Effects of 6-(methylsulfinyl)hexyl isothiocyanate on cyclooxygenase-2 expression induced by lipopolysaccharide, interferon-γ and 12-*O*-tetradecanoylphorbol-13-acetate

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Abstract. 6-(methylsulfinyl)hexyl isothiocyanate (6-MITC) is a bioactive compound extracted from a typical Japanese spice, wasabi (*Wasabia japonica* (Miq.) Matsumura). In the present study, we found that 6-MITC suppressed the expression of cyclooxygenase-2 (COX-2) induced by lipopolysaccharide (LPS), interferon-γ (IFN-γ), but did not suppress that induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), in murine macrophage RAW264. Molecular mechanisms were investigated by targeting the transcriptional factors including activator protein-1 (AP-1), CCAAT/enhancer-binding protein δ (C/EBPδ), CRE-binding protein (CREB) and nuclear factor κB (NF-κB), which bind to the core element of *COX-2* promoter. LPS induced activation of all of these factors and 6-MITC suppressed LPS-induced activation of AP-1, C/EBPδ, CREB, but not NF-κB. IFN-γ did not induce any activation of these factors, but 6-MITC suppressed IFN-γ-induced COX-2 expression, suggesting that the upstream region of the core element is linked for this suppression. Finally, TPA stimulated the activation of CREB and AP-1, but 6-MITC did not block TPA-induced COX-2 expression. These results suggest that LPS, IFN-γ and TPA regulate COX-2 expression through different mechanisms, and 6-MITC acts as a potent inhibitor of COX-2 expression induced by LPS or IFN-γ.

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Abbreviations: 6-MITC, 6-(methylsulfinyl)hexyl isothiocyanate; AP-1, activator protein-1; C/EBP, CCAAT/enhancer-binding protein; COX-2, cyclooxygenase-2; CREB, CRE-binding protein; IFN-γ, interferon-γ; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

Key words: 6-(methylsulfinyl)hexyl isothiocyanate, macrophages, cyclooxygenase-2, lipopolysaccharide, 12-*O*-tetradecanoylphorbol-13-acetate, interferon-γ

Introduction

Numerous studies have demonstrated that high intake of cruciferous vegetables such as broccoli, watercress, cabbage, and cauliflower, can prevent cancer (1). The bioactive compounds involved in these vegetables are isothiocyanates (ITCs), which have been reported to have cancer-preventive effect in the carcinogenesis of lung, breast and colon (2). 6-(methylsulfinyl) hexyl isothiocyanate (6-MITC) is an ITC, present as a major allyl isothiocyanate in a typical Japanese spice, wasabi (*Wasabia japonica* (Miq.) Matsumura). Oral administration of 6-MITC showed the inhibitory effects on mouse skin tumor induced by 9,10-dimethyl-1,2-benzanthracene as an initiator and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as a promoter (3), and on mouse lung tumor induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (4). 6-MITC also has been demonstrated to specifically suppress cell proliferation of breast cancer and melanoma cell lines (5). These results suggest that 6-MITC is a potent chemopreventive compound.

Cyclooxygenase (COX-2) is an enzyme catalyzing the conversion of arachidonic acid to prostaglandins. There are two isoforms of COX, designated COX-1 and COX-2. COX-1 is constitutively expressed in most tissues to maintain normal physiological functions (6). In contrast, COX-2 is undetectable or present at very low levels under basal conditions, but is rapidly induced by inflammatory stimuli, cytokines, growth factors, tumor promoters, and carcinogens (7,8). Several lines of evidence suggest that COX-2 plays an important role in the process of carcinogenesis because COX-2 is overexpressed in a variety of transformed cells and tumors (9,10). Enhanced induction of COX-2 promotes the growth of malignant cells by increasing cell proliferation (11), promoting angiogenesis (12), and inhibiting immune surveillance (13) and apoptosis (14). Various chemopreventive strategies have focused on the identification of COX-2 inhibitors because the targeted inhibition of COX-2 is an effective approach for suppressing inflammation and carcinogenesis.

