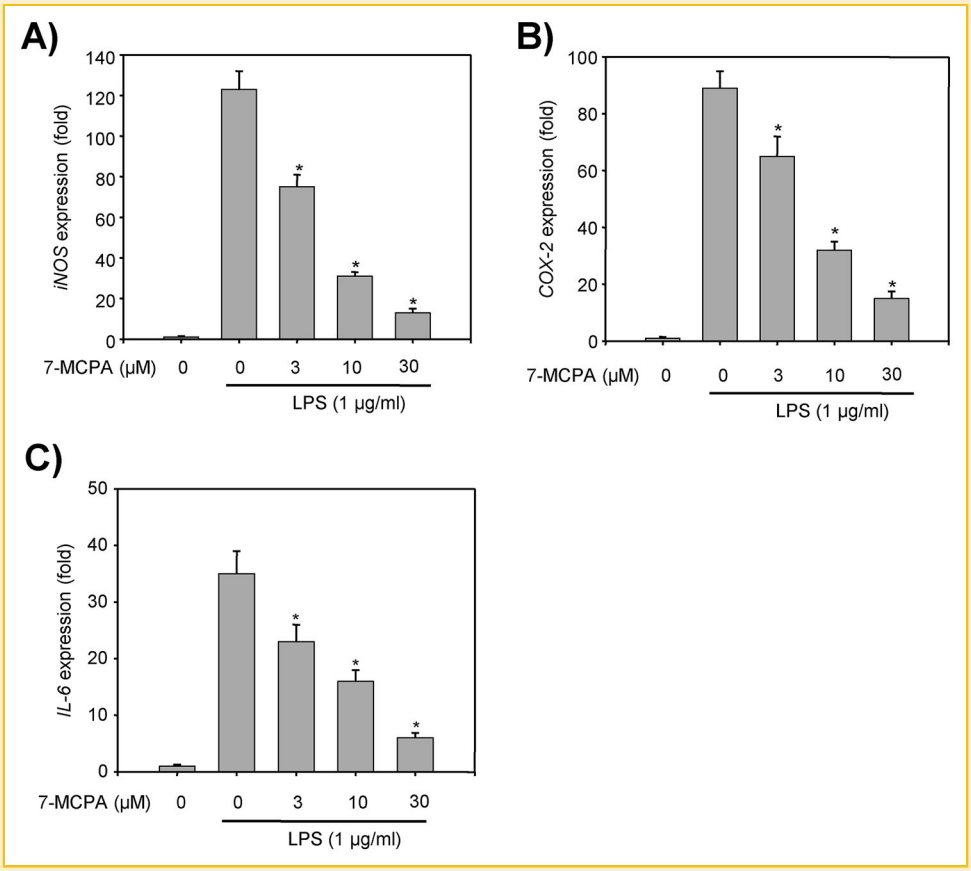

Fig. 3. 7-MCPA inhibits LPS-induced iNOS, COX-2, and IL-6 expression in RAW264.7 cells. A–C: RAW264.7 cells were incubated for 30 min with the indicated concentrations of 7-MCPA prior to stimulation with LPS (1 μg/ml) for 8 h. Subsequently, total RNAs were prepared and then performed real-time qPCR analysis to determine the mRNA expression levels of iNOS (A), COX-2 (B), and IL-6 (C). Data are presented as mean ± SEM ($^*P < 0.01$ compared with LPS only, n = 5).

suppressed LPS-induced activation of NF-κB. 7-MCPA concentration-dependently inhibited LPS-induced expression of an NF-κB reporter gene construct (Fig. 8A). We examined whether 7-MCPA suppressed LPS-induced degradation and phosphorylation of the IκBα protein, which is an essential step to activate NF-κB. 7-MCPA prevented phosphorylation and degradation of the IκBα protein in LPS-treated RAW264.7 cells after 15 min stimulation (Fig. 8B). We also found that pretreating RAW264.7 cells with 7-MCPA significantly inhibited LPS-induced phosphorylation p38 MAPK and JNK in a time- and concentration-dependent manner (Fig. 8C and 8D).

DISCUSSION

7-MCPA is a β-carboline alkaloid isolated from *E. longifolia*. In the present study, we demonstrated the potential involvement of the Nrf2/HO-1 pathway in the anti-inflammatory activity of 7-MCPA. This compound activated Nrf2 and subsequent Nrf2 target gene expression, such as HO-1, NQO1, and GCLC, via a ROS-dependent p38 MAPK pathway in RAW264.7 cells. Induction of this pathway was correlated with suppressing NO, PGE_2, and IL-6 production in LPS-stimulated RAW264.7 cells. 7-MCPA also protects C57BL/6 mice from LPS-induced death. This is the first report describing that a β-carboline alkaloid suppresses the LPS-induced inflammatory response by activating Nrf2.

