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Ebselen inhibits p38 mitogen-activated protein kinase-mediated endothelial cell death by hydrogen peroxide

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Abstract

Ebselen (2-phenly-1, 2-benzisoselenazol-3[2H]-one) is a seleno-organic compound exhibiting both glutathione peroxidase and antioxidant activity. Although it has been reported that ebselen is effective against hydrogen peroxide (H_2O_2)-induced cell death in several cell types, its effect on endothelial cell damage has not yet been elucidated. In the present study, we examined the effect of ebselen on H_2O_2-induced human umbilical vein endothelial cells (HUVECs) death, and its intracellular mechanism. Our findings showed that pretreatment of HUVECs with ebselen resulted in a significant recovery from H_2O_2-induced cell death in a concentration-dependent manner. In addition to the inhibition of lactate dehydrogenase (LDH) leakage, ebselen inhibited H_2O_2-induced cytochrome *c* release and caspase-3 activation and the resultant apoptosis in HUVECs. Moreover, it was observed that H_2O_2 significantly stimulated activation of mitogen-activated protein (MAP) kinases, i.e., p38 MAP kinase, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK1/2). Ebselen inhibited H_2O_2-induced p38 MAP kinase, but not JNK or ERK1/2 activation. Furthermore, SB203580 (4-[4-fluorophenyl]-2-[4-methylsulfinyl-phenyl]-5-[4-pyridyl]-1H-imidazole), a specific p38 MAP kinase inhibitor, inhibited H_2O_2-induced p38 MAP kinase phosphorylation, cytochrome *c* release, caspase-3 activation, as well as cell death in HUVECs. These findings suggest that ebselen attenuates H_2O_2-induced endothelial cell death through the inhibition of signaling pathways mediated by p38 MAP kinase, caspase-3, and cytochrome *c* release. Thus, inhibition of p38 MAP kinase by ebselen may imply its usefulness for prevention and/or treatment of endothelial cell dysfunction, which was suggested to be the first step in the development of atherosclerosis.

Keywords: Hydrogen peroxide; Ebselen; p38 MAP (mitogen-activated protein) kinase; Apoptosis; HUVEC (human umbilical vein endothelial cell)

1. Introduction

Apoptosis is one of the forms of cell death that plays a fundamental role in the development of multicellular organisms and numerous physiological processes. Imbalance between cell growth and apoptosis can lead to many pathological phenomena, including atherosclerosis, hypertension, as well as myocardial and brain infarction (Dimmeler and Zeiher, 2000). Endothelial cells represent the lining of blood vessles and the heart (Choy et al., 2001). In addition, endothelial cells regulate the permeability of blood vessels to leukocytes and inflammatory mediators during inflammation (Gimbrone et al., 1997). Damage to the endothelium, either by the immune system, infection or other factors may be an early causative event of atherosclerosis, since it will compromise endothelial cell capabilities to regulate vascular function and homeostasis (Ross, 1999). It has been reported that enhanced production of reactive oxygen species in the vascular endothelium occurs very early in the atherogenic process, even preceding atherosclerotic lesion formation, suggesting a link between reactive oxygen species and endothelial cell apoptosis. (Ohara et al., 1993; Alexander, 1995; Dimmeler and Zeiher, 2000).

Hydrogen peroxide (H_2O_2), one of the main reactive oxygen species, is known to cause lipid peroxidation and DNA damage in cells (Halliwell and Aruoma, 1991). Caspase-3, a cysteine-dependent aspartate protease known to be involved as a key executor in cell death (Jeon et al., 1999; Hartman et al., 2000), has been reported to be

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activated by H_2O_2 as a final effector in apoptotic cell death in vitro (Dipietrantonio et al., 1999; Matsura et al., 1999). In addition, H_2O_2 has the ability to stimulate cytochrome *c* release from the mitochondria (Li et al., 2000b).

Three subfamilies of mitogen-activated protein (MAP) kinases, extracellular signal-regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 MAP kinase, were shown to be activated in response to reactive oxygen species generation (Sundaresan et al., 1995).

Ebselen (2-phenyl-1, 2-benzisoselenazol-3[2H]-one) is a heterocyclic seleno-organic compound that was developed originally as an anti-inflammatory drug (Cotgreave et al., 1988, 1989). Its glutathione peroxidase like activity was first described in 1984 (Parnham and Kindt, 1984) and since that time ebselen's antioxidant property has attracted much attention as this enzyme represent one of the major lines of defense against damage by hydroperoxides including hydrogen peroxide and lipid peroxides (Parnham et al., 1987). It was reported that, ebselen acts as a scavenger of reactive oxygen species including peroxyl radical and peroxynitrite (Sies and Masumoto, 1997). Furthermore, ebselen has been suggested to have a potential to protect the brain damage due to ischemia in which reactive oxygen species may be involved in its pathogenesis (Johshita et al., 1990; Takasago et al., 1997). Previous studies showed that the cerebral infarct size was reduced in rats with cerebral artery occlusion by ebselen pretreatment (Dawson et al., 1995).

Although it was reported that ebselen has a protective effect against H_2O_2-induced cell death in various cell types including $HepG_2$ (Yang et al., 1999), human HL-60 (Li et al., 2000a), and PC12 cells (Yoshizumi et al., 2002), the protective mechanism of ebselen on endothelial cells has not yet been elucidated. In the present study, we examined the effect of ebselen on H_2O_2-induced human umbilical vein endothelial cells (HUVECs) death, as well as the mechanism of this protective effect, especially focusing on the roles of caspase-3, cytochrome *c*, and MAP kinases.

