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Ebselen attenuates oxidative stress-induced apoptosis *via* the inhibition of the c-Jun N-terminal kinase and activator protein-1 signalling pathway in PC12 cells

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1 Ebselen (2-phenyl-1,2-benzisoselenazol-3[2H]-one) is a selenoorganic compound exhibiting both glutathione peroxidase activity and antioxidant activity. Although it has been reported that ebselen is effective for oxidative stress-induced neuronal damage both *in vivo* and clinically, the precise mechanisms of the efficacy have not yet been elucidated. Thus, we hypothesized that ebselen may affect reactive oxygen species-induced mitogen-activated protein (MAP) kinase activation in cultured PC12 cells.

2 Our findings showed that hydrogen peroxide (H_2O_2) stimulated rapid and significant activation of extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK) and p38 in PC12 cells, which is a model of catecholamine-containing neurons.

3 H_2O_2-induced JNK activation was inhibited by ebselen, whereas ERK1/2 and p38 activation by H_2O_2 were not affected by ebselen.

4 Inhibition by ebselen of H_2O_2-induced hydroxyl radical generation in PC12 cells was observed using electron paramagnetic resonance measurements. Ebselen also inhibited H_2O_2-induced increases in DNA binding activity of activator protein-1 (AP-1), a downstream transcription factor of JNK, composed of the c-Jun homo/heterodimer.

5 Finally, pretreatment of cells with ebselen resulted in a significant recovery from cell death including apoptosis by H_2O_2 in PC12 cells.

6 These findings suggest that ebselen attenuates oxidative stress-induced neuronal cell death through the inhibition of the JNK and AP-1 signalling pathway. Thus, inhibition of JNK by ebselen may imply its usefulness for treatment of ischaemic cerebral diseases relevant to neuronal cell death. *British Journal of Pharmacology* (2002) **136,** 1023–1032

Keywords: Reactive oxygen species; ebselen; c-Jun N-terminal kinase (JNK); apoptosis; PC12 cell

Abbreviations: AP-1, activator protein-1; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DTPA, diethylenetriaminepentaacetic acid; DTT, dithiothreitol; EPR, electron paramagnetic resonance; ERK, extracellular signal-regulated kinase; H_2O_2, hydrogen peroxide; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; MEKK, MAP kinase/ERK kinase kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NGF, nerve growth factor; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; SDS, sodium dodecylsulphate

Introduction

Reactive oxygen species (ROS) have been proposed to be involved in the pathogenesis of cerebral ischaemia and reperfusion injury (Chan, 1994; Kontos, 1985). ROS including hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$), and peroxynitrate ($ONOO^-$) have been shown to increase upon ischaemia and reperfusion of the brain (Chan, 1996). In the ischaemic and reperfused brain, many alterations such as change in neurotransmitter release, change in gene expression, and neuronal cell death have been observed (Chan, 2001; Mattson *et al*., 2000). These alterations in the brain during ischaemia-reperfusion were suggested to be attributable to the change in the intracellular signalling mechanisms. Among many intracellular signalling molecules, ROS-induced cellular events have been implicated, in part, to the activation of mitogen-activated protein (MAP) kinases (Herdegen *et al*., 1998; Ozawa *et al*., 1999).

Three subfamilies of MAP kinases that are sensitive to ROS have been identified: extracellular-signal regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK), p38 kinase (Abe & Berk, 1998). Each subfamily may be regulated *via* different signal transduction pathways and modulate specific cell functions (Widmann *et al*., 1999). It has been recognized that ERK1/2 plays a major role in cell proliferation and differentiation as well as survival by various growth factors (Cobb & Goldsmith, 1995). However, JNK and p38 are activated by various

