RESEARCH ARTICLE

# Se-methylselenocysteine inhibits lipopolysaccharide-induced NF-κB activation and iNOS induction in RAW 264.7 murine macrophages

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**Scope**: Se-methyl-L-selenocysteine (MSC), a naturally occurring organoselenium compound, has shown cancer chemopreventive activity against several types of cancer. Herein, the effect of MSC on the inflammatory response in lipopolysaccharide (LPS)-activated murine RAW 264.7 macrophage cells was investigated.

**Methods and results**: The present results demonstrated that MSC markedly inhibited LPS-induced production of NO in a dose-dependent pattern with decreased mRNA and protein levels of inducible nitric oxide synthase (iNOS). MSC also reduced nuclear translocation of p65 and p50 subunits of nuclear factor-κB (NF-κB), a critical transcription factor necessary for iNOS expression, accompanied with downregulation of LPS-triggered NF-κB-dependent gene expression evaluating by a luciferase reporter. Inhibition of nuclear translocation by MSC might result from the prevention of the inhibitor of NF-κB from phosphorylation and consequent degradation *via* suppression inhibition of phosphorylation of IκB kinase α/β. Exploring the action mechanism involved, MSC can reduce the phosphorylation/activation of mitogen-activated protein kinases (MAPKs) related to NF-κB activation induced by LPS, including p38 MAPK and c-Jun N-terminal kinase in RAW 264.7 cells.

**Conclusion**: MSC might contribute to the potent anti-inflammatory effect in LPS-activated RAW 264.7 cells *via* downregulation of NF-κB activation and iNOS expression, suggesting that MSC may be considered as a therapeutic candidate for chronic inflammatory diseases.

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**Abbreviations**: **GAPDH,** glyceraldehyde-3-phosphate dehydrogenase; **GMSC,** *N*-γ-(L-glutamyl)-Se-methyl-L-selenocysteine; **GSeM,** *N*-γ-(L-glutamyl)-L-selenomethionine; **IκB,** inhibitor of NF-κB; **IKKα/β,** IκB kinase α/β; **iNOS,** inducible nitric oxide synthase; **JNK,** c-Jun N-terminal kinase; **LDH,** lactate dehydrogenase; **MAPKs,** mitogen-activated protein kinases; **MSC,** Se-methyl-L-selenocysteine; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **NF-κB,** nuclear factor-κB; **NO,** nitric oxide; **TNF,** tumor necrosis factor; **p38 MAPK,** p38 mitogen-activated protein kinase; **p-IκBα,** phosphorylated IκBα

 

## 1 Introduction

Inflammation is a defense response to eliminate foreign pathogens or damage cells and to initiate tissue healing. Extensive and growing evidences indicate that excessive inflammation may be involved in the pathogenesis of chronic diseases, such as atherosclerosis, inflammatory arthritis and cancer [1–3]. This correlation may stem from overproduction of pro-inflammatory cytokines, enzymes and inflammatory mediators, which are released by a diverse population of immune cells in an inflammatory process. Among the extent of inflammatory infiltrates, macrophages are central to the progression of immunopathological changes during inflammation. Once activated in response to infectious agents or pro-inflammatory stimuli, macrophages secrete a variety of cytokines, growth factors and inflammatory mediators including interleukin-1β, interleukin-6, tumor necrosis factor (TNF)-α, nitric oxide (NO) and prostaglandin E2 to build up an inflammatory microenvironment required for tissue healing and remodeling [4, 5]. If the inflammatory process is prolonged and unlimiting, persistence of these mediators eventually causes oxidative DNA damage, tissue injury, organ failure, precancerous lesions and even neoplasia [6].

The enzyme-inducible NO synthase (iNOS) responsible for NO generation is a crucial effector of inflammation and has been suggested as a contributor for the pathogenesis of chronic inflammatory diseases [7, 8]. The expression of iNOS can be found in activated phagocytic cells, including neutrophils and macrophages, but does not occur in resting cells. Its regulation is mainly controlled at the transcriptional levels *via* the induction of certain nuclear factors, such as the nuclear factor-κB (NF-κB) and the activator protein-1 [9, 10]. In general, NF-κB is sequestered and kept silent in the cytoplasm by forming the complex with inhibitor of NF-κB (IκB). When macrophages are activated by endotoxins, cytokines or mitogens, IκB kinase (IKK) will phosphorylate IκB to tag it for degradation by the ubiquitin-proteasome pathway followed by liberation and nuclear translocation of NF-κB [11]. The free NF-κB in nucleus induces the transcription of a large number of inflammation-related genes, including *iNOS*, of which the encoded protein synthesizes NO in high levels and for long-term periods of time by conversion of L-arginine into L-citrulline [10, 12]. The iNOS-derived NO functions as a cytostatic or cytotoxic agent against invading pathogens of immune response and inflammation. However, the high-level expression of iNOS and excessive amounts of NO produced are believed to be pathogenic, as demonstrated by a number of experimental findings [7, 8]. The detrimental actions of iNOS-derived NO may be attributed to NO itself and its reactive nitrogen species being formed *via* the reaction of NO and superoxide anion, which result in oxidative damage of bioconstituents and alteration of gene regulation, and thereby cause tissue injures [10, 13]. Therefore, suppression of iNOS activity or gene expression may be a feasible tactic for the treatment of certain inflammatory diseases [7, 9].

