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6-(Methylsulfinyl)hexyl isothiocyanate suppresses inducible nitric oxide synthase expression through the inhibition of Janus kinase 2-mediated JNK pathway in lipopolysaccharide-activated murine macrophages

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Abstract

6-(Methylsulfinyl)hexyl isothiocyanate (6-MITC) is an active ingredient of Wasabi (*Wasabia japonica* (Miq.) Matsumura), which is a very popular pungent spice in Japan. To clarify the cellular signaling mechanism underlying the anti-inflammatory action of 6-MITC, we investigated the effects of 6-MITC on the expression of inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS)-activated murine macrophage RAW264 cells. 6-MITC showed a dose-dependent inhibition of LPS-induced nitric oxide (NO), iNOS mRNA and protein. LPS caused the c-Jun phosphorylation (a major component of AP-1) and IκB-α degradation. 6-MITC suppressed LPS-induced c-Jun phosphorylation, but did not inhibit IκB-α degradation. Cellular signaling analysis using MAPK-(U0126 for MEK1/2, SB203580 for p38 kinase and SP600125 for JNK) and Jak2-specific (AG490) inhibitors demonstrated that LPS stimulated iNOS expression via activating Jak2-mediated JNK, but not ERK and p38, pathway. 6-MITC suppressed iNOS expression through the inhibition of Jak2-mediated JNK signaling cascade with the attendant to AP-1 activation. In addition, the structure–activity study revealed that the inhibitory potency of methylsulfinyl isothiocyanates (MITCs) depended on the methyl chain length. These findings provide the molecular basis for the first time that 6-MITC is an effective agent to attenuate iNOS production.

Keywords: 6-(Methylsulfinyl)hexyl isothiocyanate; Inducible nitric oxide synthase; Lipopolysaccharide; Janus kinase 2; c-Jun N-terminal kinase; Activator protein-1; Macrophage

1. Introduction

Nitric oxide (NO) plays an important role in the regulation of many physiological functions, such as vasodilatation, neurotransmission and inflammation [1,2]. NO is produced from L-arginine by a chemical reaction catalyzed by NO synthase (NOS) in living systems [3]. There are three distinct isoforms of NOSs. Endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) are constitutively expressed in endothelium and neural tissues, respectively [4]. On the other hand, inducible nitric oxide synthase (iNOS) is only induced by various inflammatory stimuli, such as bacterial endotoxic lipopolysaccharide (LPS) and inflammatory cytokines in macrophages, hepatocytes and endothelial cells [4–6]. iNOS catalyzes the formation of a large amount of NO, which plays a key role in the various forms of inflammation and carcinogenesis [6–8]. Therefore, NO production by iNOS may reflect the degree of inflammation, and provides a measure to assess the effect of chemopreventive agents on the inflammatory process.

In the iNOS gene promoter, two transcription factors including activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) have been identified to bind the *cis*-acting elements, which regulate the transcription. AP-1 is one of the major transcription factors regulating iNOS expression [9,10], and minimally activated under normal physiologic

Abbreviations: AP-1, activator protein-1; ERK, extracellular signal-regulated kinase 1/2; iNOS, inducible nitric oxide synthase; Jak2, Janus kinase 2; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; 6-MITC, 6-(methylsulfinyl)hexyl isothiocyanate; NF-κB, nuclear factor κB; NO, nitric oxide

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conditions, but is dramatically activated by inflammatory stimuli, such as LPS [11]. NF-κB is another critical regulator involved in the induction of iNOS and activated by the inflammatory responses during viral and bacterial infections [9,10].

LPS is the principal component of the outer membrane of Gram-negative bacteria, and can activate a wide variety of immunological responses [11]. Several lines of reports indicate that LPS activates pathways of mitogen-activated protein kinases (MAPKs) to induce iNOS, tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) [12–14]. Furthermore, recent reports indicate that Janus kinase 2 (Jak2), a protein tyrosine kinase (PTK), is also involved in LPS-induced activation of macrophages [15], and may link to the production of iNOS in RAW264.7 cells [16]. Although both MAPK pathway and Jak2 pathways show important roles in LPS-induced iNOS expression, the cellular signaling between these two events is still unclear.

Wasabi (*Wasabia japonica* (Miq.) Matsumura) is a very popular pungent spice in Japan. Accumulated studies showed that Wasabi has a lot of physiological functions, such as appetite enhancement [17], anti-microbial activity [18], inhibition of platelet aggregation [19] and the suppression of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine-induced rat gastric carcinogenesis [20]. The active ingredients of Wasabi were identified as allyl isothiocynates [21]. Of which, 6-(Methylsulfinyl)hexyl isothiocyanate (6-MITC) is a major compound [22]. Several lines of studies have showed that 6-MITC has potency of anticancer effects. Oral administration of 6-MITC reduced the development of experimental mouse skin tumor in vivo [23]. Feeding with 6-MITC containing water prevented papilloma induction by a two-stage process with DMBA as an initiator and TPA as a promoter. Treatment with 6-MITC in human stomach tumor cell line (MKN-28) suppressed cell growth [21].

In the present study, we investigated the effects of MITCs on NO production and iNOS expression in murine macrophage cell line RAW264, which can be stimulated with LPS to mimic a state of infection and inflammation. Our results demonstrated that 6-MITC suppressed LPS-induced iNOS expression through the inhibition of Jak2-mediated JNK pathway with the attendant to AP-1 activation.