Accumulated data have indicated that there are three *cis*-acting elements including nuclear factor κB (NF-κB), CCAAT/enhancer-binding protein (C/EBP) and cAMP-responsive element (CRE) present in the promoter (15). The tran-

scriptional factors such as NF-κB, C/EBPs, CRE binding protein (CREB) and activator protein-1 (AP-1) bind to these *cis*-acting elements and transactivate *COX-2* gene expression. Various different inflammatory stimuli have been reported to induce *COX-2* gene transcription (7,8). However, the precise mechanisms underlying *COX-2* gene expression triggered by these stimuli have not been fully elucidated.

In the present study, we investigated the effect of 6-MITC on COX-2 expression activated by LPS, INF-γ, and TPA in murine macrophage RAW264 cells. Our study revealed that LPS, IFN-γ and TPA induced COX-2 expression through different pathways, and 6-MITC acted as a potent inhibitor for intervening LPS- and IFN-γ-activated COX-2 expression.

Materials and methods

Materials and cell culture. 6-MITC purified by reverse-phase HPLC to >99% were obtained from Hakucho Pharmaceutical Co., Ltd (Japan). 6-MITC was dissolved in dimethyl sulfoxide (DMSO, final concentration was 0.2%). Antibodies against phospho-CREB, phospho-c-Jun, CREB, c-Jun and IκB-α were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against COX-2, COX-1, C/EBPß, C/EBPδ, p65 and α-tubulin 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). 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.

Western blot analysis. Western blot assay was performed as described previously (16). In brief, RAW264 ($1x10^6$ cells) were pre-cultured in a 6-cm dish for 24 h, and then starved by being cultured in serum-free medium 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 LPS, IFN-γ, TPA, interleukin-ß (IL-ß), tumor necrosis factor-α (TNF-α), or H_2O_2 for different times. The harvested cells were lysed and the supernatants were boiled for 5 min. Protein concentration was determined 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 Western 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 each specific antibody was quantified using the Lumi Vision Imager software (Taitec Co.).

Plasmids. The *COX-2* promoter-luciferase deletion constructs (-1432/+59, -327/+59) were generous gifts of Dr Hiroyasu Inoue (Nara Women's University, Nara, Japan) and have been described previously (17-19).

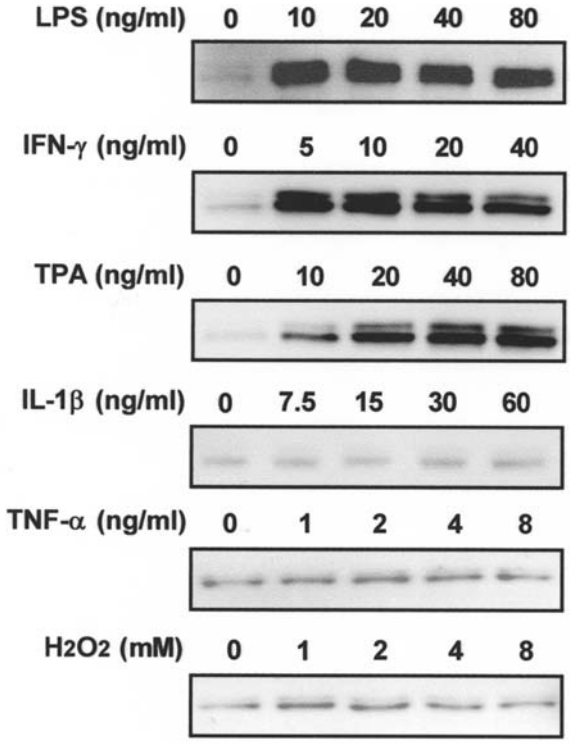

Figure 1. Effects of inflammatory stimuli on COX-2 expression in RAW264 cells. After RAW264 cells ($1x10^6$) were starved in serum-free medium for 3 h, the cells were treated with the indicated concentrations of LPS, INF-γ, TPA, IL-1ß, TNF-α, or H_2O_2 for 12 h. COX-2 was detected with its antibody by Western blot analysis.