The organoselenium Se-methyl-L-selenocysteine (MSC) was first identified in *Astragalus bisulcatus* grown on selenium-rich soils [14] and thought to be as a chemopreventive agent tested in a variety of cell culture studies and animal carcinogenesis models [15, 16]. MSC has anti-cancer activity that induces apoptotic cell death of some cancer cell lines [17–19] and blocks cell cycle progression at G1 to S phase by modulating the activity and expression of cyclin-dependent kinase 2 in mouse mammary epithelial tumor cells [20, 21]. MSC was also known to attenuate carcinogen-induced premalignant lesions in a rat mammary carcinogenesis model [22, 23] and inhibits growth of LNCaP human prostate cancer xenografts in nude mice [24]. An additional mechanism relevant for the anti-angiogenic effect of MSC *in vivo* is that MSC significantly reduced intratumoral microvessel density and vascular endothelial growth factor expression in mammary carcinomas [25]. Despite numerous analyses of the past years reveal action mechanisms of anti-cancer activity of MSC, the anti-inflammatory potential of MSC has remained elusive. In the present study, we examined the effect of MSC on LPS-induced inflammatory responses of RAW 264.7 murine macrophages and determined its possible signaling pathways involved. Our study indicated that MSC was able to protect against LPS-induced inflammation and NO formation by blocking the activation of NF-κB, p38 mitogen-activated protein kinase (p38 MAPK) and c-Jun N-terminal kinase (JNK), and thereby suppressing iNOS expression.

## 2 Materials and methods

### 2.1 Materials

Lipopolysaccharide (LPS) (*Escherichia coli* 0127:E8), sulfanilamide, naphthylethylenediamine dihydrochloride, and DTT were purchased from Sigma Chemical (St. Louis, MO). Se-methyl-L-selenocysteine, *N*-γ-(L-glutamyl)-Se-methyl-L-selenocysteine, L-selenomethionine and *N*-γ-(L-glutamyl)-L-selenomethionine were gifts from Sabinsa (Piscataway, NJ). The purity of these compounds is >99% by HPLC. RT-PCR reagents were purchased from Promega (Madison, WI).

### 2.2 Cell culture

RAW 264.7 murine macrophage cells obtained from the American Type Culture Collection (Rockville, MD) were cultured in the Roswell Park Memorial Institute (RPMI) 1640 media (without phenol red) supplemented with 10% endotoxin-free, heat-inactivated fetal calf serum (GIBCO, Grand Island, NY), 100 units/mL penicillin, and 100 μg/mL streptomycin and kept at 37°C in a humidified atmosphere of 5% $CO_2$ in air according to ATCC recommendations.

 

When the cells reached a density of 2–3 $\times$ $10^6$ cells/mL, they were activated by incubation in medium containing *E. coli* LPS (100 ng/mL). Various concentrations of test compounds dissolved in DMSO were added together with LPS. Cells were treated with 0.05% DMSO as the vehicle control.

### 2.3 Nitrite quantification

The nitrite concentration in the culture medium was measured as an indicator of NO production, according to the Griess reaction as described previously [26]. The cells ($5 \times 10^5$ cells/mL) were treated with LPS (100 ng/mL) for 24 h in the presence of seleno-amino compound or vehicle. The conditional medium (100 μL) was taken, mixed with an equal volume of the Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water), and incubated at room temperature for 10 min. Nitrite production was determined by the absorbance at 550 nm. A standard curve was generated with $NaNO_2$.

### 2.4 Cell viability assay

RAW 264.7 cells were seeded at a density of $5 \times 10^3$ cells/mL into 96-well plates and grown overnight. Then, the cells were treated with various concentrations of the test compound for the indicated times. Control cells were treated with DMSO to yield a final concentration of 0.05% v/v. After incubation, the proliferating cell numbers were determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as follows: 20 μL of MTT solution (5 mg/mL, Sigma, St. Louis, MO) was added to each well and incubated for 24 h at 37°C. Then the supernatant was aspirated, and the MTT–formazan crystals formed by metabolically viable cells were dissolved in 200 μL of DMSO. Finally, the absorbance was monitored by a microplate reader at a wavelength of 570 nm.

### 2.5 Cytotoxicity assay

RAW 264.7 cells were seeded at a density of $5 \times 10^3$ cells/well in U-bottomed 96-well microplates, cultured for 18 h, and the medium was then replaced by 50 μL fresh phenol red-free RPMI 1640 medium (Gibco BRL, Grand Island, NY) containing 10% FCS. After treating LPS and test compounds, cytotoxicity was assessed by measuring the intracellular lactate dehydrogenase (LDH) leakage, a cytotoxic end-point indicator for damage of plasma membrane, using a CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega) according to the manufacturer's instructions. The release of LDH into the supernatant was quantified by recording the absorbance at 490 nm and the LDH activity was expressed as a percentage of the LDH release in control cultures.