2. Materials and methods

2.1. Cells and chemicals

HUVECs and endothelial cell basal medium-2 (EBM-2) were purchased from Clonetics (San Diego, CA). Ebselen (2-phenyl-1, 2-benzisoselenazol-3[2H]-one) was kindly donated from Daiichi Pharmaceutical (Tokyo, Japan). Hydrogen peroxide (H_2O_2) was purchased from Wako (Osaka, Japan). Cell counting Kit-8 was from Dojindo (Tokyo, Japan) and cytochrome *c* assay kit was from Quantikine M, R&D systems (Minneapolis, MN). Caspase assay kit was purchased from Clontech laboratories (Paloalto, CA). Phospho-p38 MAP kinase (Thr^{180}/Tyr^{182}), JNK (Thr^{183}/Tyr^{185}) and ERK1/2 (Thr^{202}/Tyr^{204}) antibodies were from Cell Signaling Technology (Beverly, MA). SB203580 (4-[4-fluorophenyl]-2-[4-methylsulfinylphenyl]-5-[4-pyridyl]-1H-imidazole) was from the Sigma Chemical Co. (St. Louis, MO). Lactate dehydrogenase (LDH)-cytotoxic test was from Wako. All other chemicals were commercial products of reagent grade.

2.2. Cell culture

HUVECs were grown in endothelial cell basal medium-2 (EBM-2), supplemented with 10% (v/v) fetal bovine serum, gentamicin sulphate (50 μg/ml)/amphotericin-B (50 ng/ml) in addition to human recombinant fibroblast growth factor-B (hFGF-B; 10 ng/ml), human recombinant epidermal growth factor (hEGF; 20 ng/ml), human recombinant vascular endothelial growth factor (hVEGF; 1 ng/ml), insulin-like growth factor-1 in aqueous solution cell culture tested (IGF-1; 1 ng/ml), ascorbic acid (1 μg/ml), heparin (3 ng/ml), hydrocortisone (0.4 μg/ml). Cells were grown in T-75 flasks (IWAKI, Osaka, Japan). The cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C, until reaching 80–90% confluence in 35-mm and 60-mm collagen coated dishes (IWAKI), then growth was arrested by incubation in serum free EBM-2 medium for 12 h. Serum free EBM-2 medium was replaced by serum free RPMI 1640 medium before each experiment to avoid interference of EBM-2 medium with H_2O_2. All experiments were performed using growth-arrested cells to minimize the basal activity of the kinases.

2.3. Cell viability assay

When HUVECs reached 80–90% confluence in 35-mm collagen coated dishes, growth was arrested using serum free EBM-2 meduim, EBM-2 was replaced by serum free RPMI 1640 before the experiment. Different concentrations of ebselen or SB203580 was added and incubated for 30 min. Following 24 h incubation with H_2O_2 (50 μM), 60 μl Kit-8 reagent was added to each 1 ml of the medium, and after a further 2 h incubation, 0.1 M HCl was added to stop the reaction. Kit-8 reduction was read at 450-nm using spectrophotometer.

2.4. Hoechst 33248 staining, and LDH-cytotoxic test

In order to examine the effect of ebselen on H_2O_2-induced apoptotic cell death, HUVECs were preincubated for 30 min with different concentrations of ebselen, this was followed by incubation of cells with H_2O_2 (50 μM) for 24 h. HUVECs were then stained with Hoechst 33248 stain as described before to evaluate apoptosis (Koyama et al., 1998). In brief, the cells were cultured on a cover glass in a 35-mm dish and the cell-attached cover glass was taken out, washed in phosphate-buffered saline (PBS), transferred into MeOH/Acetic acid (3:1) and submerged for 2 min, cells were dried up at room temperature. Thereafter, the cells were fixed on a cover glass at room temperature and stained

with Hoechst 33248 working solution (0.05 μg/ml) for 10 min. Next, the cover glass was washed with distilled water and mounted in buffered glycerol. Fluorescence was visualized using a fluorescent microscope (Olympus, Tokyo, Japan). The cells on the cover glass were observed under high magnification (× 400) and the percentage of apoptotic cells were calculated in 10 different fields of five separate samples. In addition, LDH leakage was determined using LDH-cytotoxic test kit (Wako). The test was performed according to the manufacture's protocol. Briefly, at the end of the incubation an aliquot of the medium (50 μl) was added to the kit reagent, incubated for 45 min, the reaction was then stopped and the absorbance was measured at 560 ± 10-nm using microplate reader.

2.5. Cytochrome c release assay

This assay is a quantitative sandwich enzyme immunoassay, and all reagents required for performing the assay are included within the assay kit. The assay was performed according to the manufacture's instructions. In brief, HUVECs in serum free RPMI 1640 were preincubated with ebselen or SB203580 for 30 min, followed by 2 h incubation with H_2O_2 (50 μM). Medium was sucked out and the cells were washed with cold PBS, and then resuspended in 0.4 ml cell lysis buffer and incubated in room temperature for 1 h. The immunoreactive cytochrome *c* was measured using a microplate reader with an optical density at 450-nm.

