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Selenoorganic Compound, Ebselen, Inhibits Nitric Oxide and Tumor Necrosis Factor-α Production by the Modulation of Jun-N-terminal Kinase and the NF-κB Signaling Pathway in Rat Kupffer Cells

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Abstract In response to the bacterial endotoxin, LPS, Kupffer cells are induced to express NO and TNF-α. These compounds are involved in hepatic inflammation/injury, especially that associated with endotoxic shock. In this study, we demonstrate that ebselen (2-phenyl-1,2-benzisoselenazol-3[2H]one), a selenoorganic compound, blocks LPS-induced NO and TNF-α production by cultured rat liver Kupffer cells. LPS can activate both the NF-κB signaling pathway and MAPK signal transduction pathways such as JNK and p38 MAPK. We find that ebselen inhibits LPS-induced NF-κB nuclear translocalization, and also suppresses the LPS-induced phosphorylation of JNK, but not the phosphorylation of p38 MAPK. This inhibition of signal transduction leads to a decrease in the transcription of TNF-α and the inducible isoform of NO. Furthermore, ebselen inhibits LPS-induced COX-2 expression, which is responsible for proinflammatory prostaglandin production, without affecting constitutive COX-1 expression. These data suggest the mechanism by which ebselen acts as an antiinflammatory agent, and also suggest that ebselen may be potent in preventing hepatic injury such as endotoxic shock, in which Kupffer cell activation has been implicated. J. Cell. Biochem. 78:595–606, 2000.

Key words: ebselen; nitric oxide; TNF-α; JNK; NF-κB; rat; Kupffer cells; COX

Monocytes and macrophages contribute to the process of inflammation through the production of several molecules, including NO and inflammatory cytokines such as TNF-α. Kupffer cells, the resident macrophages in the sinusoids of the liver, play an important role in hepatic inflammation/injury, especially that associated with endotoxic shock [Nolan, 1981]. In response to the bacterial endotoxin, LPS, Kupffer cells are stimulated to produce NO and TNF-α, which are involved in the pathogenesis of liver injury. LPS activates both the NF-κB signaling pathway and the MAPK signal transduction pathways such as JNK and p38 MAPK [Muller et al., 1993; Waskiewicz and Cooper, 1995; Kyriakis and Avruch, 1996; Lee and Young, 1996]. Analysis of the promoters of TNF-α and the iNOS has revealed binding sites for activator protein 1 (AP-1) and NF-κB that regulate the expression of NO and TNF-α [Xie et al., 1993; Trede et al., 1995]. In addition, LPS selectively stimulates mitogen-inducible COX-2, but not COX-1, in macrophages [Lee et al., 1992; O'Sullivan et al., 1992; Hempel et al., 1994; Feng et al., 1995]. COX-2 is responsible for initiating the synthesis of mediators such as thromboxane B2 (TXB2) in inflammatory conditions, which modulate a variety of physiological processes including

Abbreviations used: LPS, lipopolysaccharide; NO, nitric oxide; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor kappa B; JNK, Jun-N-terminal kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; COX, cyclooxygenase; iNOS, inducible isoform of nitric-oxide synthase; NADPH, nicotinamide adenine dinucleotide phosphate.

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the production of inflammatory cytokines [O'Sullivan et al., 1992]. Although the signaling pathways that lead to the expression of COX-2 in LPS-stimulated macrophages are not well understood, it has been reported that activation of MAPKs and NF-κB leads to promotion of COX-2 expression in addition to the involvement of reactive oxygen species [Feng et al., 1995; Hwang et al., 1997].

Ebselen (2-phenyl-1,2-benzioselenazol-3[2H]-one) is a lipid-soluble selenoorganic compound that has an array of pharmacological effects including antiinflammatory and antioxidant activities [Müller et al., 1984; Hayashi and Slater, 1986]. Ebselen exhibits glutathione (GSH) peroxidelike activity, reducing hydrogen peroxide with thiol cosubstrates such as GSH and *N*-acetylcysteine [Müller et al., 1985; Cotgreave et al., 1987]. In addition, ebselen has been reported to inhibit both NADPH oxidase [Cotgreave et al., 1989] and iNOS [Hattori et al., 1994]. Ebselen inhibits ADP-iron-induced lipid peroxidation in isolated hepatocytes [Müller et al., 1985] and protects against diquat cytotoxicity [Wendel and Tiegs, 1986]. In rat Kupffer cells, ebselen inhibits the production of superoxide anion and nitric oxide, while protecting grafts against reperfusion injury [Wang et al., 1992]. Recently, ebselen has been reported to prevent reperfusion injury to the liver, brain, heart, and stomach [Johshita et al., 1990; Ueda et al., 1990; Hoshida et al., 1994; Ozaki et al., 1997]. Although its activity as an antiinflammatory agent has been established, the mechanism by which ebselen inhibits LPS-induced production of NO remains to be elucidated. Furthermore, little is known about the effects of ebselen on cytokine production.

In this study, we demonstrate that ebselen suppresses TNF-α production, as well as NO production, by LPS-activated Kupffer cells. Ebselen inhibits LPS induction of both NF-κB nuclear translocation and phosphorylation of JNK, but not LPS-induced phosphorylation of p38 MAPK. Furthermore, ebselen inhibits LPS-induced COX-2 expression, which is responsible for initiating prostaglandin synthesis, without affecting constitutive COX-1 expression. These findings suggest that ebselen can inhibit LPS-induced production of TNF-α and NO by the suppression of specific signal transduction pathways. Also, these results suggest that ebselen may be useful in the prevention of hepatic injury from endotoxic shock, in which Kupffer cell activation has been implicated.