2. Materials and methods

2.1. Materials

2-, 4-, 6- and 8-MITC purified by reverse-phase HPLC to >99% were obtained from Hakucho Pharmaceutical Co. Ltd. (Japan). U0126 and antibodies against phospho-Jak2, phospho-ERK1/2, phospho-p38 kinase, phospho-c-Jun (Ser73), phospho-JNK, ERK1/2, p38 kinase, JNK and c-Jun were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against iNOS, phospho-Jak2, Jak2 and α-tubulin were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Fetal bovine serum (FBS) was from Equitech-Bio (Kerrville, TX). LPS (*Escherichia coli* Serotype 055:B5) was from Sigma (St. Louis, MO). SB203580 and AG490 were from Calbiochem (Nottingham, UK), and SP600125 was from Biomol Research Lab. (Plymouth Meeting, PA).

2.2. Cell culture

Murine macrophage-like RAW264 cells were obtained from RIKEN BioResource Center Cell Bank (Japan), and cultured at 37 °C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. All of the MITCs were dissolved in dimethyl sulfoxide (DMSO) and added directly to the culture media. Control cells were treated only with DMSO, the final concentration of which never exceeded 0.2%, and this concentration did not have any noticeable effect on the growth of cells.

2.3. Measurement of nitrite concentration

Nitrite concentrations in cultural supernatant were determined by Griess reaction [24]. Briefly, cells (3×10^5 cells) were seeded in 48-wells plates. After pre-incubation for 24 h, cells were 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 12 h. One hundred microliters of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% *N*-(1-naphthyl)ethylenediamide dihydrocholide in distilled water), and the absorbance was measured at 550 nm wave length.

2.4. Cell extraction and Western blotting analysis

Western blotting assay was performed as described previously [25]. In brief, RAW264 (1×10^6 cells) was 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 lysates 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 blotting, the membrane was incubated for 1 h 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., Japan).

2.5. RNA extraction and RT-PCR

RAW264 (1×10^6 cells) was 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 6 h. Total RNA was extracted with an Isogen RNA Kit (Nippon Gene Co., Japan) as described in manufacture manual. The oligonucleotide primers was used to amplify iNOS (forward primer 5′-CCC TTC CGA AGT TTC TGG CAG CAG C-3′ (2944–2968) and reverse primer 5′-GGC TGT CAG AGA GCC TCG TGG CTT TGG-3′(3416–3440)) and mouse GAPDH (forward primer 5′-GAC CCC TTC ATT GAC CTC AAC-3′ (143–162) and reverse primer 5′-CAT ACC AGG AAA TGA GCT TG-3′ (965–984)) as house keeping gene. The RT-PCR was performed with Ready-to-Go RT-PCR beads (Amersham Pharmacia Biotech, Little Chalfont, UK) as described previously [26]. The template- and cycle-dependences of the PCR products were confirmed for two pairs of primers used in the present study (data not shown). The cycle numbers of PCR for iNOS and GAPDH were 33 and 21 cycles, respectively. The PCR products were separated on a 2% agarose gel, and visualized under UV light after staining with ethidium bromide. The relative density of the PCR products was quantified using a Imager Software (TAITEC Co., Japan).

2.6. 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 NO production in LPS-activated macrophages

To examine the possibility that 6-MITC inhibits LPS-induced NO production, the accumulation of NO in culture media was measured using Griess reaction [24]. RAW264 cells were treated with 2–16 μM of 6-MITC for 30 min before exposure to 40 ng/ml LPS. As shown in Fig. 1, LPS caused a dramatic increase from the basal level of 3.2 ± 0.5 μM to 35.1 ± 2.3 μM of NO, and 6-MITC inhibited NO generation in a dose-dependent manner.

3.2. 6-MITC inhibits LPS-induced iNOS expression at mRNA and protein levels

To investigate whether the inhibitory effect of 6-MITC on NO production were related to iNOS expression, we examined the iNOS protein expression using Western blotting analysis. As shown in Fig. 2A, expression of iNOS

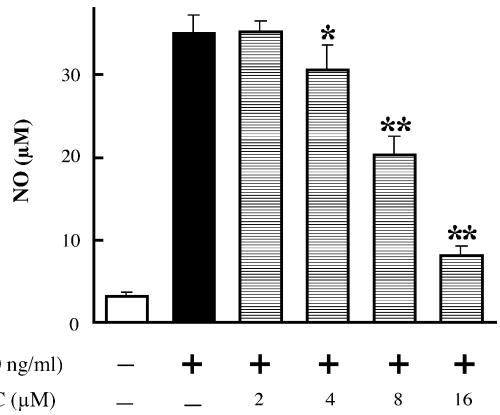

Fig. 1. 6-MITC suppresses LPS-induced NO production. RAW264 cells (3×10^5 cells) were pretreated with 2–16 μM of 6-MITC for 30 min, and then exposed to 40 ng/ml LPS for 12 h. The culture medium was subsequently isolated and analyzed for nitric levels. NO concentration was determined by Griess reaction. Each value represents the mean ± S.D. of triplicate tests. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. LPS (40 ng/ml).

