Selenium Attenuates Lipopolysaccharide-Induced Oxidative Stress Responses Through Modulation of p38 MAPK and NF-κB Signaling Pathways

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Lipopolysaccharide (LPS) produces reactive oxygen species (ROS) and nitric oxide (NO) in macrophages. These molecules are involved in inflammation associated with endotoxic shock. Selenium (Se), a biologically essential trace element, modulates the functions of many regulatory proteins involved in signal transduction and affects a variety of cellular activities, including cell growth and survival. We demonstrate that Se attenuated LPS-induced ROS and NO production in murine macrophage cultures in vitro. This Se-decreased production of NO was demonstrated by decreases in both mRNA and protein expression for inducible NO synthase (iNOS). The preventive effects of Se on iNOS were p38 mitogen-activated protein kinase– and nuclear factor–κB–dependent. Se specifically blocked the LPS-induced activation of p38 but not that of c-jun–N-terminal kinase and extracellular signal–regulated kinase; the p38-specific pathway was confirmed using p38 inhibitor SB 203580. These results suggest that the mechanism by which Se may act as an anti-inflammatory agent and that Se may be considered as a possible preventive intervention for endotoxemia, particularly in Se-deficient locations. However, the efficacy and safety of Se need to be further investigated, because long-term intake >0.4 mg Se/day in adults can produce adverse effects. Exp Biol Med 229:203–213, 2004

Key words: selenium; glutathione; iNOS; lipopolysaccharide; mitogen-activated protein kinases; nitric oxide; nuclear factor–κB; reactive oxygen species

Macrophages play a crucial role in the initiation and maintenance of inflammation. During endotoxemia and inflammation, macrophages are activated by lipopolysaccharide (LPS) and cytokines. Several intermediates are involved in LPS-induced signal transduction, such as protein kinase C, mitogen-activated protein kinases (MAPKs), transcription factors nuclear factor (NF)–κB, and AP-1 (1–4). Stimulation of macrophages by LPS results in the expression of inducible nitric oxide synthase (iNOS), which catalyzes the production of NO (5, 6). NO acts as an intracellular messenger and regulates cellular functions such as vasorelaxation and inflammation. NO has an important role in the elimination of pathogens and tumor cells; however, overproduced NO is oxidized to reactive nitrogen species and results in the disruption of cell signaling and uncontrolled systemic inflammation and septic shock. The expression of iNOS is regulated by pathways that involve MAPKs and NF-κB in macrophages (6, 7). NF-κB is a transcription factor that modulates the expression of variety of genes involved in immune and inflammatory responses, including iNOS and tumor necrosis factor (TNF)–α (6, 8). MAPKs have important functions as mediators of cellular responses to extracellular signals. MAPKs important to mammalian cells include p38, c-jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK; Ref. 9). NF-κB is an important intermediary of LPS-induced signal transduction in macrophages. P38 MAPK is thought to play an important role in the regulation of inflammatory molecules (10); specifically, it plays a major role in LPS-induced NO production and iNOS induction (11).

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Reactive oxygen species (ROS) are mediators of cellular injury and are involved in the onset of cellular damage during endotoxemia (12, 13). ROS are involved a in a variety of cellular stress mechanisms. Glutathione (GSH) is the major intracellular antioxidant with multiple biological functions, including the maintenance of the thiol moieties of proteins and the reduced form of many other biologically active molecules (14).

Selenium (Se) is an essential trace element for mammalian cells. The antioxidant and other beneficial effects of Se have been recognized for some time. Se has regulatory functions in cell growth and cellular death and modulates signal transduction in various cells (15–17). Se is a component of selenoproteins such as Se-dependent glutathione peroxidase (Se-GPx) and thioredoxin reductase (18). Se, as an essential constituent of GPx, plays an important role in scavenging ROS. On the other hand, several reports have suggested the possible induction of ROS by Se; for example, Se induced oxidative stress by stimulating ROS and catalyzing the oxidation of thiol groups (19, 20). Although it is known that ROS and GSH are closely involved in Se metabolism and bioactivity of various cells, the exact mechanism remains unclear. The antioxidant property of Se is concentration-related for *in vitro* systems; the exact relationship with dose *in vivo* is not well characterized. Se has been known to regulate the activation of NF-κB by various stimuli, including LPS, TNF-α, and ovalbumin (16, 18, 21, 22). The NF-κB and MAPKs (including p38) are involved in stress-related signaling pathways and are involved in the LPS-mediated modulation of gene expression (23, 24). Previous studies have suggested modulatory effects of Se on MAPKs (17, 22); however, the types and precise mechanism have not been investigated. On the basis of this prior information, we hypothesized that Se would antagonize the inflammatory responses induced by endotoxin and that such antagonism would involve signaling via MAPKs in murine macrophages. The principal aim of the present study therefore was to examine the effect of Se on endotoxin-induced NO and ROS production and associated signaling pathways in a murine macrophage line.

Materials and Methods

Cell Cultures. BALB/c murine macrophage cells (J774A.1; American Type Culture Collection TIB-67) were grown in Dulbecco's modified Eagle's medium, with 2 m*M* glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and supplemented with 10% non–heat-inactivated fetal bovine serum (Atlanta Biologicals, Atlanta, GA) in 5% CO_2 at 37°C.

Assay for Viable Cells and Lactate Dehydrogenase (LDH) Release. Cell viability was determined by 3(4,5-dimethyl thiazolyl-2)2,5 diphenyl tetrazolium bromide (MTT; Sigma, St. Louis, MO) assay, as described elsewhere (25). Cells were seeded at 2×10^4 cells/well in 96-well microplates and treated with Se (sodium selenite; Sigma). Mitochondrial enzyme activity, an indirect measure of the number of viable respiring cells, was determined using MTT after 24 hrs of treatment with Se. The MTT absorbance was read using a Spectra SLT microplate reader (Tecan, Durham, NC). Cell viability was calculated as the relative absorbance compared with that of control cultures.

