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Inhibition of lipopolysaccharide-induced cyclooxygenase-2 transcription by 6-(methylsulfinyl) hexyl isothiocyanate, a chemopreventive compound from *Wasabia japonica* (Miq.) Matsumura, in mouse macrophages

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Abstract

6-(Methylsulfinyl)hexyl isothiocyanate (6-MITC) is a chemopreventive compound occurring in Wasabi (*Wasabia japonica* (Miq.) Matsumura), which is a very popular pungent spice in Japan. We investigated the effects of 6-MITC on the expression of cyclooxygenase-2 (COX-2) in lipopolysaccharide (LPS)-activated murine macrophage RAW264 cells. Treatment with 6-MITC suppressed LPS-mediated induction of COX-2 protein in a dose-dependent manner. Transfections with various *COX-2* promoter reporter constructs revealed that the inhibitory effects of 6-MITC on *COX-2* gene expression were directed by the core promoter elements including nuclear factor κB (NF-κB), CCAAT/enhancer-binding protein (C/EBP) and cyclic AMP-response element (CRE) sites. Western blotting analysis showed that 6-MITC inhibited LPS-induced activation of MAPK (ERK, p38 kinase and JNK) and transcriptional factors (CREB, c-Jun and C/EBPδ) binding the core elements of *COX-2* promoter, substantiating the involvement of these signal transduction pathways in the regulation of COX-2 expression by 6-MITC. Moreover, Western blotting experiments with MAPK-specific inhibitors (U0126 for MEK1/2, SB203580 for p38 kinase and SP600125 for JNK) demonstrated that 6-MITC suppressed LPS-induced COX-2 expression by blocking the activation of JNK-mediated AP-1 and ERK/p38 kinase-mediated CREB or C/EBPδ. Finally, the structure–activity study revealed that the inhibitory potency of methylsulfinyl isothiocyanates (MITCs) depended on the methyl chain length. These findings demonstrate for the first time that 6-MITC is an effective agent to attenuate COX-2 production, and enhance our understanding of the anti-inflammation properties of 6-MITC.

Keywords: 6-(Methylsulfinyl)hexyl isothiocyanate; Cyclooxygenase-2; Lipopolysaccharide; Mitogen-activated protein kinase; Macrophage

1. Introduction

Wasabi (*Wasabia japonica* (Miq.) Matsumura), called Japanese horseradish, is a member of the Brassicaceae family of vegetables. Its rhizome is a very popular pungent spice in Japan. Several studies have shown that Wasabi has multiple biological activities, such as appetite enhancement [1], anti-microbial activity [2], inhibition of platelet aggregation [3] and the suppression of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine-induced rat gastric carcinogenesis [4]. By studying fractions from Wasabi, allyl isothiocynates are the active compounds of Wasabi [5], and 6-(methylsulfinyl)hexyl isothiocyanate (6-MITC) are present as major allyl isothiocyanate [6]. The results of animal experiments showed that oral administration of 6-MITC had inhibitory effects on mice skin tumor induced by DMBA as an initiator and TPA as a promoter [7] and on mice lung tumor induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone [8]. These results suggest that 6-MITC may be implicated in cancer chemoprevention.

Cyclooxygenase (COX) catalyzes the synthesis of prostaglandins from arachidonic acid. There are two isoforms

Abbreviations: AP-1, activator protein-1; C/EBP, CCAAT/enhancer-binding protein; COX, cyclooxygenase; CRE, cyclic AMP-response element; CREB, CRE binding protein; ERK, extracellular signal-regulated kinase 1/2; JNK, c-Jun NH_2-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; 6-MITC, 6-(methylsulfinyl)hexyl isothiocyanate; NF-κB, nuclear factor κB

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of COX, designated COX-1 and COX-2, which are encoded by different genes. COX-1 is constitutively expressed in most tissues and believed to be responsible for normal physiological functions [9]. In contrast, COX-2 is not detectable in most normal tissues or resting immune cells, but it could be induced by lipopolysaccharide (LPS), inflammatory cytokines, growth factors and carcinogens [10,11]. Many cell types associated with inflammation, such as macrophages, endothelial cells and fibroblasts, express the COX-2 upon induction [12]. COX-2 overexpression is also found in a variety of transformed cells and tumors [13,14]. Enhancement of COX-2 can stimulate the growth of malignant cells by increasing cell proliferation [15], promoting angiogenesis [16], and inhibiting immune surveillance [17] and apoptosis [18]. These effects were reversed by the nonsteroidal anti-inflammatory drugs (NSAID), such as etodolac, meloxicam and celecoxib, which are known as COX-2-specific inhibitors. Thus, COX-2 plays an important role in inflammation and carcinogenesis, and the identification of COX-2 inhibitors is considered to be a promising approach to prevent cancer.

In the *COX-2* gene, *cis*-acting elements including nuclear factor κB (NF-κB, −223/−214), CCAAT/enhancer-binding protein (C/EBP, −132/−124) and cyclic AMP-response element (CRE, −59/−53) have been found to play a critical role in regulating transcription. NF-κB is a transcription factor involved in LPS-mediated induction of many cytokines and inflammatory products, and the inhibition of NF-κB activity has been reported to impair *COX-2* mRNA induction [19,20]. C/EBP is generally believed to play an important role in COX-2 induction in macrophages as well as in other cell types, mainly through interactions with the two C/EBP family members, C/EBPδ and β [21–23]. Finally, the cyclic AMP-response element (CRE) is essential for both basal and induced *COX-2* transcription in most cells through the binding with CRE binding protein (CREB) and activator protein-1 (AP-1) [24–27]. However, the relative contribution of transcription factors and signaling cascades in regulating *COX-2* transcription is not clear yet.

In the present study, we investigated the effects of 6-MITC on the *COX-2* gene expression in murine macrophage cell line RAW264, which can be stimulated with LPS to mimic a state of infection and inflammation. Our results demonstrate that 6-MITC suppressed LPS-induced COX-2 expression by blocking the signal transduction cascades of mitogen-activated protein kinase (MAPK) leading to the activations of CREB, C/EBPδ and AP-1.

2. Materials and methods

2.1. Materials and cell culture

2-, 4-, 6- and 8-MITC purified by reverse-phase HPLC to >99% were obtained from Hakucho Pharmaceutical Co., Ltd. (Japan). All of the MITCs used were dissolved in dimethyl sulfoxide (DMSO, final concentration was 0.2%). U0126 and antibodies against phospho-CREB, phospho-ERK1/2, phospho-p38 kinase, phospho-c-Jun (Ser73), phospho-JNK, ERK1/2, p38 kinase, JNK, IκB-α and phospho-IκB-α were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against COX-2, COX-1, C/EBPβ, C/EBPδ and p65 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Fetal bovine serum (FBS) was from Equitech-Bio (Kerrville, TX). LipofectAMINE was from Life Technologies, Inc. (Grand Island, NY). LPS (*Escherichia coli* Serotype 055:B5) was from Sigma (St. Louis, MO). SB203580 was from Calbiochem (Nottingham, UK), and SP600125 was from Biomol Research Lab. (Plymouth Meeting, PA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (Poole, UK). Murine macrophage-like RAW264 cells were obtained from RIKEN BioResource Center Cell Bank (Cell No. RCB0535), and cultured at 37 °C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. Because FBS contains numerous compounds, such as LPS and growth factors, which influence the biological characteristics of macrophages [28–30], we performed all of experiments under serum-free or low-serum conditions.

2.2. Western blotting analysis

Western blotting assay was performed as described previously [31]. In brief, RAW264 (1×10^6 cells) were pre-cultured in 6-cm dish for 24 h, and then starved by being cultured in serum-free for another 2.5 h to eliminate the influence of FBS. The cells were treated with or without 6-MITC for 30 min before exposure to 40 ng/ml LPS for the different times. The harvested cells were lysed and the supernatants were boiled for 5 min. Protein concentration was determined by using dye-binding protein assay kit (Bio-Rad Hercules, CA) according to the manufacturer's manual. Equal amounts of lysate protein (∼40 μg) were run on 10% SDS-PAGE and electrophoretically transferred to PVDF membrane (Amershan Pharmacia Biotech, Little Chalfont, UK). After blotting, the membrane was incubated with specific primary antibody overnight at 4 °C, and further incubated for 1 h with HRP-conjugated secondary antibody. Bound antibodies were detected by ECL system with a Lumi Vision PRO machine (TAITEC Co., Japan). The relative amount of proteins associated with specific antibody was quantified using the Lumi Vision Imager software (TAITEC Co.).