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inflammatory cytokines and environmental stresses that lead to cellular hypertrophy or apoptosis (Kyriakis & Avruch, 1996). PC12 cells are useful for studying the intracellular signalling mechanisms, and are regarded as a model for catecholamine-containing neurons. Recently, several studies reported the roles of MAP kinase activation in both the protection and the injury of postischaemic neuronal cells including PC12 cells (Tsuji *et al*., 2000; Xia *et al*., 1995; Zhang *et al*., 1998). Much evidence is accumulating that activation of three MAP kinase family members, ERK1/2, JNK, and p38 kinase was observed after ischaemia and reperfusion of neurons (Herdegen *et al*., 1998; Ozawa *et al*., 1999). These studies suggested that the activation of ERK1/2 made neuronal cells protective against ischaemia and reperfusion, whereas the activation of JNK and p38 kinase led to apoptosis (Xia *et al*., 1995; Zhang *et al*., 1998). However, the precise roles of these three MAP kinase signalling pathways in the regulation of cellular phenotypic modulation are still unclear and may be cell type specific (Liu *et al*., 1996).

Ebselen (2-phenyl-1,2-benzisoselennazol-3[2H]-one) is a lipid-soluble selenoorganic compound that potently inhibits lipid peroxidation through a glutathione peroxidase-like action (Muller *et al*., 1984; Wendel *et al*., 1984). Since ebselen is active against membrane hydroperoxides such as phospholipid hydroperoxide but not glutathione peroxidase (Maiorino *et al*., 1988), this agent effectively inhibits lipid peroxidation *in vitro* (Safayhi *et al*., 1985). In addition, ebselen has been suggested to have a potential to protect the brain damage due to ischaemia in which ROS 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 transient middle cerebral artery occlusion by ebselen pretreatment (Dawson *et al*., 1995). Another report showed that ebselen improved the outcome of acute ischaemic stroke in a placebo-controlled, double blind clinical trial (Yamaguchi *et al*., 1998). However, the precise mechanisms of its efficacy against ischaemic cerebral diseases have not yet been elucidated. Thus, we hypothesized that ebselen may exert its preventing effect against ROS-induced neuronal damage through affecting MAP kinase signalling cascades.

In the present study, we first examined the effect of hydrogen peroxide (H_2O_2), as an oxidative stress, on MAP kinase activities, i.e. ERK1/2, JNK and p38 kinase in PC12 cells. Thereafter, we investigated the effect of ebselen on the change in MAP kinase activities and their downstream transcription factors in the cells. The findings of this study showed that ebselen specifically inhibited H_2O_2-induced JNK activation, but not ERK1/2 or p38 activation in PC12 cells. In addition, ebselen inhibited H_2O_2-induced increase in the DNA binding activity of activator protein-1 (AP-1), a downstream transcription factor of JNK. Ebselen scavenged $^{\cdot}OH$ radicals generated by H_2O_2 stimulation and attenuated PC12 cell death including apoptosis. Although ROS-induced JNK activation has been reported in many cells (Fei *et al*., 2000; Lo *et al*., 1996; Mansat-de Mas *et al*., 1999), we discovered that ebselen inhibited ROS-induced JNK activation and the resultant PC12 cell death probably through the scavenging effect of $^{\cdot}OH$ radicals.

Methods

Chemicals

Ebselen (2-phenyl-1,2-benzisoselenazol-3[2H]-one) was from Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan). All other chemicals were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.) except where indicated. H_2O_2 was from Wako Pure Chemicals (Osaka, Japan). Anti-pan- and phospho-ERK1/2 (Thr202/Tyr204) and p38 (Thr180/Tyr182) antibodies, the JNK assay kit and pan-JNK antibody were from Cell Signaling Technology Inc. (Beverly, MA, U.S.A.). All other chemicals were commercial products of reagent grade.

Cell culture

Rat pheochromocytoma (PC12) cells were grown in RPMI 1640 medium supplemented with 10% (v v^{-1}) donor horse serum, 5% (v v^{-1}) new born calf serum and antibiotics (100 units ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin) in flasks precoated with collagen. The culture was replaced after 2 months of passage by thawing a fresh aliquot of frozen cells. The cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. Cells at 70–80% confluence in 60-mm and 100-mm dishes were growth arrested by incubation in serum-free RPMI 1640 medium for 24 h prior to use. All experiments were performed with growth-arrested cells to minimize basal MAP kinase activity.