Both released and total LDH concentrations were determined as described elsewhere (26). The release of LDH from cells is a pathological manifestation of the increased plasma membrane permeability associated with cell death. The LDH release was expressed as the percentage of LDH released (LDH activity in medium/total LDH activity × 100). The absorbance was read kinetically using a PowerWave$_x$ Microplate Scanning spectrophotometer (Bio-Tek Instrument, Inc., Winooski, VT).

Se and DNA Synthesis. DNA synthesis was used as an index of proliferation of macrophages exposed to Se, as described elsewhere (27). After 24 hrs of treatment with Se, each well was pulsed with 0.5 μCi of [methyl-^{3}H] thymidine (DuPont NEN Products, Boston, MA) and incubated for an additional 18 hrs. Cells were harvested, lysed, and counted with a liquid scintillation counter (Pharmacia, Turku, Finland). Proliferative response (uptake of [^{3}H] thymidine) was expressed as net disintegrations per min.

ROS Generation. The production of ROS was measured by detecting the fluorescence intensity of oxidant-sensitive probe dihydrorhodamine 123 (DHR, 10 μ*M*; Molecular Probes, Eugene, OR), which is a cell-permeable, nonfluorescent molecule (28). On interaction with free radicals, DHR is oxidized, which results in the liberation of rhodamine, a highly fluorescent mitochondrial-specific marker. The cells were preincubated for 24 hrs with Se (indicated in the Results section), followed by 18 hrs with LPS, and the fluorescent intensity was recorded using Spectramax Germini fluorescence plate reader (Molecular Devices, Irvine, CA). The DHR fluorescence was detected at excitation of 507 nm and emission 529 nm. The fluorescence values were digitized using SoftMax Pro Version 3.1.1 (Molecular Devices). The results were similar in at least three independent replications, and data from a representative experiment ($n = 5$ wells) have been presented.

Western Blot Analyses. Samples of protein (cell lysates) were electrophoresed using 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, as described elsewhere (29), and then transferred to nitrocellulose membranes. The membranes were stained with the reversible Ponceau S (Sigma), to ascertain equal loading of samples in the gel. The iNOS and p65 NF-κB were assayed using anti-iNOS antibody and anti–NF-κB (p65) antibody (Santa Cruz Biotech, Santa Cruz, CA). The p38, JNK, and ERK activation was determined using anti–phospho-p38, -JNK, and -ERK antibodies (Cell Signaling, Beverly, MA). Immunodetection was done using enhanced chemiluminescence detection kit (Amersham Pharmacia, Piscataway, NJ).

Immunofluorescent Localization of NF-κB. Cells were cultured in sterile four-chambered slides (Nalge Nunc International, Naperville, IL) with Se for 24 hrs prior to an additional 4-hr treatment of LPS. After washing, cells were fixed and permeabilized in 1:1 cold methanol:acetone for 30 mins, washed twice with phosphate-buffered saline (PBS), and blocked for 50 mins with 1% normal goat serum in PBS that contained 0.1% Triton X-100. Mouse monoclonal anti–NF-κB–p65 subunit primary antibody diluted in PBS that contained 2 mg/ml fatty acid–free bovine serum albumin (Sigma) and 0.1% Triton X-100 was applied to the slides and incubated for 1 hr at 37°C in a humidified chamber. Cells were washed with PBS, followed by 1 hr incubation with fluorescein isothiocyanate–conjugated anti–mouse secondary antibody. Slides were washed, and cover slips were mounted and viewed with an Olympus IX71 (Melville, NY) inverted fluorescence microscope equipped with appropriate optics. Images from the microscope were recorded by Olympus digital camera using Olympus Magnifier SP software.

NO Assay. The amount of stable nitrite, the end product of NO generation by activated macrophages, was determined by a colorimetric assay, as described elsewhere (25). In brief, 50 μl of culture supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrocholoride, and 2.5% H_3PO_4) and incubated at room temperature for 10 mins. The absorbance at 540 nm was read in an SLT microplate reader (Tecan, Durham, NC). The nitrite concentration was determined by extrapolation from a sodium nitrite standard curve.

Semiquantitative Analysis of iNOS Expression. Reverse-transcription polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for iNOS and β-actin (internal control). The condition for RT and PCR steps were done as reported elsewhere (25). The cycle number was optimized to ensure product accumulation in the exponential range. Amplified products were separated by electrophoresis on a 2% agarose gel and documented using a Kodak DC 290 digital camera and digitized using UN-SCAN-IT software (Silk Scientific, Orem, UT). Band intensities for the respective cytokine were normalized to that of β-actin in the same sample.

GSH Assay. Total GSH, reduced and oxidized (GSH + GSSG), was measured in cell lysates using recycling GSH with GSH reductase and NADPH (30). The GSH was detected by the color change at 412 nm that is associated with 5,5-dithiobis-(2-nitrobenzoic acid) reduction. This assay was adapted for use in a microplate using a PowerWave$_X$ Microplate Scanning spectrophotometer at 405 nm. Cells were lysed by sonication, and the absorbance was read kinetically. GSSG was measured using the above procedure after cell lysates had been treated with 2-vinylpyridine (2-VP; 2 μl/100 μl sample for 60 mins; Ref. 31). GSH levels were quantified using standards of GSSG with and without 2-VP and expressed as GSSG equivalents. GSH levels were calculated from the difference between total GSH and GSSG. Validation of this procedure was carried out by processing 20–100 pmol of GSH and GSSG as described above and also by including 66 pmol of GSH and GSSG standard in cell extracts. Samples were then assayed with or without 2-VP derivatization. There was no effect on GSH and GSSG measurements resulting from sample processing, compared with standards prepared and assayed immediately.

Replication, Data Presentation, and Statistical Analysis. All experiments were repeated two to three times with similar trends; however, data from a representative trial are depicted in the Results section. Statistical analyses were done using SAS statistical software (SAS Institute, Cary, NC). Treatment effects were analyzed using one-way ANOVA, followed by Duncan's multiple range test. $P < 0.05$ was used to indicate significance.