Transient transfection assay. Transient transfection was performed according to the modified method as described previously (20). RAW264 cells ($1x10^5$) 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-ß-galactosidase plasmids using Lipofectamine 2000. After 5 h of 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, IFN-γ, or TPA for 6 h. The activities of luciferase and ß-galactosidase in cell lysate were measured with a luminometer (Berthold) according to the supplier's recommendations. Luciferase activity is normalized to transfection efficiency monitored by ß-galactosidase expression, and *COX-2* transcription activity was expressed as fold induction relative to the control cells without LPS, IFN-γ, or TPA treatment.

Nuclear extracts. Nuclear extracts were prepared using the nuclear extract kit from Active Motif (Carlsbad, CA). Briefly, RAW264 cells ($3x10^6$) were pre-cultured in a 10-cm dish for 24 h, and then starved by being cultured in serum-free medium 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, IFN-γ, or TPA. The cell pellet was suspended in 1 x hypotonic buffer supplemented with complete protease inhibitor cocktail, incubated for 15 min at 4˚C, voltexed with detergents and centrifuged briefly. The nuclear pellet was washed twice with the cytoplasmic buffer

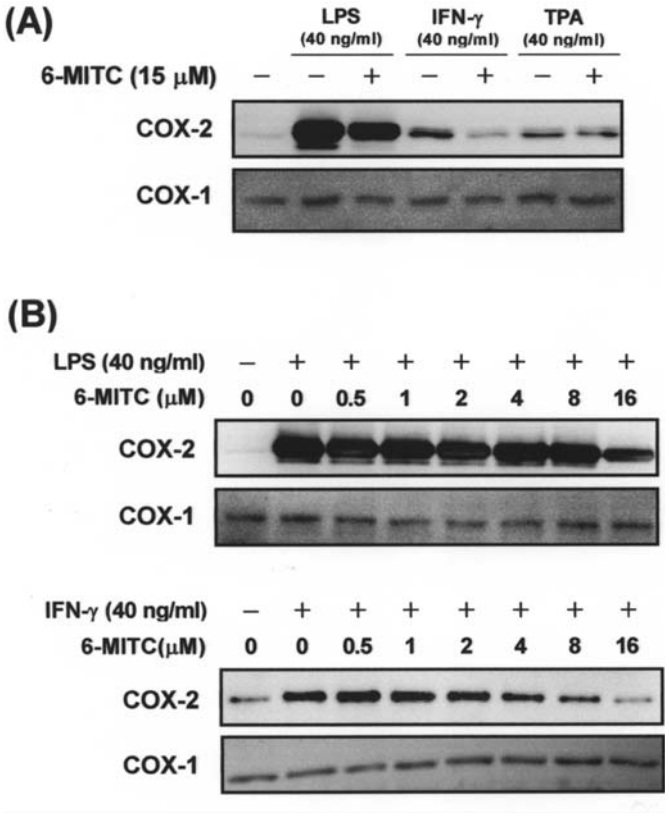

Figure 2. Effect of 6-MITC on COX-2 expression induced by LPS, IFN-γ or TPA. (A) After RAW264 cells ($1x10^6$) were starved in serum-free medium for 2.5 h, the cells were treated with 15 μM 6-MITC for 30 min, and then exposed to 40 ng/ml LPS, IFN-γ, TPA for 12 h. (B) The cells were treated with the indicated concentrations of 6-MITC for 30 min, and then exposed to 40 ng/ml LPS or IFN-γ for 12 h. COX-2 and COX-1 were detected by Western blot analysis with their antibodies, respectively.