Preparation of cell lysates for MAP kinase activity assay

PC12 cells in the serum-free medium were treated with or without H_2O_2 at each indicated time or dose-point. After treatment, the cells were washed once with ice-cold PBS, then lysed with 0.5 ml of lysis buffer (mM), Tris-HCl 20, pH 7.4, NaCl 150, Na_2EDTA 1, EGTA 1, 1% (v v^{-1}) Triton, sodium pyrophosphate 2.5, β-glycerophosphate 1, Na_3VO_4 1, 1 $\mu g\ ml^{-1}$ leupeptin and 1 mM PMSF, and flash frozen using liquid nitrogen. After allowing the cells to thaw, cells were harvested and the lysates were sonicated (Handy Sonic UR-20 P; Tomy Seiko Co., Ltd., Tokyo, Japan) on ice for 10 s and then centrifuged at $14{,}000 \times g$ for 20 min at 4°C. The protein concentrations of the supernatants were measured using a Bradford protein assay (Bio-Rad, Hercules, CA, U.S.A.).

Measurements of ERK1/2 and p38 kinase activities in PC12 cells

Previously, we measured each MAP kinase activity using in-gel kinase assay with specific substrates. However, we found that the activation of ERK1/2 or p38 by in-gel kinase assay and immunoblotting for phospho-ERK1/2 or phospho-p38 were highly correlated ($R^2 = 0.90$) in various cell types (unpublished data). Therefore, we used immunoblotting for phospho-ERK1/2 and phospho-p38 to evaluate ERK1/2 and p38 activation as described previously (Yoshizumi *et al*., 2000; Kyaw *et al*., 2001). Using pan-ERK1/2 and p38 antibodies, total ERK1/2 and p38 protein expression were also measured. For immunoblot analysis, cell lysates (30 μg of protein) were subjected to SDS-polyacrylamide gel

electrophoresis, and proteins were transferred to nitrocellulose membranes (Hybond™-ECL, Amersham Pharmacia Biotech, Buckinghamshire, U.K.) as described previously (Yoshizumi *et al*., 2000; Kyaw *et al*., 2001). The membrane was blocked for 1 h at room temperature with a commercial blocking buffer from Amersham Pharmacia Biotech. The blots were then incubated for 12 h with phospho-ERK1/2 or p38 antibodies with a dilution rate of 1 to 1000 (Cell Signaling Technology Inc.), followed by incubation for 1 h with secondary antibody (horseradish peroxidase conjugated). Immunoreactive bands were visualized using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) and were quantified by densitometry in the linear range of film exposure using a UMAX Astra 2200 scanner and NIH image 1.60.

JNK activity assay

As we could not obtain high quality phospho-JNK antibodies, we utilized *in-vitro* kinase assay to measure JNK activity. JNK activity was measured with a commercially available kit based on phosphorylation of recombinant c-Jun (Cell Signaling Technology Inc.). After treatment, cells were rinsed twice with ice-cold PBS, scraped off the plates into lysis buffer (included in the kit), and sonicated three times on ice. After removing the cell debris by centrifugation ($14{,}000 \times g$, 20 min, 4°C), the protein content in the supernatant was measured using a Bradford protein assay kit (Bio-Rad). Equal amounts of protein (300 μg) were then immunoprecipitated with c-Jun (1–89) fusion protein beads overnight. After washing the beads, kinase assays were performed according to the instructions of the manufacturer. Beads were loaded on a 10% (w v^{-1}) SDS-polyacrylamide gel, and immunoblotting was performed with an antibody against phospho-c-Jun with a dilution rate of 1 to 1000 (Yoshizumi *et al*., 2000; Kyaw *et al*., 2001). Using pan-JNK antibody, total JNK protein expression were also measured.