2.3. Determination of PGE_2

PGE_2 in culture medium was measured with a PGE_2 enzyme immunoassay kit (Cayman Co., St. Louis, MO) according to manufacturer's manual. Briefly, RAW264 cells (5×10^5 cells) were seeded into each well of 6-well

plates. After incubation for 24 h, the cells starved by being cultured in serum-free for another 2.5 h to eliminate the influence of FBS. The cells were then treated with or without 6-MITC for 30 min before exposure to 40 ng/ml LPS for 12 h. The level of PGE_2 released into culture medium was determined by measuring absorbance at 405 nm with a microplate reader.

2.4. Cell survival assay

RAW264 cells (2×10^4 cells/well) were plated into each well of 96-well microtiter plates. After incubating for 24 h, the cells were starved by being cultured in serum-free for another 2.5 h to eliminate the influence of FBS, and then treated with or without 16 μM 6-MITC. After further incubation for 30 min, cells were treated with or without 40 ng/ml LPS for 12 h. In the case of 40 ng/ml LPS plus 16 μM 6-MITC, the cells were treated with 16 μM 6-MITC for 30 min, and then exposed to 40 ng/ml LPS for 12 h. MTT solution was added to each well and the cells were incubated for another 4 h. The resulting MTT-formazan product was dissolved by the addition of 100 μl of 0.04N HCl-isopropanol. The amount of formazan was determined by measuring the absorbance at 595 nm with a microplate reader (Bio-Rad, Model 550). The results are expressed as the optical density ratio of the treatment to control.

2.5. Plasmids

The human *COX-2* promoter-luciferase deletion constructs (−1432/+59, −327/+59, −220/+59, −124/+59 and −52/+59) and mutant constructs (KBM, ILM and CRM) were generous gifts of Dr. Hiroyasu Inoue (Nara Women's University, Nara, Japan) and have been described previously [20,23,25,26]. KBM represents the −327/+59 *COX-2* promoter construct in which the NF-κB site was mutagenized; ILM represents the −327/+59 *COX-2* promoter construct in which the C/EBP site was mutagenized; CRM represents the −327/+59 *COX-2* promoter construct in which the CRE site, ILM–CRM represents the −327/+59 *COX-2* promoter construct in which the C/EBP and CRE sites.

2.6. Transient transfection assay

Transient transfection was performed according to the modified method as described previously [32]. RAW264 cells (1×10^5) were plated into each well of 12-well plates, and cultured for 24 h. The cells were then co-transfected with 0.5 μg *COX-2* promoter-luciferase plasmids and 0.12 μg CMV-β-galactosidase plasmid using LipofectAMINE2000. After 5 h incubation, the medium was replaced and cultured for another 20.5 h. The cells were treated with or without 6-MITC for 30 min before exposure to 40 ng/ml LPS for 6 h. The activities of luciferase and β-galactosidase in cell lysate were measured with a luminometer (Berthold) according to the supplier's recommendations. Luciferase activity values were normalized to transfection efficiency monitored by β-galactosidase expression, and *COX-2* transcription activity was expressed as fold induction relative to the control cells without LPS treatment.

2.7. Nuclear extracts

Nuclear extracts were prepared as described previously [33]. Briefly, RAW264 cells (1×10^6) were pre-cultured in 6-cm dish for 24 h, and then starved by being cultured in serum-free for another 2.5 h to eliminate the influence of FBS. The cells were treated with or without 6-MITC for 30 min before exposure to 40 ng/ml LPS. Harvested cells were lysed by incubation in buffer A (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol and 0.2 mM phenylmethylsulfonyl fluoride) on ice for 15 min, and then centrifuged at 13,500 × *g* for 10 min at 4 °C. The nuclear pellets were resuspended in buffer B (20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol and 0.2 mM phenylmethylsulfonyl fluoride) for 15 min at 4 °C, and then centrifuged at 13,500 × *g* for 15 min at 4 °C. The supernatants containing nuclear extracts were stored at −80 °C until Western blotting analysis.

2.8. Statistical analyses

Difference between the treated and the control was analyzed by Student's *t*-test. A probability of $P < 0.05$ was considered significant.

3. Results

3.1. 6-MITC suppresses LPS-induced COX-2 expression and PGE_2 release

To investigate the inhibitory effect of 6-MITC on COX-2 expression, RAW264 cells were treated with the different concentrations of 6-MITC for 30 min before exposure to 40 ng/ml LPS for 12 h. As shown in Fig. 1A, 6-MITC suppressed LPS-induced expression of COX-2, but not COX-1, in a dose-dependent manner at the concentration range of 2–16 μM. Furthermore, 6-MITC also suppressed LPS-induced PGE_2 release with the same fashion of COX-2 expression (Fig. 1B). As shown in Fig. 1C, there is no significant difference in the cell viability between the treatment of 40 ng/ml LPS alone, or 16 μM 6-MITC alone, or 40 ng/ml LPS plus 16 μM 6-MITC. Thus, the inhibitory action by 6-MITC was not caused by its cytotoxicity.

3.2. 6-MITC inhibits LPS-induced transcription activity of COX-2 gene

To determine whether the suppression of COX-2 expression by 6-MITC was due to transcription regulation, we