Results

Cytotoxicity of Se in Murine Macrophage Cultures. Cell viability and DNA synthesis were investigated to determine the threshold Se concentration above which Se reduced cell viability and growth. Exposure to 10 μ*M* of Se for 24 hrs did not affect the viability of cells (Fig. 1A). The viability of cells exposed to 50 μ*M* Se for 24 hrs was reduced to 60% of control cultures. Cells exposed to 10 μ*M* Se showed no changes in DNA synthesis as indicated by [^{3}H]thymidine incorporation; however, 50 μ*M* Se almost completely blocked DNA synthesis (Fig. 1B). Cells treated with Se showed a similar dose response for LDH release (Fig. 1C). These data indicate that concentrations of Se $\leq$10 μ*M* did not affect the viability or proliferation of J774A.1 macrophages, whereas $\geq$50 μ*M* were cytotoxic. LPS induced LDH release in a concentration-dependent manner (Fig. 1D).

Selenium Inhibited LPS-Induced ROS Generation. LPS has been shown to increase the cellular oxidative stress (12). To assess whether LPS directly induces the production of ROS, cells were placed in a black plate in the presence of LPS (30 ng/ml) and DHR (10 μ*M*) for 100 mins. LPS did not increase the oxidation of the DHR during 100 mins, which indicates no increase in ROS during such short-term exposure (Fig. 2A). To assess whether prolonged exposure to LPS induces ROS, cells were placed in a six-well plate and incubated with LPS for 18 hrs in the presence of DHR, as reported elsewhere (2). LPS increased the oxidation of DHR in a concentration-dependent fashion (Fig. 2B), and the increased DHR fluorescence intensity was abolished by 1 m*M* *N*-acetylcysteine (data not shown). Se itself did not alter ROS production, but Se decreased LPS-induced ROS production (Fig. 2C).

Selenium Inhibited LPS-Induced Activation of p38 MAPK. LPS activates MAPKs signal-transduction pathways (9, 11). Therefore, we assayed the effect of Se on the activation of MAPKs signaling pathways by examining

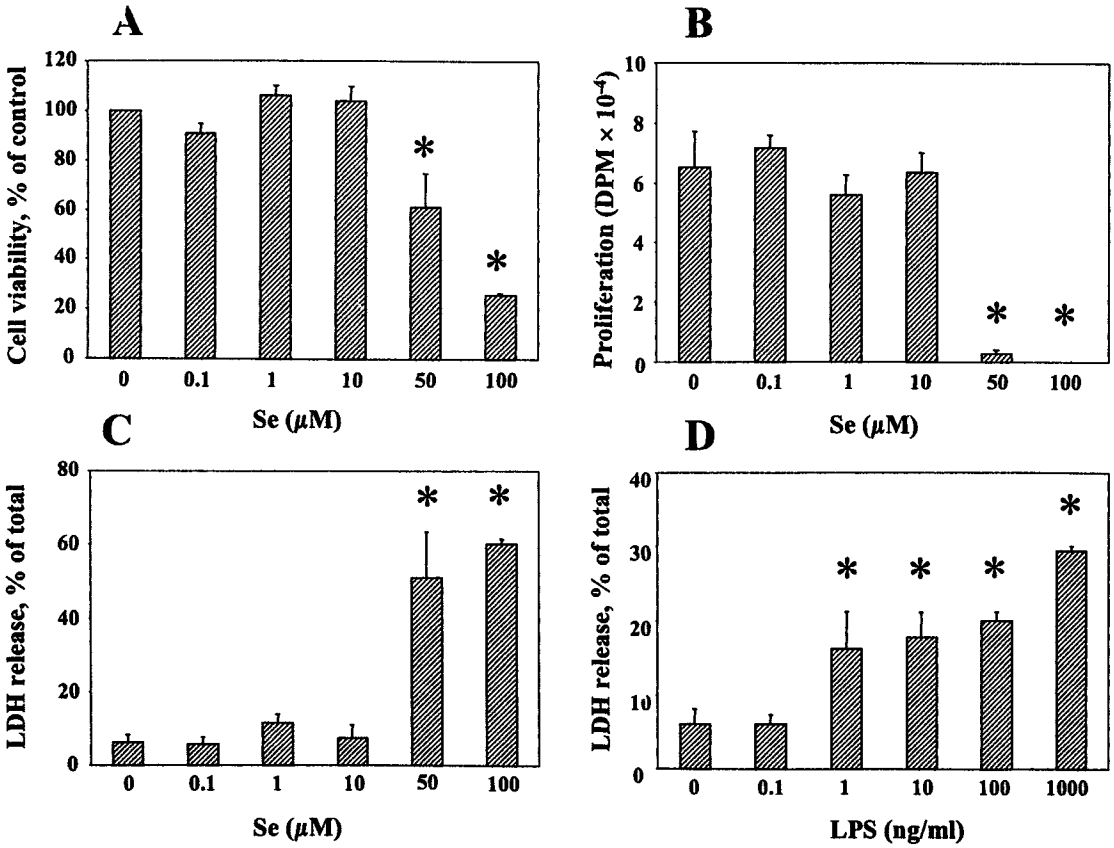

Figure 1. Effects of Se on cell viability (A), proliferation (B), and LDH release (C) in J774A.1 cells. Effects of LPS on LDH release (D). Cells were treated with various concentration of Se for 24 hrs. Cell viability was represented by relative absorbance compared with controls. The LDH release was expressed as a percentage of total cellular LDH. DNA synthesis of lymphocytes was measured by [^{3}H]thymidine incorporation. Results from a representative experiment are expressed as mean ± SE (n = 4 in a single experiment). *Significantly different than the control cultures at $P < 0.05$.

the phosphorylation of p38, JNK, and ERK by Western blot analyses. Stimulation of cells with LPS resulted in increased phosphorylation of all three MAPKs, and this phosphorylation peaked at 15–30 mins (data not shown). Se alone had no effect on any of the three MAPKs; however, Se decreased the LPS-induced activation of p38 but did not affect the phosphorylation of JNK and ERK (Fig. 3). Oxidative stress is known to induce the activation of p38 (32). To further elucidate the role of p38 on Se effect, we used specific p38 inhibitor SB 203580, as indicated below.