Electron paramagnetic resonance (EPR) measurements of hydroxyl radical (·OH) generation in PC12 cells

EPR spin trapping technique was adopted to clarify the ROS production from PC12 cells. PC12 cells were suspended with DTPA (1 mM) added PBS (1×10^6 cells ml^{-1}), then aliquots were mixed with 0.9 M DMPO and H_2O_2 with or without ebselen (10 μM). Each sample was incubated for 5 min at 37°C, then transferred to three pieces of glass capillary (10 μl, Drummond Co., Broomall, PA, U.S.A.) and set to the EPR cavity for the measurement. To avoid interference from the extracellular EPR signal of H_2O_2, an excess amount of catalase (100 units) was added to the incubation medium immediately before measurements. A JEOL EPR spectrometer (JES-TE 300, JEOL Co., Ltd., Tokyo, Japan) with an X-band cavity was employed to collect all EPR spectra. Typical instrument conditions were: 8 mW microwave power, 1.0 Gauss modulation amplitude, 0.03 s time constant, 120 s scan time, and 100 Gauss scan range. Hyperfine coupling constants and spectral simulations were obtained using a computer program, WINSIM (http://epr.niehs.nih.gov) (Duling, 1994).

Gel mobility shift assay

For gel mobility shift assay, nuclear protein extracts were prepared from PC12 cells in 100-mm dishes after stimulation with H_2O_2 at the indicated time points. The cells were washed once with ice-cold PBS and were scraped off the plates into 1 ml PBS, then centrifuged at $5000 \times g$ for 5 min and supernatants were removed. The samples were homogenized in 0.4 ml of 10 mM HEPES (pH 7.9) containing: KCl 10 mM, EDTA 0.1 mM EGTA 0.1 mM, PMSF 0.5 mM, DTT 1 mM; chymostatin 10 μg μl^{-1}, aprotinin 1 $\mu g\ ml^{-1}$, pepstatin 1 $\mu g\ ml^{-1}$ and leupeptin 1 $\mu g\ ml^{-1}$; incubated on ice for 15 min; and then added 1/100 volume of 10% (v v^{-1}) Nonidet NP-40 and centrifuged at $5000 \times g$ for 30 s at 4°C. The resulting precipitations were homogenized in 50 μl of HEPES 20 mM (pH 7.9), NaCl 0.4 M, EDTA 1 mM, EGTA 1 mM, PMSF 1 mM, DTT 1 mM, chymostatin 10 μg μl^{-1}, aprotinin 1 $\mu g\ ml^{-1}$, pepstatin 1 $\mu g\ ml^{-1}$ and leupeptin 1 $\mu g\ ml^{-1}$; stirred at 4°C for 15 min. The resulting supernatant was assayed for protein concentrations and stored at −80°C before use. The procedure for the gel mobility shift assay was described previously (Yoshizumi *et al*., 1998). In brief, the gel mobility shift assay of PC12 cells nuclear activator protein-1 (AP-1) binding activity was performed with an oligonucleotide probe containing the AP-1 binding sequence (5′-CGCTTGATGACTCAGCCGGAA-3′) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.). The probe was end-labelled with γ-^{32}P-ATP (Amersham) using T4 polynucleotide kinase, and purified by chromatography on a Bio-Spin column (Roche Molecular Biochemicals, Mannheim, Germany). For the DNA-protein binding reaction, the samples of PC12 cell nuclear extracts (10 μg protein) were incubated with 10 fmol of a ^{32}P-labelled oligonucleotide containing the consensus AP-1 binding site at room temperature for 30 min, in 20 μl of binding buffer consisting of (mM): HEPES (pH 7.9) 20, EDTA 0.2, EGTA 0.2, NaCl 80, $MgCl_2$ 0.3, DTT 1, PMSF 0.2, 6% (v v^{-1}) glycerol and 2 μg of polydeoxyinosinic deoxycytidylic acid (poly[dI-dc])(Roche). The DNA-protein complexes were separated from the free DNA probe using electrophoresis on 7% (w v^{-1}) nondenaturing polyacrylamide gels in 6.7 mM Tris-HCl (pH 7.5), 3.3 mM sodium acetate, 0.1 mM EDTA, and 2.5% (v v^{-1}) glycerol. Gels were run at 160 V at 4°C for 3 h, dried and subjected to autoradiography and analysed with a bioimage analyser (BAS-1500; Fuji Film, Tokyo, Japan). To determine the specificity of DNA-protein binding, binding reactions were performed as described above, in the presence of a 100 fold molar excess of a non-labelled AP-1 consensus oligonucleotide competitor followed by electrophoresis. Furthermore, supershift assays were performed with rabbit polyclonal pan-c-Jun antibody (Santa Cruz Biotechnology). Anti c-Jun antibody was added to samples after the initial binding reaction between PC12 cells nuclear protein extracts and ^{32}P-labelled consensus AP-1 oligonucleotide, the reaction was allowed to proceed at room temperature for 1 h, and then the samples were subjected to electrophoresis, as described above.