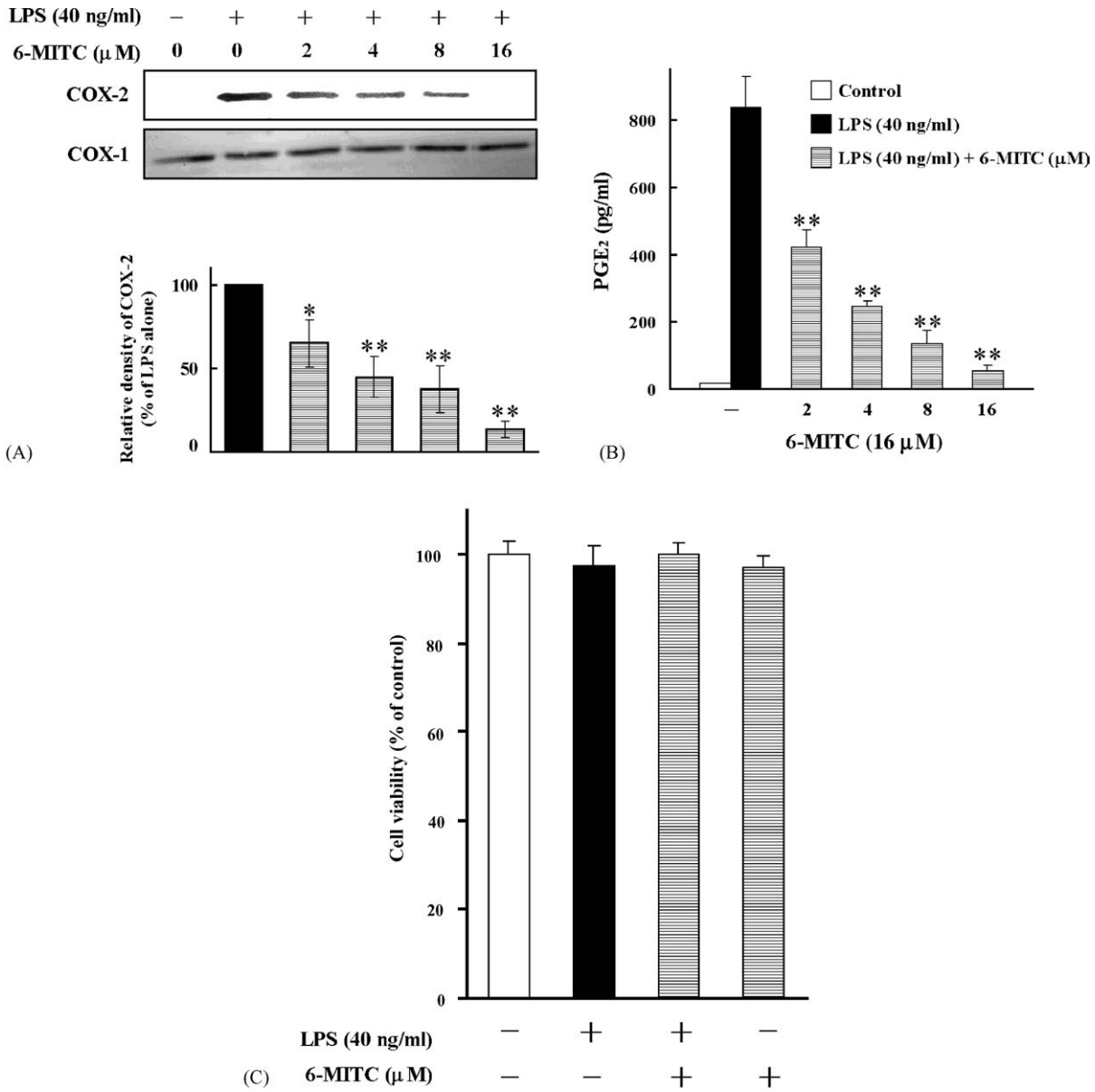

Fig. 1. 6-MITC suppresses LPS-induced COX-2 expression and PGE_2 release in RAW264 cells. (A) After RAW264 cells (1×10^6 cells) were starved in serum-free medium for 2.5 h, the cells were treated with the indicated concentrations of 6-MITC for 30 min, and then exposed to 40 ng/ml LPS for 12 h. COX-2 and COX-1 were detected by Western blotting analysis with their antibodies, respectively. Histograms show the densitometric analysis of COX-2 protein expression normalized to COX-1. The data represent the mean ± S.D. of three to four separate experiments, and the figure is a representative of those experiments each with similar results. (B) After RAW264 cells (5×10^5 cells) were starved in serum-free medium for 2.5 h, the cells were treated with the indicated concentrations of 6-MITC for 30 min, and then exposed to 40 ng/ml LPS for 12 h. The amount of PGE_2 in medium was measured as described in Section 2. Each value represents the mean ± S.D. of triplicate tests. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. LPS. (C) The effects of LPS and 6-MITC on cell viability of RAW264 cells. After RAW264 cells (2×10^4 cells/well) were starved in serum-free medium for 2.5 h, the cells were treated with or without 16 μM 6-MITC. After further incubation for 30 min, cells were treated with or without 40 ng/ml LPS for 12 h. In the case of 40 ng/ml LPS plus 16 μM 6-MITC, the cells were treated with 16 μM 6-MITC for 30 min, and then exposed to 40 ng/ml LPS for 12 h. The cell density was assessed calorimetrically after staining with MTT and expressed as optical density ratio of the treatment to control at 595 nm. Values are means of eight data; bar, S.D. There were no significant difference between control and each treatment ($P > 0.05$).

performed a promoter activity assay of *COX-2* gene with a full *COX-2* promoter-luciferase plasmid (−1432/+59). As shown in Fig. 2A, 6-MITC suppressed LPS-induced *COX-2* promoter activity in a dose-dependent manner. It has been reported that the *cis*-elements, such as NF-κB (−223/−214), C/EBP (−132/−124) and CRE (−59/−53) sites are present in *COX-2* promoter and play a critical role in the regulation of *COX-2* transcription [23,25,26]. To further define the regions of the *COX-2* promoter that responded to inhibitory action by 6-MITC, transient transfections were performed using a series of *COX-2* promoter deletion constructs (Fig. 2B). The inhibitory effects of 6-MITC on LPS-mediated *COX-2* promoter activity were observed in all of *COX-2* promoter deletion constructs except the

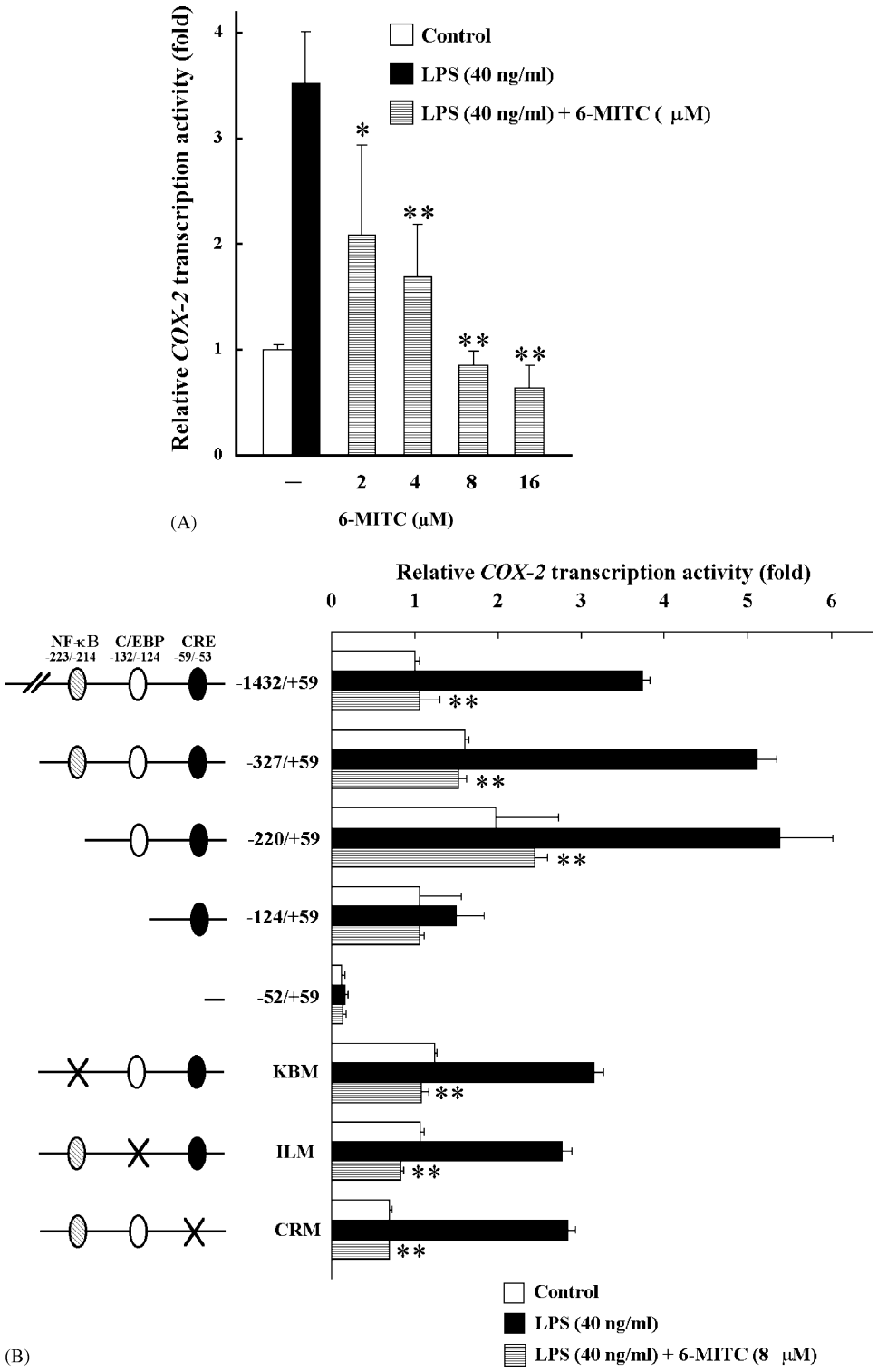

Fig. 2. 6-MITC suppresses LPS-induced transcription activity of *COX-2* gene. (A) 6-MITC suppresses LPS-induced *COX-2* transcription activity in a dose-dependent manner. RAW264 cells (1×10^5 cells) seeded into each wells of 12-well plates were transfected with 0.5 μg of −1432/+59 *COX-2* promoter-luciferase reporter constructs and 0.12 μg CMV-β-galactosidase plasmid. (B) Localization of region of *COX-2* promoter that mediates the effects of LPS and 6-MITC. RAW264 cells were transfected with 0.5 μg of a series of deleted *COX-2* constructs ligated to luciferase (−1432/+59, −327/+59, −220/+59, −124/+59 and −52/+59) or a series of mutated *COX-2* promoter-luciferase constructs (KBM, ILM and CRM) and 0.12 μg CMV-β-galactosidase plasmid. After 5 h incubation, the medium was replaced with complete medium and cultured for another 20.5 h. The cells were then treated with or without 6-MITC for 30 min before they were exposed to 40 ng/ml LPS for 6 h. The luciferase activity values were normalized to transfection efficiency monitored by β-galactosidase expression, and *COX-2* promoter activity is expressed as fold induction to the −1432/+59 construct without LPS treatment. Each value represents the mean ± S.D. of three to four separate experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. LPS.

−52/+59 construct, suggesting that the promoter region between −327 and −52 including NF-κB, C/EBP and CRE sites may be essential for *COX-2* promoter activity. To test this notion, we next examined the transcription activity of *COX-2* gene using the mutated constructs (KBM, ILM or CRM) in which specific enhancer elements were mutated. The results show that none of these mutated promoter elements were essential to both the inductive effect of LPS and the suppressive effect of 6-MITC. Thus, the existence of at least two *cis*-elements in the promoter is required for LPS-induced *COX-2* gene expression, and 6-MITC may inhibit LPS-induced COX-2 expression by suppressing transcriptional factors binding to at least two of these *cis*-elements.

3.3. 6-MITC inhibits LPS-induced COX-2 expression through the blockage of MAPK signaling cascades

Accumulated data indicate that LPS stimulation in RAW264 macrophages triggers the activation of MAPK including ERK, p38 kinase and JNK, and then induces COX-2 expression [27,33,34]. Therefore, we investigated the effect of 6-MITC on the activation of ERK, p38 kinase and JNK. As shown in Fig. 3A, 6-MITC caused a dose-dependent inhibition of LPS-induced phosphorylation of ERK, p38 kinase and JNK in the concentration ranges from 2 to 16 μM, suggesting that 6-MITC may suppress COX-2 expression by blocking the activation of MAPK.