2.6. Measurement of caspase-3 activity

HUVECs in serum free RPMI 1640 medium (60-mm collagen coated dishes) were preincubated with different concentrations of ebselen or SB203580 for 30 min, 50 μM H_2O_2 was added and incubated for 4 h. The assay was performed according to the manufacture's protocol. In brief, cells were resuspended in 50 μl chilled cell lysis buffer and incubated for 10 min on ice. Cells were harvested and the lysates were centrifuged in a microcentrifuge at 25,000 × *g* for 3 min at 4 °C to precipitate cell debris. Thereafter, 50 μl of 2 × reaction buffer/Dithiothreitol (DTT) mix (10 μl of 1M Dithiothreitol (DTT)/ml 2 × reaction buffer) was added to the supernatant, finally 5 μl of 1 mM caspase-3 substrate (DEVD) conjugated to 7-amino-4-trifluoromethyl coumarin (AFC) was added to each tube and incubated for 1 h in 37 °C water bath. After transferring samples and standard solution to 96-well microplate, fluorescent activity was measured using fluorometer with 400-nm excitation filter and 505-nm emission filter.

2.7. Preparation of cell lysate for MAP kinases phosphorylation assay

HUVECs in serum free RPMI 1640 was treated with or without ebselen or SB203580 and incubated for 30 min, after incubation with H_2O_2 for 10 min, medium was sucked out and the cells were washed once with cold PBS. Thereafter, the cells were harvested using 0.5 ml cell lysis buffer (20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 mM phenyl methyl sulfonyl fluoride and incubate on ice. After thawing, cells were harvested and cell lysates were sonicated (Handy Sonic UR-20 P, Tomy Seiko, Tokyo, Japan) on ice for 15 s, and then centrifuged at 25,000 × *g* for 20 min at 4 °C to precipetate cell debris. The supernatant was transferred to new microtubes and the protein concentration was determined using Bradford protein assay (Bio-Rad, Hercules, CA) and stored in − 80 °C until assayed for MAP kinase phosphorylation.

2.8. Measurements of p38 MAP kinase, JNK and ERK1/2 phosphorylation in HUVECs

Previously, we measured each MAP kinase activity using an in-gel kinase assay with specific substrates. However, we found that the activities of p38 MAP kinase, JNK, and ERK1/2, as measured by the in-gel kinase assay, and their phosphorylation states, as assayed by immunoblotting, were highly correlated ($R^2 = 0.90$) in HUVECs. Therefore, we used immunoblotting for detection of phospho-p38 MAP kinase, phospho-JNK and phospho-ERK1/2 to evaluate p38 MAP kinase, JNK and ERK1/2 phosphorylation, respectively, as described previously (Yoshizumi et al., 2000, 2001; Kyaw et al., 2001). For immunoblot analysis, cell lysates were subjected to SDS-PAGE (using 30 μg protein for p38 MAP kinase and ERK1/2, and 40 μg protein for JNK), the proteins were transferred to nitrocellulose membranes (Hybond™–ECL, Amersham Pharmacia Biotech, Buckinghamshire, England), as described previously (Yoshizumi et al., 2000, 2001; Kyaw et al., 2001). The membranes were blocked for 1 h at room temperature with a commercial blocking buffer from Amersham Pharmacia Biotech. The blots were incubated for 12 h with antiphosphospecific p38 MAP kinase, JNK or ERK1/2 antibodies (Cell Signaling), followed by incubation for 1 h with a secondary antibody conjugated to horseradish peroxidase. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) and were quantified by densitometry in linear range of film exposure using a UMAX Astra 2200 scanner (UMAX Technologies, Brockwood, Dallas, TX) and NIH image 1.60 software.

2.9. Statistical analysis

Values are presented as mean ± S.E. for five separate experiments. One-way analysis of variance was used to determine significance among groups, after which post-hoc test with the Bonferroni correction was used for

comparison between individual groups. A value at $P<0.05$ was considered to be significant.

3. Results

3.1. Ebselen inhibited H_2O_2-induced HUVEC death

The protective effect of ebselen against H_2O_2-induced cell death in several cell types has been reported (Yang et al., 1999; Yoshizumi et al., 2002; Li et al., 2000a). In the present study, we examined the effect of ebselen on H_2O_2-induced HUVEC death by measuring Kit-8 reduction. As shown in Fig. 1, incubation with 50 μM H_2O_2 for 24 h resulted in HUVEC death of 16.5%. Ebselen pretreatment inhibited H_2O_2-induced cell death in a concentration-dependent manner, although ebselen itself had no significant effect on cell viability. The protective effect of ebselen against H_2O_2-induced cell death was almost complete and cell viability was able to return back to the control level by 0.3 μM ebselen pretreatment.