MATERIALS AND METHODS

Isolation and Primary Culture of Rat Kupffer Cells

Kupffer cells were isolated by the dish-adhesion technique after collagenase perfusion and pronase digestion of the liver tissue, as described previously [Motomura et al., 1996, 1997]. The viability of the cell preparation was >95%, as determined by trypan blue dye exclusion. Freshly isolated Kupffer cells were maintained in RPMI 1640 culture medium (Life Technology, Rockville, MD) supplemented with 10% heat-inactivated fetal calf serum (FCS) in either 24-well plates or 60-mm tissue culture dishes. The medium was changed after 3 h and 24 h of culture. For these experiments, Kupffer cells were used on the 3rd day of culture. Ebselen (Daiichi Pharmaceutical Co., Tokyo, Japan) was dissolved in dimethyl sulfoxide (DMSO) and applied to cultures at <0.5% of the total volume of the media. In all experiments, the media was supplemented with 0.5% DMSO as a control for the possible effects of DMSO on the cells, and we confirmed that 0.5% DMSO did not affect the production of LPS-induced production of TNF-α and NO. The cell viability was >95% in all experiments, as determined by trypan blue exclusion. All animal experiments were conducted in accordance with the ethical guidelines of Kyushu University.

Measurement of Nitrite and TNF-α

Kupffer cells were cultured in 24-well plastic tissue culture plates at a density of 6×10^5 cells/ml. The medium was changed after 2 days, and the cells were stimulated with LPS (100 ng/ml) in either the presence or absence of ebselen for 24 h. In some experiments, cells were pretreated with ebselen and washed three times with medium before LPS stimulation. Production of NO was quantified by measuring the accumulation of nitrite in the culture medium using the Griess reaction, as reported previously [Motomura et al., 1996, 1997]. Samples were mixed with an equal volume of Griess reagent and were incubated at room temperature for 10 min. The absorbance of each sample was measured spectrophoto-

metrically at 540 nm by an ImmunoReader MJ2000 (InterMed, Tokyo, Japan) using sodium nitrite as the standard. The concentration of TNF-α in the supernatant was measured by an enzyme-linked immunosorbent assay using a monoclonal hamster anti-murine TNF-α antibody and a polyclonal rabbit anti-mouse TNF-α antibody (Genzyme Diagnosis, Cambridge, MA), as reported previously [Motomura et al., 1996, 1997].

Semiquantitative Reverse Transcriptase Polymerase Chain Reaction Assay for iNOS and TNF-α mRNA

Total cellular RNA was extracted from Kupffer cells using an Isogen kit (Nippon Gene, Inc., Tokyo, Japan). After treatment with RNase-free DNase I (Stratagene, La Jolla, CA), 1 μg of total RNA was reverse transcribed into first-strand cDNA using AMV reverse transcriptase (TaKaRa, Tokyo, Japan) with 0.5 μg random primer (9 mer). Negative control reactions either without RNA or without reverse transcriptase were performed to check for RNA carryover and contamination with genomic DNA, respectively.

The polymerase chain reaction (PCR) primers were constructed based on the published nucleotide sequences of the rat TNF-α gene (GenBank accession number, L00981) [Kwon et al., 1993] (5′-CCC AGA CCC TCA CAC TCA GA-3′ [sense]; 5′-GCC ACT ACT TCA GCA TCT CG-3′ [antisense]); the rat iNOS gene (GenBank accession number, U03699) [Galea et al., 1994] (5′-TGG GCA CCG AGA TTG GAG TC-3′ [sense]; 5′-AAA TAC CGC ATA CCT GAA GG-3′ [antisense]); and the rat glycerol aldehyde 3-phosphate dehydrogenase (GAPDH) (GenBank accession number, M17701) [Tso et al., 1985] (5′-GGC AAG TTC AAT GGC ACA GT-3′ [sense]; 5′-AAG GTG GAG GAA TGG GAG TT-3′ [antisense]). In the competitive PCR of TNF-α, iNOS, and GAPDH, the TNF-α cDNA sequence with a 74-bp deletion, the iNOS cDNA sequence with an 82-bp deletion, and the GAPDH cDNA sequence with a 146-bp deletion, respectively, were used as the competitor DNA sequences. The competitor DNA sequence for TNF-α PCR and that for iNOS PCR were generated by PCR using the following composite 3′ primer and the respective 5′ primer noted above: 3′ TNF-α composite primer (5′-GCC ACT ACT TCA GCA TCT CG = G TTA GAA GGA CAC AGA TTG-3′), 3′ iNOS composite primer (5′-AAA TAC CGC ATA CCT GAA GG = T GTC ACC ACC AGC AGT AGT T-3′). The competitor DNA sequence for GAPDH PCR was generated by PCR using the following composite 5′ primer and the respective 3′ primer noted above: 5′ GAPDH composite primer (5′-GGC AAG TTC AAT GGC ACA GT = G TGG AGT CTA CTG GCG TCT T-3′). In the PCR for generation of each competitor DNA, the PCR reaction mixture contained 1 × PCR buffer II (Perkin-Elmer, Forester City, CA), 2.5 mM $MgCl_2$, 200 mM of each dNTP, 0.3 mM of each sense and antisense primer, and 1.5 U Amplitaq (Perkin-Elmer). The PCR conditions were preincubation at 95°C for 5 min, followed by 35 cycles of 95°C for 45 s, 58°C for 45 s, and 72°C for 45 s. The PCR products for the amplification of the competitor DNA sequence were subcloned into pGEM-T (Promega, Madison, WI) using a pGEM-T easy vector system (Promega), and sequenced. The series of competitive PCR for amplification of TNF-α, iNOS, or GAPDH was performed using the same amount of cDNA and various concentrations of the respective competitor DNA sequence. The cDNA PCR products and the competitor PCR products can be distinguished by size on electrophoresis (TNF-α, 694 bp vs. 620 bp; iNOS, 752 bp vs. 670 bp; GAPDH, 725 bp vs. 579 bp).