E. longifolia extract can be consumed as a tonic drink or is available in the health-food market either in a raw crude powder form or a capsule mixed with other aphrodisiac herbs. In Vietnam, a decoction and an alcoholic extract of *E. longifolia* roots are used to treat rheumatism [Bich et al., 2004; Tran et al., 2014]. This plant is a rich source of bioactive compounds, predominated by alkaloids and quassinoids [Bhat and Karim, 2010; Park et al., 2014; Tran et al., 2014]. Some quassinoids and alkaloids from *E. longifolia* extracts inhibit TNF-α-induced activation of NF-κB [Tran et al., 2014], although the molecular mechanisms by which these compounds inhibits NF-κB activation have not been identified. In the present study, we showed that the β-carboline alkaloid 7-MCPA inhibited the LPS-induced inflammatory response in RAW264.7 cells by activating Nrf2 via a ROS-dependent p38 pathway.

β-carboline alkaloids are of interest due to their diverse pharmacological activities, including sedative, anxiolytic, hypnotic, anticonvulsant, antitumor, antiviral, antiparasitic as well as antimicrobial activities [Cao et al., 2007]. Only a few investigations

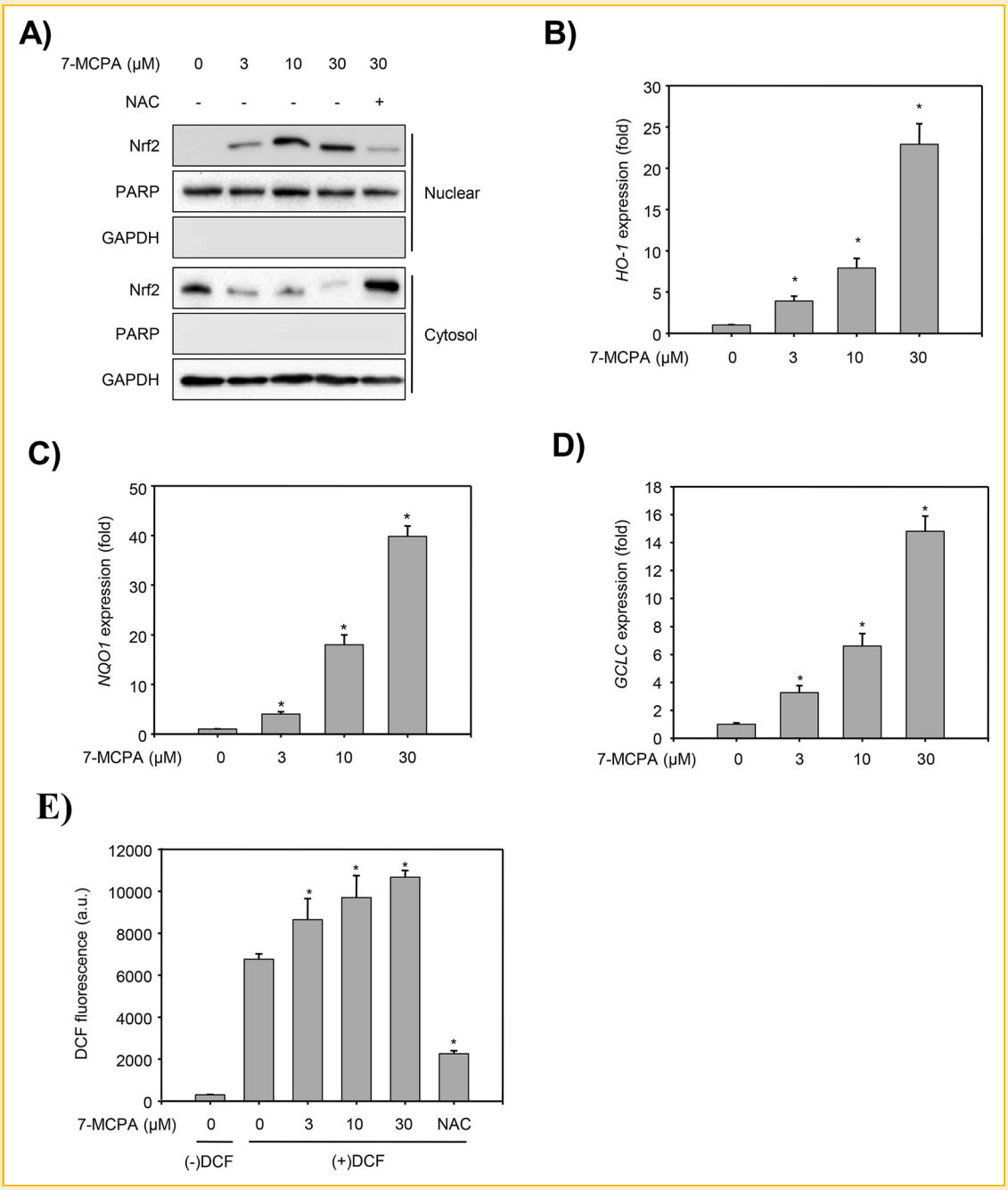