Selenium Inhibited LPS-Induced Nuclear Translocation of NF-κB. NF-κB controls the transcription of many signaling genes, including iNOS (6). LPS treatment induced the nuclear translocation of p65 NF-κB after 4 hrs of incubation, as determined by immunostaining (Fig. 4A). Exposure of cells to LPS showed intensified nuclear staining, compared with the diffuse cytosolic staining in untreated cells. Treatment of J744A.1 cells with LPS caused an apparent distortion in the shape of nuclei. Pretreatment of cells with Se attenuated the LPS-induced nuclear localization of NF-κB. To further confirm the inhibitory effect of Se on the LPS-induced nuclear translocation of NF-κB, Western blots were conducted (Fig. 4B). Pretreatment with Se inhibited the LPS-induced nuclear translocation of p65 NF-κB, but Se alone had no effect.

Se inhibited the LPS-induced NO production and iNOS. Incubation of cells with LPS (30 ng/ml) caused nitrite accumulation in the cell culture medium during the 24-hr observation period, which indicated NO production (Fig. 5A). Se by itself had no effect on NO production in these cultures. There was a difference in the pattern of suppression between pretreatment and simultaneous treatment with Se. Simultaneous treatment with Se did not prevent LPS-induced NO production. However, pretreatment with Se decreased the production of NO in response to LPS with maximal efficacy after a 24-hr preincubation with Se.

To determine whether the altered synthesis of iNOS was responsible for the observed effects on NO production, we analyzed the expression of iNOS mRNA and protein by RT-PCR and Western blot analyses, respectively. Incubation of cells with LPS (30 ng/ml) increased cellular iNOS mRNA (Fig. 5B) and protein (Fig. 5C) after 12- and 24-hr treatments, respectively. Pretreatment with Se for 24 hrs significantly attenuated both LPS-induced iNOS mRNA and protein. Treatment of cells with specific p38 inhibitor (SB 203580) decreased LPS-induced NO production and

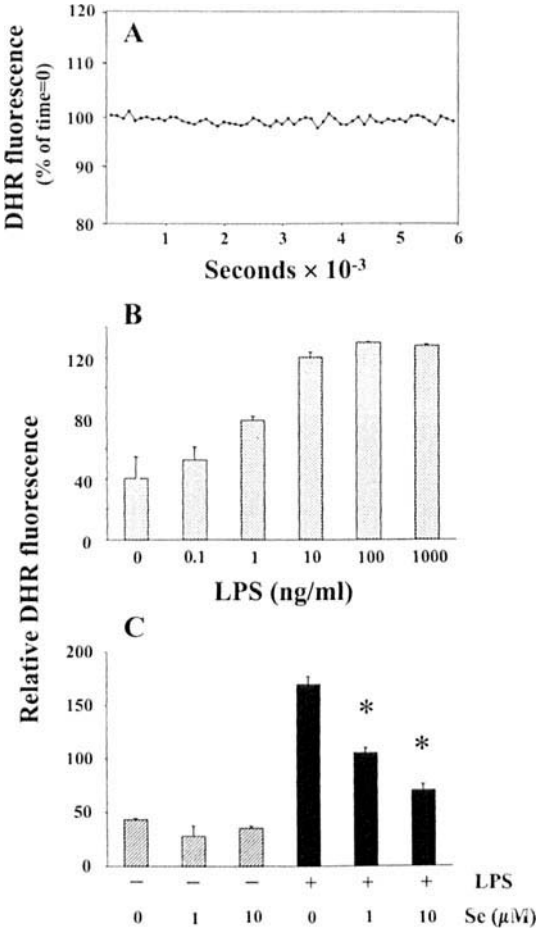

Figure 2. Effect of Se on LPS-induced ROS production. (A) DHR fluorescence in J774A.1 cells exposed to LPS (30 ng/ml). (B) DHR fluorescence in J774A.1 cells exposed to different concentration of LPS for 18 hrs. (C) DHR fluorescence in J774A.1 cells exposed to LPS (30 ng/ml) at 18 hrs after 24 hrs of pretreatment with Se. Results are expressed as mean ± SE of one representative experiment (n = 5). *Significantly different than the LPS alone group at $P < 0.05$.