Before quantitation of TNF-α and iNOS by competitive PCR, we performed the competitive PCR for GAPDH to ensure the integrity of the procedure and that comparable amounts of cDNA were applied in a series of experiments. Each PCR was performed with different known amounts of GAPDH competitor (0.2–200 fg) and with the same reaction parameters as described above. After ensuring that equal amounts of cDNA were applied to each reaction mixture in the PCR series for TNF-α and iNOS, the competitive PCRs were performed using the same reaction parameters described above. Four microliters of each sample was electrophoresed on a 2% agarose gel impregnated with ethidium bromide, and the bands were visualized under ultraviolet light. The intensity of ethidium bromide luminescence was measured by a CCD image sensor (Densitograph AE6900F, Atto, Tokyo, Japan). The amount of either TNF-α- or iNOS-mRNA in each experiment was expressed as a ratio of the amount of either TNF-α- or iNOS-PCR product

to the amount of the respective competitor PCR product.

Immunofluorescence

Kupffer cells were grown on Permanox chamber slides in culture medium. Cells were washed with phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. Then, the cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min, and incubated with 10% FCS in PBS overnight at 4°C. Cells were subsequently incubated with a rabbit antibody for NF-κB p65 (no. sc-372, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 2 h, followed by incubation with a secondary antibody, rhodamine-conjugated goat anti-rabbit immunoglobulin G (Jackson ImmunoResearch, West Grove, PA) for 2 h, as described elsewhere [Zandi et al., 1997]. Finally, cells were viewed and photographed using a Zeiss Axiophot microscope.

Western Blot Analysis of Phospho- and Nonphospho-MAPKs, COX-1, and COX-2

Whole cell lysates were prepared in solubilizing buffer containing 25 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol, 1 mM EDTA, and 4% sodium dodecyl sulfate (SDS). Protein lysates (20 μg) were subjected to reducing 10% SDS-polyacrylamide gel electrophoresis, transferred onto BA-S-83-reinforced nitrocellulose membranes (Schleicher & Schuell, Keene, NH), and immunoblotted for 1 h at room temperature with the primary antibody for JNK, phospho-JNK (Thr183/Tyr185), p38 MAPK, phospho-p38 MAPK (Thr 180/Tyr185) (New England Biolabs, Beverly, MA), COX-1, or COX-2 (Santa Cruz Biotechnology) after blocking with 5% skim milk overnight at 4°C. Antibody binding was detected using alkaline phosphatase–conjugated avidin–biotin complex. The levels of JNK, phosphorylated-JNK, p38 MAPK, and phosphorylated-p38 MAPK were quantitated by densitometry using an optical scanner system. For comparison, the ratios of phosphorylated JNK and p38 MAPK to nonphosphorylated JNK and p38 MAPK, respectively, were calculated from the densitometric data.

Statistical Analysis

All results are shown as the mean ± SD. Comparisons were made using one-way analysis of variance followed by Scheffe's test or the Mann-Whitney test.

RESULTS

Ebselen Inhibits LPS-Induced NO and TNF-α Production by Kupffer Cells

Unstimulated Kupffer cells exhibited low basal levels of NO and TNF-α production, whereas the addition of LPS to the culture medium resulted in a dramatic increase of NO and TNF-α production during the 24-h observation period (Fig. 1). Treatment of the cells with increasing concentrations of ebselen led to concentration-dependent inhibition of LPS-induced NO and TNF-α production. LPS-induced NO and TNF-α production in Kupffer cells was almost completely blocked by the addition of 100 μM ebselen (Fig. 1). This inhibition was not caused by a cytotoxic effect of ebselen on the cells, because the viability of the cells in the presence of 100 μM of ebselen was >95% as determined by trypan blue exclusion.

Ebselen Pretreatment Suppresses LPS-Induced NO Production by Kupffer Cells

To exclude the possibility that ebselen directly scavenges NO in the culture medium, we treated cells with 50 μM ebselen for 24, 12, or 3 h, and washed the cells with medium three times before exposure to LPS. Pretreatment for 3 h with 50 μM ebselen suppressed LPS-induced NO and TNF-α production. No difference was detectable in the pattern of suppression between pretreatment and simultaneous treatment (Fig. 2). Furthermore, allowing 3 h between pretreatment with ebselen and LPS stimulation did not diminish the inhibitory effect of pretreatment (Fig. 2).

Ebselen Inhibits LPS-Induced iNOS and TNF-α mRNA Expression in Kupffer Cells

To assess the level of inhibition of iNOS and TNF-α mRNA transcription by ebselen treatment, we examined the level of iNOS and TNF-α mRNA in LPS-stimulated Kupffer cells. Kupffer cells were incubated with or without LPS in the presence or absence of ebselen, and the level of TNF-α and iNOS mRNA transcripts was assessed by semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) at 3 h after LPS stimulation. In the Kupffer cells that were incubated in the absence of LPS and ebselen, RT-PCR in the presence of 0.2 fg competitor detected only competitor-specific bands in both the competitive TNF-α and the competitive iNOS RT-PCR