Determination of cell viability with MTT reduction

Measurement of cellular MTT reduction was carried out as described previously (Hansen *et al*., 1989). PC12 cells were

cultured at 70–80% confluently. Thereafter, cells were growth arrested by serum-free RPMI 1640 medium. Following 24 h incubation with H_2O_2, MTT was added to a final concentration of 0.5 mg ml^{-1}, and after a further 1 h incubation PC12 cells were lysed with isopropanol that contained 0.04 M HCl. MTT reduction was read at 550 nm by a spectrophotometer.

Nuclear Morphology-Hoechst 33248 staining

PC12 cells were stained with Hoechst 33248 dye (Koyama *et al*., 1998). The cells were cultured on a coverglass in a 35-mm dish and the cell-attached coverglass was taken out, washed in PBS, transferred into MeOH/Acetic acid (3:1) and submerged for 2 min, then cells were dried up at room temperature. Then, the cells were fixed on a coverglass at room temperature and stained with Hoechst 33248 working solution (0.05 μg ml^{-1}) for 10 min. Next, the coverglass was washed with distilled water and mounted in buffered glycerol. Fluorescence was visualized using a fluorescent microscope (Olympus, Tokyo, Japan). The cells on the coverglass were observed under high magnification ($\times$400) and the percentage of apoptotic cells were calculated in ten different fields of five separate samples.

Agarose gel electrophoresis for DNA fragmentation

DNA fragmentation was detected by the methods of Wyllie *et al*. (1980) with minor modifications. Cells were pelleted at 400 $\times$ *g* and washed twice with ice-cold Tris-buffered saline (NaCl 137 mM, KCl 2.7 mM, Tris, pH 7.0 25 mM). The pellets were resuspended in 50 ml of Tris-EDTA (EDTA 1 mM, Tris, pH 8.0 10 mM) and lysed with 0.5 ml of an extraction buffer (0.1 M EDTA, 0.5% (w v^{-1}) SDS, 10 mM Tris, pH 8.0) containing 0.5 mg ml^{-1} proteinase K. After overnight incubation at 50°C, DNA was extracted from the samples and ethanol preincubated for agarose gel electrophoresis. The DNA separated in the 2% agarose gel was visualized by UV fluorescence.

Statistics

Values are presented as means $\pm$ s.d. for five separate experiments. Two-way ANOVA, followed by Scheffe's multiple comparison test was used to compare individual groups. A value at $P<0.05$ was considered to be significant. n = number of observations, d.f. = degrees of freedom in the figure legends.

Results

Time courses for the activation of ERK1/2, JNK, and p38 in PC12 cells stimulated by H_2O_2

To evaluate the relative magnitude of MAP kinase activation by H_2O_2, growth-arrested PC12 cells were exposed 300 μM H_2O_2. Activation of ERK1/2, JNK and p38 in the cell lysates was determined as described in Methods. ERK1/2 was activated rapidly (peak at 5–10 min) and then gradually declined (Figure 1A, top). JNK was activated within 5 min and sustained for at least 60 min after the stimulation by H_2O_2 in PC12 cells (Figure 1A, middle). p38 activity showed a gradual increase by H_2O_2 treatment, peaked at 10 min, then decreased (Figure 1A, bottom). No differences in the amounts of ERK1/2, JNK and p38 were observed in samples by Western blot analysis with pan-ERK1/2, JNK and p38 antibodies (data not shown). These findings are summarized in Figure 1B.