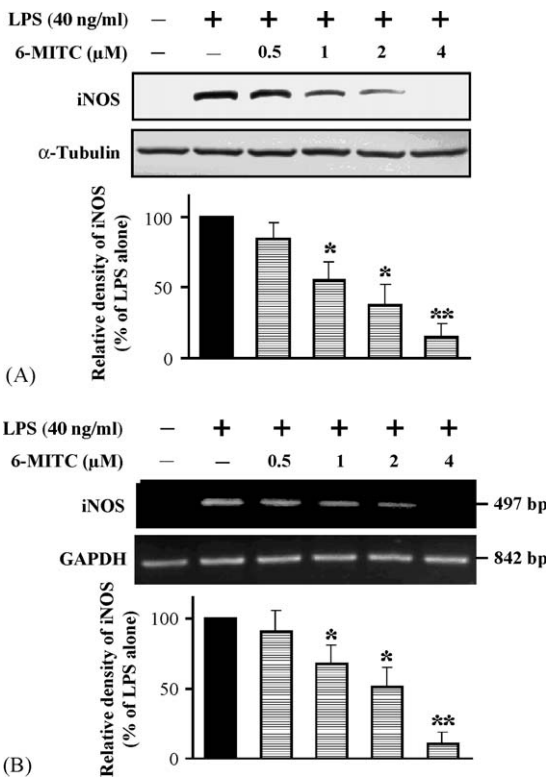

Fig. 2. 6-MITC suppresses LPS-induced expression of iNOS protein and mRNA. (A) After RAW264 cells (1×10^6 cells) were starved in serum-free medium for 2.5 h, the cells were treated with the indicated concentrations of 6-MITC for 30 min, and then exposed to 40 ng/ml LPS for 12 h. iNOS and α-tubulin were detected by Western blotting analysis with their antibodies, respectively. Histograms show the densitometric analysis of iNOS protein expression normalized to α-tubulin. (B) After 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 6 h. Total RNA was prepared and RT-PCR was performed as described in Section 2. The PCR products were separated on 2% agarose gel and stained with ethidium bromide. Histograms show the densitometric analysis of iNOS mRNA expression normalized to GAPDH. The data represent the mean ± S.D. of three to four separate experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. LPS (40 ng/ml).

protein was markedly increased upon exposure to LPS for 12 h. Pretreatment with 6-MITC showed a dose-dependent inhibition of LPS-induced iNOS protein. The amount of α-tubulin, which was presented as an internal control, remained uncharged. Furthermore, we examined the level of iNOS mRNA by RT-PCR analysis (Fig. 2B). LPS dramatically induced iNOS mRNA, and 6-MITC suppressed LPS-induced iNOS mRNA in a dose-dependent manner. As a housekeeping gene control, GAPDH mRNA was not affected by LPS and 6-MITC treatment. These results suggest that 6-MITC suppressed NO production by blocking iNOS expression at the transcription level.

3.3. 6-MITC inhibits LPS-induced c-Jun phosphorylation, but does not block IκB-α degradation

It has been reported that AP-1 regulates iNOS expression by binding to iNOS promoter [9]. Thus, we tested the effect of 6-MITC on LPS-induced c-Jun phosphorylation, a major component of AP-1 complex [27]. RAW264 cells were pretreated with 6-MITC for 30 min before exposure to LPS for 30 min, and then examined the phosphorylated and total c-Jun by Western blotting analysis. As shown in Fig. 3A, 6-MITC inhibited LPS-induced c-Jun phosphorylation in a dose-dependent manner, suggesting 6-MITC might inhibit LPS-induced iNOS expression by blocking AP-1 activation.

NF-κB is another critical factor for iNOS expression induced by LPS [9,10]. NF-κB is inactivated in the cytosol by binding to IκB, and become active through translocation to the nucleus preceded by LPS-induced proteolytic degradation of IκB [28,29]. To examine whether 6-MITC can directly inhibit degradation of IκB, the level of IκB-α protein was assessed in RAW264 cells treated with or without 6-MITC (Fig. 3B). LPS dramatically reduced the IκB-α protein at 30 min, and 6-MITC did not suppress the degradation of IκB-α. As a positive control, delphinidin suppressed the degradation of IκB-α. These findings indicate that 6-MITC has no inhibitory effect on LPS-induced degradation of IκB-α.

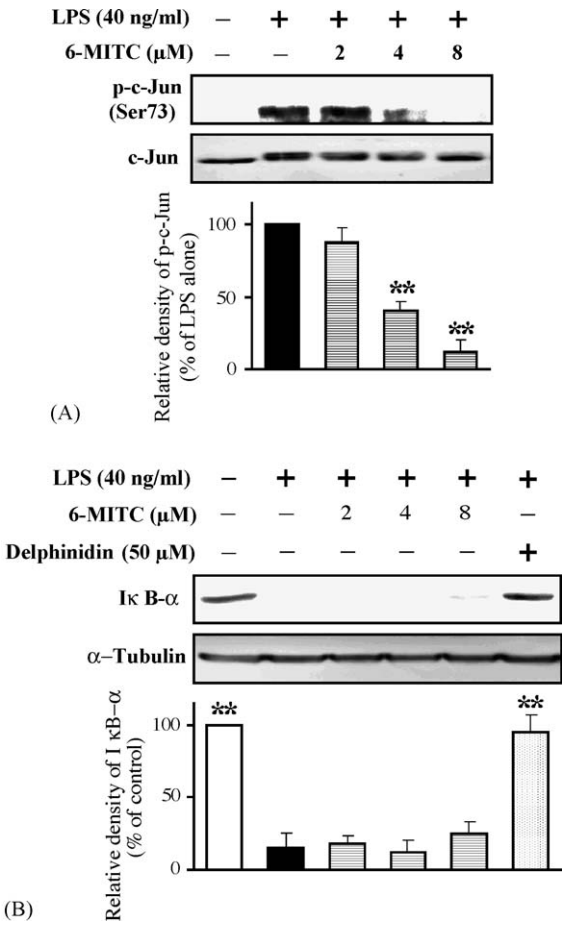

Fig. 3. 6-MITC inhibits LPS-induced c-Jun phosphorylation, but does not decrease IκB-α degradation. Cell culture and Western blotting analysis were done as described in Fig. 2A. (A) Cells were treated with 6-MITC (2–8 μM) for 30 min, and then exposed to 40 ng/ml LPS for 30 min. Histograms show the densitometric analysis of phosphorylated c-Jun normalized to total c-Jun. (B) Cells were treated with 6-MITC (2–8 μM) and delphinidine (50 μM) for 30 min, and then exposed to 40 ng/ml LPS for 30 min. Histograms show the densitometric analysis of IκB-α normalized to α-tubulin. The data represent the mean ± S.D. of three to four separate experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. LPS (40 ng/ml).