To identify which factor of MAPK is more critical to block LPS-induced COX-2 expression, we used MAPK-specific inhibitors (U0126 for MEK1/2, SB203580 for p38 kinase and SP600125 for JNK) to block LPS-induced COX-2 expression (Fig. 3B). LPS-induced COX-2 expression was partially suppressed by treatment with U0126, SB203580 and SP600125 alone. Treatment with the combination of two inhibitors strongly inhibited COX-2 expression. In particular, treatment with three inhibitors completely inhibited COX-2 expression. These data indicate that there are cooperative actions in MAPK pathways leading to activate COX-2 expression, and 6-MITC inhibited LPS-induced COX-2 expression by blocking all of three MAPK pathways.

3.4. Different effects of 6-MITC on the transcriptional factors regulating COX-2 gene expression

It has been reported that transcription factors including CREB, AP-1, C/EBP and NF-κB can bind the responding *cis*-elements (see Fig. 2) in *COX-2* promoter, and regulate *COX-2* transcription [23,25,27,34]. To identify the effects of 6-MITC on these transcriptional factors, we pretreated RAW264 cells with 6-MITC for 30 min before exposure to 40 ng/ml LPS, and then examined these transcription factors by Western blotting analysis. As shown in Fig. 4A, 6-MITC almost completely inhibited LPS-induced phosphorylation of CREB and c-Jun, which is a major component of AP-1 in c-Jun/c-Fos heterodimer form. The control protein, α-tubulin, showed no change. LPS also can cause COX-2 expression by stimulating the expression of C/EBP [23,35,36]. Thus, we determined the level of C/EBPδ and β at whole cellular lysate and nuclear fraction. As shown in Fig. 4B, 6-MITC markedly blocked LPS-induced C/EBPδ at both whole and nuclear. However, 6-MITC did not block the potential C/EBPβ at either whole or nuclear. As a positive control, LPS stimulated nuclear translocation of p65 with no change in whole p65. Thus, 6-MITC suppressed LPS-induced the expression and subsequent nuclear translocation of C/EBPδ. Taken together, 6-MITC might inhibit COX-2 expression, at least, by suppressing LPS-mediated activation of CREB, AP-1 and C/EBPδ, but not C/EBPβ.

NF-κB is another critical factor for COX-2 expression induced by LPS or proinflammatory cytokines [19,20]. The translocation of NF-κB to nucleus is preceded by phosphorylation, ubiquitination and proteolytic degradation of IκB [37]. To identify whether 6-MITC can directly inhibit degradation of IκB, we examined the effect of 6-MITC on IκB-α degradation and phosphorylation. As shown in Fig. 4C, LPS reduced the IκB-α protein at 30 min, but 6-MITC did not suppress the degradation of IκB-α. Rather, 6-MITC retarded de novo synthesis of IκB-α, which reappeared at 180 min after LPS stimulation in the control. Next, IκB-α phosphorylation was observed after exposure to LPS for 10 min, which was not blocked by 6-MITC. Moreover, LPS strongly evoked the nuclear translocation of p65 into nucleus, but 6-MITC did not inhibit it (Fig. 4D). These data indicate that 6-MITC appeared to has no inhibitory effect on LPS-induced phosphorylation and degradation of IκB-α, and subsequent nuclear translocation of p65.

These data suggested that the *cis*-elements of CRE and C/EBP, but not NF-κB, are required for the inhibition of COX-2 transcription by 6-MITC. To confirm this conclusion, we transfected a plasmid with double mutation at CRE and C/EBP sites. The results showed that the transcription activity was completely abolished when transfection with the double-mutated plasmids (Fig. 4E). As controls, the transcription activity with full or core elements was significantly inhibited by 6-MITC. Thus, 6-MITC might inhibit COX-2 expression by suppressing LPS-mediated activation of CREB, AP-1 and C/EBPδ, which bind to CRE and C/EBP sites.

3.5. Signaling from MAPK to the transcription factors in LPS-mediated COX-2 expression

We further investigated the inter-relation between MAPK signaling pathways and the transcription factors in LPS-mediated COX-2 expression because 6-MITC suppressed the activation of both MAPK (ERK, p38 kinase and JNK) and the transcription factors (CREB, C/EBPδ and AP-1). RAW264 cells were pretreated with MAPK

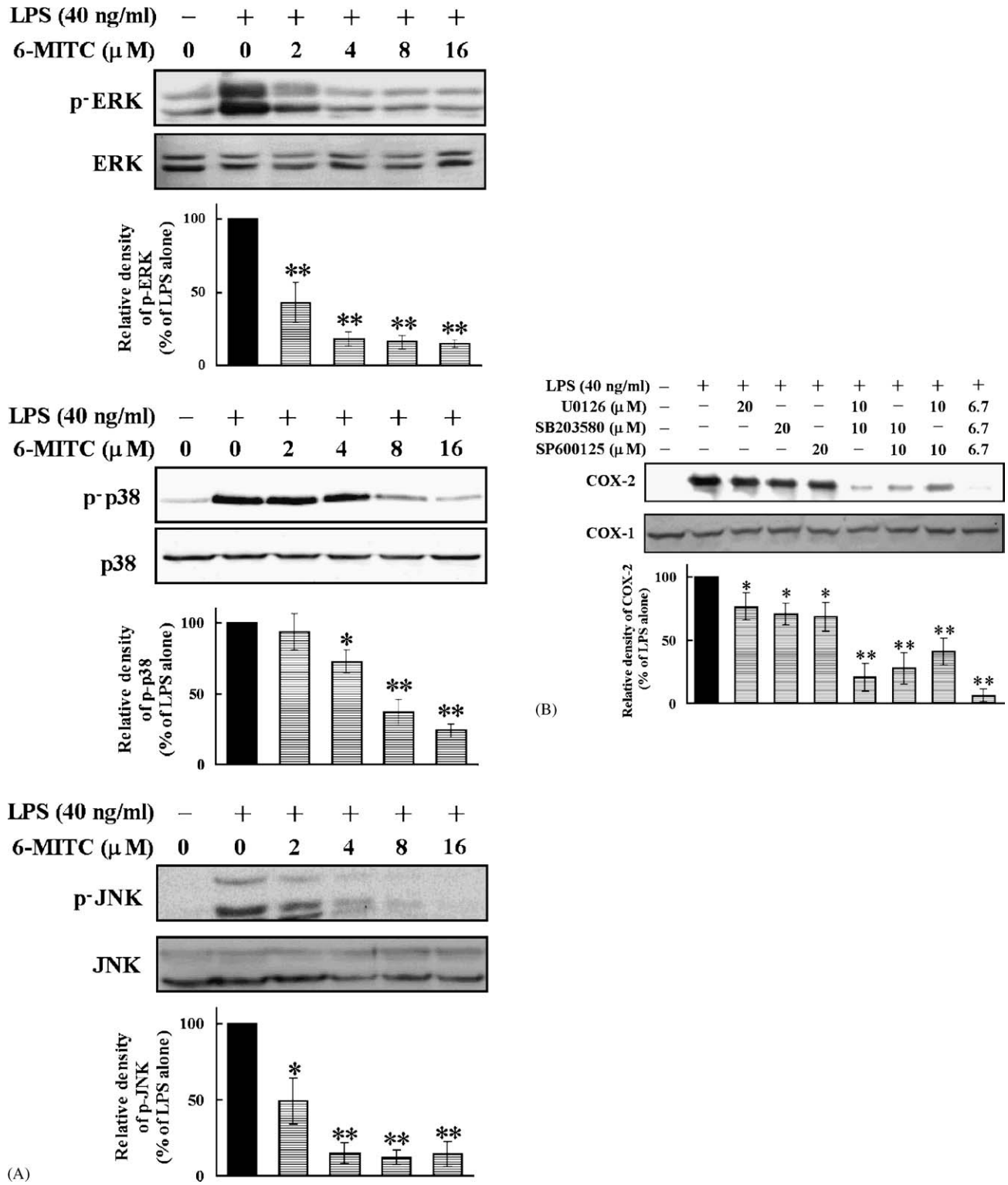

Fig. 3. 6-MITC inhibits LPS-induced COX-2 expression by suppressing MAPK phosphorylation. (A) 6-MITC inhibits LPS-induced MAPK phosphorylation. Cell culture and Western blotting analysis were done as described in Fig. 1. RAW264 cells were treated with 6-MITC (2–16 μM) for 30 min, and then exposed to 40 ng/ml LPS for 30 min. Histograms show the densitometric analysis of phosphorylated MAPK normalized to total MAPK, respectively. (B) MAPK inhibitors suppress LPS-induced COX-2 expression. Cell culture and Western blotting analysis were done as described in Fig. 1. RAW264 cells were treated with U0126, SB203580 and SP600125 (20 μM in lanes 3–5, 10 μM in lanes 6–8, 6.7 μM each in lane 9 for each inhibitor) for 30 min, and then exposed to 40 ng/ml LPS for 12 h. Histograms show the densitometric analysis of COX-2 protein normalized to COX-1. The data represent the mean ± S.D. of three to four separate experiments, and the figure is a representative of those experiments each with similar results. $^{*}P < 0.05$; $^{*}P < 0.01$ vs. LPS.