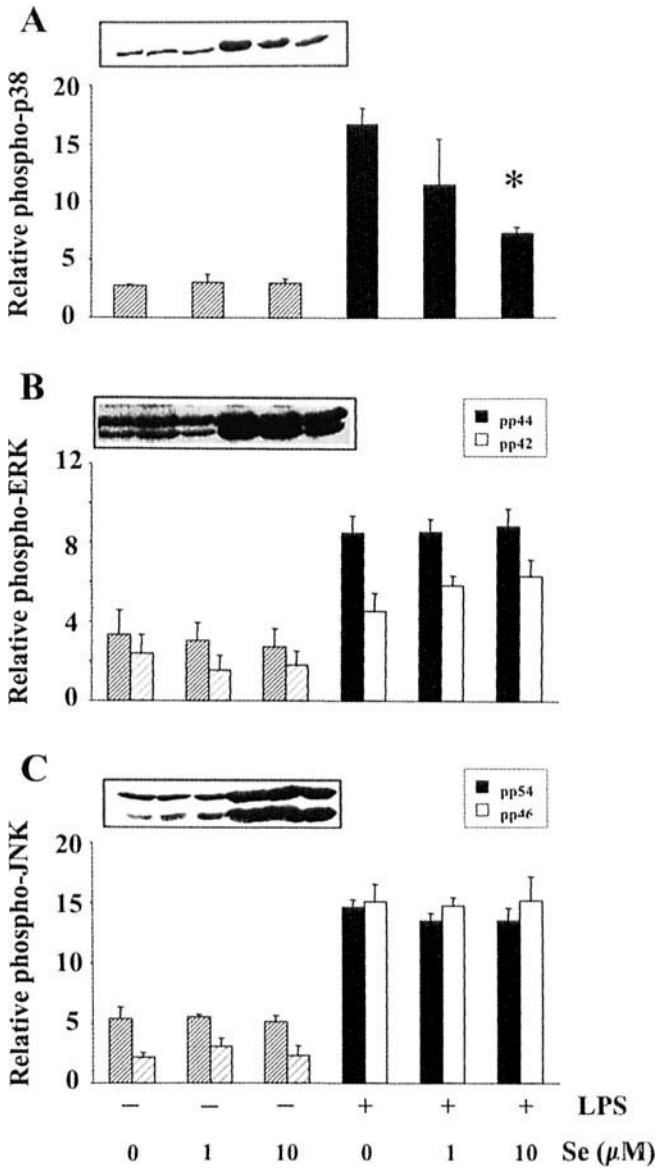

Figure 3. Effect of Se on phosphorylation of p38 (A), ERK (B), and JNK (C) MAPKs. After pretreatment with Se for 24 hrs, J774A.1 cells were stimulated by LPS (30 ng/ml) 15 mins. Extracts of cells (25 μg) were analyzed by Western blot. Results are expressed as mean ± SE (n = 3 of a representative trial). *Significantly different than the LPS alone group at $P < 0.05$. Insert, a representative Western blot in the same order.

concomitant iNOS mRNA and protein in murine macrophages (Fig. 6). Inhibitors of JNK or ERK did not influence the iNOS expression (data not shown).

Selenium Decreased Intracellular GSH. Incubation of cells with Se (10 μ*M*) decreased total intracellular GSH levels (Table 1). The exposure of cells to LPS alone did not affect the intracellular GSH; however, cotreatment of cultures with Se and LPS decreased total GSH to a greater extent than Se alone. The GSH synthase inhibitor buthionine sulfoximine (BSO; 50 μ*M*) decreased total intracellular GSH levels and was used as a positive control.

Discussion

In the present study, we provide experimental support for the hypothesis that Se, an integral part of GPx, may have a pivotal role in anti-inflammatory responses, in part by significantly inhibiting the production of NO, an important mediator in the pathophysiology of inflammatory diseases. On the basis of previous literature and the current results, it is apparent that the inflammatory responses of LPS in macrophages includes the initial induction of ROS, which leads to the activation of MAPKs and NF-κB and to the induction of iNOS and inflammatory cytokines. This last step would lead to the production of NO in toxic amounts, together with toxic cytokines. Se can alleviate these effects by its antioxidant action and by modulating the downstream pathway. Our results suggest that the Se-modulated pathway is p38 specific; ERK and JNK showed little involvement in it, if any. Our experiments showed that the pretreatment of macrophages with Se decreased ROS production and prevented LPS-induced iNOS production. We used in-