3.4. 6-MITC inhibits LPS-induced iNOS expression by blocking JNK phosphorylation

MAPK signaling pathways play a critical role in the regulation of inflammatory response and coordinate the induction of many genes encoding inflammatory mediators [11]. Thus, we investigated the influence of 6-MITC on the activation of MAPK, such as JNK, ERK and p38 kinase. As shown in Fig. 4A, LPS markedly induced the phosphorylation of JNK, ERK and p38 kinase, and 6-MITC suppressed LPS-induced phosphorylation of all of MAPKs in a dose-dependent manner.

To determine which MAPK signaling pathway is involved in LPS-induced iNOS expression, we pretreated the cells with MAPK-specific inhibitors (U0126 for MEK1/2, SB203580 for p38 kinase and SP600125 for JNK), respectively, for 30 min before exposure to LPS. Fig. 4B shows that SP600125 significantly inhibited LPS-induced iNOS expression. But U0126 and SB203580 did not. Moreover, phosphorylation of c-Jun, a substrate of JNK, was also significantly suppressed by SP600125 (Fig. 4C). Thus, LPS-induced iNOS expression would be regulated by JNK, but not ERK and p38 kinase, and the suppression of JNK phosphorylation by 6-MITC was associated with the inhibitory effect on iNOS expression.

3.5. 6-MITC inhibits LPS-induced Jak2 phosphorylation with the attendant to iNOS expression

Recent reports suggest that the signaling pathway of Jak2, a protein tyrosine kinase (PKT), may be involved in