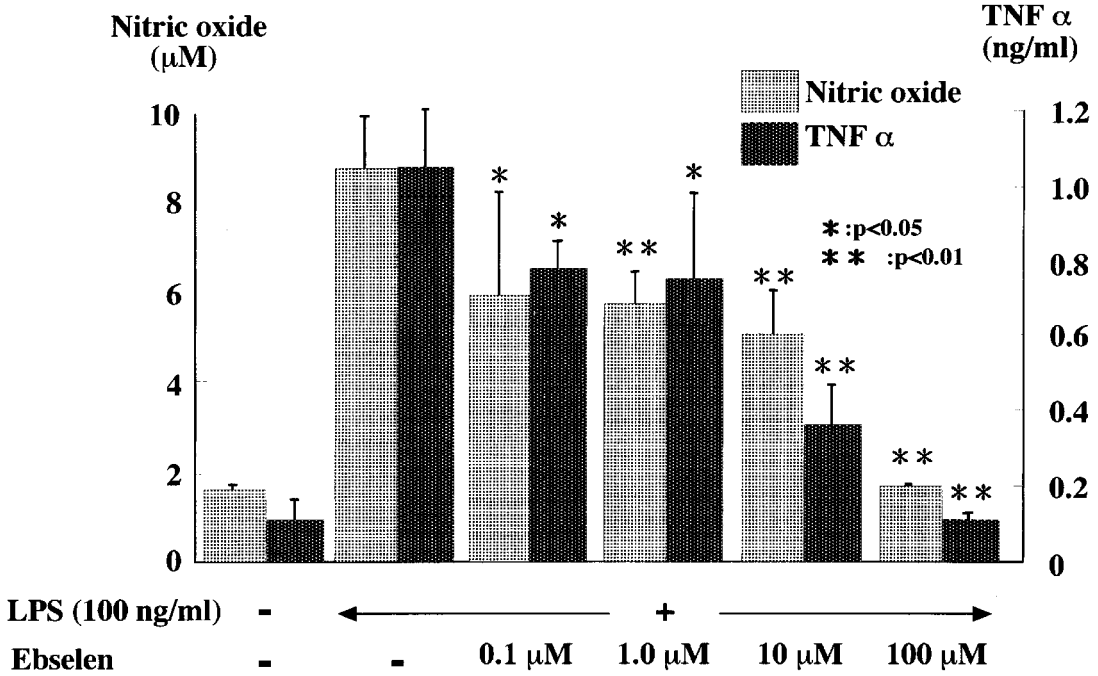

Fig. 1. The inhibitory effect of ebselen on LPS-induced NO and TNF-α production by Kupffer cells. Two days after plating 6×10^5 Kupffer cells/well in tissue culture dishes, 100 ng/ml of LPS was added in the presence or absence of various concentrations of ebselen. Culture supernatant was collected after 24 h, and the levels of NO and TNF-α were determined as described in the Materials and Methods section. Control incubations of Kupffer cells without either LPS or ebselen addition are also shown. Ebselen inhibited NO and TNF-α production in a concentration-dependent manner to a minimum inhibitory concentration of 0.1 μM. The values shown are the mean and SD of triplicate determination (*Significantly different from the level of NO production and TNF-α production in Kupffer cells with LPS stimulation alone, $P < 0.05$. **Significantly different from the level of NO production and TNF-α production in Kupffer cells with LPS stimulation alone, $P < 0.01$).

(Fig. 3, lane 4 in the left panel). LPS stimulation enhanced the transcription of both the TNF-α and iNOS genes, and, even in the presence of 2 fg competitor, resulted in intense bands of TNF-α and iNOS PCR product (Fig. 3, lane 3 in the right panel). Ebselen treatment led to a more intense competitor band, and abolished bands of the PCR product of TNF-α and iNOS in the presence of 2 fg competitor (Fig. 3, lane 3 in the middle panel). In the presence of 0.2 fg competitor, PCR resulted in a product band in the competitive iNOS PCR that was more intense than the competitor band, but resulted in only a competitor band in the competitive TNF-α PCR (Fig. 3, lane 4 in the middle panel). These results reveal that the treatment of Kupffer cells with ebselen suppresses LPS-induced TNF-α and iNOS mRNA expression.

Ebselen Suppresses LPS-Dependent NF-κB Nuclear Translocation

As described earlier, LPS activates the NF-κB signaling pathway, and both the iNOS and TNF-α promoter regions contain several sets of NF-κB-binding consensus sequences. Therefore, we examined the effect of ebselen on NF-κB nuclear translocation using the RelA (p65) antibody. LPS treatment led to NF-κB nuclear translocation in Kupffer cells during a 1-h observation period (Fig. 4A). In contrast, the addition of ebselen prevented LPS-dependent NF-κB nuclear translocation completely (Fig. 4B). In addition, nuclear translocation was inhibited by pretreatment with ebselen in a similar manner (data not shown).

Ebselen Inhibits LPS-Dependent Phosphorylation of JNK But Not p38 MAPK

LPS strongly activates MAPK signal transduction pathways, in addition to that of NF-κB. Therefore, we evaluated the effect of ebselen on the activity of MAPK signaling pathways by examining the activity of p38 MAPK and JNK, respectively. To assess the phosphorylation status of p38 MAPK and JNK, we performed Western blot analysis using antibodies specific