3.2. Effect of ebselen on H_2O_2-induced apoptosis and LDH leakage

To determine the type of H_2O_2-induced cell death measured by Kit-8, H_2O_2-treated HUVECs, with or without ebselen, were stained with Hoechst 33248 to examine the apoptotic nuclei. As shown in Fig. 2A, apoptotic nuclei staining with Hoechst 33248 revealed that 15.4% of cells showed apoptosis by treatment with 50 μM H_2O_2 for 24 h. Ebselen pretreatment inhibited H_2O_2-induced apoptosis in a concentration-dependent manner. On the other hand, LDH-leakage was also measured. Fig. 2B shows that H_2O_2 caused

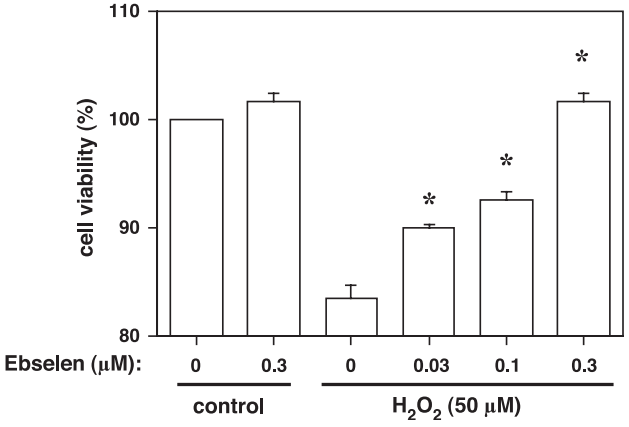

Fig. 1. The effects of ebselen on H_2O_2-induced HUVEC death. Cells were pretreated with ebselen at the indicated concentrations for 30 min prior to incubation with 50 μM H_2O_2 for 24 h. Cell viability was measured as described in Materials and methods. Values are expressed as % of control, which was defined as untreated cells (values are means ± S.E., $n=5$). The asterisks represent significant differences compared with H_2O_2 stimulation ($^*P<0.05$).

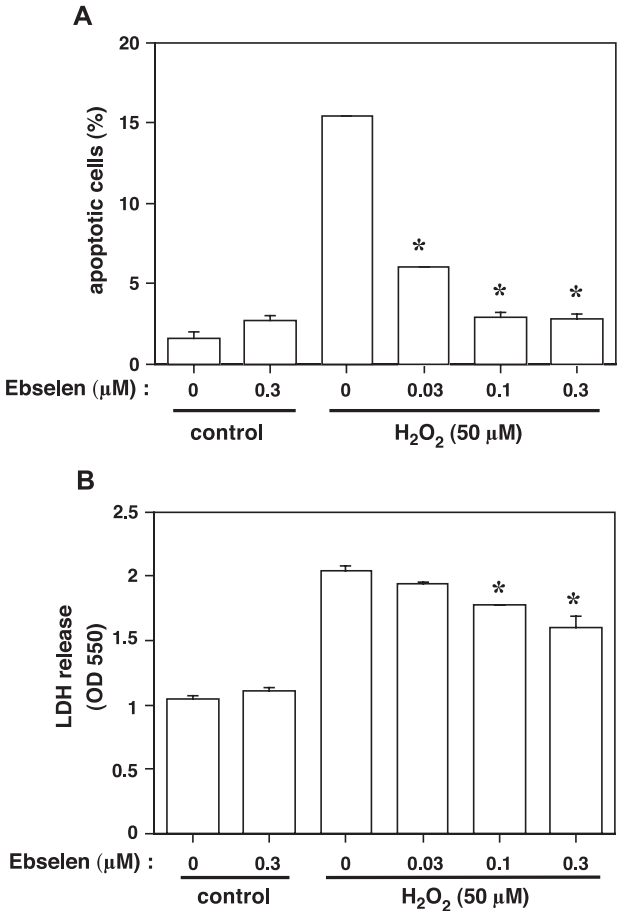

Fig. 2. Inhibition by ebselen of H_2O_2-induced apoptosis (A), and LDH leakage (B) in HUVECs. Cells were pretreated with ebselen at the indicated concentrations for 30 min prior to incubation with 50 μM H_2O_2 for 24 h, this was followed by cell staining with Hoechst 33248 (A) or measurement of LDH leakage (B) as described in Materials and methods. Values are expressed as means ± S.E. $n=5$. The asterisks represent significant differences compared with H_2O_2 stimulation ($^*P<0.05$).

significant LDH leakage after 24 h incubation. Pretreatment of HUVECs with different concentrations of ebselen (0–0.3 μM) inhibited H_2O_2-induced LDH leakage in a concentration-dependent manner. Although ebselen showed significant inhibition of LDH leakage, its effect on the prevention of apoptosis seems to be more prominent because Hoechst 33248 staining revealed that most of the cell death (15.4%) induced by 50 μM H_2O_2 was evaluated to be apoptotic cell death.

3.3. Ebselen inhibited H_2O_2-induced cytochrome c release in HUVECs

Cytochrome *c* plays an important role in apoptotic cell death. It is released from the mitochondria in response to apoptotic signals (Skulachev, 1998; Tschopp et al., 1998; Hakem, 1998). In the present study, we examined the effect of ebselen pretreatment on H_2O_2-induced cytochrome *c* release in HUVECs, by using cytochrome *c* assay kit. As