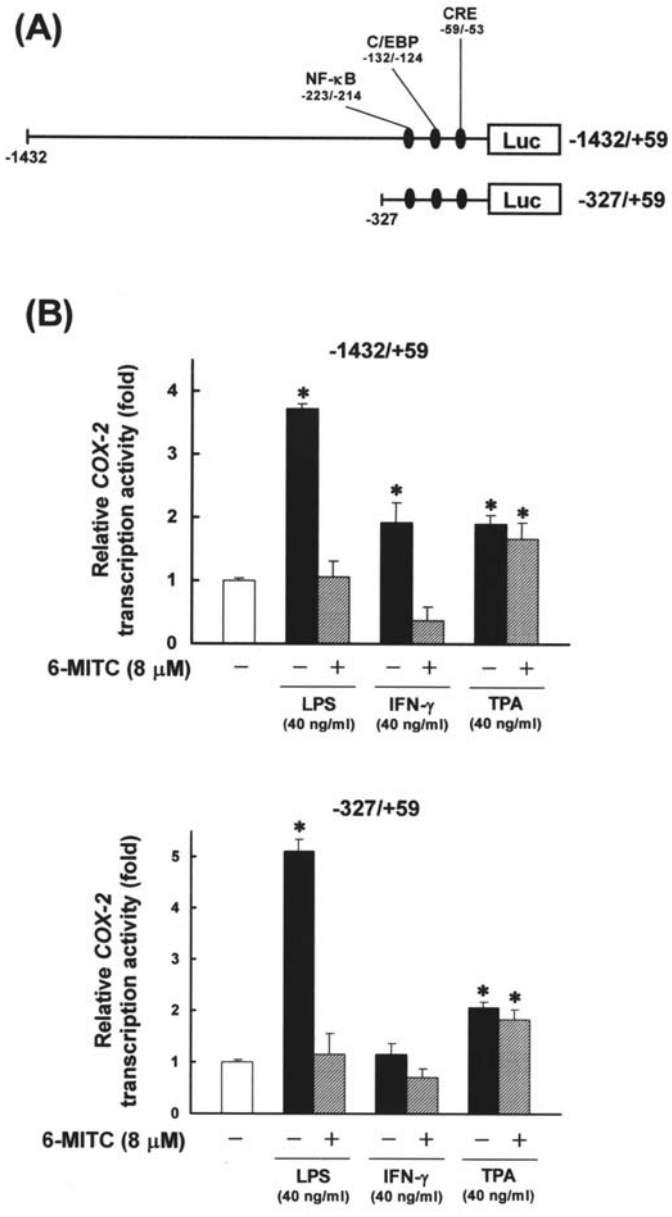

Figure 3. Different effects of 6-MITC on *COX-2* promoter activity induced by LPS, IFN-γ or TPA. (A) Schematic diagram of *COX-2* promoter-luciferase reporter constructs used. (B) RAW264 cells were transfected with 0.5 μg of *COX-2* promoter constructs (-1432/+59 or -327/ +59) and 0.12 μg CMV-ß-galactosidase plasmid. After 5 h of 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, IFN-γ and TPA for 6 h. The luciferase activity values were normalized to transfection efficiency monitored by ß-galactosidase expression, and *COX-2* promoter activity was expressed as fold induction to control. Each value represents the mean ± SD of three to four separate experiments. *P<0.05 versus control.

followed by resuspension in the lysis buffer supplemented with 1 mM DTT and protease inhibitors. The suspension was incubated on a rocking platform at 4˚C for 30 min. The suspension was then voltexed briefly and centrifuged for 10 min at 14,000 x g at 4˚C. The supernatant (nuclear fraction) was collected, and nuclear extracts were stored at -80˚C until Western blot analysis.

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

Results

Effects of inflammatory stimuli on COX-2 expression. To determine the effects of inflammatory stimuli on COX-2 protein expression in mouse macrophages, RAW264 cells were incubated with the indicated concentrations of LPS, INF-γ, TPA, interleukin-1ß (IL-1ß), tumor necrosis factor-α (TNF-α), or H_2O_2 for 12 h. As shown in Fig. 1, the expression of COX-2 protein was increased after incubation with LPS, INF-γ or TPA. On the other hand, IL-1ß and TNF-α did not induce COX-2 expression although the used concentrations could induce COX-2 expression in Caco-2 colon carcinoma cells (21), human airway smooth muscle (HASM) cells (22,23), L929 fibroblast cells (24), and lung adenocarcinoma A549 cells (24). Thus, LPS, INF-γ and TPA are potent inducers of COX-2 expression in RAW264 cells.