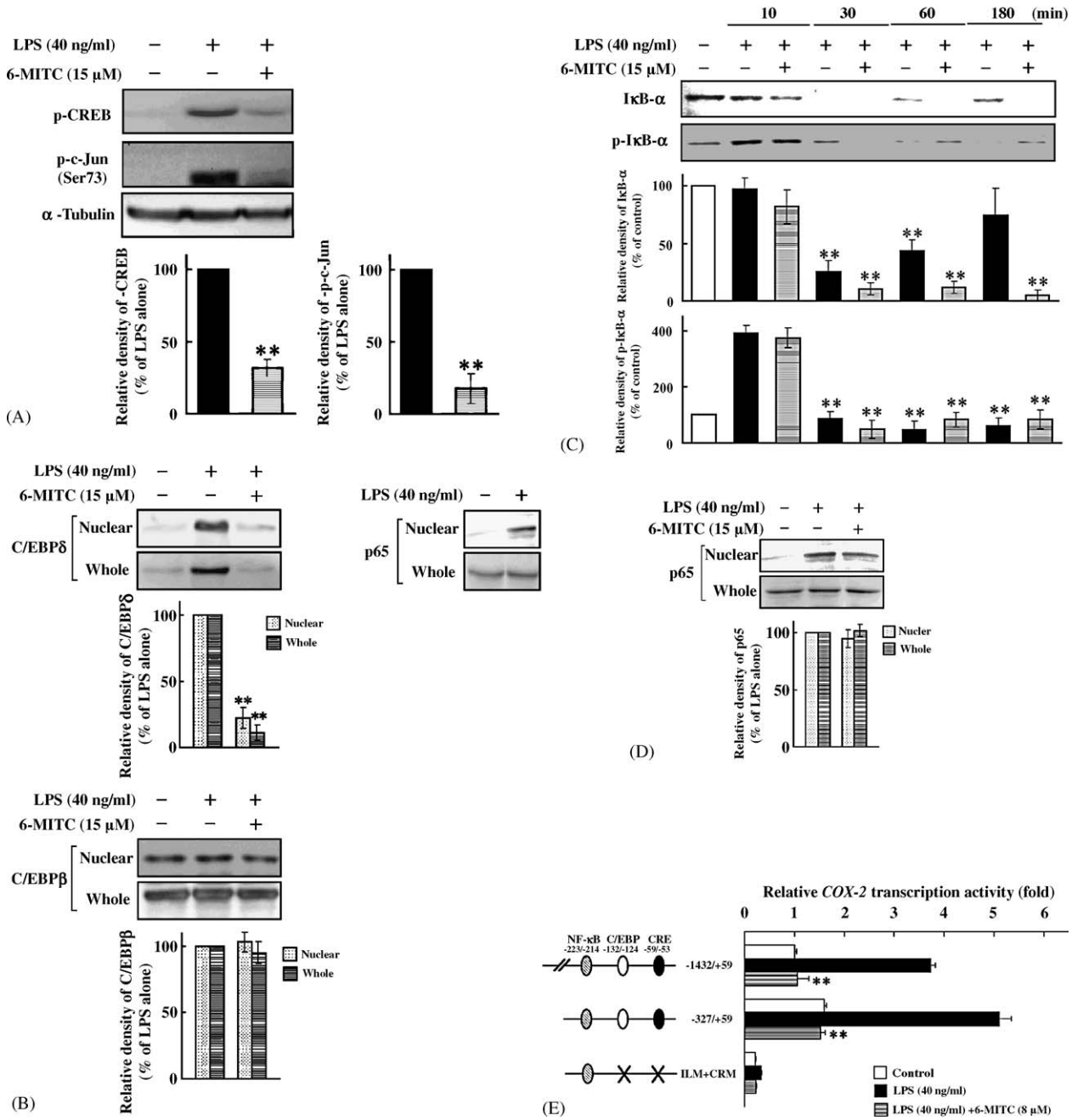

Fig. 4. Effects of 6-MITC on LPS-induced activation of the transcriptional factors. (A) 6-MITC inhibits LPS-induced phosphorylation of CREB and c-Jun. Cell culture and Western blotting analysis were done as described in Fig. 1. RAW264 cells were treated with 15 μM 6-MITC for 30 min, and then exposed to 40 ng/ml LPS for 30 min. Histograms show the densitometric analysis of phosphorylated CREB and c-Jun normalized to α-tubulin. (B) 6-MITC inhibits LPS-induced nuclear translocation and expression of C/EBPδ, but not C/EBPβ. The cells were treated with or without 15 μM 6-MITC for 30 min, and then exposed to 40 ng/ml LPS for 4 h. The nuclear proteins were extracted, and C/EBPδ and β were detected by Western blotting analysis. Histograms show the densitometric analysis of C/EBPδ and C/EBPβ. The nuclear p65 was used as a positive control to confirm the preparation of nuclear protein. (C) 6-MITC has no influence on LPS-induced degradation and phosphorylation of IκB-α. The cells were treated with or without 15 μM 6-MITC for 30 min, and then exposed to 40 ng/ml LPS for the indicated times. Histograms show the densitometric analysis of IκB-α and phosphorylated IκB-α protein. (D) 6-MITC did not suppress the nuclear translocation of p65. The cells were treated with or without 15 μM 6-MITC for 30 min, and then exposed to 40 ng/ml LPS for 30 min. The nuclear proteins and whole cell lysates were extracted, and p65 was detected by Western blotting analysis. Histograms show the densitometric analysis of nuclear p65 normalized to p65 of whole cell lysates. The data represent the mean ± S.D. of three to four separate experiments, and the figure is a representative of those experiments each with similar results. (E) Promoter activity of double mutant construct at C/EBP and CRE sites (ILM–CRM). Cells were transfected with 0.5 μg of −1432/+59, −327/+59, or double mutant construct at C/EBP and CRE sites and 0.12 μg CMV-β-galactosidase plasmid. The values of luciferase activities were normalized to transfection efficiency monitored by β-galactosidase expression, and *COX-2* promoter activity is expressed as fold induction to the −1432/+59 construct without LPS treatment. Each value represents the mean ± S.D. of three to four separate experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. LPS.

inhibitors alone or combinative for 30 min before exposure to LPS, and the phosphorylation of these transcription factors were then examined by Western blotting analysis. As shown in Fig. 5A, LPS-induced CREB phosphorylation was partially inhibited by U0126 or SB203580 alone, and was completely suppressed in the presence of both U0126 and SB203580. But, SP600125 could not suppress LPS-induced CERB phosphorylation, and the combinations of SP600125 and U0126 or SB203580 could not completely suppress CERB phosphorylation. Thus, the cascades of ERK and p38 kinase, but not JNK, might link to CREB-mediated COX-2 expression. On the other hand, LPS-induced c-Jun phosphorylation was completely inhibited by U0126 or SP600125 alone, or their combination. However, SB203580 could not suppress c-Jun phosphorylation. These data suggest that LPS-induced c-Jun phosphorylation, which leads to COX-2 expression, is regulated through the cascades of both JNK and ERK, but not p38 kinase.

We also examined the effects of MAPK inhibitors on the expression and nuclear translocation of C/EBPδ and β. As shown in Fig. 5B, the level of whole C/EBPδ was partially inhibited by U0126 or SB203580 alone, and completely suppressed in the presence of both U0126 and SB203580. However, SP600125 did not suppress C/EBPδ expression. We further determined the level of nuclear C/EBPδ. The inhibitors showed the same inhibitory effect on nuclear C/EBPδ as the result of whole C/EBPδ. On the other hand, whole and nuclear levels of C/EBPβ in such treatments were steadily constant. These results suggest that LPS-induced expression and nuclear translocation of C/EBPδ, but not C/EBPβ, might be mediated through the cascades of both ERK and p38 kinase, but not JNK.

Taken together, our results indicate that LPS-induced CREB phosphorylation and the expression of C/EBPδ are mediated by ERK and p38 kinase pathways, while c-Jun (AP-1) phosphorylation is mediated by ERK and JNK pathways. Thus, there is a redundancy in the signaling pathways and transcription factors regulating LPS-mediated COX-2 expression. 6-MITC blocked all of these steps.