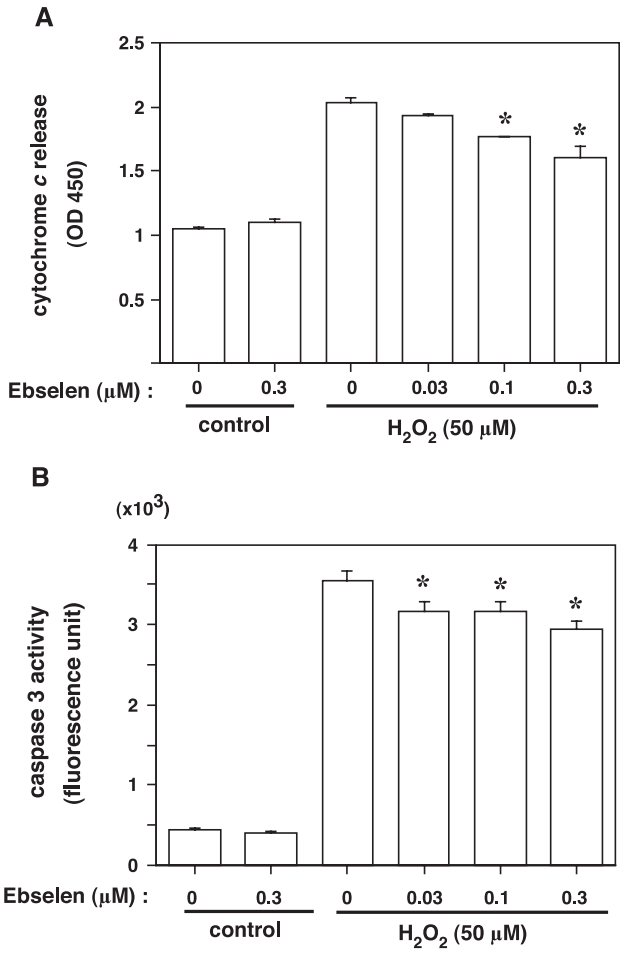

Fig. 3. Inhibition by ebselen of H_2O_2-induced cytochrome *c* release (A), and caspase-3 activation (B) in HUVECs. (A) Cells were preincubated with ebselen at the indicated concentrations for 30 min. Cells were then stimulated with H_2O_2 (50 μM) for 2 h. Cytochrome *c* release was assayed as described in Materials and methods. (B) After 30 min preincubation with ebselen at the indicated concentrations, HUVECs were stimulated with H_2O_2 (50 μM) for 4 h. Caspase-3 activity was measured as described in Materials and methods. Values are expressed as means ± S.E., $n=5$. The asterisks represent significant differences compared with H_2O_2 stimulation (*$P<0.05$).

shown in Fig. 3A, ebselen inhibited H_2O_2-induced cytochrome *c* release in a concentration-dependent manner.

3.4. Effect of ebselen on H_2O_2-induced caspase-3 activation in HUVECs

Since caspase-3 has been shown to be an important regulator of apoptotic cell death (Green and Reed, 1998), we next examined the effect of H_2O_2 (50 μM) on caspase-3 activity in HUVECs. As shown in Fig. 3B, incubation of HUVECs with H_2O_2 (50 μM) for 4 h significantly increased caspases-3 activity. Preincubation with different concentrations of ebselen for 30 min significantly inhibited H_2O_2-induced caspase-3 activation in a concentration-dependent manner. These findings coincide with those of cell counting Kit-8 assay (Fig. 1), and cytochrome *c* release assay (Fig. 3A).

3.5. Effect of ebselen on H_2O_2-induced p38 MAP kinase, JNK and ERK1/2 phosphorylation in HUVECs

To clarify the effect of ebselen on H_2O_2-induced MAP kinase phosphorylation, HUVECs were pretreated with various concentrations of ebselen for 30 min before the addition of H_2O_2 (50 μM) for 10 min. H_2O_2-induced phosphorylation of p38 MAP kinase was inhibited by ebselen in a concentration-dependent manner. In contrast, ebselen showed no significant effect on H_2O_2-induced JNK and ERK1/2 phosphorylation (Fig. 4). These findings indicate that H_2O_2-induced p38 MAP kinase phosphorylation is specifically sensitive to ebselen in HUVECs.

3.6. Effect of SB203580, a specific p38 inhibitor, on H_2O_2-induced p38 MAP kinase phosphorylation and HUVEC death

To verify that H_2O_2-induced p38 MAP kinase phosphorylation is responsible for HUVECs death pathway, we