Effect of 6-MITC on COX-2 expression induced by LPS, IFN-γ or TPA. To investigate the inhibitory effect of 6-MITC on COX-2 expression induced by LPS, IFN-γ, or TPA, RAW264 cells were treated with 15 μM 6-MITC for 30 min before exposure to 40 ng/ml LPS, IFN-γ, or TPA for 12 h. As shown in Fig. 2A, 6-MITC suppressed LPS- and IFN-γ-induced

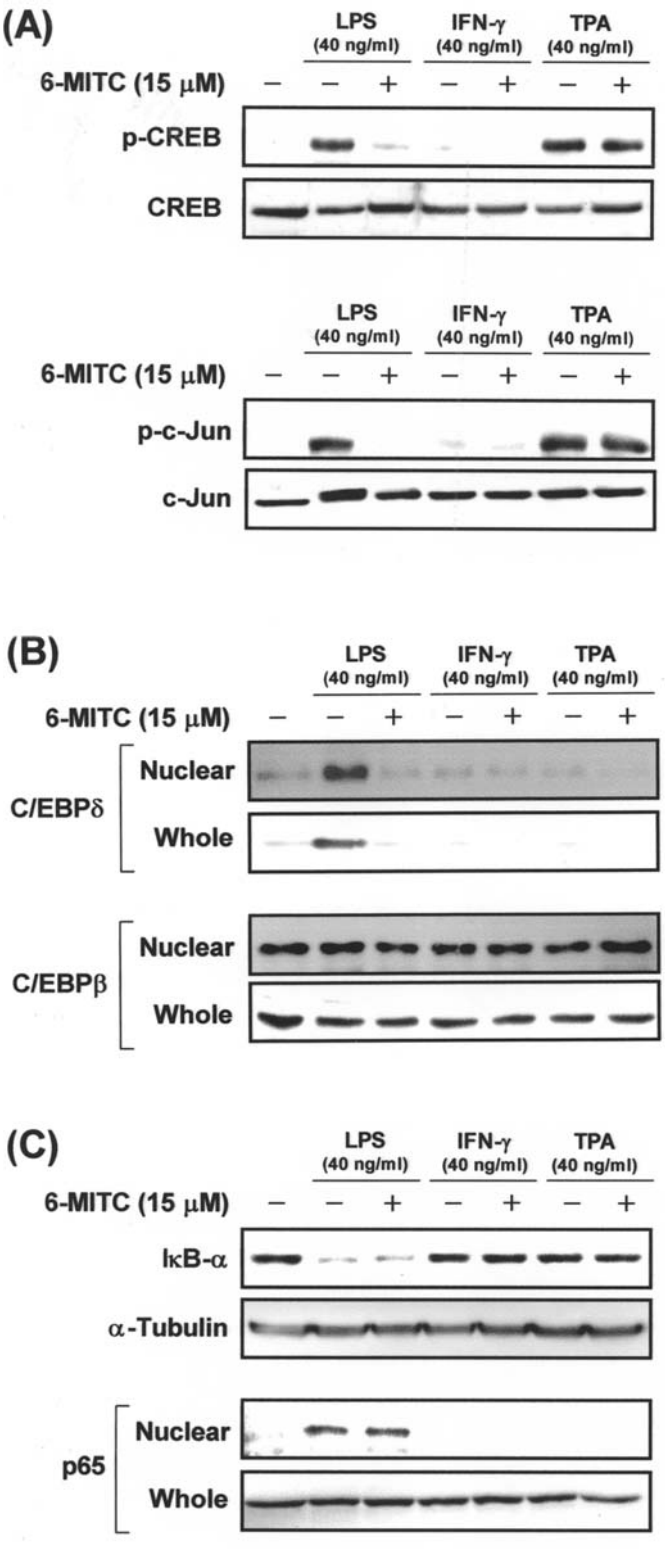

Figure 4. Effects of 6-MITC on the activation of the transcriptional factors induced by LPS, IFN-γ or TPA. (A) Cell culture and Western blot analysis were done as described in Fig. 2. RAW264 cells were treated with 15 μM 6-MITC for 30 min, and then exposed to 40 ng/ml LPS, IFN-γ or TPA for 30 min. Phosphorylated and total c-Jun or CREB were detected by Western blot analysis with their antibodies, respectively. (B) The cells were treated with or without 15 μM 6-MITC for 30 min, and then exposed to 40 ng/ml LPS, IFN-γ or TPA for 4 h. The nuclear proteins and whole cell lysates were extracted, and C/EBPδ and ß were detected by Western blot analysis. (C) The cells were treated with 15 μM 6-MITC for 30 min, and then exposed to 40 ng/ml LPS, IFN-γ and TPA for 30 min. The nuclear proteins and whole cell lysates were extracted, and IκB-α, p65 and α-tubulin were detected by Western blot analysis.

COX-2 expression. However, 6-MITC did not suppress TPA-induced COX-2. The amount of COX-1, which was constitutive protein, remained uncharged. To further examine the inhibitory effects of 6-MITC on COX-2 expression induced by LPS or IFN-γ, the cells were incubated with various concentrations of 6-MITC before the treatment with LPS or IFN-γ. Fig. 2B shows that 6-MITC inhibited COX-2 expression induced by LPS or IFN-γ in a dose-dependent manner. Our previous results have shown that the concentration range of 6-MITC used in these experiments had no significant effect on the cellular viability as measured by MTT assay (data not shown) (25). Thus, the inhibitory actions of 6-MITC on COX-2 expression were not caused by its toxicity.