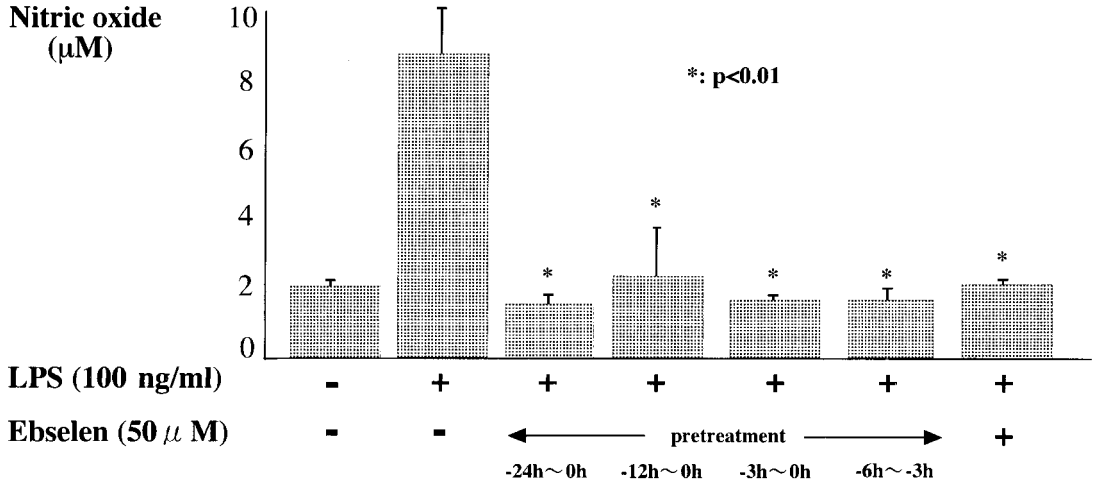

Fig. 2. The inhibitory effect of ebselen pretreatment on LPS-induced NO production by Kupffer cells. After 2 days' incubation of 6×10^5 Kupffer cells/well, 100 ng/ml of LPS was added in the presence or absence of ebselen pretreatment (50 μM). Culture supernatant was collected after 24 h incubation in the presence of LPS, and the level of NO was determined as described in the Materials and Methods section. Control incubations of Kupffer cells without either LPS or ebselen addition are also shown. Kupffer cells were treated with 50 μM ebselen for 24 h, 12 h, or 3 h, and the cells were washed with medium three times before exposure to LPS. Pretreatment with ebselen for 3 h was sufficient to suppress LPS-induced NO production. Allowing 3 h between the pretreatment with ebselen and LPS stimulation did not diminish the inhibitory effect of pretreatment. The values shown are the mean and SD of triplicate determination (*significantly different from the level of NO production in Kupffer cells with LPS stimulation alone, $P < 0.01$).

for either MAPK, phospho-MAPK, JNK, or phospho-JNK. Although LPS induced the phosphorylation of both p38 MAPK and JNK, ebselen was able to block the LPS-induced phosphorylation of JNK by ~80%, but did not affect the phosphorylation of p38 MAPK (Fig. 5).

Ebselen Inhibits LPS-Induced Expression of COX-2

Because LPS selectively stimulates the expression of mitogen-inducible COX-2 in macrophages but does not affect constitutively expressed COX-1 [Lee et al., 1992], we examined the effect of ebselen on the LPS-induced activation of COX-2. LPS (100 ng/ml) induced COX-2 expression, and this induction was partially suppressed by the addition of ebselen (50 μM). Conversely, COX-1 expression was not affected by the presence of either LPS or ebselen (Fig. 6).

DISCUSSION

In this study, we demonstrate that ebselen inhibits LPS-dependent NO and TNF-α production in cultured rat Kupffer cells. Ebselen inhibits NO and TNF-α production in a concentration-dependent manner with 1 μM ebselen inhibiting production by 40%. Pretreatment with ebselen, for 3 h before LPS stimulation, also inhibits NO and TNF-α production to the same degree as simultaneous treatment. This result suggests that ebselen may be retained in the cytoplasm or the cytoplasmic membrane. Ebselen can mimic the catalytic activity of GSH peroxidase in vitro [Müller et al., 1985; Cotgreave et al., 1987], and has been reported to inhibit NADPH oxidase [Cotgreave et al., 1989], NO synthase [Hattori et al., 1994], and COX [Safayhi et al., 1985]. Inhibition of NADPH oxidase by ebselen has been described for Kupffer cells [Wang et al., 1992], as well as for peritoneal macrophages [Parnham and Kindt, 1989] and granulocytes [Ichikawa et al., 1987]. Previously, we confirmed this direct inhibition by an in vitro assay using recombinant NADPH oxidase (data not shown) [Hata et al., 1998], although the possibility that the effect may result from O_2 scavenging could not be excluded completely. Our observations and previous reports suggest that there are at least two possible mechanisms of inhibition by ebselen: ebselen interfering with target enzymes such as NADPH oxidase and NO synthase; and ebselen acting via H_2O_2 scavenging.

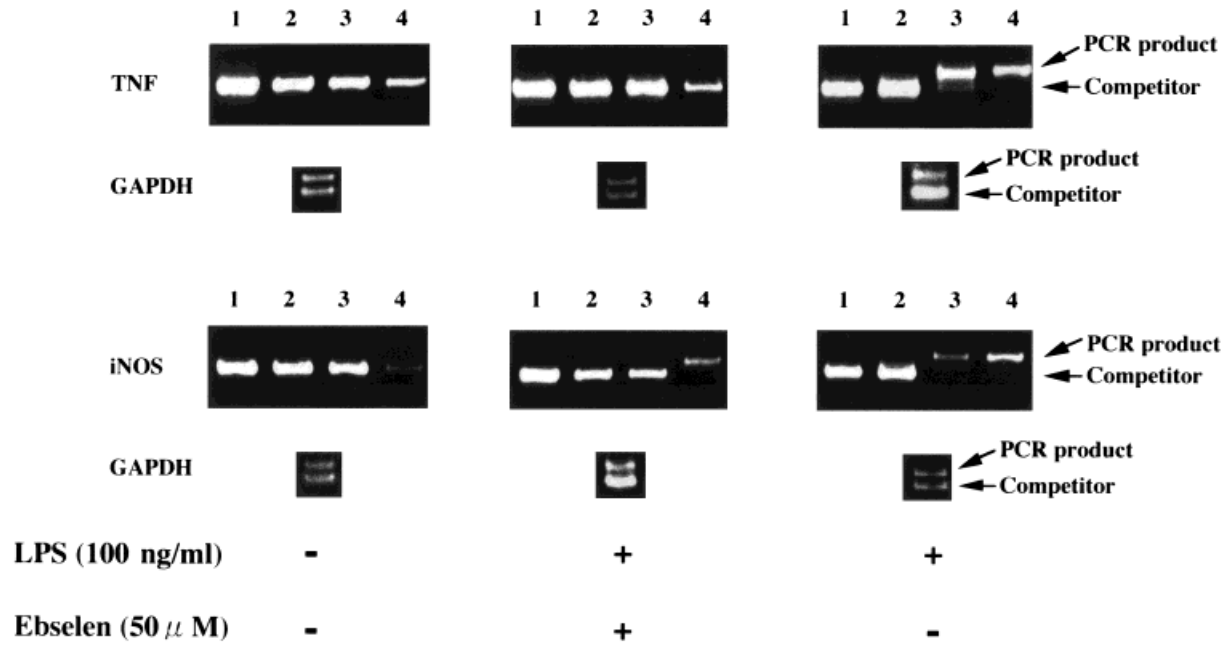

Fig. 3. Semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of iNOS and TNF-α mRNA expression. Kupffer cells (6×10^6/dish) were treated with 100 ng/ml of LPS in the presence or absence of 10^{-7} M of ebselen. At 3 h of stimulation, samples were collected for analysis of the levels of TNF-α, iNOS, and glycerol aldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Samples were analyzed by competitive RT-PCR using primer pairs specific for TNF-α or iNOS, respectively, after adjusting and ensuring that equal amounts of cDNA were present in each PCR based on the results of the competitive PCR of GAPDH. The series of competitive PCRs for TNF-α and iNOS was performed using the same amount of cDNA in each PCR with the following amounts of the competitor of TNF-α or iNOS : 200 fg (lane 1), 20 fg (lane 2), 2 fg (lane 3), and 0.2 fg (lane 4). The LPS-induced increase in iNOS and TNF-α mRNAs was inhibited by ebselen. Each result is representative of three independent experiments.