3.6. Inhibitory activity of MITC on LPS-induced COX-2 expression depends on the methyl chain length

Depending on the length of the methyl chain of methylsulfinyl isothiocyanates, there are a number of analogues of methyl sulfinyl isothiocyanates (MITCs) in Wasabi extracts (Fig. 6A). To investigate the structure–activity relationship of MITCs, we tested the effects of 2-, 4-, 6- and 8-MITC on LPS-induced COX-2 expression at the same concentration that did not affect cellular viability as measured by MTT assay (data not shown). As shown in Fig. 6B, all four MITCs suppressed LPS-induced COX-2 expression, and the potency of inhibition was observed to be proportional to the length of their methyl chain. On the other hand, the expression levels of COX-1 in such treatment were steadily constant. Thus, an increase in the methyl chain length of MITCs appears to be important for their inhibitory activities on LPS-induced COX-2 expression.

4. Discussion

Accumulated data suggest that COX-2 overexpression is strongly related to inflammation and cancer. On the basis of this information, specific inhibition of COX-2 is a promising approach to preventing or targeting cancer and inflammation [38,39]. In the present study, we found that 6-MITC, a major compound in Wasabi, could reduce LPS-induced COX-2 protein expression and transcription activity without affecting the constitutive COX-1 expression (Figs. 1 and 2), suggesting that 6-MITC is a potent inhibitor of COX-2 expression.

Many previous studies have indicated that the inductive effects of LPS on COX-2 expression are mediated by the first 327 base pairs in the 5′ flanking regions of *COX-2* gene including NF-κB, C/EBP and CRE sites [20,23,25,33,34]. Moreover, single site of NF-κB, C/EBP or CRE cannot sufficiently response to induce *COX-2* transcription activity, and two of these *cis*-acting elements are at least recruited to achieve maximal induction of transcription [33]. In the present study, our data indicated that 6-MITC inhibited LPS-induced COX-2 expression by suppressing transcriptional factors binding to the first 327 base pairs in the 5′ flanking regions of *COX-2* gene (Fig. 2). Moreover, mutation of a single NF-κB, C/EBP or CRE promoter element did not abrogate the 6-MITC effect. Thus, the inhibition of at least two of these *cis*-elements is required to achieve the maximal inhibitory action of 6-MITC on *COX-2* gene expression, suggesting that the inhibitory effect of 6-MITC on COX-2 expression could be obtained by targeting the signaling pathways leading to at least two promoter elements including NF-κB, C/EBP and CRE sites.

Although many signaling pathways may contribute to *COX-2* gene expression, MAPK pathways play critical role on LPS-induced COX-2 expression [27,33,34]. Our results from the experiments of MAPK-specific inhibitors demonstrate that there are cooperative actions in MAPK pathways leading to stimulate COX-2 expression (Fig. 3B). 6-MITC blocked LPS-induced phosphorylation of MAPK including ERK, p38 kinase and JNK (Fig. 3A), and also blocked the phosphorylation of MAPKK, such as MEK (an ERK kinase), MKK (a p38 kinase kinase) and SEK (a JNK kinase) (data not shown). Thus, our data suggest that 6-MITC might inhibit LPS-induced COX-2 expression by blocking all of three MAPK signaling pathways.

We further investigated the effects of 6-MITC on the transcription factors including CREB, AP-1 and C/EBP. Because LPS-induced phosphorylation of CREB and AP-1 can regulate *COX-2* gene expression through binding CRE site in *COX-2* promoter [27,34,35], and LPS-induced nuclear translocation of C/EBP can stimulate *COX-2* gene

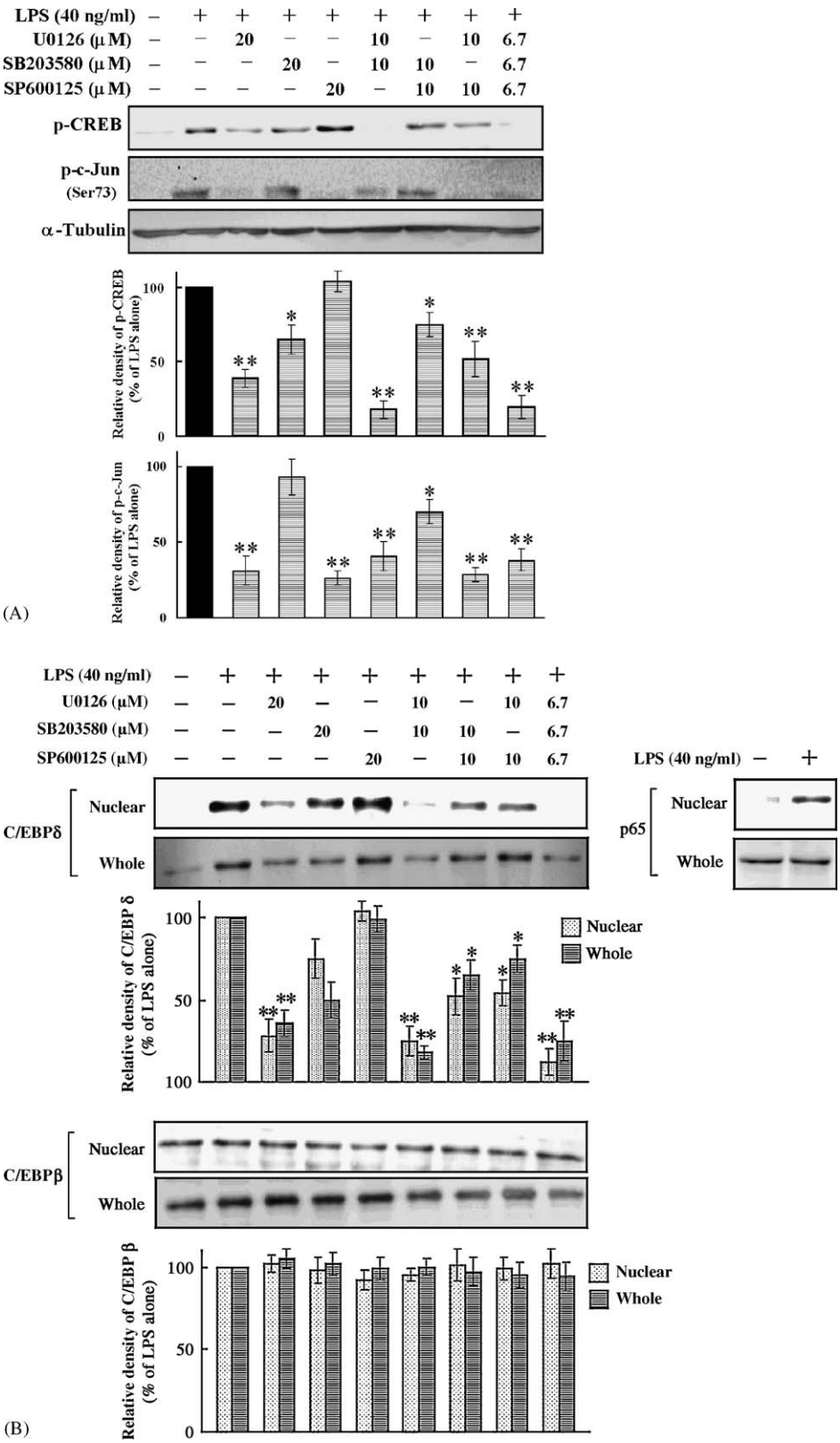

Fig. 5. Effects of MAPK inhibiters on LPS-induced activation of the transcriptional factors. (A) LPS-induced CREB phosphorylation is suppressed by U0126 and SB203580, and c-Jun phosphorylation is suppressed by U0126 and SP600125. Cell culture, treatment and Western blotting analysis were done described in Figs. 3 and 4. Histograms show the densitometric analysis of phosphorylated CREB and c-Jun normalized to α-tubulin. (B) LPS-induced nuclear translocation and expression of C/EBPδ is suppressed by U0126 and SB203580. Cell treatment, nuclear extraction and Western blotting analysis were done as described in Figs. 3 and 4. The nuclear p65 was used as a positive control to confirm the preparation of nuclear protein. Histograms show the densitometric analysis of C/EBPδ and C/EBPβ. The data represent the mean ± S.D. of three to four separate experiments, and the figure is a representative of those experiments each with similar results. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. LPS.