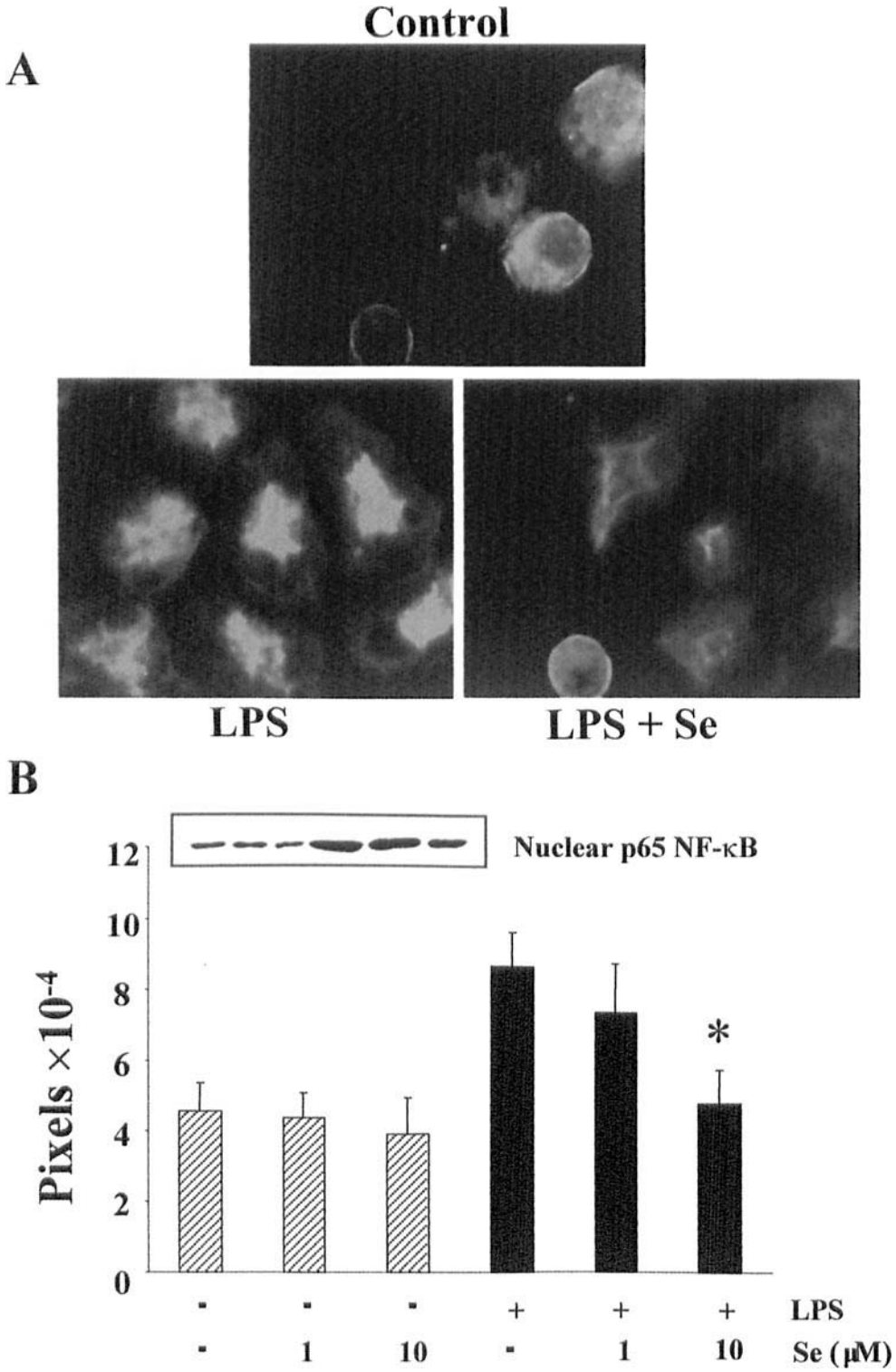

Figure 4. Effect of Se on translocation of p65 NF-κB levels shown by immunofluorescent staining (A) and Western blotting of nuclear extracts (B) in J774A.1 cells. Cells were pretreated with Se for 24 hrs before stimulation by LPS (30 ng/ml) for 4 hrs. Untreated cells displayed diffuse cytosolic staining, whereas the intensified staining in the nucleus was observed in LPS-treated cells is indicative of NF-κB nuclear translocation. The cells treated with LPS alone showed mitogenic response to LPS—irregular shaped nuclei with appearance of spindle-like shapes. Pretreatment of cells with Se (10 μ*M*) attenuated the LPS-induced translocation of NF-κB. Nuclear extracts (5 μg) were analyzed by Western blot (B). Results are expressed as mean ± SE (n = 3 of a representative trial). *Significantly different than the LPS alone group at $P < 0.05$. Insert, a representative Western blot in the same order.

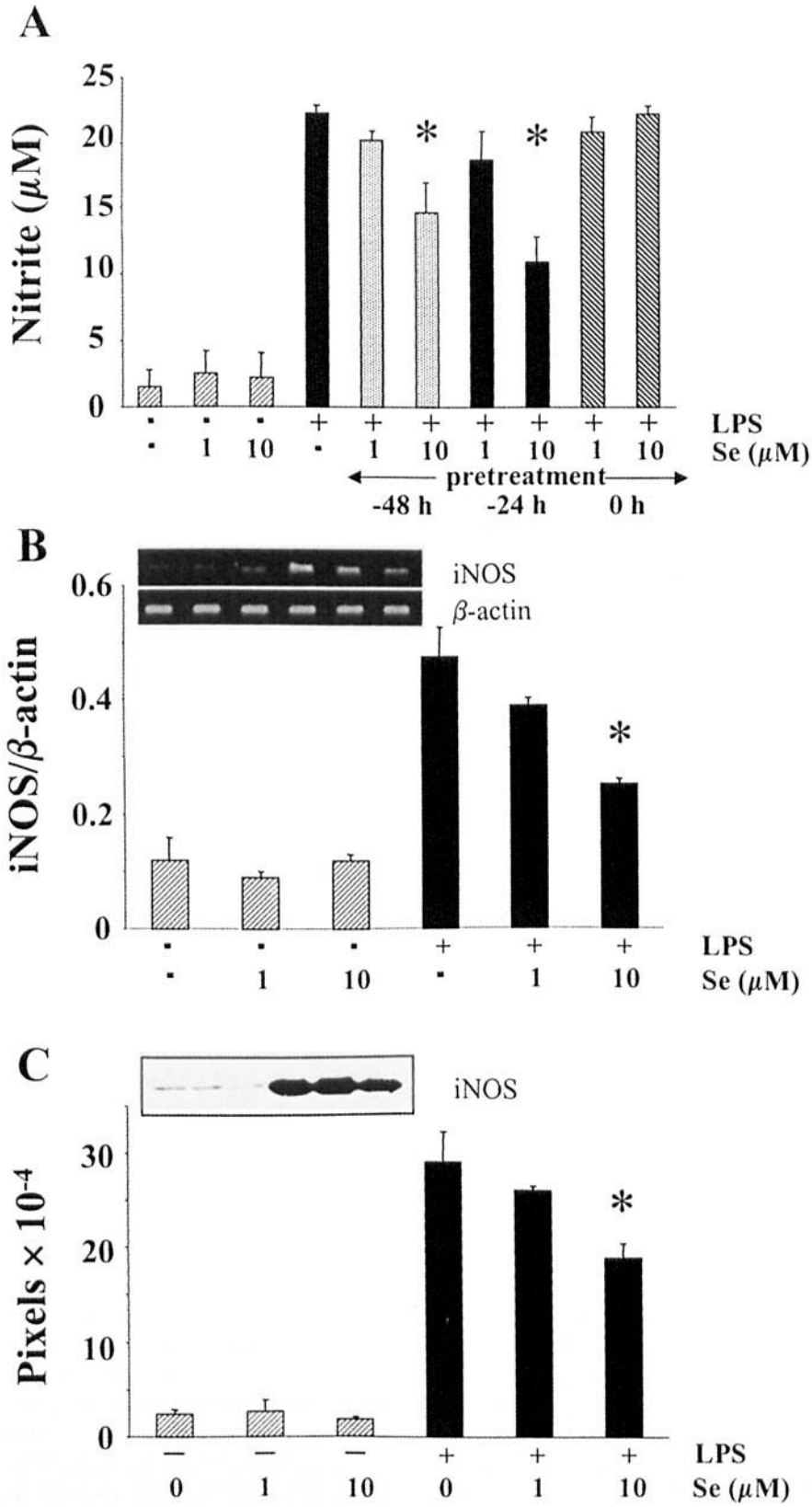

Figure 5. Effect of Se on NO production (A), iNOS mRNA (B), and protein (C) in J774A.1 cells. Cells were pretreated for 24 hrs with Se and stimulated by LPS (30 ng/ml) for 12 hrs for mRNA and 24 hrs for protein. iNOS mRNA expression was quantified by RT-PCR and normalized against β-actin. Extracts of cells (5 μg) were analyzed by Western blot. Results from a representative experiment are expressed as mean ± SE ($n = 3$ of a representative trial). *Significantly different than the LPS alone group at $P < 0.05$. Insert, a representative RT-PCR and Western blot gel in the same order.