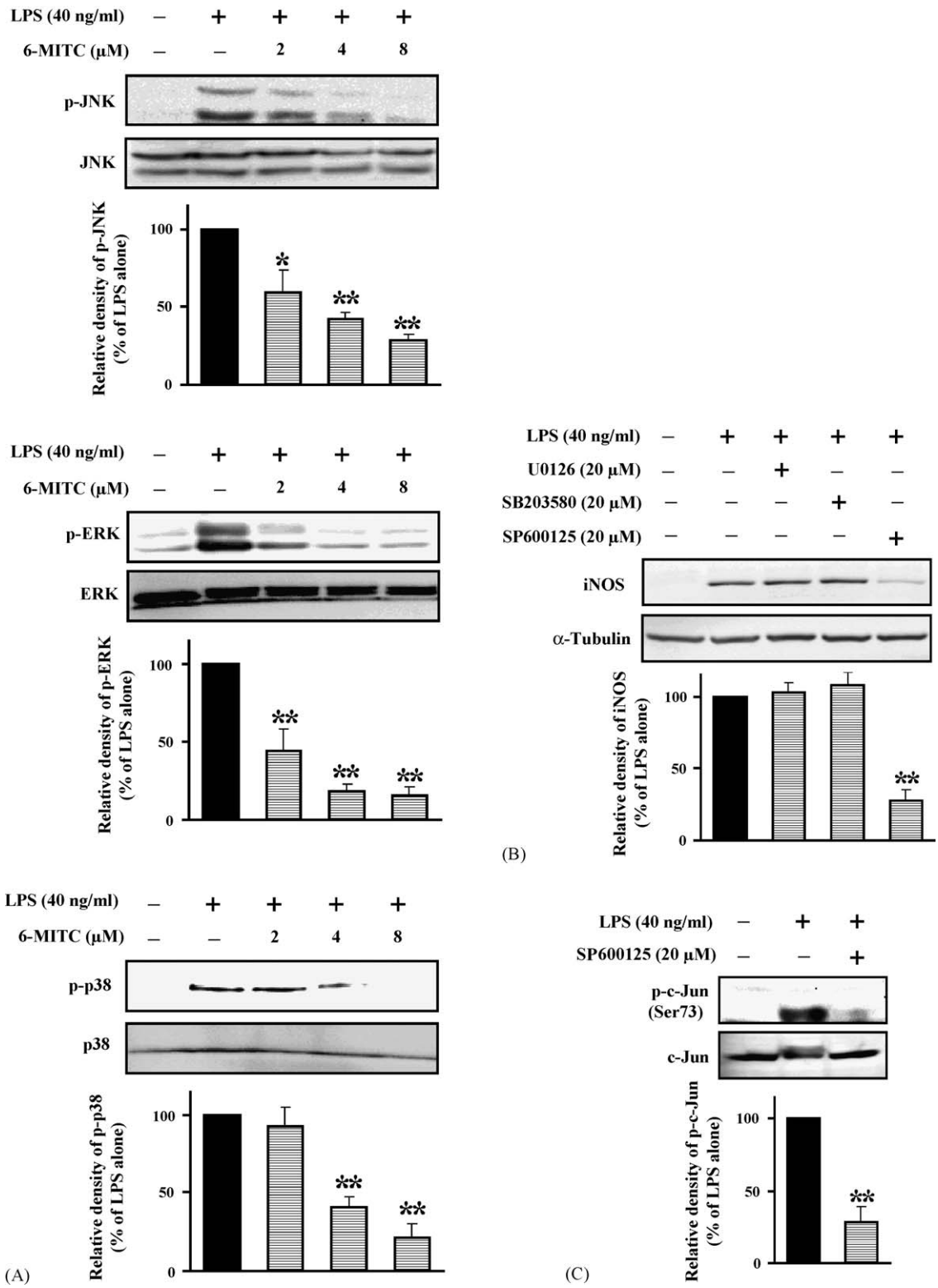

Fig. 4. 6-MITC inhibits LPS-induced iNOS expression by blocking JNK phosphorylation. Cell culture and Western blotting analysis were done as described in Fig. 2A. (A) Cells were treated with 6-MITC (2–8 μM) for 30 min, and then exposed to 40 ng/ml LPS for 30 min. Histograms show the densitometric analysis of phosphorylated MAPK normalized to total MAPK, respectively. (B) Cells were treated with 20 μM of U0126, SB203580 or SP600125 for 30 min, and then exposed to 40 ng/ml LPS for 12 h. Histograms show the densitometric analysis of iNOS normalized to α-tubulin. (C) Cells were treated with 20 μM of SP600125 for 30 min, and then exposed to 40 ng/ml LPS for 30 min. Histograms show the densitometric analysis of phosphorylated c-Jun normalized to total c-Jun. The data represent the mean ± S.D. of three to four separate experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. LPS (40 ng/ml).

LPS-induced inflammation response [15]. To investigate whether the inhibitory effect of iNOS expression by 6-MITC involves a Jak2 pathway, we treated RAW264 cells with 6-MITC and AG490, a specific inhibitor of Jak2 tyrosine phosphorylation, for 30 min before exposure to LPS. As shown in Fig. 5A, LPS markedly induced phosphorylation of Jak2. AG490 and 6-MITC significantly inhibited the phosphorylation of Jak2. We also tested the effect of AG490 on the expression of iNOS in LPS-treated cells. LPS-mediated iNOS expression was also significantly blocked by AG490 (Fig. 5B). These data suggest that a Jak2 pathway would be involved in LPS-mediated iNOS expression, and 6-MITC might suppress iNOS expression through the inhibition of Jak2-mediated signaling pathway.

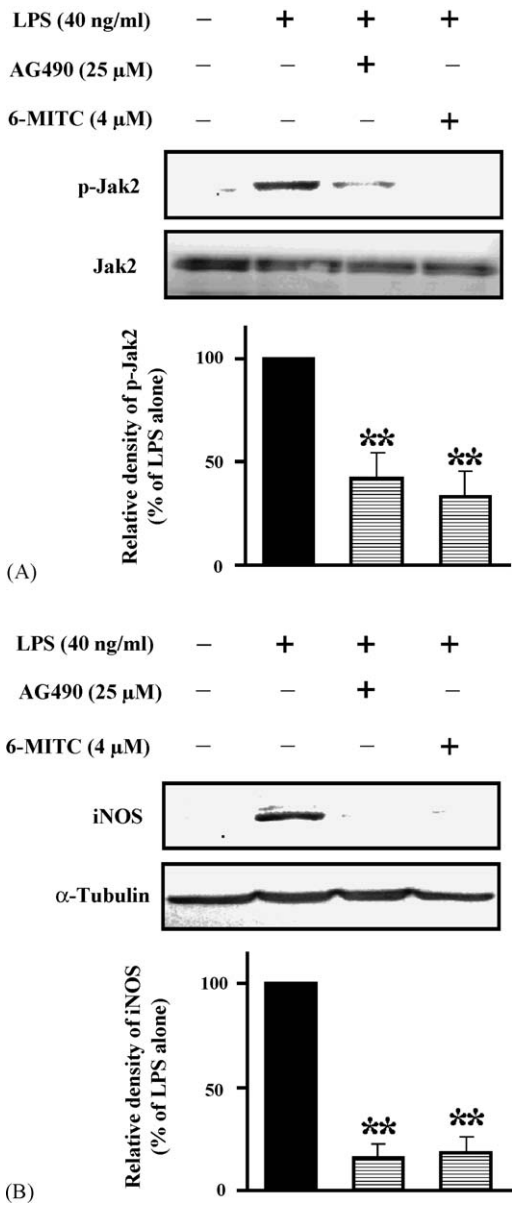

Fig. 5. Effects of 6-MITC and AG490 on LPS-induced Jak2 phosphorylation and iNOS expression. Cell culture and Western blotting analysis were done as described in Fig. 2A. (A) Cells were treated with AG490 (25 μM) or 6-MITC (4 μM) for 30 min, and then exposed to 40 ng/ml LPS for 30 min. Histograms show the densitometric analysis of Jak2 phosphorylation normalized to total Jak2. (B) Cells were treated with AG490 (25 μM) or 6-MITC (4 μM) for 30 min, and then exposed to 40 ng/ml LPS for 12 h. Histograms show the densitometric analysis of iNOS protein normalized to α-tubulin. The data represent the mean ± S.D. of three to four separate experiments. $^{**}P < 0.01$ vs. LPS (40 ng/ml).

3.6. A Jak2-mediated JNK pathway leads to iNOS expression

Our data showed that both JNK and Jak2 pathways are involved in the inhibition of iNOS expression by 6-MITC. To clarify the cellular signaling between these two factors, we used AG490, to investigate the effect on LPS-induced phosphorylation of MAPKs. As shown in Fig. 6A, LPS-induced JNK phosphorylation was significantly inhibited when pretreatment with AG490 for 30 min. However, LPS-induced phosphorylation of ERK and p38 kinase was not inhibited by AG490. As the same fashion, LPS-induced c-Jun phosphorylation was also strongly blocked by AG490 (Fig. 6B). Next, we used SP600125, to investigate the influence on LPS-induced Jak2 phosphorylation. As shown in Fig. 6C, SP600125 did not inhibit LPS-induced Jak2 phosphorylation while AG450 inhibited Jak2 phosphorylation. Thus, Jak2 might act in the upstream of JNK signaling pathway. Taken together, the data suggest that LPS stimulated the expression of iNOS through Jak2-mediated JNK, but not affect ERK and p38 kinase, pathway. 6-MITC suppressed iNOS expression by blocking Jak2 phosphorylation with the attendant to JNK-mediated AP-1 activation.