Our study also demonstrates that ebselen can suppress iNOS and TNF-α mRNA expression. In human pancreatic islets, ebselen prevents the expression of iNOS induced by a combination of cytokines (interleukin- [IL-] 1β, TNF-α, and interferon-γ) [de-Mello et al., 1996]. Many cytokine genes, including TNF-α, are controlled at the posttranscriptional level (selective decay of mRNA), as well as at the transcriptional level. The selective destabilization of these mRNAs is facilitated by an AU-rich element (ARE) in the mRNA 3′ untranslated region [Sachs, 1993]. ARE-mRNAs can be transiently stabilized by stimulation of the JNK signaling pathway [Chen et al., 1998]. Thus, ebselen may suppress the stabilization of TNF-α mRNA by inhibiting activation of the JNK signal cascade.

It is important to develop a better understanding of how ebselen affects signaling pathways to suppress inflammation. LPS treatment results in the activation of macrophages, including Kupffer cells, by binding to cell surface receptor complexes, GPI-anchored protein CD14 and Toll-like receptor 2 (TLR2) [Yang et al., 1998]. TLR2 is a transmembrane protein with an intracellular portion containing a motif associated with the IL-1 receptor [Belvin et al., 1996]. A recent study demonstrated that LPS signaling uses a molecular framework analogous to that of IL-1 signaling [Zhang et al., 1999]. The response involves activation of genes in the cytokine cascade, particularly NF-κB [Ulevitch and Tobias, 1995]. In most cells, NF-κB exists in an inactive form in the cytoplasm that is bound to the inhibitory protein IκB. After specific extracellular stimuli, such as LPS, NF-κB dissociates from IκB, translocates to the nucleus, and activates its target genes [Baeuerle and Henkel, 1994; Finco and Baldwin, 1995]. Recently, an IκB kinase (IKK) has been identified that phosphorylates IκB at sites that trigger its degradation [DiDonato et al., 1997]. Furthermore,

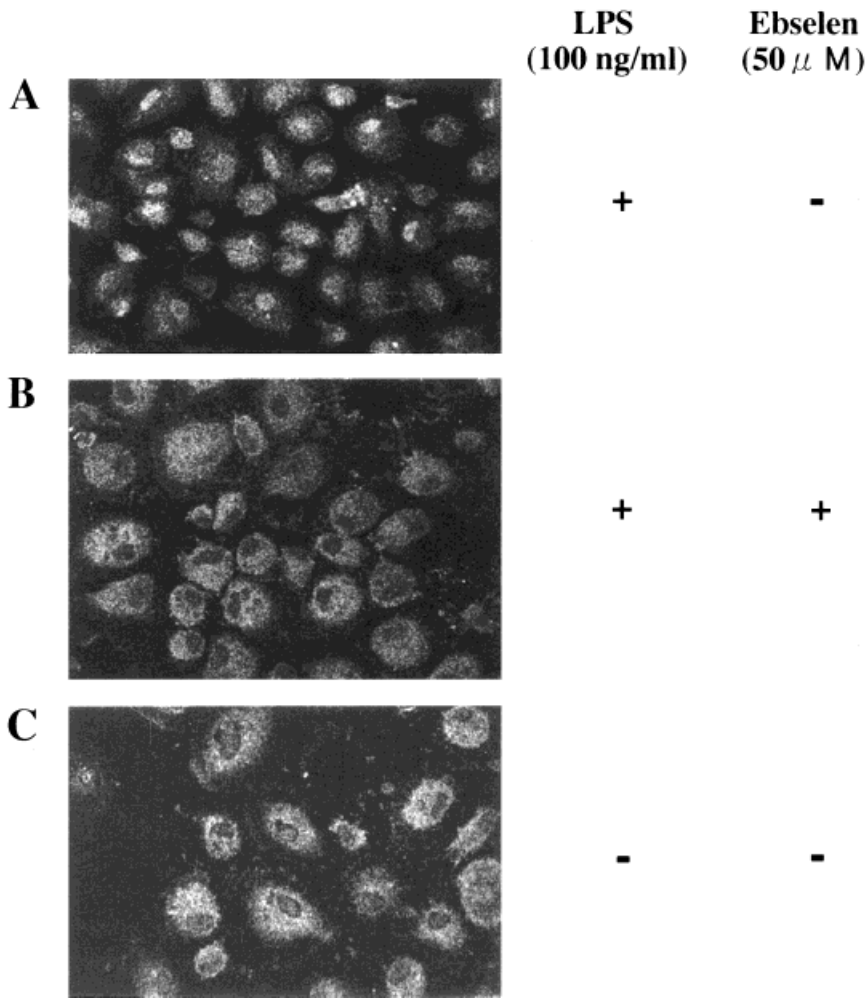

Fig. 4. Nuclear translocation of NFκB by LPS stimulation. Isolated Kupffer cells were treated with 100 ng/ml of LPS in the presence or absence of 50 μM of ebselen. After stimulation for 60 min, translocation of NFκB was examined using an anti-NFκB p65 antibody, followed by rhodamine-conjugated IgG fraction of goat anti-rabbit IgG, and observed as described in the Materials and Methods section. **A:** LPS stimulation only. **B:** LPS stimulation in the presence of ebselen. **C:** Without LPS stimulation. LPS stimulation induced the translocation of NFκB. Ebselen inhibited the LPS-induced translocation of NFκB. Each result is representative of three independent experiments.