Different effects of 6-MITC on LPS-, IFN-γ- or TPA-induced COX-2 promoter activity. The core element (-327/-52) in *COX-2* promoter contains three *cis*-acting elements including NF-κB, C/EBP and CRE sites, which are involved in the regulation of *COX-2* gene expression (15). To investigate whether the inhibition of COX-2 expression by 6-MITC is mediated through the core element, transient transfections were performed using full (-1432/+59) and core (-327/+59) *COX-2* promoter-luciferase constructs (Fig. 3A). As shown in Fig. 3B, 1) LPS induced *COX-2* promoter activity in both -1432/+59 and -327/+59 constructs, and 6-MITC reduced them to control level. 2) IFN-γ induced *COX-2* promoter activity of -1432/+59 construct, which was suppressed by 6-MITC. IFN-γ did not induce the promoter activity of -327/+59 construct. 3) TPA also induced the *COX-2* promoter activity in both -1432/+59 and -327/+59, but 6-MITC did not block the transcriptional activity. These results suggest that the core element is required for LPS- and TPA-induced *COX-2* gene expression, and 6-MITC might block LPS-induced COX-2 expression by suppressing the core element. On the other hand, the upstream region of the core element may respond to the IFN-γ-induced COX-2 expression, and 6-MITC may block COX-2 expression through the upstream region.

Effects of 6-MITC on the transcriptional factors regulating COX-2 expression. Transcription factors including CREB, AP-1, C/EBP and NF-κB can regulate *COX-2* transcription by binding the core elements in *COX-2* promoter (15,18,26). To elucidate the effects of 6-MITC on these transcriptional factors, we pretreated RAW264 cells with 6-MITC for 30 min before exposure to LPS, IFN-γ, or TPA, and then examined the activation of these transcription factors by Western blot analysis.

As shown in Fig. 4A, LPS and TPA strongly induced the phosphorylation of CREB and c-Jun, which is a key component of AP-1 in c-Jun/c-Fos heterodimer form. 6-MITC blocked the phosphorylation of CREB and c-Jun induced by LPS, but not TPA. In contrast, IFN-γ did not induce phosphorylation of CREB and c-Jun.

LPS also markedly induced expression and nuclear translocation of C/EBPδ, which were blocked by 6-MITC (Fig. 4B). However, IFN-γ and TPA did not induce C/EBPδ at either whole or nuclear fraction. The C/EBPß levels at either whole or nuclear were not affected by three inducers and 6-MITC. These results suggest that 6-MITC suppressed LPS-activated

expression and subsequent nuclear translocation of C/EBPδ leading to COX-2 expression. In contrast, C/EBPδ was not involved in IFN-γ- and TPA-induced COX-2 expression.