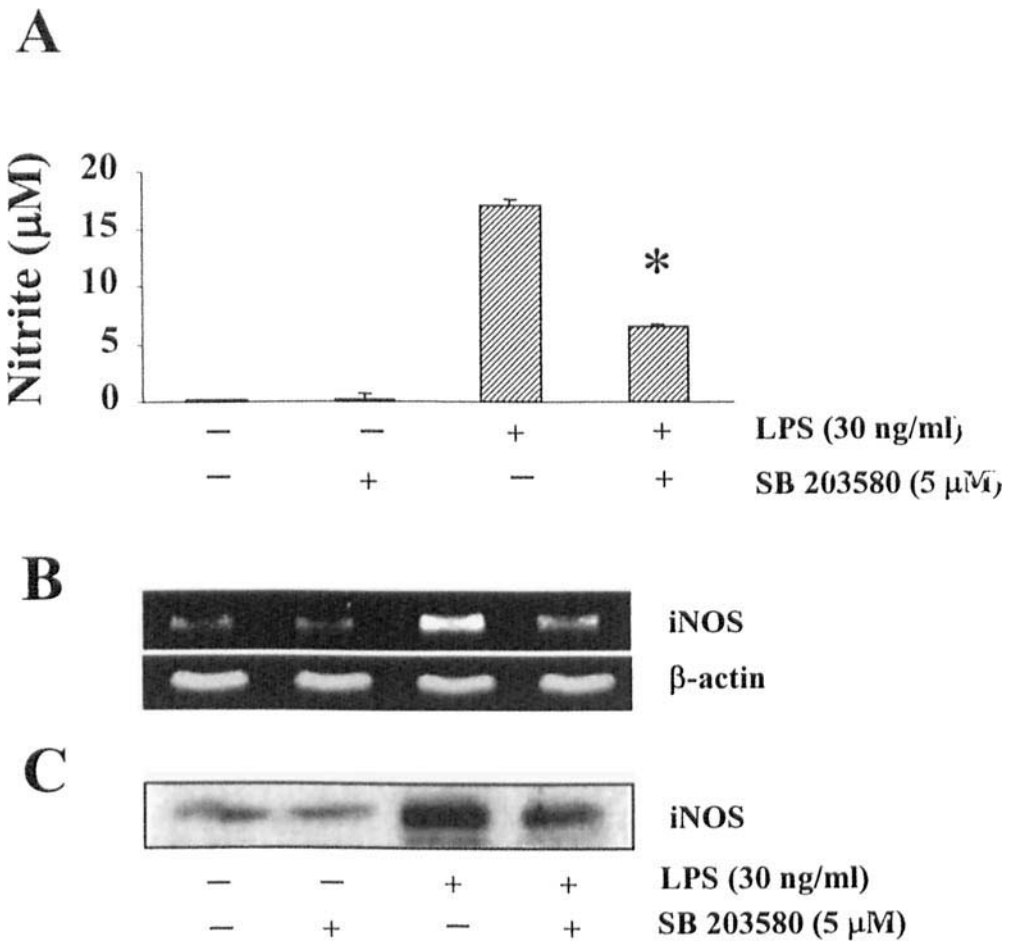

Figure 6. Effect of specific p38 inhibitors on NO production (A), iNOS mRNA (B), and protein (C) in J774A.1 cells. Cells were pretreated for 30 mins with SB 203580 and stimulated by LPS (30 ng/ml) for 24 hrs for NO production, 12 hrs for iNOS mRNA, and 24 hrs for iNOS protein. iNOS mRNA expression was quantified by RT-PCR and normalized against β-actin. Extracts of cells (5 μg) were analyzed by Western blot. Results from a representative experiment are expressed as mean ± SE (n = 4 for A and n = 3 for B and C from a single experiment). *Significantly different than the LPS alone group at $P < 0.05$. Insert (B and C), a representative RT-PCR and Western blot in the same order.

organic Se instead of the organic forms because, in our previous studies, sodium selenite, but not seleno-L-methionine, was effective on the immune responses *in vivo* (27).

Selenium is an important element for eukaryotes and many bacteria but is also toxic at higher levels (33, 34). It has been reported that Se inhibits LPS-induced NO production and iNOS protein in human T cells, mouse macrophages, and rat Kupffer cells (16, 22, 35). Additionally, Prabhu *et al.* (18) reported that Se deficiency increased iNOS expression in RAW macrophages. During the condition of Se deficiency, macrophages produce more NO and ROS than during Se supplementation. The authors explained that Se deficiency caused a decrease of GPx activity and an increase in overall oxidative stress, leading to increased iNOS induction and subsequent NO production.

LPS induces lipid peroxidation and hydroperoxide formation, and this effect can be prevented by the addition of antioxidants (36). Treatment with Se itself decreased cellular GSH, and treatment with Se plus LPS decreased GSH to an even greater extent. Se alone perhaps decreased GSH by increasing GPx activity. GPx uses GSH to reduce cellular peroxides, thus consuming reduced GSH. After LPS treatment, Se reduced LPS-induced NO and ROS by increasing GPx activity, therefore resulting in a greater decrease of GSH. Of interest, only the pretreatment of cells with Se showed antagonism to LPS responses. Simultaneous or post-treatment with Se did not block LPS effects. This result can exclude the possibility that Se directly scavenges NO and ROS in the culture media and supports the synthesis of new GPx as a possible mechanism.

The MAPK cascade is one of the important signaling pathways in immune responses. LPS activates all three types of MAPKs—p38, JNK, and ERK—in mouse macrophages and human monocytes (4, 11, 37). The exact signaling pathways among three types of MAPKs are still unclear; however, there is a cross-talk and signal convergence among the MAPKs. Many of the upstream kinases and downstream substrates are the same for each of the major cascades (38). According to the present results, LPS simultaneously activated all three MAPKs in J774A.1 cells; the maximal activation occurred 15–30 mins after stimulation in J774A.1 cells (data not presented). Among the MAPKs, Se only

inhibited the activation of p38 but not of JNK or ERK. It has been reported that inorganic Se decreased ultraviolet-stimulated activation of p38 and JNK but not ERK in human embryonic kidney cells (17), and organic Se decreased the LPS-induced activation of JNK but not p38 in rat Kupffer cells (22). Other reports have shown that, in the differential regulation of MAPKs on LPS-induced NO signaling, the p38 but not ERK was responsible for the LPS-mediated induction of NO production in murine macrophages (11, 37, 39). These paradoxical results may arise from the differences of gene expression in a receptor- and cell type–dependent manner. Rao (40) reported that, even among the macrophages, cells from different compartments showed different expression of receptors and signal transduction molecules, and these differences may account for differences in MAPK activation.