IKK is phosphorylated by an NF-κB-inducing kinase (NIK) [Régnier et al., 1997]. We demonstrate that ebselen prevents NF-κB nuclear translocation, suggesting that ebselen inhibits a step upstream of IKK activation in the NF-κB signaling pathway. Further study of the phosphorylation states of IKK and NIK will be needed.

The LPS–CD14–TLR2 complex also activates stress-induced MAP-kinase (JNK and p38 MAPK) signal transduction pathways in addition to the NF-κB signaling pathway [Muller et al., 1993; Waskiewicz and Cooper, 1995; Kyriakis and Avruch, 1996; Lee et al., 1996]. p38 MAPK has been implicated in the expression of cytokines, whereas p38 MAPK inhibitors such as pyridinyl imidazole compounds can strongly prevent LPS-induced TNF-α and IL-1β synthesis [Han et al., 1994; Lee et al., 1994; Rouse et al., 1994]. It is intriguing that ebselen selectively inhibits LPS-induced phosphorylation of JNK, but not that of p38 MAPK. This selective inhibition suggests that ebselen can interfere specifically with a protein involved in the JNK pathway. In the IL-1 signaling cascade, MAPK kinase kinase, i.e., transforming growth factor-β activated kinase (TAK1), links both the MAPK kinase 4–

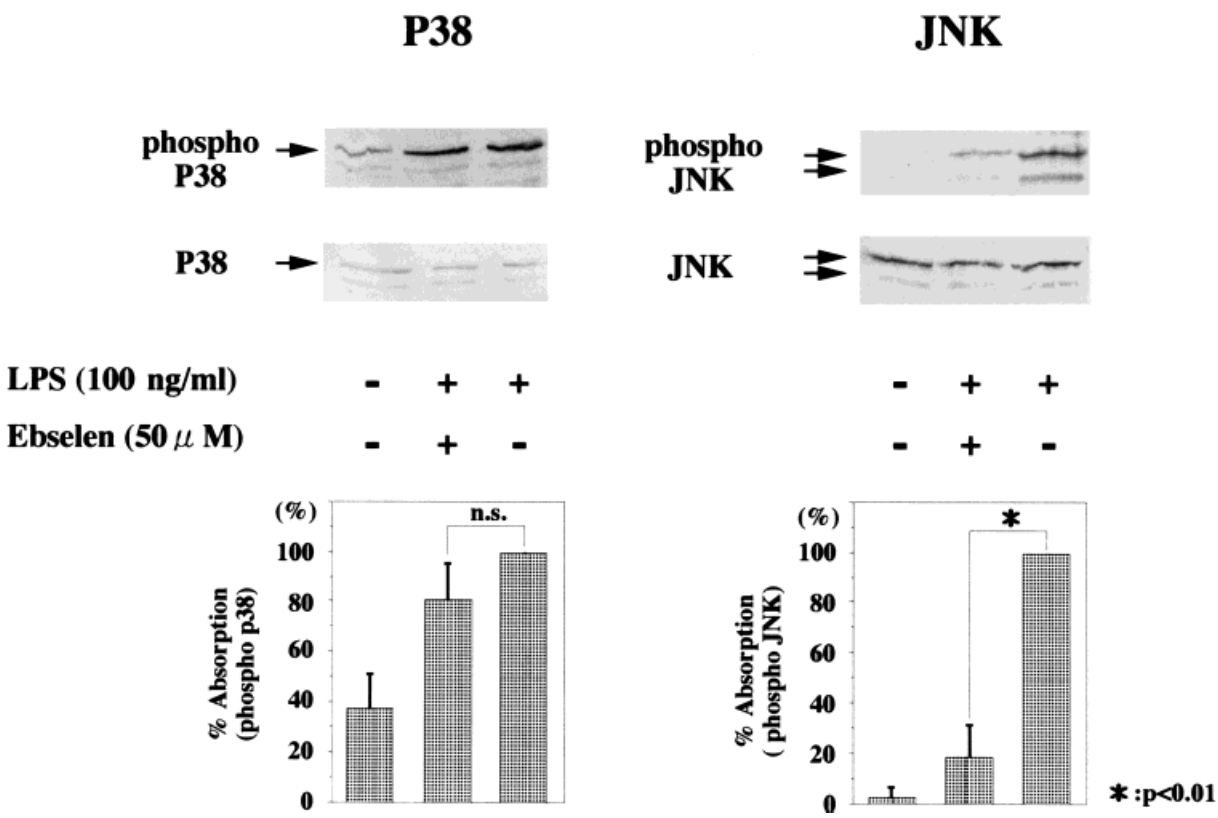

Fig. 5. Western blot analysis of p38 MAPK, phospho-p38 MAPK, JNK, and phospho-JNK. Kupffer cells (6×10^6/dish) were treated with 100 ng/ml of LPS in the presence or absence of 50 μM of ebselen. After 20 min, the cells were placed in lysis buffer containing 4% sodium dodecyl sulfate (SDS). Twenty micrograms of each cell lysate was separated by 10% SDS-polyacrylamide gel electrophoresis. A control lysate derived from Kupffer cells that were not incubated in LPS was also examined. Western blot analysis of Kupffer cells stimulated with LPS revealed the expression of phosphorylated forms of p38 MAPK and SAPK/JNK. Ebselen prevented LPS-induced phosphorylation of JNK significantly, but it did not affect the phosphorylation of p38 MAPK. Each figure is representative of three independent experiments. To compare the ratio of phosphorylation of JNK and p38 MAPK, we measured the intensity of each band as described in Materials and Methods. The ratio of phosphorylation in the presence of LPS alone was used as a control (100%). The values shown are the mean and SD of triplicate determination (*significantly different from the level of phosphorylation of JNK with LPS stimulation alone, $P < 0.01$. n.s., not significant).