### 2.6 Total protein extraction and subcellular fractionation

Total protein extracts (for iNOS, β-actin, phosphorylated IκBα, nonphosphorylated IκBα, phosphorylated MAP kinases and nonphosphorylated MAP kinases) were prepared in a lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 0.5 mM PMSF and 0.5 mM DTT) for 30 min at 4°C. Cytosolic fraction (for β-actin, NF-κB p65 and p50) and nuclear fraction (for histone H1, NF-κB p65 and p50) were prepared in a hypotonic buffer (10 mM HEPES pH 7.6, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1.5 mM $MgCl_2$, 1 mM PMSF, 1 μg/mL aprotinin and 1 μg/mL leupeptin). The nuclei were pelleted by centrifugation at 3000 $\times$ *g* for 5 min. The supernatants containing cytosolic proteins were collected. Nuclei lysis was performed with hypertonic buffer containing 30 mM HEPES, 1.5 mM $MgCl_2$, 450 mM KCl, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1 μg/mL aprotinin and 1 μg/mL leupeptin. The supernatants containing nuclear proteins were obtained by centrifugation at 12 000 $\times$ *g* for 20 min.

### 2.7 Western blotting

Proteins from (50 μg) whole-cell lysates, cytosolic extracts or nuclear extract were resolved by 10% SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Immobilon P, Millipore, Bedford, MA), and then probed with primary antibody followed by secondary antibody conjugated with horseradish peroxidase. The immunocomplexes were visualized with enhanced chemiluminescence kits (Amersham, UK).

### 2.8 Reverse transcription-PCR

The level of iNOS mRNA expression was measured by RT-PCR. Total RNA was isolated using TRIzol reagent (Sigma) as recommended by the manufacturer's instructions. Briefly, total RNA (5 μg) was reverse-transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase and oligo (dT) 18 primer by incubating the reaction mixture (25 μL) at 40°C for 90 min. Amplification of cDNA was performed byPCR in a final volume of 50 μL containing 2 μL of RT product, dNTPs (each at 200 μM), 1 $\times$ reaction buffer, a 1 μM concentration of each primer and 50 units/mL Pro Taq DNA polymerase. The specific PCR primers used in this experiment are listed in Table 1. The PCR conditions were as follows: after an initial denaturation for 5 min at 95°C, 30 cycles of amplification (denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s and extension at 72°C for 1 min) were performed, followed by 72°C for 10 min. A 5 μL sample of each PCR product was electrophoresed on a 2% agarose gel and visualized by ethidium bromide

 

**Table 1.** The sequences of primers used for iNOS and GAPDH RT-PCR

| mRNA source | Primer sequence |
|---|---|
| iNOS | Sense: 5′-CTGCTTTGTGCGAAGTGTCAGT-3′ |
| | Antisense: 5′-GGCACCCAAACACCAAGCTC-3′ |
| GAPDH | Sense: 5′-CAACTTTGTCAAGCTCATTTCCTG-3′ |
| | Antisense: 5′-CCTCTCTTGCTCAGTGTCCTT-3′ |

The *GAPDH* gene was used as an internal standard to normalize the amount of total RNA present in each reaction.

staining. Each value was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Values presented are the mean ± SE of at least triplicate measurements.

### 2.9 Transient transfection and luciferase assay

RAW 264.7 cells were seeded in a 60 mm dish. When the cells reached confluence, the medium was replaced with serum-free Opti-MEM (Gibco BRL). The cells were then transiently transfected with the pNFκB-Luc plasmid reporter gene (Stratagene, Jalla, CA) or the pGL2-iNOS-Luc plasmid reporter gene and the control plasmid β-galactosidase using Lipofectamine 2000 in accordance with the instructions of the manufacturer (Life Technologies, Invitrogen, CA). pGL2-iNOS-Luc plasmid reporter gene, kindly donated by Dr Biing-Hui Liu (Chung Shan Medical University, Taichung, Taiwan), comprises a 1588-bp segment from the 5′-promoter region of the iNOS gene followed by a luciferase reporter gene. After 24 h, the cells were coincubated with 100 ng/mL LPS with or without MSC for an additional 24 h. Each well was then washed twice with cold PBS and harvested in 150 μL of lysis buffer (0.5 M HEPES pH 7.8, 1% Triton N-101, 1 mM $CaCl_2$ and 1 mM $MgCl_2$). Luciferase activity was assayed by means of the LucLite luciferase reporter gene kit (Promega), with 100 μL of cell lysate used in each assay. Luminescence was measured on a top counter microplate scintillation and luminescence counter (Packard 9912 V) in single photon counting mode for 0.1 min/well, following a 5 min adaptation in the dark. The luciferase activity was normalized to β-galactosidase activity to control for transfection efficiency.

### 2.10 Statistical analysis

Quantitative data represent mean values with the respective standard error of the mean (SE) corresponding to three or more replicates. One-way Student's *t*-test was used to assess the statistical significance between the LPS- and seleno-amino compound plus LPS-treated cells. Data were considered statistically significant at $p < 0.05$.