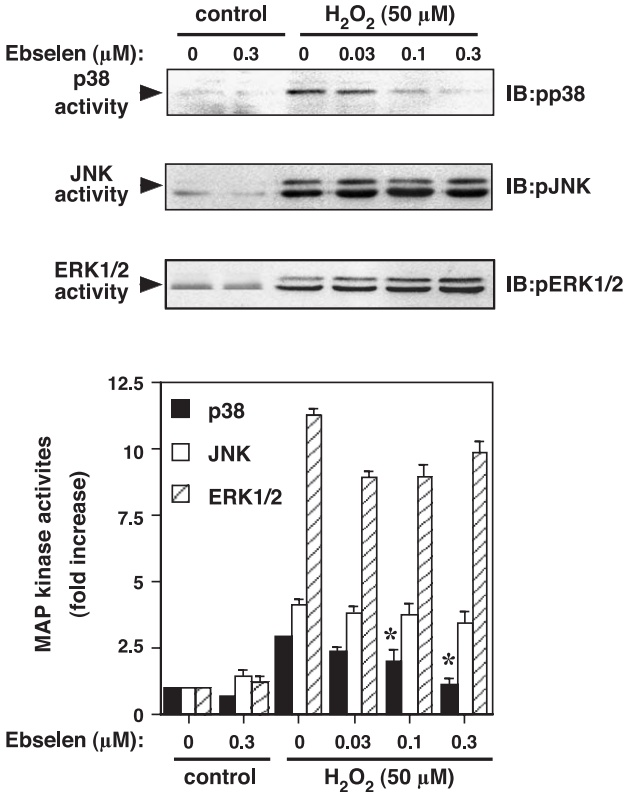

Fig. 4. Inhibition by ebselen of H_2O_2-induced p38 MAP kinase activation, but not JNK and ERK1/2 in HUVECs. Cells were pretreated with ebselen at the indicated concentrations for 30 min. After 10 min incubation with H_2O_2 (50 μM), the phosphorylation of p38 MAP kinase, JNK and ERK1/2 were measured as described in Materials and methods. No significant differences in the amounts of p38 MAP kinase, JNK and ERK1/2 were observed in samples by immunoblot analysis with anti-p38 MAP kinase, JNK and ERK1/2 antibodies (data not shown). Values were normalized by arbitrarily setting the densitometry of control cells (values are means ± S.E., $n=5$). The asterisks represent significant differences compared with H_2O_2 stimulation (*$P<0.05$).

examined the effect of different concentrations (0.01, 0.1, 1 μM) of SB203580, a specific p38 MAP kinase inhibitor, on H_2O_2-induced p38 MAP kinase phosphorylation and HUVEC death. Results in Fig. 5A show that, SB203580 inhibited H_2O_2-induced p38 MAP kinase phosphorylation in a concentration-dependent manner. Fig. 5B shows that H_2O_2-induced HUVEC death was significantly inhibited by SB203580 in a concentration-dependent manner.

3.7. Effect of SB203580, a specific p38 inhibitor, on H_2O_2-induced cytochrome c release and caspase-3 activation in HUVECs

Since our findings in Figs. 4 and 5 strongly suggest the role of p38 MAP kinase in H_2O_2-triggered death pathway in HUVECs, we examined the effect of different concentrations (0.01, 0.1, 1 μM) of SB203580, a specific p38 MAP kinase inhibitor, on H_2O_2-induced cytochrome *c* release and caspase 3 activation in HUVECs. Cells were perincubated with various concentrations of ebselen for 30 min before the addition of H_2O_2 (50 μM) for 2 h or 4 h, thereafter, cytochrome *c* release and caspase 3 activity were measured, respectively. Fig. 6A,B shows the inhibitory effect of SB203580, a specific p38 inhibitor, on H_2O_2-induced cytochrome *c* release and caspase 3 activation, which is more pronounced than that of ebselen, suggesting the role of p38 MAP kinase in H_2O_2-triggered apoptotic pathway in HUVECs.

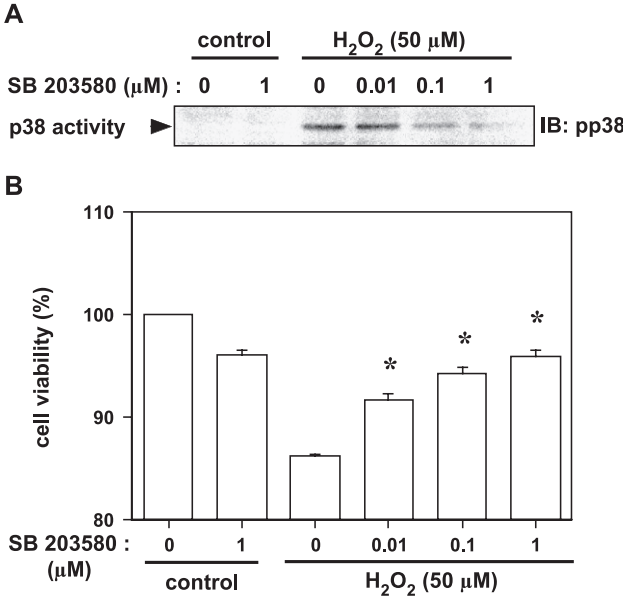

Fig. 5. Inhibitory effect of SB203580 on H_2O_2-induced p38 MAP kinase activation (A), and cytotoxicity (B) in HUVECs. (A) Cells were preincubated with SB203580 at the indicated concentrations for 30 min. Then the cells were stimulated with 50 μM H_2O_2 for 10 min. p38 MAP kinase activity was measured as described in Materials and methods. (B) After pretreating cells with SB203580 at the indicated concentrations for 30 min, cells were stimulated with H_2O_2 (50 μM) for 24 h. Cell viability was determined as described in the Materials and methods. Values are expressed as % of the controls, and are means ± S.E., $n = 5$. The asterisks represent significant differences compared with H_2O_2 stimulation ($^*P < 0.05$).