Fig. 4. 7-MCPA activates Nrf2 in RAW264.7 cells. A: The cells were treated for 2 h with the indicated concentrations of 7-MCPA with or without NAC (100 μM). Cytoplasmic and nuclear extracts were prepared and then performed Western blot analysis with the indicated antibodies. PARP and GAPDH were used as a marker protein for nuclear and cytosolic fractions, respectively. B–D: RAW264.7 cells were incubated for 8 h with the indicated concentrations of 7-MCPA. Subsequently, total RNAs were prepared and performed real-time qPCR analysis to determine the mRNA expression levels of HO-1 (B), NQO1 (C), and GCLC (D). Data are presented as mean ± SEM ($^*P < 0.01$ compared with LPS only, n = 5). E: 7-MCPA increases intracellular ROS level. The cells were incubated for 30 min with the indicated concentrations of 7-MCPA or NAC (100 μM). Intracellular ROS levels were measured by DCF-DA staining. Data are presented as mean ± SEM ($^*P < 0.01$ compared with vehicle only, n = 6).

have reported on the anti-inflammatory activities of β-carboline derivatives. For example, quassidine F, 6-methoxy-3-vinyl-β-carboline, and 6,12-dimethoxy-3-vinyl-β-carboline isolated from *Picrasma quassioides* inhibit the LPS-induced inflammatory response in RAW264.7[Zhao et al., 2012]. Two β-carboline alkaloids isolated from *Melia azedarach*, such as 4,8-dimethoxy-1-vinyl-beta-carboline and 4-methoxy-1-vinyl-beta-carboline, also inhibit the LPS-induced inflammatory response in RAW264.7 [Lee et al., 2000]. In the present study, we identified 7-MCPA as a potent Nrf2 activator, and 7-MCPA-mediated activation of Nrf2 was correlated