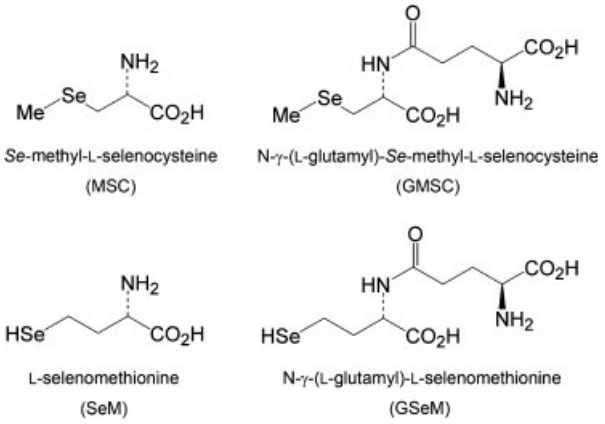


**Figure 1.** Chemical structures of selenium-containing amino compounds.

## 3 Results

### 3.1 MSC suppresses LPS-induced nitrite production in RAW 264.7 macrophages

To investigate and compare the anti-inflammatory effects of selenium-containing amino acids and peptides including MSC, *N*-γ-(L-glutamyl)-Se-methyl-L-selenocysteine (GMSC), L-selenomethionine (SeM) and *N*-γ-(L-glutamyl)-L-selenomethionine (GSeM) (Fig. 1), nitrite, a stable end-product of NO released in the culture media of LPS-activated macrophages, was assayed by the Griess reaction. As shown in Fig. 2, the nitrite production induced by LPS (100 ng/mL) was clearly reduced in the presence of MSC by >50% at 30 μM, whereas the other tested compounds GMSC, SeM and GSeM had a less inhibitory effect at the same concentration. We also found that MSC, at a concentration range 20–50 μM, dose dependently inhibited LPS-evoked nitrite production with an approximate $IC_{50}$ value of ~30 μM (Fig. 3A). To exclude the possibility that the decrease in nitrite production by MSC was due to growth inhibition and general cellular toxicity, the effects of MSC on cell viability and cytotoxicity were determined by MTT assay and LDH release assay, respectively. Although exposure of LPS (100 ng/mL) alone led to the survival rate drop to ~85.4% compared with the unstimulated control, MSC did not influence viability of LPS-treated cells even at the highest concentration (50 μM), as shown in Figs. 3B and C.

### 3.2 MSC blocks LPS-induced iNOS protein and mRNA expression

Since iNOS is responsible for NO generation in the inflammatory process, we next determined iNOS protein and mRNA expression in macrophages exposed to four selenium-containing amino acids and peptides. We examined the effects of tested compounds on protein expression by Western blotting. MSC caused an inhibition of iNOS protein expression in

 

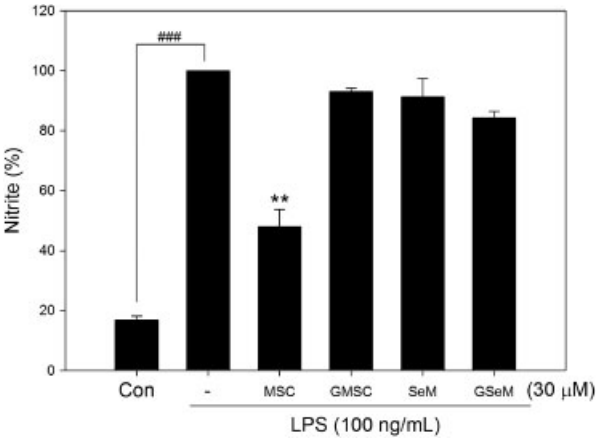


**Figure 2.** Effects of tested compounds on LPS-induced nitrite production. RAW 264.7 cells were exposed to various tested compounds (30 μM) with LPS (100 ng/mL) for 24 h. At the end of incubation time, 100 μL of the culture medium was collected for the nitrite assay using the Griess reagent. $^{**}p<0.01$ compared with LPS alone. $^{\#\#\#}p<0.001$ compared with the control group.

LPS-stimulated RAW 264.7 cells, whereas GMSC, SeM and GSeM had no effect on the protein level of iNOS expression elicited by LPS (Fig. 4A). RT-PCR analysis was done to investigate whether MSC suppressed LPS-mediated induction of iNOS *via* a pretranslational mechanism. The results indicated that the lower levels of iNOS mRNA were expressed in the presence of MSC in LPS-activated macrophages, but did not occur in the presence of GMSC, SeM or GSeM (Fig. 4B). Furthermore, MSC caused a dose-related suppression of *iNOS* gene expression in LPS-activated macrophages (Fig. 4C). These results indicate that inhibition of iNOS expression by MSC occurred in parallel with the comparable inhibition of NO production. The difference in LPS-triggered iNOS mRNA expression in Figs. 4B and C might possibly result from different lot numbers of LPS applied, which might affect the LPS inducibility in RAW 264.7 cells [27].