3.7. Inhibitory activity of MITCs on LPS-induced NO production and iNOS expression depends on the methyl chain length

Depending on the length of the methyl chain of methylsulfinyl isothiocyanates (MITCs), there are a number of analogues of MITC in Wasabi extracts (Fig. 7A). To investigate the structural-activity relationship of MITCs on LPS-induced NO production, RAW264 cells were treated with 2-, 4-, 6- and 8-MITC for 30 min before exposure to LPS for 12 h. As shown in Fig. 7B, all of four MITCs suppressed LPS-induced NO production in a dose-dependent manner, and the inhibitory potency of 2-, 4-, 6- and 8-MITC were strong in the order of increasing in the methyl chain length. The inhibitory actions by MITCs were not caused by their cytotoxicity because the concentration that inhibited NO production did not affect cellular viability as measured by MTT assay (data not shown). We also examined the effect of four MITCs on LPS-induced iNOS expression after pretreatment with same concentration of MITCs. As shown in Fig. 7C, the inhibitory potency of MITCs on iNOS expression was also observed to be proportional to the

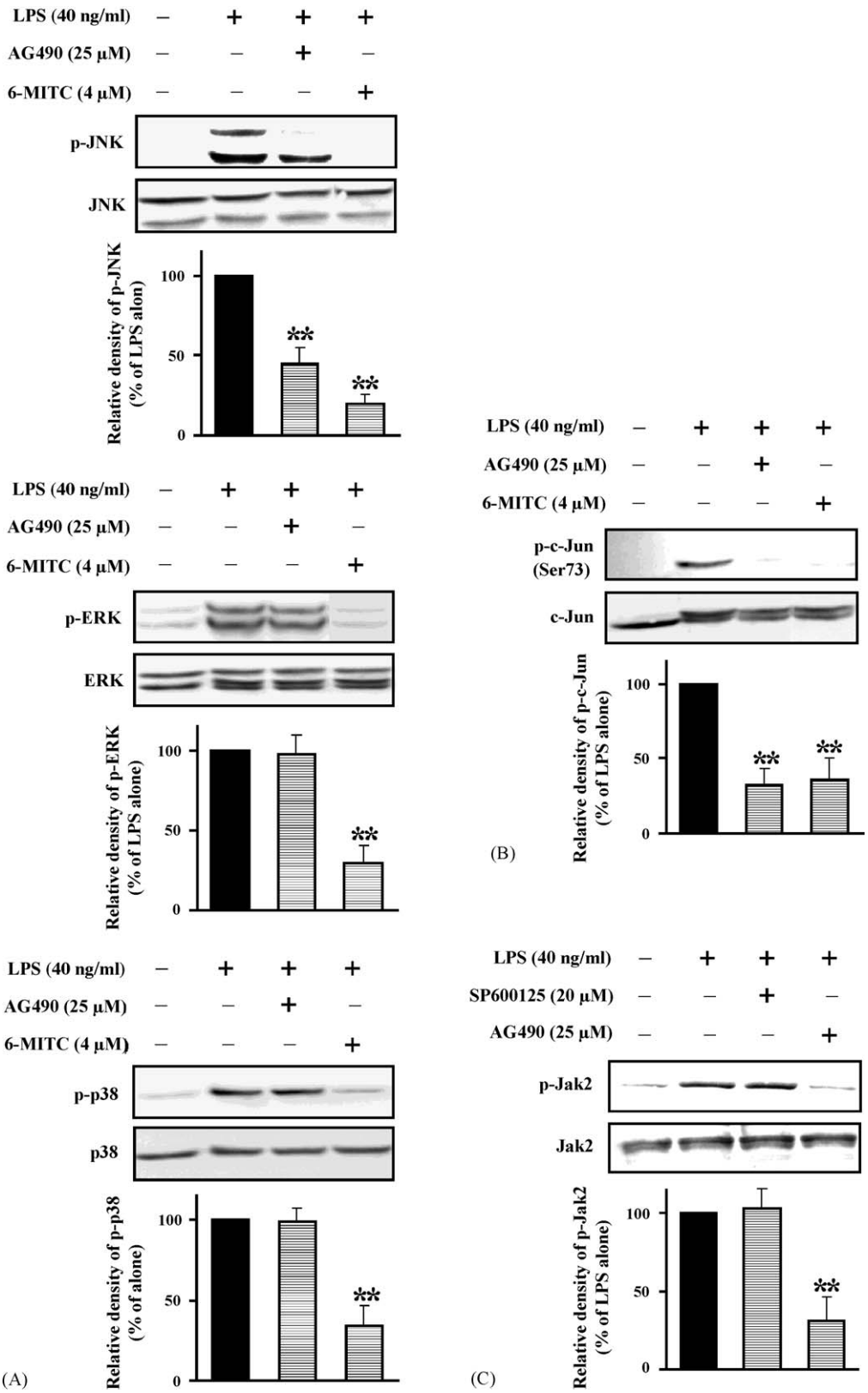

Fig. 6. Effects of AG490 on LPS-induced phosphorylation of MAPK and c-Jun. Cell culture and Western blotting analysis were done as described in Fig. 2A. (A) Cells were treated with AG490 (25 μM) or 6-MITC (4 μM) for 30 min, and then exposed to 40 ng/ml LPS for 30 min. Histograms show the densitometric analysis of phosphorylated MAPK normalized to total MAPK, respectively. (B) Cells were treated with AG490 (25 μM) or 6-MITC (4 μM) for 30 min, and then exposed to 40 ng/ml LPS for 30 min. Histograms show the densitometric analysis of phosphorylated c-Jun normalized to total c-Jun. (C) Cells were treated with SP600125 (20 μM) or AG490 (25 μM) for 30 min, and then exposed to 40 ng/ml LPS for 30 min. Histograms show the densitometric analysis of phosphorylated Jak2 normalized to total Jak2. 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. The data represent the mean ± S.D. of three to four separate experiments. $^{**}P < 0.01$ vs. LPS (40 ng/ml).