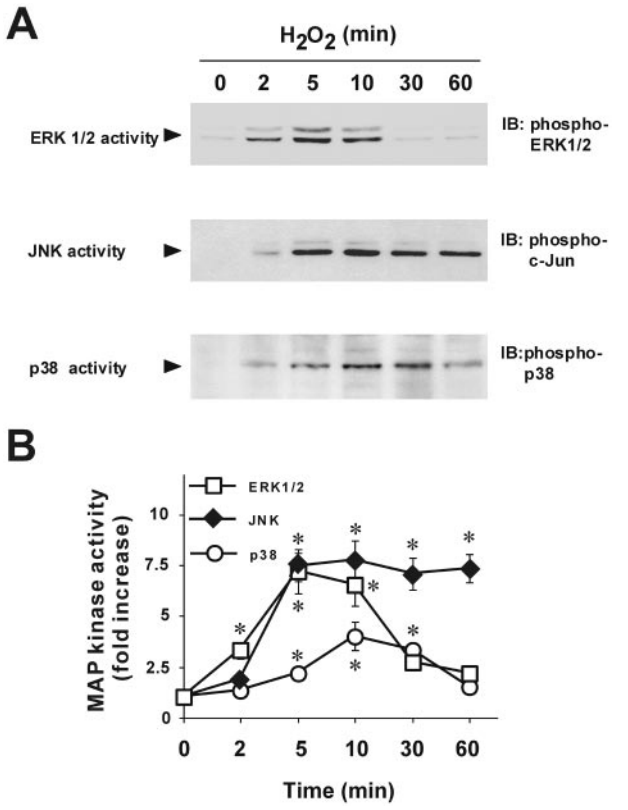

Figure 1 Time course of H_2O_2-induced ERK 1/2, JNK and p38 activation in PC12 cells. Cells were stimulated with 300 μM H_2O_2 for the indicated periods of time. Cells were harvested, lysed, and used for subsequent analysis. The activities of ERK1/2, JNK and p38 were measured as described in Methods. (A) Representative blots are shown. (B) Densitometric analysis of ERK 1/2 (sum of the ERK1 and ERK2 bands), JNK and p38 activation. Values were normalized by arbitrarily setting the densitometry of control cells (time = 0) to 1.0 (values are the means $\pm$ s.d., $n=5$). No obvious MAP kinase activation was observed in vehicle-treated samples. Two-way ANOVA detected significant differences between groups with or without H_2O_2 treatment: ERK1/2 ($F=65.859$, $P<0.0001$, d.f. = 5), JNK ($F=109.645$, $P<0.0001$, d.f. = 5) and p38 ($F=68.570$, $P<0.0001$, d.f. = 5). *$P<0.05$ when compared with vehicle-treated groups at each time point (ANOVA followed by Scheffe's test).

Concentration-response curve for the activation of ERK1/2, JNK, p38 in PC12 cells stimulated by H_2O_2

We also determined the concentration-dependence for MAP kinase activation by H_2O_2 in PC12 cells. ERK1/2 activation was determined by a 5-min incubation period, and JNK and p38 were by 10 min incubation (Figure 2). ERK1/2 and p38 activation were observed only at higher concentrations of H_2O_2, i.e. more than 300 μM (Figure 2A, top and bottom). In contrast, JNK was activated at lower concentrations of H_2O_2