### 3.3 MSC reduces nuclear NF-κB level and NF-κB activation

Because the production of NO by iNOS induction in activated RAW 264.7 cells is regulated by transcription factor NF-κB, the translocation and activation of NF-κB as well as the distribution of NF-κB p65 and p50 subunits and NF-κB-dependent luciferase reporter assay were monitored. Nuclear and cytosolic extracts were isolated and NF-κB in the nuclear and cytosolic fractions was quantified by Western blotting for the p65 and p50 subunits. As shown in Fig. 5A, LPS sharply increased the translocation of p65 and p50 from cytosol to nucleus and the increase was inhibited by coincubation of the cells with MCS in a dose-dependent manner. In an additional study, transient transfection with pGL2-iNOS-Luc reporter plasmid or pNFκB-Luc reporter plasmid was done to confirm whether MSC inhibited NF-κB

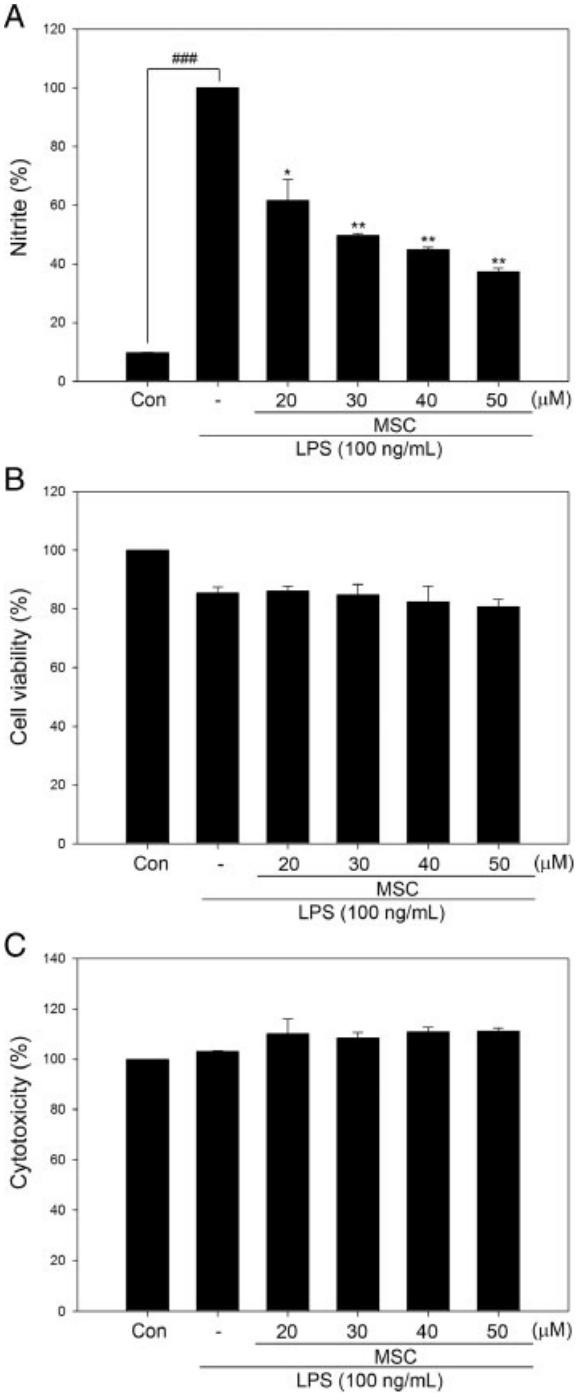


**Figure 3.** Effects of MSC on LPS-stimulated NO production, cell viability and cytotoxicity of RAW 264.7 macrophage cells in culture. The cells were treated with LPS (100 ng/mL) only or with different concentrations of MSC as indicated for 24 h. (A) Nitrite in the medium was measured using the Griess reagent. (B) Cell viability was determined by the MTT assay. (C) Cell cytotoxicity was assessed using a LDH release assay, as described in Section 2. $^{*}p<0.05$ or $^{**}p<0.01$, indicating statistically significant differences from the LPS-treated group. $^{\#\#\#}p<0.001$ compared with the control group.

 