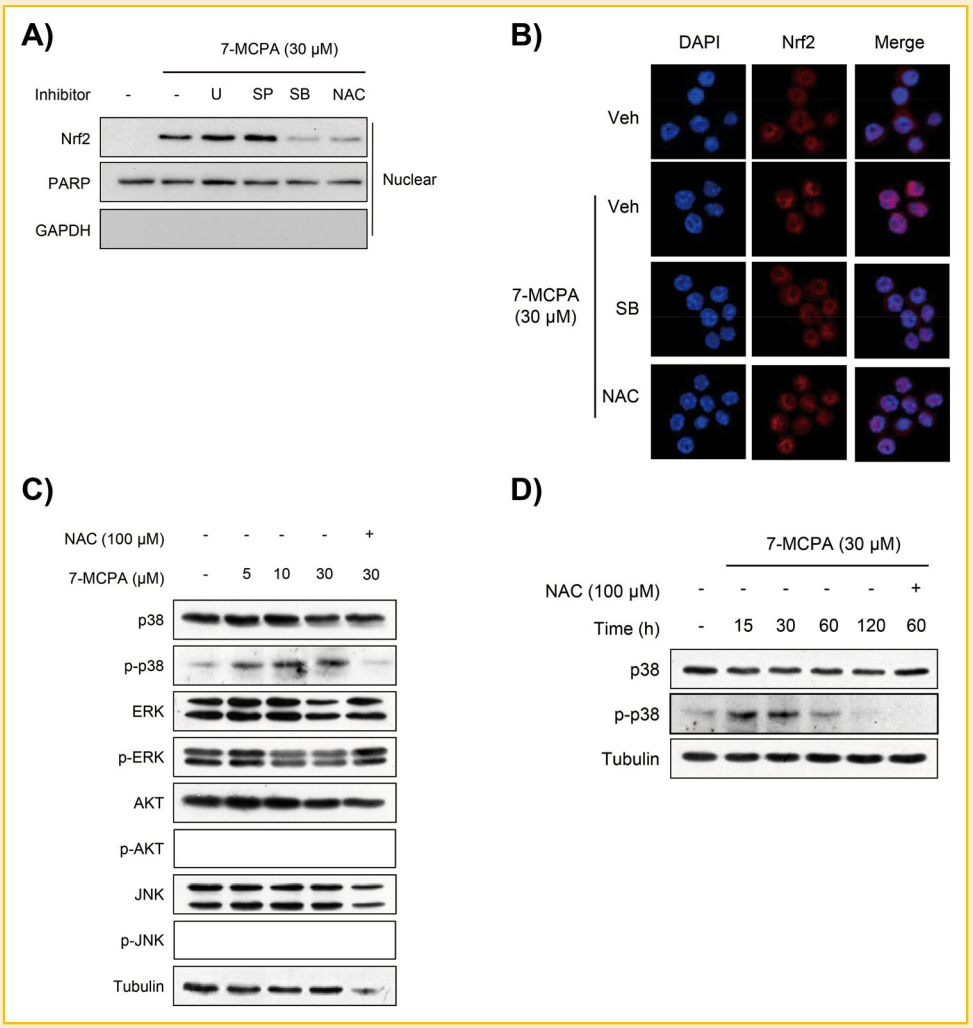

Fig. 5. 7-MCPA activates Nrf2 via a ROS-dependent p38 MAPK pathway. A: RAW264.7 cells were pretreated for 30 min with U0126 (U, 10 μM), SP600125 (SP, 10 μM), SB203580 (SB, 10 μM), or NAC (100 μM) and then further incubated with 7-MCPA (30 μM) for 2 h. Nuclear extracts were prepared and then performed Western blot analysis with the indicated antibodies. PARP and GAPDH were used as a marker protein for nuclear and cytosolic fractions, respectively. B: RAW264.7 cells were incubated for 30 min with SB203580 (SB, 10 μM) or NAC (100 μM), and then further incubated for 2 h with 7-MCPA. Nrf2 was stained with an anti-Nrf2 antibody and then visualized with a secondary antibody conjugated with Alexa Fluor 546. DAPI was used to stain nuclei. Veh, vehicle. C: RAW264.7 cells were incubated for 30 min with the indicated concentrations of 7-MCPA alone or together with NAC (100 μM), and total cell lysates were prepared and performed Western blot analysis with the indicated antibodies. D: RAW264.7 cells were incubated for the indicated periods of time with 7-MCPA (30 μM) alone or together with NAC (100 μM), and total cell lysates were prepared and then performed Western blot analysis with the indicated antibodies.

with suppression of the inflammatory response in LPS-stimulated RAW264.7 cells. Thus, our results provide an important understanding the anti-inflammatory mechanism of β-carboline alkaloids.

Induction of HO-1 is widely recognized as an effective cellular strategy to counteract cellular damage and inflammation [Hwangbo et al., 2009; Motterlini and Foresti, 2014]. There is a growing body of evidence that induction of HO-1 efficiently represses the inflammatory response by inhibiting production of various inflammatory cytokines [Hwangbo et al., 2009; Boutten et al., 2011; Motterlini and Foresti, 2014]. Many plant-derived compounds, such as curcumin, resveratrol, tussilagone, and 2′-hydroxychalcone, induce HO-1 and exert anti-inflammatory activities in different cells types [Motterlini et al., 2000; Juan et al., 2004; Abuarqoub et al., 2006; Hsu et al., 2008; Hwangbo et al., 2009]. Our data also show that inhibition of HO-1 activity by SnPP abrogated the anti-inflammatory activities of 7-MCPA in RAW264.7 cells. These results suggest that induction of HO-1 by 7-MCPA is at least partially responsible for the anti-inflammatory effects of 7-MCPA. The anti-inflammatory actions of HO-1 are attributed to several factors, including degradation of the pro-oxidant heme, formation of the antioxidants biliverdin/bilirubin, and release of anti-inflammatory/anti-apoptotic carbon monoxide [Ryter et al., 2006; Abraham and Kappas, 2008; Choo et al., 2015]. Although the exact mechanisms involved in the anti-inflammatory effects of HO-1 have not been fully elucidated, one or more enzymatic HO-1 by-products have been evaluated as factors