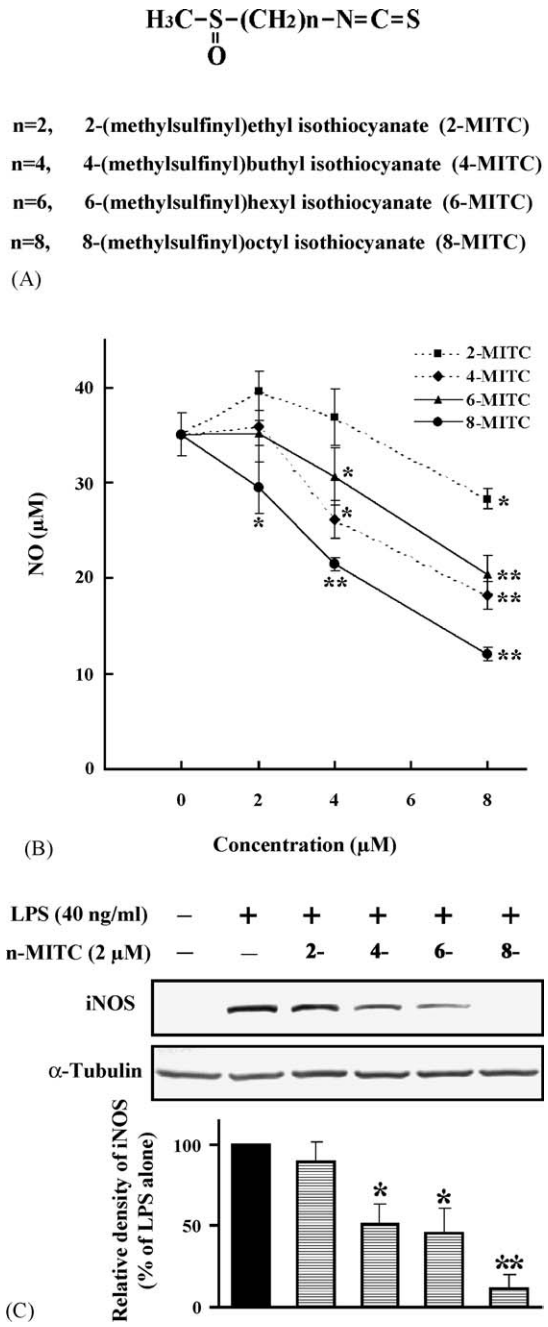

Fig. 7. Structure–activity relationship of MITCs on the inhibition of LPS-induced NO production and iNOS expression. (A) Chemical structure of MITCs used in this study. (B) Cell treatment and determination of NO concentration were done as described in Fig. 1. RAW264 cells were treated with 2-, 4-, 6-, or 8-MITC (2–8 μM) for 30 min, respectively, and then exposed to 40 ng/ml LPS for 12 h. Each value represents the mean ± S.D. of triplicate tests. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. LPS (40 ng/ml). (C) Cell treatment and Western blotting analysis were done as described in Fig. 2A. Cells were treated with 2 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 iNOS protein normalized to α-tubulin. The data represent the mean ± S.D. of three to four separate experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. LPS (40 ng/ml).

length of their methyl chain. Thus, an increase in the methyl chain length of MITCs appears to be important for their inhibitory activities on LPS-induced NO production and iNOS expression.

4. Discussion

iNOS is one of the potent mediators of pro-inflammation and carcinogenesis because iNOS-generated NO is known to be involved in promotional stage of tumorigenesis or neoplastic transformation [30–32], and can cause mutagenesis and carcinogenesis [32,33]. Therefore, the inhibitors that interfere in iNOS activity or expression are considered to have ability of cancer chemoprevention. 6-(Methylsulfinyl)hexyl isothiocyanate is an active ingredient of a traditional Japanese spice Wasabi, and has been shown to have anti-inflammatory effects [34,35]. In the present study, we chose iNOS as a target molecule to investigate the molecular mechanism behind the anti-inflammatory action. Our data demonstrated that 6-MITC suppressed iNOS expression through the inhibition of Jak2-mediated JNK signaling cascade with the attendant to AP-1 activation.

Although multiple signaling pathways are known to be activated in macrophages upon LPS exposure, MAPK signaling pathways can regulate many gene expression in LPS-activated macrophages [11]. However, the cellular signaling between MAPK and iNOS expression is not clear. In this study, we investigated the effect of 6-MITC on the LPS-induced MAPK activation and iNOS expression. Although 6-MITC caused a dose-dependent inhibition of the phosphorylation of JNK, ERK and p38 kinase (Fig. 4A), the data from MAPK-specific inhibitors showed that only JNK is required to LPS-induced iNOS expression, because only JNK-specific inhibitor SP600125 suppressed iNOS expression while ERK-specific inhibitor U0126 and p38-specific inhibitor SB203580 did not (Fig. 4B). Thus, 6-MITC might suppress iNOS expression by blocking JNK phosphorylation. This finding is consistent with the results reported by Caivano [36] and Paul et al. [37]. They showed that p38 and ERK pathway are not essential for NO production and iNOS expression [36,37] because LPS-induced iNOS expression could not be blocked by SB203580 and PD98059. On the other hand, our finding is not consistent with results reported by Kim et al. [38], in which, they showed that activation of ERK and p38 is involved in LPS-induced iNOS expression [38]. The reason for inconsistency is still unknown because the times and concentrations of the treatment were almost same each other.