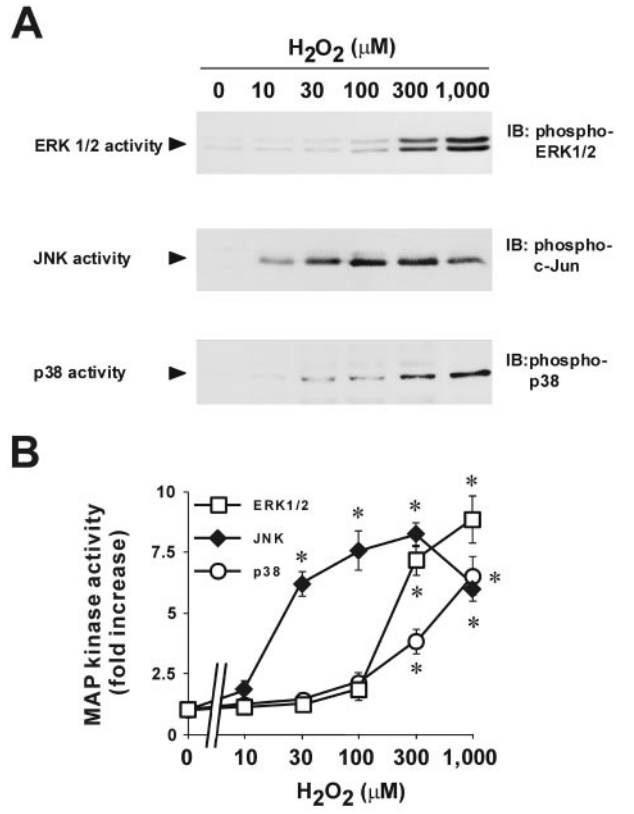

Figure 2 H_2O_2 stimulates a concentration-dependent increase in ERK 1/2, JNK and p38 activities in PC12 cells. Cells were stimulated with the indicated concentrations of H_2O_2 for 5 min for ERK1/2 activation and for 10 min for JNK and p38 activation. Cells were harvested, lysed, and used for subsequent analysis. The activities of ERK1/2, JNK and p38 were measured as described in Methods. (A) Representative blots are shown. (B) Densitometric analysis of ERK1/2 (sum of the ERK1 and ERK2 bands), JNK and p38 activation. Values were normalized by arbitrarily setting the densitometry of control cells (without H_2O_2) to 1.0 (values are the means ± s.d., $n=5$). No obvious MAP kinase activation was observed in vehicle-treated samples. Two-way ANOVA detected significant differences between groups with or without H_2O_2 treatment: ERK1/2 ($F=129.504$, $P<0.0001$, d.f. = 5), JNK ($F=107.909$, $P<0.0001$, d.f. = 5) and p38 ($F=74.18$, $P<0.0001$, d.f. = 5). $^*P<0.05$ when compared with vehicle-treated groups at each concentration (ANOVA followed by Scheffe's test).