JNK cascade and the NIK–NF-κB cascade [Ninomiya-Tsuji et al., 1999]. Ebselen suppresses both NF-κB activation and JNK activation, suggesting that ebselen can interfere with TAK-1, which is not likely to be involved in p38 MAPK activation [Nick et al., 1999]. The p42/p44 ERK, a so-called "classic MAPK," has been reported to be activated by LPS [Waskiewicz et al., 1995; Kyriakis et al., 1996]. In Kupffer cells, LPS stimulation induces activation of p42/p44 ERK, which is suppressed by treatment with ebselen (data not shown). Although the precise mechanism by which LPS activates the "classic MAPK" (Raf–MEK–ERK) cascade is not yet well understood, further study is needed to assess the effects of ebselen on this cascade.

We also demonstrate that ebselen can suppress LPS-induced COX-2 expression, but does not affect COX-1 expression. It is evident that two forms of COX exist: an inducible isoform known as COX-2 and a constitutive enzyme known as COX-1 [Feng et al., 1993; Herschman, 1996]. COX-2 is induced by a variety of stimuli including LPS, oxidative stress, and certain cytokines [Lee et al., 1992; O'Sullivan et al., 1992; Hempel et al., 1994; Feng et al., 1995], whereas COX-1 regulates prostanoid synthesis under normal physiologic conditions [Feng et al., 1993]. One of the major eicosanoid products resulting from COX-2 induction is proinflammatory TXB2 [O'Sullivan et al., 1992]. Although the signaling pathways that lead to the expression of COX-2 in either LPS-

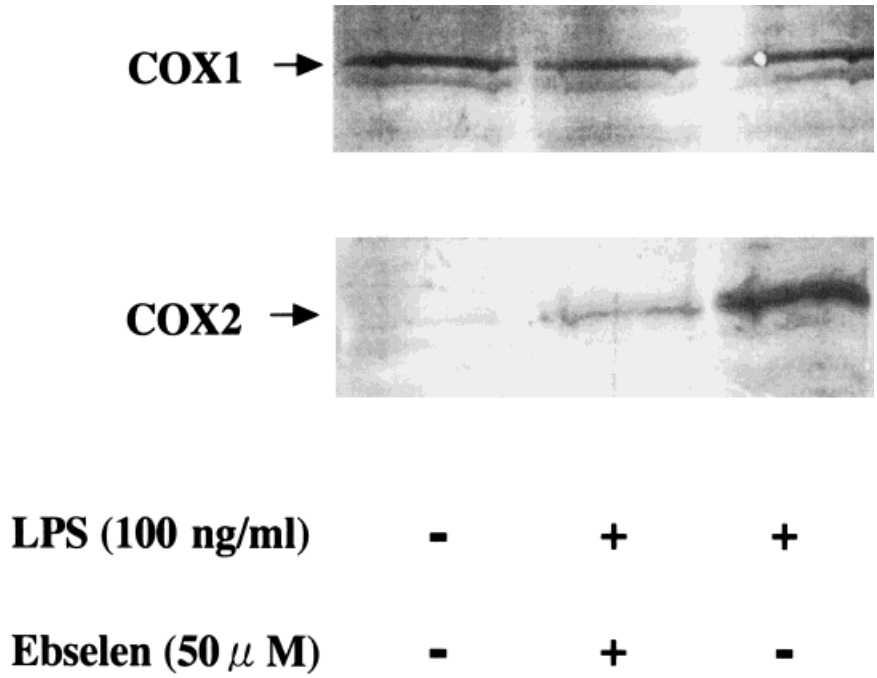

Fig. 6. Western blot analysis of COX-1 and COX-2. Kupffer cells (6×10^6/dish) were treated with 100 ng/ml of LPS in the presence or absence of 50 μM ebselen. After stimulation for 20 min, cell lysates were obtained with lysis buffer containing 4% sodium dodecyl sulfate (SDS). Twenty micrograms of each sample was separated by 10% SDS-polyacrylamide gel electrophoresis. A control lysate derived from Kupffer cells not incubated in LPS was also examined. LPS induced COX-2 expression, and this induction was suppressed by ebselen. COX-1 was not induced by LPS, and ebselen had no effect on COX-1 expression. Each result is representative of three independent experiments.

stimulated macrophages or Kupffer cells are not well understood, it has been reported recently that MAPKs and NF-κB activation leads to COX-2 expression [Hwang et al., 1997]. Analysis of the COX-2 gene promoters has revealed binding sites for NF-κB and the cyclic AMP response element [Kosaka et al., 1994]. Inhibition of NF-κB and JNK by ebselen may contribute to suppression of COX-2 expression in addition to its direct inhibition of cyclooxygenase [Safayhi et al., 1985]. In monocytes, IL-1β treatment leads to reactive oxygen intermediate-dependent NF-κB activation, which requires NADPH oxidase activity [Bonizzi et al., 1999]. Thus, direct inhibition of NADPH oxidase by ebselen may also lead to suppression of COX-2 expression.

Our results suggest that ebselen may have therapeutic applications in diseases, such as hepatic inflammation/injury associated with endotoxemia, where activated Kupffer cells play a prominent pathogenic role. Recently, it was reported that aspirin inhibits the activation of JNKs, but not p38 MAPK [Yin et al., 1998]. Aspirin also inhibits NF-κB signaling by the direct binding to IKK, resulting in reduced ATP binding [Yin et al., 1998]. Furthermore, aspirin is a well-known COX inhibitor that binds directly to the COX active site [Vane, 1994]. We have demonstrated that ebselen inhibits the activation of JNK, NF-κB signaling, and COX-2. It is intriguing that ebselen has similar effects on signaling pathways as aspirin. We have found that the administration of ebselen suppresses liver injury induced by *Propionibacterium acnes* and LPS in rats. Clearly, further in vivo studies are required to determine whether ebselen can be used in the treatment of hepatic injury.

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