Several lines of evidence indicated that AP-1 is involved in the regulation of LPS-induced iNOS [9]. In the present study, we found that 6-MITC markedly suppressed LPS-induced c-Jun phosphorylation, a major component of AP-1 (Fig. 3A). Moreover, SP600125 also suppressed c-Jun phosphorylation (Fig. 4C). Thus, 6-MITC might inhibit iNOS expression by blocking JNK-mediated AP-1 activation. On the other hand, a recent study showed that SP600125 also inhibited several other kinases including p70 S6 kinase, AMP-dependent protein

kinase, and cyclin-dependent protein kinase 2/cyclin A [39]. Thus, we can not exclude the possibility that SP600125 exhibited the effects through the inhibition of other kinases in iNOS expression.

Additional experiments were carried out to examine the effect of 6-MITC on the activation of NF-κB, because NF-κB is one of the essential factors for iNOS expression [9,10]. The nuclear translocation and DNA binding of NF-κB is preceded by the degradation of IκB [28,29]. Many chemopreventive natural compounds, such as capsaicin [40], suchinone [41], apigenin [42] and (−)-epigallocatechin-3-gallate [43], inhibit LPS-induced iNOS expression by blocking the degradation of IκB-α in mouse macrophage cells. It is noticed that 6-MITC had no influence on the degradation of IκB-α (Fig. 3B), suggesting that 6-MITC may inhibit iNOS expression without the degradation of IκB-α, and mainly by blocking AP-1 activation. Thus, 6-MITC may a novel inhibitor, showing different molecular mechanism with that of the chemopreventive compounds described above.

Jak2 has been reported to be involved in the regulation of iNOS expression in RAW264.7 macrophages [16]. Recent studies indicated that LPS induces tyrosine phosphorylation of Jak2, but not Jak1 and Jak3 [15]. Activated Jak2 then regulates the phosphorylation of JNK through the activation of PI3K, thus, Jak2 may play a pivotal role in LPS-induced iNOS expression. But, the cellular signaling from Jak2 to transcription factors mediating iNOS transcription is not well understood. In this study, we clarified the signaling pathway of Jak2-mediated iNOS expression. First, we have shown that Jak2-specific inhibitor, AG490, strongly suppressed Jak2 phosphorylation and iNOS expression in LPS-treated cells (Fig. 5). Second, we have demonstrated that the treatment with AG490 suppressed phosphorylation of JNK and c-Jun, but not ERK and p38 kinase (Fig. 6A and B). Thus, Jak2 may regulate iNOS expression through JNK-mediated AP-1 pathway. In addition, LPS-induced Jak2 phosphorylation was inhibited by AG490, but not by SP600125 (Fig. 6C), suggesting that Jak2 is located in the upstream of JNK in LPS-induced signaling pathway. Consistent with our findings on LPS-induced Jak2-mediated JNK pathway in RAW264.7 macrophage, a recent report shows that treatment with AG490 inhibited the phosphorylation of both PI3K and JNK, and treatment with PI3K-specific inhibitor LY294002 inhibited JNK phosphorylation while JNK inhibitor did not inhibit PI3K phosphorylation, confirming that Jak2 regulates JNK phosphorylation by PI3K [15]. Finally, 6-MITC markedly suppressed phosphorylation of Jak2 (Fig. 5A) and JNK (Fig. 4A), suggesting that 6-MITC might suppress iNOS expression through the blockage of Jak2-mediated JNK signaling pathway. But, we could not identified the molecular target(s) of 6-MITC in this study although we also investigated the effect of 6-MITC on the binding of FITC-conjugated LPS to the LPS receptor by a flow cytometric assay, and found that 6-MITC did not affect the binding of LPS to the receptor in plasma membrane in RAW264 cells (data not shown). On the other hand, we also tested the cell toxicity in 12 h culture by MTT assay, and there was not significant difference in cell survival below 16 μM of 6-MITC (data not shown). Thus, the inhibitory action of 6-MITC on LPS-induced iNOS expression was not due to non-specific toxicity in cellular assays. 6-MITC may target intracellular factors between LPS receptor and Jak2 to inhibit iNOS expression although these factors need to be clarified in further work.

It has been reported that LPS also induces other pro-inflammatory mediators, such as TNF, IL1, IL6 and COX-2 [15,41,44]. In fact, we also observed an inhibitory effect of MITC on LPS-induced COX-2 and prostaglandin E_2 (Uto et al., unpublished data). Thus, MITC may also target other mediators to attenuate pro-inflammatory reaction, which are interesting to be clarified in our next study.

In the present study, we further determined the structure–activity relationship of methylsulfinyl isothiocyanates on NO production and iNOS expression. Our data indicated that all of four MITCs (2-, 4-, 6- and 8-MITC) suppressed LPS-induced NO production and iNOS expression, and the potency of inhibition was proportional to the length of their methyl chain, suggesting that an increase in the methyl chain length is important for the inhibitory activity by MITCs (Fig. 7) although amount of 8-MITC in *Wasabi* is very lower than 6-MITC. A similar relationship was also reported previously in the induction of quinone oxidoreductase by MITCs in Hepa 1c1c7 cells [24]. 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 inhibited LPS-induced iNOS expression at cellular signaling level. LPS induced iNOS expression by activating Jak2-mediated JNK signaling cascade with the attendant to AP-1 activation. 6-MITC blocked LPS-induced iNOS expression through the blockage of Jak2 signaling cascade with the attendant to JNK-mediated AP-1 activation. These findings provide the first molecular basis for the anti-inflammatory and anti-carcinogenic action of 6-MITC.

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