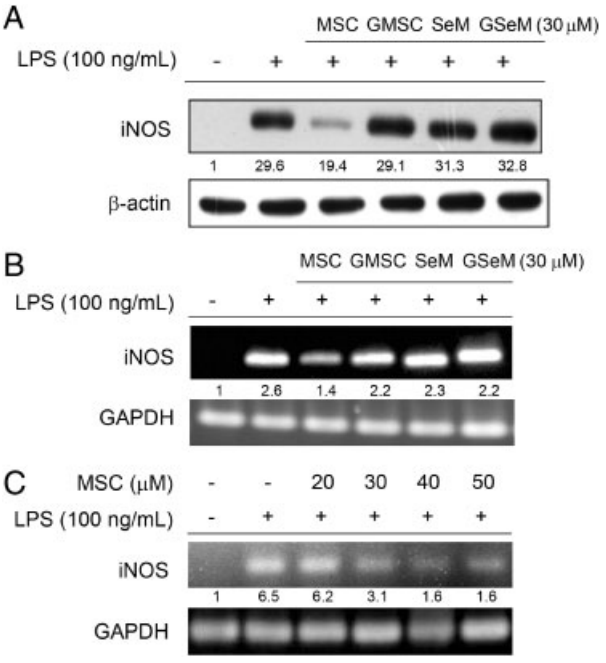


**Figure 4.** Effects of various selenium-containing amino compounds on iNOS expression in LPS-stimulated RAW 264.7 cells. Cells were treated with LPS (100 ng/mL) alone or with various tested compounds (30 μM) for 24 h. (A) Equal amounts of total proteins (50 μg) were subjected to 7.5% SDS-PAGE and the protein levels of iNOS and β-actin (loading control) were determined by Western blotting using specific antibodies against iNOS (BD Biosciences, Los Angeles, CA) and β-actin (Cell Signaling Technology, Beverley, MA), respectively. The values below the figure represent the change in protein expression normalized to β-actin. (B) Total RNA was subjected to RT-PCR analysis. The values below the figure represent the ratio of iNOS to GAPDH mRNA expression. (C) Dose-dependent decrease in LPS-induced iNOS mRNA expression by MSC. Cells were treated LPS (100 ng/mL) in the presence of various concentrations of MSC for 24 h. The mRNA expression of iNOS was performed by RT-PCR. The values below the figure represent the ratio of iNOS to GAPDH mRNA expression. All of the results are representative of three independent experiments.

transactivity in LPS-activated macrophages. As shown in Figs. 5B and C, MSC effectively and dose dependently impaired the increase in luciferase activity elicited by LPS treatment in those cells bearing pGL2-iNOS-Luc reporter or pNFκB-Luc reporter plasmid, respectively. A correlation between the effect on NO production and the inhibition of luciferase activity was also observed (Fig. 3A).

### 3.4 MSC inhibits the phosphorylation and degradation of IκBα and the phosphorylation of IKKα/β in response to LPS treatment

Because the LPS-mediated translocation of NF-κB to nucleus is preceded by the phosphorylation and proteolytic degradation of IκBα, we examined both phosphorylated and nonphosphorylated protein levels of IκBα by Western blot analysis. MSC was found to inhibit the LPS-induced phosphorylation and degradation of IκBα (Fig. 6). In parallel, the phosphorylation of IKKα/β responsible for IκBα phosphorylation and degradation was also downregulated by MSC coincubation.

### 3.5 MSC affects MAP kinase activation in LPS-stimulated RAW 264.7 cells

The three MAP kinases, p38 MAPK, ERK1/2 and JNK, have been reported to be involved in the LPS-mediated activation of NF-κB [28–30]. We further studied the effects of MSC on the induction of these kinases in RAW 264.7 macrophages using Western blot analysis techniques. Anti-phospho-p38 (Thr180/Tyr182), anti-phospho-ERK1/2 (Thr42/Tyr44) and anti-phospho-JNK (Thr183/Tyr185) antibodies were applied to detect the phosphorylated degrees of these kinases, which have direct correlation with their activation. As demonstrated in Fig. 7, MSC attenuated LPS-induced phosphorylation of p38 MAPK and JNK but not that of ERK1/2. The total protein levels of p38 MAPK, ERK1/2 and JNK were not altered by either LPS or MSC cotreatment. The results thus suggest that the inhibitory modulation of p38 MAPK and JNK pathways is an upstream event required for the anti-inflammatory activity of MSC in macrophages challenged with LPS.

## 4 Discussion

Selenium, an essential trace element, displays health benefits at low levels and its deficiency is closely linked to severe human diseases including inflammatory disorders, cancers or Keshan disease [31, 32]. Selenium and selenium compounds are found in various plants, including garlic, wild leeks, onions and broccoli, and generally thought to be chemopreventive agents based on well-designed animal experiments [33–35]. Among many forms of selenium, MSC is one of the most attractive Se compounds with relatively extensive studies due to its low toxicity and potentially anti-cancer activity. MSC has superior chemopreventive activity of breast cancer over SeM and selenite in the carcinogen-induced rat tumorigenesis model [36, 37] and may serve as a pro-drug through a β-lyse-mediated reaction by which MSC is rapidly converted in cells to methylselenol, a critical selenium metabolite for chemopreventive effects [35, 38]. MSC was also reported to sensitize the anti-cancer drug irinotecan *in vivo* by inhibiting the expression of cyclooxygenase-2, iNOS, and hypoxia-inducible factor-1α and thereby reducing angiogenesis, but these synergistic effects of MSC may not stem from the induction of apoptosis [39]. Inhibition of iNOS and COX-2 expression by MSC in this xenograft experiment raises the possibility that MSC may have

 