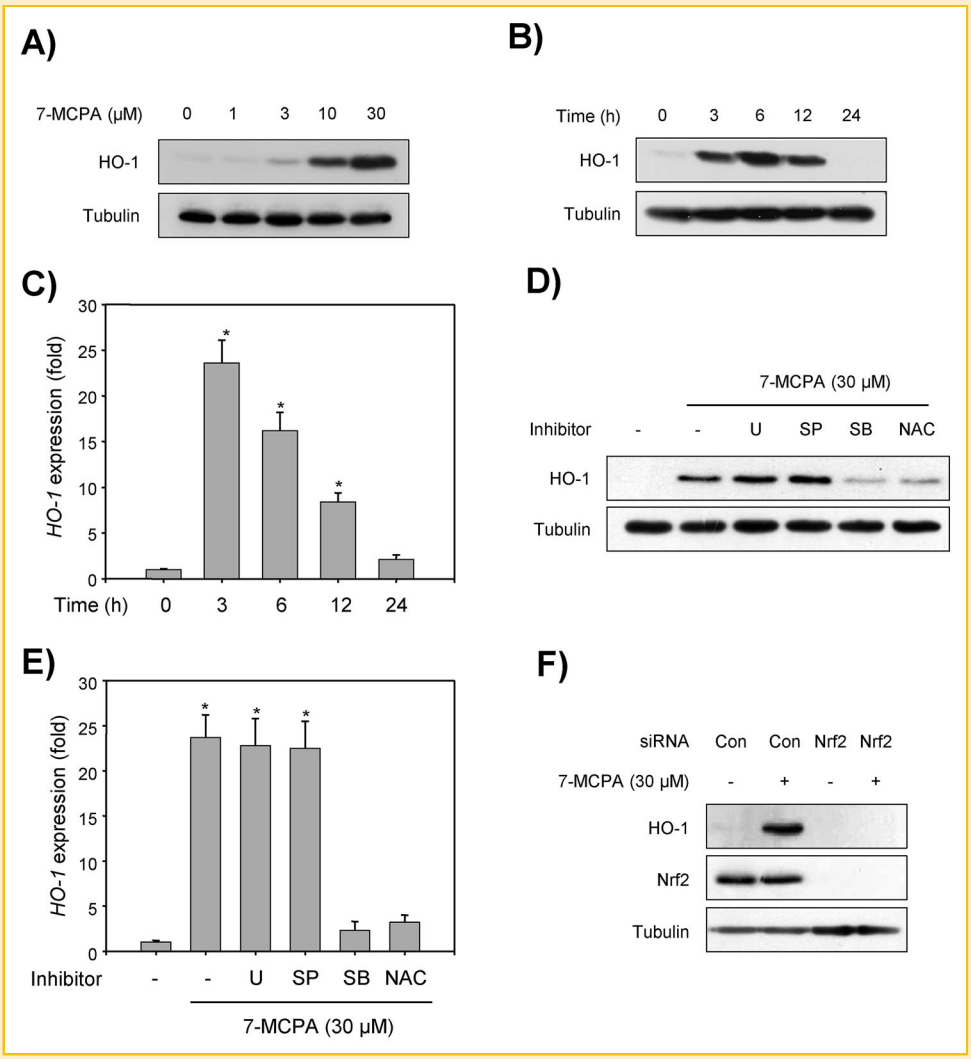

Fig. 6. Nrf2 is necessary for 7-MCPA-induced HO-1 expression. A: RAW264.7 cells were incubated for 6 h with the indicated concentrations of 7-MCPA, and total cell lysates were prepared and then performed Western blot analysis with the indicated antibodies. B: RAW264.7 cells were incubated for the indicated periods of time with 7-MCPA (30 μM), and total cell lysates were prepared and then performed Western blot analysis with the indicated antibodies. C: RAW264.7 cells were incubated for the indicated periods of time with 7-MCPA (30 μM). Total RNAs were prepared and then performed real-time qPCR analysis to HO-1 mRNA expression level. Data are presented as mean ± SEM ($^*P < 0.01$ compared with vehicle only, n = 5). (D) RAW264.7 cells were incubated for 6 h with 7-MCPA (30 μM) alone or together with U0126 (U, 10 μM), SP600125 (SP, 10 μM), SB203580 (SB, 10 μM), or NAC (100 μM). Total cell lysates were prepared and then performed Western blot analysis with the indicated antibodies. E: RAW264.7 cells were incubated for 3 h with 7-MCPA (30 μM) alone or together with U0126 (U, 10 μM), SP600125 (SP, 10 μM), SB203580 (SB, 10 μM), or NAC (100 μM). Total RNAs were prepared and performed real-time qPCR analysis to determine the HO-1 mRNA expression level. Data are presented as mean ± SEM ($^*P < 0.01$ compared with vehicle only, n = 5). F: RAW264.7 cells were transfected with the scrambled control siRNA (Con) or Nrf2-targeted siRNA (Nrf2) for 48 h and then were further incubated for 6 h with 7-MCPA. Total cell lysates were prepared and then performed Western blot analysis with the indicated antibodies.