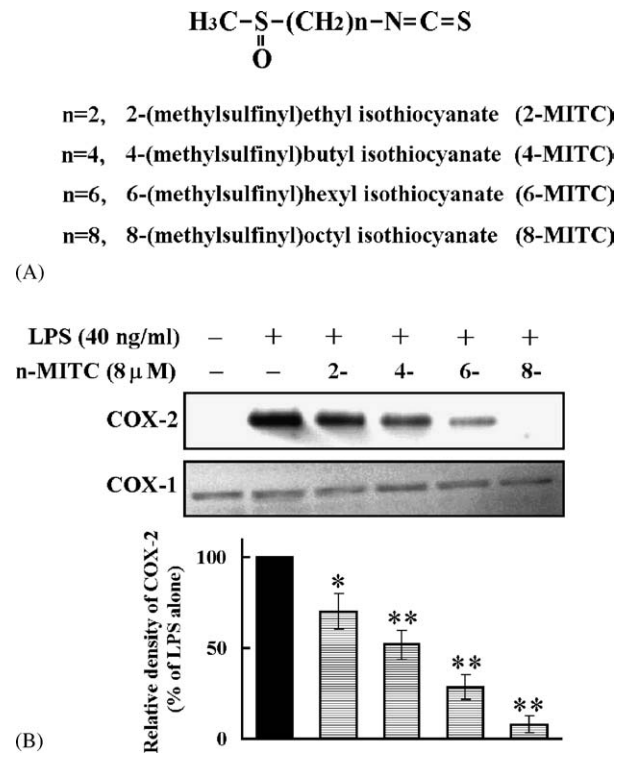

Fig. 6. Structure–activity relationship of MITCs on the inhibition of LPS-induced COX-2 expression. (A) Chemical structure of MITCs used in this study. (B) Inhibitory effects of MITCs on LPS-induced COX-2 expression. Cell treatment and Western blotting analysis were done described in Fig. 1. RAW264 cells were treated with 8 μM of 2-, 4-, 6- or 8-MITC for 30 min, respectively, and then exposed to 40 ng/ml LPS for 12 h. Histograms show the densitometric analysis of COX-2 protein normalized to COX-1. The data represent the mean ± S.D. of three to four separate experiments, and the figure is a representative of those experiments each with similar results. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. LPS.

expression through binding C/EBP site in *COX-2* promoter [23,35,36]. Several lines of studies have showed that the binding of CREB and AP-1 to CRE site depends on the phosphorylation of CREB and c-Jun, a component of the AP-1 transcription factor complex [34,40,41], and that the binding of C/EBP to *COX-2* promoter is preceded by nuclear translocation of C/EBP [34,35,42]. Thus, the inhibition of phosphorylation of CREB and c-Jun, and nuclear translocation of C/EBP result in a binding suppression of those transcriptional factors to *COX-2* promoter. Our data showed that 6-MITC inhibited LPS-induced phosphorylation of CREB and c-Jun (Fig. 4A). 6-MITC also blocked LPS-induced expression and nuclear translocation of C/EBPδ, but not C/EBPβ (Fig. 4B). Inoue et al. showed that C/EBPδ, but not C/EBPβ, is rapidly induced by LPS, and C/EBPδ regulates *COX-2* gene expression through the combination of C/EBP and CRE sites [23]. Thus, 6-MITC may suppressed the expression and nuclear translocation of C/EBPδ, leading to the inhibition of COX-2 expression.

NF-κB is one of the essential factors for COX-2 expression. Some chemopreventive compounds, such as capsaicin [43], sauchinone [35] and apigenin [44], inhibit LPS-induced COX-2 expression by blocking degradation of IκB-α in mouse macrophage cells. But, 6-MITC had no influence on phosphorylation and degradation of IκB-α (Fig. 4C), and on nuclear translocation of p65 (Fig. 4D). Moreover, the data from double-mutated promoter at CRE and C/EBP sites showed that transcription activity was completely abolished. Thus, 6-MITC may inhibit COX-2 expression by a NF-κB-independent pathway, suggesting a novel type of inhibitor for COX-2. It is noticed that 6-MITC could retard de novo synthesis. A similar result was also observed in the treatment with sulforaphane, which is an analogue of 6-MITC, and interfered the synthesis of IκB-α [45]. But the reason is still not known. It needs to be clarified in our next study.

There are multiple lines of evidence supporting that LPS regulates COX-2 expression by MAPK signaling pathways [33,34]. However, signaling from MAPK to transcription factors mediating COX-2 expression are not clear. In this study, we identified the signaling pathways. First, we have shown clearly that ERK and p38 kinase pathways cooperatively regulate COX-2 expression by activating CREB and C/EBPδ because ERK-specific inhibitor U0126 and p38 kinase-specific inhibitor SB203580 suppressed CREB phosphorylation and C/EBPδ expression, but JNK-specific inhibitor SP600125 did not (Fig. 5A and B). Second, we have identified that ERK and JNK signaling pathways cooperatively regulate COX-2 expression by activating AP-1 since SP600125 and U0126, but not SB203580, inhibited c-Jun phosphorylation (Fig. 5A). Our data demonstrated that there is a redundancy at the signaling pathways, transcription factor and promoter levels for COX-2 expression in LPS-treated RAW264 cells. The redundancy may be an important mechanism to ensure the COX-2 expression in macrophage during inflammation. Finally, we have demonstrated that 6-MITC blocked LPS-induced COX-2 expression by suppressing ERK and p38 kinase signaling cascades leading to the activation of CREB and C/EBPδ, and by inhibiting JNK cascade leading to AP-1 activation.

Although 6-MITC is one of major allyl isothiocyanate in Wasabi [5,6], there are a number of analogues of 6-MITC present in Wasabi. To investigate the structure–activity relationship, we treated RAW264 cells with four types of MITCs (Fig. 6A) for 12 h and then determined the COX-2 expression. As shown in Fig. 6B, the inhibitory potency on COX-2 protein was showed in the order of 2-, 4-, 6- and 8-MITC, suggesting that an increase in the methyl chain length is important for the inhibitory activity of LPS-induced COX-2 expression. A similar relationship was also reported previously in the induction of quinone oxidoreductase in Hepa 1c1c7 cells [46]. Thus, MITC compounds which have the long methyl chain may be more effective in anti-inflammatory and anti-carcinogenic action within the non-toxic concentrations.

In summary, we show the data for the first time that 6-MITC inhibits LPS-induced COX-2 expression at the signaling level and at the transcription factor/promoter

levels. These findings provide the first molecular basis for the anti-inflammatory action of 6-MITC.