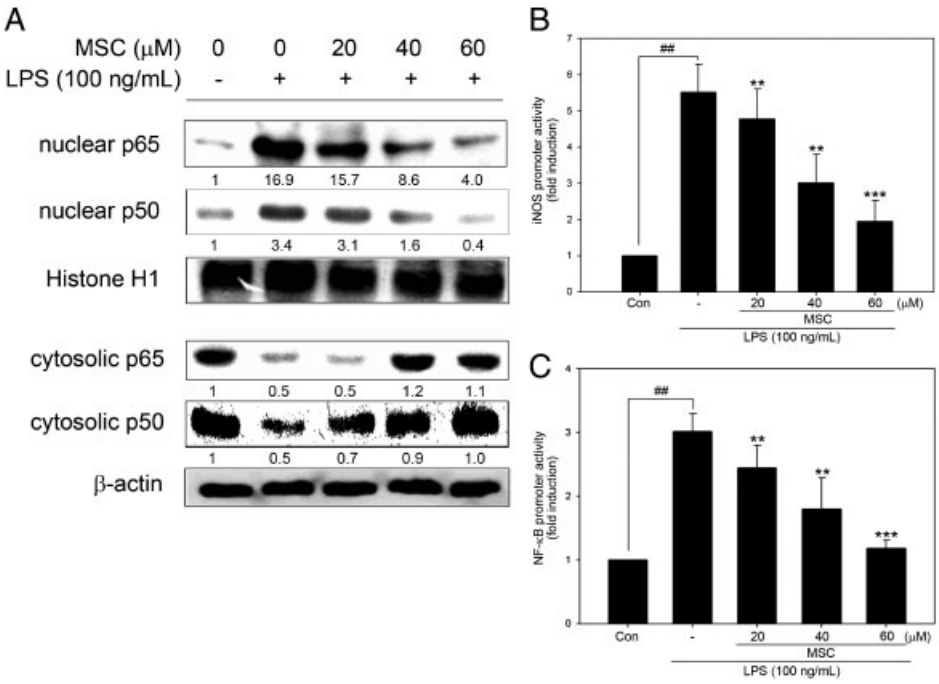


**Figure 5.** Effects of MSC on LPS-induced NF-κB nuclear translocation and activation in RAW 264.7 cells. (A) Western blots for NF-κB p65 and p50 in the nuclear and cytosolic fractions. Cells were treated with LPS (100 ng/mL) alone or with MSC (20, 40 or 60 μM) for 45 min. Then, cytosolic and nuclear fractions were prepared and analyzed by Western Blotting using specific antibodies against NF-κB p65 and p50, histone H1 and β-actin (All antibodies were purchased from Cell Signaling Technology). The values below the figure represent the change in protein expression normalized to histone H1 or β-actin. (B, C) The transcriptional activity of NF-κB in stimulated RAW 264.7 cells transiently transfected with the luciferase reporter gene. Cells were transiently transfected with 2 μg of pGL2-iNOS-Luc or pNFκB-Luc reporter gene and then treated with LPS (100 ng/mL) with or without MSC for 24 h. The transcriptional activity was detected through the luciferase activity as described in Section 2 and expressed as the comparative degree of control represented by this activity. Data represent the mean ± SD of relative luciferase activity of at least three values. Values were considered statistically significant (*versus* LPS treatment) for $^{**}p<0.01$ and $^{***}p<0.001$. $^{\#\#}p<0.05$ as compared with the control group.

the anti-inflammatory activity. Thus, in the present study, we have investigated the effects of MSC on LPS-induced NO formation and iNOS expression in activated RAW 264.7 macrophages.

In this study, MSC was demonstrated to suppress LPS-induced NO generation related to the downregulation of iNOS mRNA expression, as well as iNOS promoter-evoked luciferase activity, in a dose-dependent manner (Figs. 2, 3A, 4 and 5B), suggesting that MSC may modulate LPS-elicited iNOS expression *via* a transcriptional regulation. The predominant transcriptional factor that controls *iNOS* gene expression in response to LPS has been characterized as NF-κB. The 5′ flanking region of murine *iNOS* gene contains at least 22 transcriptional response elements, including NF-κB responsive κB element, which is activated through the Toll-like receptor 4-mediated pathway responsive to LPS stimulation [40–42]. The protection of IκBα from degradation (Fig. 6) and decreased NF-κB nuclear translocation (Fig. 5A) by MSC were well correlated with the suppression of LPS-induced iNOS expression and NO production. Hence, MSC dose dependently reduced NF-κB transcriptional activity induced by LPS, as measured by NF-κB-dependent luciferase assay (Fig. 5C), and this inhibition is positively related to its suppressive ability of NF-κB nuclear translocation. On the basis of these findings, we suggest that the anti-inflammatory activity of MSC might be, in part, attributed to modulation of NF-κB pathways.

The IKK complex, responsible for the phosphorylation and degradation of IκBα protein, is the main upstream kinase involved in NF-κB activation and therefore LPS-triggered NO generation in response to toll-like receptor 4 receptor firing by LPS [43]. The IKK complex consists of three subunits (IKKα, IKKβ and IKKγ) and its activity is enhanced by phosphorylation of specific serine sites in the activation loops of IKKα and IKKβ [44]. Our result shows that MSC led to a marked decrease in LPS-induced phosphorylation of IκBα protein (Fig. 6), implying the possibility that MSC may have an inhibitory effect on IKKα/β activity. In agreement with our assumption, MSC resulted in a dose-related inhibition of IKKα/β phosphorylation (activation). This inhibitory effect may be a result of protection of IκBα from degradation by MSC, but how MSC affects LPS-mediated IKKα/IKKβ activation remains unclear. Different types of MAP kinases including p38 MAPK, ERK1/2 and JNK have been reported to participate in the regulation of NF-κB signaling pathway in inflammatory responses

 