that could inhibit macrophage-mediated inflammation [Abraham and Kappas, 2008; Ryter et al., 2006; Choo et al., 2015]. As 7-MCPA induced HO-1 expression in RAW264.7 cells, the observed anti-inflammatory effects of 7-MCPA may be mediated by one or more enzymatic by-products of HO-1.

Nrf2-deficient mice express higher levels of NF-κB upon LPS or TNF-α stimulation [Thimmulappa et al., 2006]. Several Nrf2-activating compounds, such as sulforaphane, a well-known Nrf2 activator, inhibit activation of NF-κB [Xu et al., 2005]. Like other Nrf2-activating compounds, 7-MCPA inhibited LPS-induced activation of NF-κB. Because Nrf2 and NF-κB regulate each other negatively, our results show that 7-MCPA-mediated activation of Nrf2 may suppress NF-κB and that this could play an important role in abrogating the LPS-induced inflammatory response in RAW264.7 cells. We also found that 7-MCPA inhibited the activation of p38 MAPK and JNK in LPS-stimulated RAW264.7 cells. Thus, it is possible to speculate that 7-MCPA may inhibit LPS-induced signaling pathways via Nrf2-independent mechanisms. However, the detailed mechanisms by which 7-MCPA inhibits LPS signaling pathways remain to be elucidated.