To confirm the specific role of p38 in our model, we used a specific p38 inhibitor. Pretreatment of cells with SB 203580 blocked LPS-induced NO production and the concomitant iNOS expression and production. Moreover, specific ERK and JNK inhibitors did not prevent LPS-induced iNOS induction (data not presented). Therefore, in the model used in the current study, the prevention of endotoxin-induced iNOS induction by Se was mediated by p38 but not other MAPKs.

The interplay between NF-κB and MAPK is important in determining the signal pathways. The exact relationship between NF-κB and MAPKs is still controversial. However, NF-κB and p38 pathways may converge downstream, because p38 inhibition interferes with NF-κB–dependent gene transcription. The induction of NF-κB–dependent genes is reduced by a dominant-negative p38 MAPK expression vector and a specific p38 inhibitor. Inhibition of the p38 MAPK did not alter NF-κB activation, but it decreased the DNA binding of TATA-binding protein to the TATA box, which is a target for p38 (1). Obviously, both NF-κB and p38 are involved in inhibitory response of Se on LPS-induced NO production.

To further investigate the inhibitory effects of Se on LPS-induced NO production, we tested the transcription levels of involved intermediates. It is known that the modulation of iNOS is dependent on NF-κB activation in LPS-stimulated macrophages (6). In the current study, we found that Se blocked nuclear translocation of p65 NF-κB. The selenoorganic compound ebselen inhibited LPS-induced NF-κB translocation in Kupffer cells (22), and inorganic Se inhibits NF-κB–DNA binding in T cells (16). After extracellular stimulus, such as LPS, phosphorylation and degradation of IκB protein occur, resulting in the translocation of free NF-κB to the nucleus and the activation of target gene expression (41, 42). The inhibitory activity of Se on NF-κB translocation suggested that Se inhibits upstream of IκB kinase activation in this pathway. Further investigation will be needed to demonstrate the effect of Se on various NF-κB upstream components.

Table 1. Effects of Se on Intracellular GSH and GSSG[a]

Treatment	GSH + GSSG (μmol/10^6 cells)	GSSG (μmol/10^6 cells)
Control	12.09 ± 0.14	0.82 ± 0.04
Se 10 μ*M*	8.20 ± 0.99*	0.93 ± 0.17
LPS	11.95 ± 0.88	0.69 ± 0.05
LPS + Se 10 μ*M*	5.19 ± 1.21**	0.91 ± 0.11
BSO 50 μ*M*	1.57 ± 0.14*	0.74 ± 0.22

[a] After pretreatment with selenium (24 hrs) and BSO (30 mins), LPS (30 ng/ml) was added to cells. Amounts of GSH and GSSG were determined by enzyme assays. Values are expressed as mean ± SE ($n = 5$) of a single experiment.

* Significant difference compared with control at $P < 0.05$. ** Significant difference compared with control or Se at $P < 0.05$.

The action of LPS in macrophage cells is mediated via membrane surface receptors. Se binds to a specific Se-binding protein in circulation; this, in turn, has affinity to CD14, which is present in circulation as well as on monocyte surfaces. The resulting complex interacts with Toll-like receptor 4 (TLR4), which is associated with another protein (MD-2) and elicits signaling for NF-κB activation (43). TLR4 signaling increases the production of ROS, which causes an activation of IκB kinase. Se is a known antioxidant and therefore can interfere with ROS generation and prevent this signaling cascade. It is not known whether Se is involved with the interaction of LPS and TLR4. It also needs to be investigated whether other antioxidants will also have a similar effect on LPS-induced NF-κB activation.

Se modulates immune function and a variety of cellular responses, such as proliferation, survival, and death (15). Se at low levels is essential for the synthesis of selenoenzymes, but higher toxic levels of Se are harmful to organism by the metal reacting with essential thiol groups on enzymes to form RS-Se-SR adducts and the resulting blockage of enzyme activity. Se induces the mitochondrial permeability transition by the modification of protein thiol groups, which results in the release of cytochrome *c* and a loss of mitochondrial membrane potential (44). Kim and Stadtman (16) explained that Se is toxic by inhibiting gene expression under NF-κB regulation. Jeong *et al.* (21) showed that Se has a beneficial effect by inhibiting the allergen-induced inflammation and generation of ROS and NF-κB–induced TNF-α within similar concentrations of Se. From our present study, we propose that Se has a beneficial effect on the endotoxin-mediated inflammatory response. The doses of Se we used were not cytotoxic, as revealed by three different types of cytotoxicity tests.

In the adult U.S. population, the concentration of Se in plasma is generally 1.5 μ*M* (45). In human adults, Se at 1 μ*M* in plasma meets the body's requirements; concentrations less than that usually reflect Se deficiency (46). Se deficiency occurs in many countries of the world, and Se

supplementation may be useful in combating a variety of infectious diseases. The optimal intake of Se in adults is 55 μg/day; amounts >400 μg/day lead to Se toxicity (46).

The profound mechanisms of Se function in immune system are not fully understood. This report is the first to show that Se prevented LPS-induced inflammatory response by specifically inhibiting the p38 MAPK pathway. It should be emphasized, however, that these observations illustrate an example of an in vitro experiment and only a short-term treatment. Considering the toxicity of Se at higher doses and that treatment with Se should precede the LPS exposure, additional *in vivo* trials are warranted. For further understanding of Se effects, studies on the regulation of cytokines and the relationship between cytokines and MAPKs are needed.

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