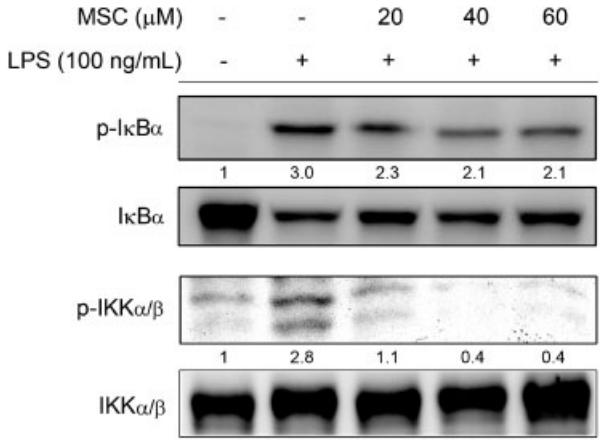


**Figure 6.** Effects of MSC on LPS-induced phosphorylation and degradation of IκBα, and phosphorylation of IKKα/β. RAW 264.7 cells were treated with LPS (100 ng/mL) for 15 min in the presence of various concentrations of MSC or vehicle. Total cellular lysates were prepared and analyzed for content of phosphorylated IκBα (p-IκBα), IκBα, phosphorylated IKKα/β (p-IKKα/β) and IKKα/β by Western Blotting using specific antibodies against p-IκBα (Ser32/36), IκBα, p-IKKα/β (Ser180/181) and IKKα/β (All antibodies were obtained from Cell Signaling Technology). The values below the figure represent the ratio of p-IκBα to IκBα or p-IKKα/β to IKKα/β, respectively. The results are representative of at least three independent experiments.

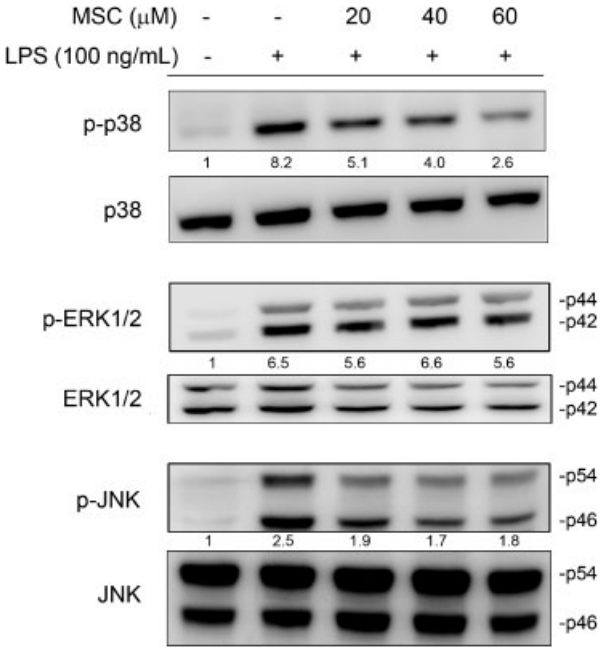


**Figure 7.** Effects of MSC on LPS-induced phosphorylation of MAP kinases. The cells were treated with LPS (100 ng/mL) with or without MSC (20, 40 or 60 μM) for 15 min. The whole-cell lysate was analyzed by Western blotting for both phosphorylated and nonphosphorylated p38 MAPK, ERK1/2 and JNK. The values below the figure represent the ratio of phosphorylated MAP kinase to nonphosphorylated one. Antibodies against phospho-p38 (p-p38, Thr180/Tyr182), p38, phospho-ERK1/2 (p-ERK1/2, Thr42/Tyr44), ERK1/2, phospho-JNK (p-JNK, Thr183/Tyr185) and JNK were from Cell Signaling Technology. These experiments were repeated three times with similar results.

[28, 30]. Our result, in accordance with these observations, shows that exposure of murine RAW 264.7 macrophages to LPS increased the phosphorylation (activation) of p38 MAPK, ERK1/2 and JNK, respectively. What we further demonstrated was that MSC markedly suppressed the phosphorylation (activation) of p38 MAPK and JNK induced by LPS without the change in the total protein levels of MAPK (Fig. 7), indicating that MSC specifically inhibited the activation of p38 MAPK and JNK but not the biosynthesis of these two MAPKs. In contrast, MSC did not affect the activation of ERK1/2 elicited by LPS in RAW 264.7 macrophages. Thus, we presume that suppression of LPS-induced NF-κB activation and subsequent iNOS expression by MSC is p38 MAPK- and JNK-dependent.

For chemoprevention, the safe dose of MSC should be seriously considered. Based on oral toxicity studies of MSC, no observed adverse effect levels (NOAELs) of MSC to rats and dogs were determined as being $<0.5$ and $<0.15$ mg/kg/day, respectively [45]. With the extrapolation to human equivalent dose using the body surface area normalization [46], this is equal to ~0.08 mg/kg/day or ~4.8 mg dose of MSC *per* day for a 60 kg person. Whether the anti-inflammatory efficacy in animal experiment is comparable to that in cell-based study under the allowable safe dose of MSC for animals requires further elucidated. In summary, the present study has revealed the potent anti-inflammatory activity of MSC in LPS-activated RAW 264.7 macrophages. The inhibitory effects of MSC on NF-κB and iNOS, both of which are critical factors in mediating inflammatory responses, provide strong evidence that this selenium-containing chemopreventive agent may also function as a therapeutically anti-inflammatory agent.

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