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References

[1] Kojima M. Pungent components and functional ingredient of wasabi. Food Process 1988;23:32–5.
[2] Isshiki K, Tokuoka K. Allyl isothiocyanate and wholesomeness of food. Jpn J Food Microbiol 1993;12:1–6.
[3] Kumagai H, Kashima N, Seki T, Sakurai H, Ishii K, Ariga T. Analysis of components in essential oil of upland wasabi and their inhibitory effects on platelet aggregation. Biosci Biotechnol Biochem 1994;58:2131–5.
[4] Tanida N, Kawaura A, Takahashi A, Sawada K, Shimoyama T. Suppressive effect of wasabi (pungent Japanese spice) on gastric carcinogenesis induced by MNNG in rats. Nutr Cancer 1991;16:53–8.
[5] Ono H, Adach K, Fuke Y, Shinohara K. Purification and structural analysis of substances in wasabi (*Eutrema wasabi* Maxim.) that suppress the growth of MKN-28 human stomach cancer cells. J Jpn Food Sci Technol 1996;43:1092–7.
[6] Morimitsu Y, Nakagawa Y, Hayashi K, Fujii H, Kumagai T, Nakamura Y, et al. A sulforaphane analogue that potently activates the Nrf2-dependent detoxification pathway. J Biol Chem 2002;277:3456–63.
[7] Fuke Y, Haga Y, Ono H, Nomura T, Ryoyama K. Anti-carcinogenic activity of 6-methylsulfinylhexyl isothiocyanates (6-MITC), and an active antiproliferative principal of wasabi (*Eutrema wasabi* Maxim.). Cytotechnology 1997;25:197–203.
[8] Yano T, Yajima S, Virgona N, Yano Y, Otani S, Kumagai H, et al. The effect of 6-methylthiohexyl isothiocyanate isolated from *Wasabia japonica* (wasabi) on 4-(methylnitrosamino)-1-(3-pyridyl)-1-buatnone-induced lung tumorigenesis in mice. Cancer Lett 2000;155:115–20.
[9] Funk CD, Funk LB, Kennedy ME, Pong AS, Fitzgerald GA. Human platelet/erythroleukemia cell prostaglandin G/H synthase: cDNA cloning, expression, and gene chromosomal assignment. FASEB J 1991;5:2304–12.
[10] Hempel SL, Monick MM, Hunninghake GW. Lipopolysaccharide induces prostaglandin H synthase-2 protein and mRNA in human alveolar macrophages and blood monocytes. J Clin Invest 1994;93:391–6.
[11] Crofford LJ, Wilder RL, Ristimaki AP, Sano H, Remmers EF, Epps HR, et al. Cyclooxygenase-1 and -2 expression in rheumatoid synovial tissues. Effects of interleukin-1 beta, phorbol ester, and corticosteroids. J Clin Invest 1994;93:1095–101.
[12] Hla T, Ristimaki A, Appleby S, Barriocanal JG. Cyclooxygenase gene expression in inflammation and angiogenesis. Ann NY Acad Sci 1993;696:197–204.
[13] Rolland PH, Martin PM, Jacquemier J, Rolland AM, Toga M. Prostaglandin in human breast cancer: evidence suggesting that an elevated prostaglandin production is a marker of high metastatic potential for neoplastic cells. J Natl Cancer Inst 1980;64:1061–70.
[14] Lupulescu A. Prostaglandins, their inhibitors and cancer. Prostaglandins Leukot Essent Fatty Acids 1996;54:83–94.
[15] Sheng H, Shao J, Morrow JD, Beauchamp RD, DuBois RN. Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. Cancer Res 1998;58:362–6.
[16] Ben-Av P, Crofford LJ, Wilder RL, Hla T. Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for inflammatory angiogenesis. FEBS Lett 1997;372:83–7.
[17] Goodwin JS, Ceuppens J. Regulation of the immune response by prostaglandins. J Clin Immunol 1983;3:295–314.
[18] Tsujii M, DuBois RN. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. Cell 1995;83:493–501.
[19] D'Acquisto F, Iuvone T, Rombola L, Sautebin L, DiRosa M, Carnuccio R. Involvement of NF-kappaB in the regulation of cyclooxygenase-2 protein expression in LPS-stimulated J774 macrophages. FEBS Lett 1997;418:175–8.
[20] Inoue H, Tanabe T. Transcriptional role of the nuclear factor kappa B site in the induction by lipopolysaccharide and suppression by dexamethasone of cyclooxygenase-2 in U937 cells. Biochem Biophys Res Commun 1998;244:143–8.
[21] Sorli CH, Zhang HJ, Armstrong MB, Rajotte RV, Maclouf J, Robertson RP. Basal expression of cyclooxygenase-2 and nuclear factor-interleukin 6 are dominant and coordinately regulated by interleukin 1 in the pancreatic islet. Proc Natl Acad Sci USA 1998;95:1788–93.
[22] Thomas B, Berenbaum F, Humbert L, Bian H, Bereziat G, Crofford L, et al. Critical role of C/EBPδ and C/EBPβ factors in the stimulation of the cyclooxygenase-2 gene transcription by interleukin-1β in articular chondrocytes. Eur J Biochem 2000;267:6798–809.
[23] Inoue H, Yokoyama C, Hara S, Tone Y, Tanabe T. Transcriptional regulation of human prostaglandin-endoperoxide synthase-2 gene by lipopolysaccharide and phorbol ester in vascular endothelial cells. J Biol Chem 1995;270:24965–71.
[24] Wadleigh DJ, Reddy ST, Kopp E, Ghosh S, Herschman HR. Transcriptional activation of the cyclooxygenase-2 gene in endotoxin-treated RAW 264.7 macrophages. J Biol Chem 2000;275:6259–66.
[25] Inoue H, Nanayama T, Hara S, Yokoyama C, Tanabe T. The cyclic AMP response element plays an essential role in the expression of the human prostaglandin-endoperoxide synthase 2 gene in differentiated U937 monocytic cells. FEBS Lett 1994;350:51–4.
[26] Subbaramaiah K, Chung WJ, Michaluart P, Telang N, Tanabe T, Inoue H, et al. Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. J Biol Chem 1998;273:21875–82.
[27] Xie W, Herschman HR. v-src induces prostaglandin synthase 2 gene expression by activation of the c-Jun N-terminal kinase and the c-Jun transcription factor. J Biol Chem 1995;270:27622–8.
[28] Kirikae T, Tamura H, Hashizume M, Kirikae F, Uemura Y, Tanaka S, et al. Endotoxin contamination in fetal bovine serum and its influence on tumor necrosis factor production by macrophage-like cells J774.1 cultured in the presence of the serum. Int J Immunopharmacol 1997;19:255–62.
[29] Yang Z, Khemlani LS, Dean DF, Carter CD, Slauson DO, Bochsler PN. Serum components enhance bacterial lipopolysaccharide-induced tissue factor expression and tumor necrosis factor-alpha secretion by bovine alveolar macrophages in vitro. J Leukoc Biol 1994;55:483–8.
[30] Jian ZJ, Yang Z, Mason GL, Slauson DO, Bochsler PN. Regulation of superoxide anion generation in bovine alveolar macrophages by bacterial lipopolysaccharide, serum proteins, and modulators of signal transduction. Inflammation 1995;19:637–50.
[31] Hou D-X, Kai K, Li JJ, Lin S, Terahara N, Wakamatsu M, et al. Anthocyanidins inhibit activator protein 1 activity and cell transformation: structure–activity relationship and molecular mechanisms. Carcinogenesis 2004;25:29–36.

[32] Hou D-X, Fukuda M, Fujii M, Fuke Y. Transcriptional regulation of nicotinamide adenine dinucleotide phosphate: quinone oxidoreductase in murine hepatoma cells by 6-(methylsulfinyl)hexyl isothiocyanate, an active principle of wasabi (*Eutrema wasabi* Maxim.). Cancer Lett 2000;20:195–200.

[33] Mestre JR, Mackrell PJ, Rivadeneira DE, Stapleton PP, Tanabe T, Daly JM. Redundancy in the signaling pathways and promoter elements regulating cyclooxygenase-2 gene expression in endotoxin-treated macrophage/monocytic cells. J Biol Chem 2001;276: 3977–82.

[34] Caivano M, Gorgoni B, Cohen P, Poli V. The induction of cyclooxygenase-2 mRNA in macrophages is biphasic and requires both CCAAT enhancer-binding protein beta (C/EBP beta) and C/EBP delta transcription factors. J Biol Chem 2001;276:48693–701.

[35] Lee AK, Sung SH, Kim YC, Kim SG. Inhibition of lipopolysaccharide-inducible nitric oxide synthase, TNF-alpha and COX-2 expression by sauchinone effects on I-kappaBalpha phosphorylation C/EBP and AP-1 activation. Br J Pharmacol 2003;139:11–20.

[36] Thomas B, Berenbaum F, Humbert L, Bian H, Bereziat G, Crofford L, et al. Critical role of C/EBPdelta and C/EBPbeta factors in the stimulation of the cyclooxygenase-2 gene transcription by interleukin-1beta in articular chondrocytes. Eur J Biochem 2000;267:6798–809.

[37] Zandi E, Rothwarf DM, Delhase M, Hayakawa M, Karin M. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. Cell 1997;91:243–52.

[38] Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, et al. Suppression of intestinal polyposis in $Apc^{\Delta 716}$ knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell 1996;87:803–9.

[39] Masferrer JL, Zweifel BS, Manning PT, Hauser SD, Leahy KM, Smith WG, et al. Selective inhibition of inducible cyclooxygenase 2 in vivo is antiinflammatory and nonulcerogenic. Proc Natl Acad Sci USA 1994;91:3228–32.

[40] Subbaramaiah K, Cole PA, Dannenberg AJ. Retinoids and carnosol suppress cyclooxygenase-2 transcription by CREB-binding protein/p300-dependent and -independent mechanisms. Cancer Res 2002;62:2522–30.

[41] Eliopoulos AG, Dumitru CD, Wang CC, Cho J, Tsichlis PN. Induction of COX-2 by LPS in macrophages is regulated by Tpl2-dependent CREB activation signals. EMBO J 2002;21:4831–40.

[42] Cho YH, Lee CH, Kim SG. Potentiation of lipopolysaccharide-inducible cyclooxygenase 2 expression by C2-ceramide via c-Jun N-terminal kinase-mediated activation of CCAAT/enhancer binding protein beta in macrophages. Mol Pharmacol 2003;63:512–23.

[43] Kim CS, Kawada T, Kim BS, Han IS, Choe SY, Kurata T, et al. Capsaicin exhibits anti-inflammatory property by inhibiting IκB-α degradation in LPS-stimulated peritoneal macrophages. Cell Signal 2003;15:299–306.

[44] Liang YC, Huang YT, Tsai SH, Lin-Shiau SY, Chen CF, Lin JK. Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. Carcinogenesis 1999;20:1945–52.

[45] Heiss E, Herhaus C, Klimo K, Bartsch H, Gerhauser C. Nuclear factor kappa B is a molecular target for sulforaphane-mediated anti-inflammatory mechanisms. J Biol Chem 2001;276:32008–15.

[46] Hou D-X, Fukuda M, Fujii M, Fuke Y. Induction of NADPH:quinone oxidoreductase in murine hepatoma cells by methyl sulfinyl isothiocyanates: methyl chain length-activity study. Int J Mol Med 2000;6:441–4.