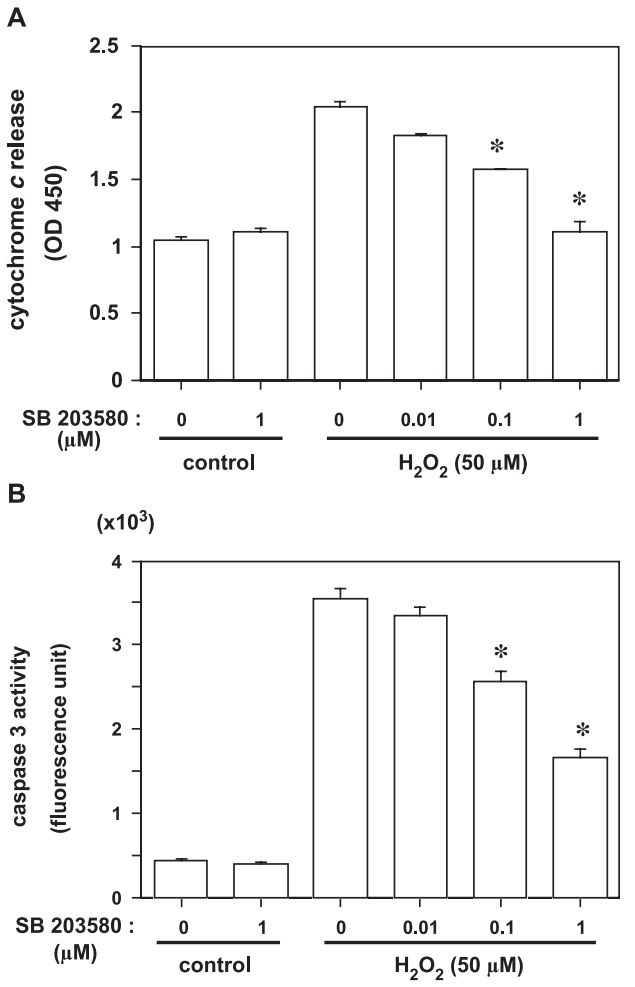

Fig. 6. Inhibitory effect of SB203580 on H_2O_2-induced cytochrome *c* release (A), and caspase-3 activation (B) in HUVECs. (A) Cells were preincubated with SB203580 at the indicated concentrations for 30 min. Cells were then stimulated with H_2O_2 (50 μM) for 2 h. Cytochrome *c* release was assayed as described in Materials and methods. (B) After 30 min preincubation with SB203580 at the indicated concentrations, HUVECs were stimulated with H_2O_2 (50 μM) for 4 h. Caspase-3 activity was measured as described in Materials and methods. Values are expressed as means ± S.E., $n = 5$. The asterisks represent significant differences compared with H_2O_2 stimulation ($^*P < 0.05$).

4. Discussion

The major findings of the present study are that ebselen protected HUVECs from H_2O_2-induced cell death. The effect of ebselen on H_2O_2-induced apoptosis is more prominent than the effect on necrosis, as revealed from Hoechst 33248 staining results. The underlying mechanisms of ebselen protective action appear to involve the inhibition of H_2O_2-induced cytochrome *c* release and caspase-3 activation in HUVECs. In addition, ebselen inhibited H_2O_2-induced p38 MAP kinase, but not JNK and ERK1/2

phosphorylation. These findings suggest that p38 MAP kinase plays an important role in HUVEC death pathway, and that p38 MAP kinase is specifically sensitive to ebselen. This was supported by the findings that SB203580, a p38 inhibitor, inhibited H_2O_2-induced p38 MAP kinase phosphorylation, cell death, as well as H_2O_2-induced cytochrome *c* release and caspase-3 activation.

It was reported that reactive oxygen species including hydrogen peroxide (H_2O_2), superoxide radical (O_2^-), hydroxyl radical (OH), and peroxynitrate ($ONOO^-$), are involved in apoptotic cell death in various cell types including endothelial cells (Jacobson, 1996). Since endothelial cell injury is thought to be the first step in the development of atherosclerotic lesions (Ross, 1995), and that apoptosis of endothelial cells can critically disturb the integrity of the endothelial monolayer (Haimovitz-Friedman et al., 1997), reactive oxygen species are thought to play an important role in the atherogenic process (Ohara et al., 1993; Alexander, 1995). Ebselen has been shown to cause protective effect against H_2O_2-induced cell death in several cell types, including PC12 cells (Yoshizumi et al., 2002), $HepG_2$ cells (Yang et al., 1999), and HL-60 cells (Li et al., 2000a), due to it's glutathione peroxidase activity, antioxidant and reactive oxygen species scavenging actions (Muller et al., 1984; Safayahi et al., 1985; Wendel et al., 1984). In the present study, we examined the effect of ebselen on H_2O_2-induced HUVEC death. It was found that ebselen protected HUVECs against H_2O_2-induced cell death in a concentration-dependent manner (Fig. 1). It is noteworthy that H_2O_2-induced HUVEC death, which was revealed from cell viability assay results (Fig. 1), is mainly due to apoptosis rather than necrosis and that ebselen almost completely inhibited apoptotic cell death (Fig. 2A).

The effects of proapoptotic signals on mitochondria have been extensively investigated, it was stated that mitochondrial cytochrome *c* is released into the cytosol in response to the apoptotic signals. The released cytochrome *c* bound to Apaf-1 (apoptotic protease activating factor-1), dATP and, Apaf-3/caspase-9 (Reed et al., 1998; Vander Heiden et al., 1997; Green and Reed, 1998), to form the apoptosoms within which caspase-9 is activated, leading to downstream activation of caspase-3 then apoptotic cell death will follow (Tschopp et al., 1998; Reed et al., 1998; Pan et al., 1998; Kuida et al., 1998). In the present study, we examined the ability of H_2O_2 to stimulate cytochrome *c* release, and we investigated the effect of ebselen on H_2O_2-induced cytochrome *c* release. As shown in Fig. 3A, ebselen inhibited H_2O_2-induced cytochrome *c* release in a concentration-dependent manner. In agreement with our results, Boireau et al. reported that Fe^{2+}/citrate-induced cytochrome *c* release from rat liver cell mitochondria was inhibited by ebselen, in addition they stated that ebselen's effect is most probably due to its antioxidant action (Boireau et al., 2000). In contrast to our results, they showed that ebselen protective effect was complete and cytochrome *c* release reached the basal level after administration of ebselen (10 μM). The discrepancy between their results and ours may be due to the difference of ebselen concentration used, as Boireau et al. used 10 μM ebselen which was cytotoxic when applied to HUVECs in our experimental conditions (data not shown).