NF-κB is also known as a critical factor for COX-2 expression induced by LPS or proinflammatory cytokines (17,27). NF-κB is inactivated in the cytosol by binding to IκB, and becomes active through translocation to the nucleus preceded by LPS-induced proteolytic degradation of IκB (28). To determine whether 6-MITC inhibit degradation of IκB, the level of IκB-α protein was assessed in the treated RAW264 cells. As shown in Fig. 4C, LPS reduced IκB-α protein, and stimulated nuclear translocation of p65, a major component of NF-κB, into nucleus. However, 6-MITC did not prevent the degradation of IκB-α and nuclear translocation of p65. On the other hand, IFN-γ and TPA had no influence on IκB-α degradation and nuclear translocation of p65. These data suggest that the inhibitory effect of 6-MITC on LPS-induced COX-2 expression is not mediated through the suppression of NF-κB activation, and IFN-γ- and TPA-induced COX-2 expression in RAW264 cells is not mediated by NF-κB activation.

Discussion

Multiple lines of evidence suggest that COX-2 has a critical role in inflammation and carcinogenesis. Thus, the targeted inhibition of COX-2 by chemopreventive agents is a promising approach to prevent inflammation and cancer. In the present study, 6-MITC, a major compound in wasabi, strongly blocked the expression of COX-2 induced by LPS or IFN-γ, but not TPA, suggesting that LPS, IFN-γ and TPA might induce COX-2 expression through distinct pathways in mouse macrophage cells.

The inductive effects of LPS on COX-2 induction are mediated by transcriptional factors including AP-1, C/EBP and NF-κB, which bind to the core elements of COX-2 (15). Moreover, there is redundancy in the three *cis*-acting elements, and two of these *cis*-acting elements are at least recruited to achieve maximal transcription of *COX-2* gene in mouse macrophage RAW264.7 cells (29). In the present study, our data indicated that the 6-MITC blocked LPS-induced COX-2 expression by suppressing the activation of CREB, AP-1 and C/EBPδ, which lead to the binding to the core element of *COX-2* gene.

IFN-γ is a potent inducer of inflammatory responses (30). The present study demonstrated that IFN-γ induced COX-2 expression, which was suppressed by 6-MITC (Fig. 2). IFN-γ did not activate the transcriptional factors including NF-κB, C/EBP, CREB and AP-1, which bind to the core element of *COX-2* promoter (Fig. 4). Blanco *et al* reported that a member of the IRF family, IRF-1, regulates IFN-γ-induced *COX-2* gene expression through two ISRE sites in mouse peritoneal macrophages (31). ISRE II sites are located in the -1354/-1345 region of *COX-2* promoter, and are efficiently bound with IRF-1 and IRF-2. In this study, transient transfections using *COX-2*-luciferase constructs indicated that IFN-γ increased *COX-2* gene expression through the region between -1432 and -327 in *COX-2* promoter (Fig. 3). Therefore, the inhibitory action of 6-MITC on IFN-γ-induced COX-2 expression is probably associated with IRF-1/2-ISRE II pathway, which is worthy of further study.

TPA has been shown to upregulate COX-2 expression in several cell lines. In the present study, we found that TPA activated CREB and AP-1, and induced COX-2 expression. However, 6-MITC could not block them. On the other hand, 6-MITC strongly blocked LPS-induced activation of CREB and AP-1. These results indicate that the signaling pathway leading to the activation of CREB and AP-1 by TPA is different from that induced by LPS. It has been reported that TPA acted as an activator of protein kinase C in several cell lines including RAW264.7 cells (32-34), and TPA-induced PKC activation is strongly linked with COX-2 induction. However, further study is needed to clarify this.

In summary, we report for the first time that 6-MITC suppressed COX-2 expression induced by LPS or IFN-γ, but not TPA, in murine macrophages. Molecular analysis revealed that LPS, IFN-γ and TPA might induce COX-2 expression through different signaling pathways. These findings are very helpful to further understand the property of 6-MITC as an anti-inflammatory and anti-cancer agent.

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