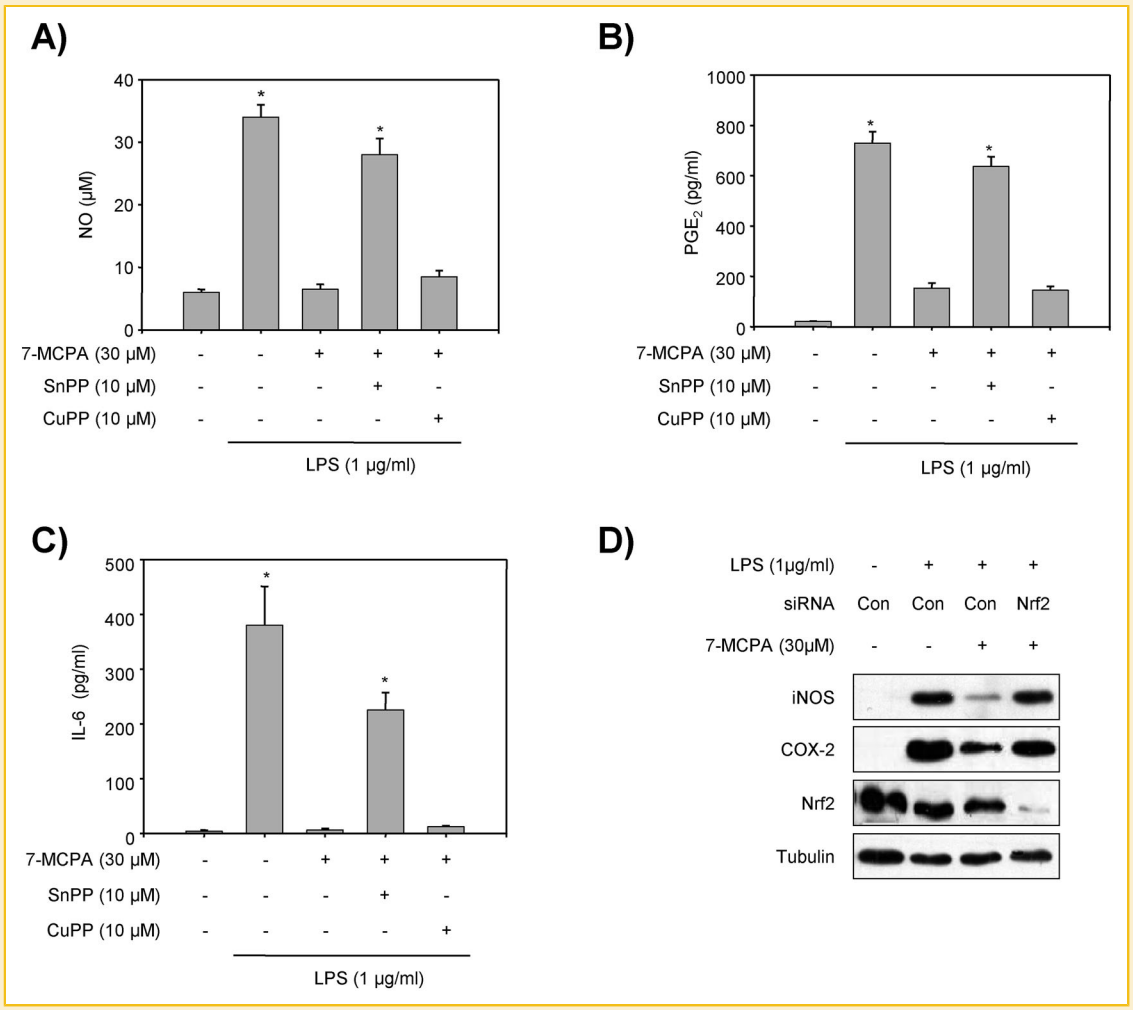

Fig. 7. Inhibiting HO-1 or Nrf2 expression reverses the anti-inflammatory effects of 7-MCPA in RAW264.7 cells. A–C: RAW264.7 cells were incubated for 30 min with 7-MCPA together with SnPP or CuPP prior to stimulation with LPS (1 μg/ml) for 24 h. The amounts of NO (A), PGE_2 (B), IL-6 (C) in culture supernatants were determined. Data are presented as mean ± SEM (* $P < 0.01$ compared with LPS only, n = 3). D: RAW264.7 cells were transfected with the scrambled control siRNA (Con) or Nrf2-targeted siRNA (Nrf2) for 48 h and then further incubated for 30 min with vehicle or 7-MCPA prior to stimulation with LPS (1 μg/ml) for 24 h. Total cell lysates were prepared and then performed Western blot analysis with the indicated antibodies.

Many phytochemicals induce HO-1 expression via activating Nrf2 through the signaling pathways involving MAPKs, protein kinase C, and phosphoinositide 3-kinase (PI3K) [Motterlini et al., 2000; Chen et al., 2005; Keum et al., 2006; Ríos et al., 2009; Shan et al., 2010]. Several lines of evidence show that activation of Nrf2 is regulated by the p38 pathway. For example, sulforaphane induces Nrf2 activation via the p38 pathway [Keum et al., 2006; Shan et al., 2010]. In the present study, we showed that the potent antioxidant NAC blocked 7-MCPA-mediated Nrf2 activation and the intracellular ROS level was increased by 7-MCPA in RAW264.7 cells, suggesting that ROS play the major role in 7-MCPA-mediated Nrf2 activation. Moreover, we showed that 7-MCPA induced activation of p38 MAPK and NAC blocked 7-MCPA-mediated inhibition of p38 activation, suggesting that ROS-mediated activation of p38 MAPK is involved in activating Nrf2 by 7-MCPA. Our finding that 7-MCPA activated Nrf2 suggests that 7-MCPA or a 7-MCPA-enriched extract from *E. longifolia* could prevent or help treat Nrf2-dependent pathological conditions, such as inflammatory diseases and cancers.

Together, we for the first time demonstrated that the β-carboline alkaloid 7-MCPA isolated from *E. longifolia* hairy-root cultures activated Nrf2 via a ROS-dependent p38 MAPK pathway and that the 7-MCPA-induced activation of the Nrf2/HO-1 pathway was associated with the 7-MCPA anti-inflammatory effects. Our results extend our understanding of the molecular mechanisms underlying the anti-inflammatory activities of β-carboline alkaloids and *E. longifolia*. Our results also imply that 7-MCPA or 7-MCPA-enriched *E. longifolia* extracts may be useful to prevent or treat inflammatory diseases