Earnshaw et al. (1999) reported that the central components of the apoptosis machinery are proteases called caspases. Caspases are present as proenzymes that are cleaved and activated during apoptosis (Cohen, 1997; Thornberry and Lazebnik, 1998). Interestingly, it has been shown that caspase-3 can be activated by H_2O_2 exposure as a final effector in apoptotic death in vitro (Dipietrantonio et al., 1999; Matsura et al., 1999). In agreement with this view, our results showed that H_2O_2 markedly increased caspase-3 activity in HUVECs. Moreover, we investigated the effect of ebselen on H_2O_2-induced effect, where we found that ebselen inhibited H_2O_2-induced caspase-3 activation as shown in Fig. 3B. These results are consistent with the findings of Holl et al. who showed the inhibitory effect of ebselen against HN2 (a bifunctional DNA-reactive alkylating agent)-induced caspase-3 activation in normal and transformed lymphocytes. Although they used higher concentration (10 μM) of ebselen, it inhibited caspase activity incompletely (Holl et al., 2000). Based on our results, 0.3 μM ebselen completely inhibited H_2O_2-induced HUVEC death (Fig. 1). However, since the inhibitory effect of 0.3 μM ebselen on H_2O_2-induced cytochrome *c* release and caspase-3 activation was incomplete (Fig. 3A and B), involvement of other mechanisms of ebselen protective effect against H_2O_2-induced HUVEC death cannot be denied. Further studies are required to define the exact role of caspase-3 in the mechanism of H_2O_2-induced HUVEC death.

The role of MAP kinase cascades as an apoptotic signal transduction machinery has recently attracted attention. MAP kinases, a family of serine/threonine kinases, that mediate intracellular signal transduction in response to various stimuli (Tibbles and Woodgett, 1999). Three major MAP kinases have been identified, i.e., ERK1/2, JNK, and p38 MAP kinase. ERK1/2 are activated mainly by growth factors and are critically involved in the regulation of mitogenesis (Seger and Krebs, 1995; Xia et al., 1995). On the other hand, JNK and p38 MAP kinase are activated mainly by cytotoxic insult and are often associated with apoptosis (Raingeaud et al., 1995; Callsen and Brune, 1999). Moreover, p38 MAP kinase was shown to be an important intracellular signaling molecule in zinc-induced apoptosis in leukemia cells (Truong-Tran et al., 2000), this in agreement with our results suggests the role of p38 MAP kinase in the regulation of apoptosis in HUVECs. As shown in Fig. 4, although H_2O_2 increased p38 MAP kinase, JNK, and ERK1/2 phosphorylation, ebselen inhibited H_2O_2-induced p38 MAP kinase, but not JNK or ERK1/2 phosphorylation in a concentration-dependent manner. Conversely, we previously reported that ebselen inhibited H_2O_2-induced JNK but not ERK1/2 or p38 MAP kinase phosphorylation in PC12 cells in a concentration-dependent manner (Yoshi-

zumi et al., 2002). It is difficult to explain the discrepancies regarding the sensitivities of the three MAP kinases to ebselen action, it may be cell type specific, in addition to the different concentrations of ebselen and H_2O_2 used in both studies. From the above findings, it was suggested that p38 MAP kinase plays a role in HUVEC death pathway. For further confirmation, the effect of SB203580, a specific p38 inhibitor, on H_2O_2-induced p38 MAP kinase phosphorylation, HUVEC death, cytochrome *c* release and caspase-3 activation was studied. In agreement with our results shown in Figs. 5A,B and 6A,B, Harada and Sugimoto (1999) and Kondoh et al. (2002) postulated the ability of SB203580 to prevent apoptotic cell death in cerebellar granule neurons and HL-60 cells, respectively, which is highly suggestive of the role of p38 MAP kinase in the apoptotic pathway. In addition, it was reported that p38 MAP kinase phosphorylation might act as an upstream molecule for caspase-3 activation (Harada and Sugimoto, 1999; Kondoh et al., 2002). However, further studies are needed to define the target molecule(s) of ebselen that link signals from reactive oxygen species to p38 activation in HUVECs to understand the entire mechanism of ebselen inhibition of endothelial cell damage caused by reactive oxygen species.

From the present findings, we concluded that ebselen has a protective effect against H_2O_2-induced HUVEC death. To our knowledge, our study is the first to show the inhibitory effect of ebselen on H_2O_2-induced cytochrome *c* release, caspase-3 and, p38 MAP kinase activation, suggesting the intracellular mechanism of ebselen protective effect. It is noteworthy that the findings of the present study may shed light on the pharmacological basis for the clinical application of seleno-organic compounds for treatment of atherosclerosis, which is relevant to endothelial cell death.

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