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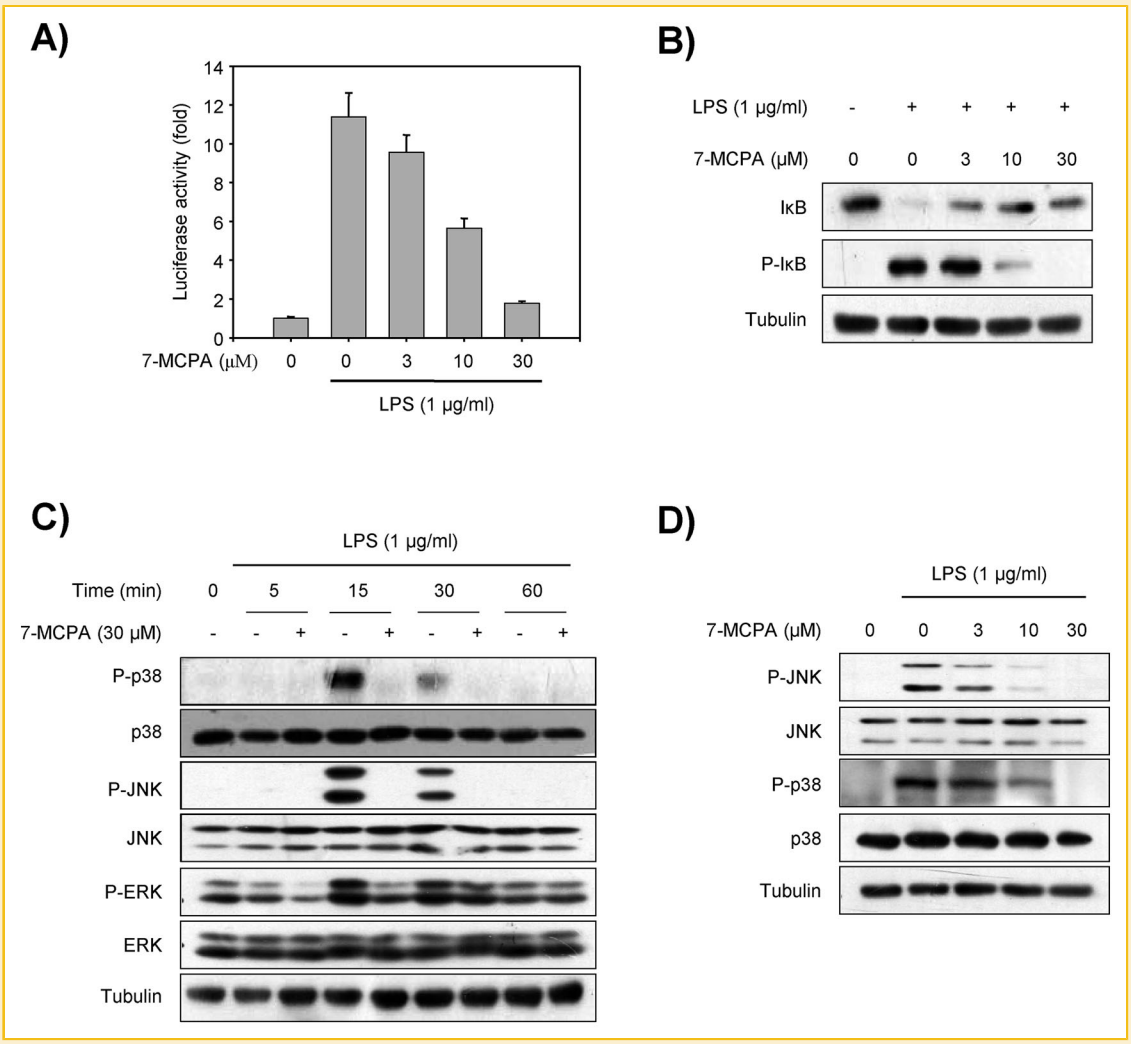

Fig. 8. 7-MCPA inhibits NF-κB activation in LPS-stimulated RAW264.7 cells. A: RAW264.7 cells transfected with a NF-κB-dependent reporter gene construct were incubated for 30 min with the indicated concentrations of 7-MCPA prior to stimulation with LPS (1 μg/ml) for 12 h. The cells were lysed, and then luciferase activities were determined. Data are presented as mean ± SEM ($^{*}P < 0.01$ compared with the LPS only, n = 5). B: RAW264.7 cells were incubated for 30 min with the indicated concentrations of 7-MCPA and then stimulated with LPS (1 μg/ml) for 15 min. Total cell lysates were prepared and then performed Western blot analysis with the indicated antibodies. C: RAW264.7 cells were incubated for 30 min with 7-MCPA (30 μM), and then stimulated with LPS (1 μg/ml) for the indicated time points. Total cell lysates were prepared and then performed Western blot analysis with the indicated antibodies. D: RAW264.7 cells were incubated for 30 min with for the indicated concentrations of 7-MCPA and then stimulated with LPS (1 μg/ml) for 15min. Total cell lysates were prepared and then performed Western blot analysis with the indicated antibodies.

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