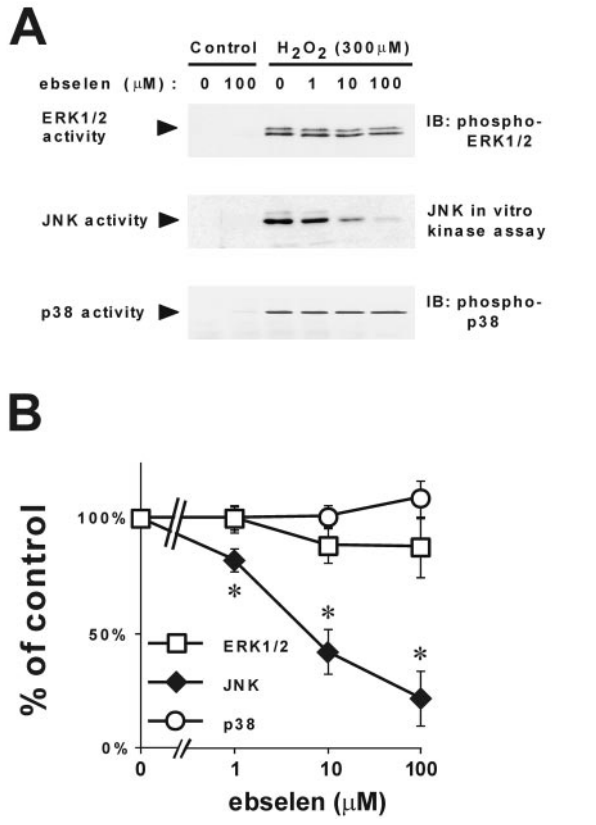

Figure 3 Inhibition by ebselen of H_2O_2-induced JNK activation in a concentration-dependent manner, but not ERK1/2 and p38 activation in PC12 cells. Cells were pretreated with ebselen at the indicated concentrations for 30 min. Controls with 0 μM ebselen represent vehicle-treated samples. Then the cells were stimulated with 300 μM H_2O_2 for 5 min for ERK activity and 10 min for JNK and p38 activities. Cells were harvested, lysed, and used for subsequent analysis. The activities of ERK1/2, JNK and p38 were measured as described in Methods. (A) Representative blots are shown. (B) Densitometric analysis of the effect of ebselen on ERK1/2, JNK and p38 activation. Values are expressed as % of controls which was defined from each MAP kinase activity stimulated by 300 μM H_2O_2 (values are the means ± s.d., n = 5). Two-way ANOVA detected significant differences between groups with or without H_2O_2 treatment for JNK ($F=77.746$, $P<0.0001$, d.f. = 3), but not for ERK1/2 ($F=3.104$, $P=0.0562$, d.f. = 3) and p38 ($F=0.849$, $P=0.4873$, d.f. = 3). $^*P<0.05$ when compared with H_2O_2-treated groups (ANOVA followed by Scheffe's test).

from 30 μM, peaked at 300 μM, and kept activated up to 1 mM (Figure 2A, middle). No differences in the amounts of ERK1/2, JNK and p38 were observed in samples by Western blot analysis with pan-ERK1/2, JNK and p38 antibodies (data not shown). These findings are summarized in Figure 2B. It was suggested that JNK is more sensitive than ERK1/2 and p38 to oxidative stress in PC12 cells.

Effect of ebselen on H_2O_2-induced ERK1/2, JNK and p38 activation in PC12 cells

To clarify whether ebselen affects H_2O_2-induced MAP kinase activation, we examined the effect of various concentrations of ebselen on H_2O_2-induced ERK 1/2, JNK and p38 activation (Figure 3A,B). The cells were pretreated with ebselen for 30 min before the addition of H_2O_2 (300 μM) for 5 min for ERK1/2 activation and for 10 min for JNK and p38 activation. H_2O_2-induced JNK activation was inhibited by ebselen in a concentration-dependent manner (1 – 100 μM) with an IC_{50} value of ≈10 μM. In contrast, ERK1/2 and p38 activation was not influenced by ebselen (Figure 3A,B). No significant differences in the amounts of ERK1/2, JNK and p38 were observed in samples by Western blot analysis with pan-ERK1/2, JNK and p38 antibodies (data not shown). These findings suggest that H_2O_2-induced JNK activation, but not ERK1/2 and p38 activation, is specifically sensitive to ebselen in PC12 cells.

Electron paramagnetic resonance (EPR) measurements of H_2O_2-induced ·OH generation and its inhibition by ebselen in PC12 cells

Since ebselen has been reported to possess an antioxidant activity through the inhibition of $O_2^{\cdot-}$ and $ONOO^-$

generation (Ichikawa *et al*., 1987; Masumoto & Sies, 1996), we examined its effect on H_2O_2-induced $^{\cdot}OH$ generation in PC12 cells. $^{\cdot}OH$ is readily generated from H_2O_2 by the Fenton reaction and originally derived from $O_2^{\cdot-}$ within the cells (Yoshizumi *et al*., 2001). As shown in Figure 4, application of 300 μM H_2O_2 to the cells resulted in a rapid increase in $^{\cdot}OH$ generation, which was almost abolished by 10 μM ebselen pretreatment. Since an excess amount of catalase was added to the cell incubation medium before measurements, it is unlikely that EPR signals of extracellular H_2O_2 interfered with the $^{\cdot}OH$ signals of the cells. These findings suggest that ebselen has a radical scavenging activity for $^{\cdot}OH$ generated by H_2O_2 stimulation of PC12 cells.

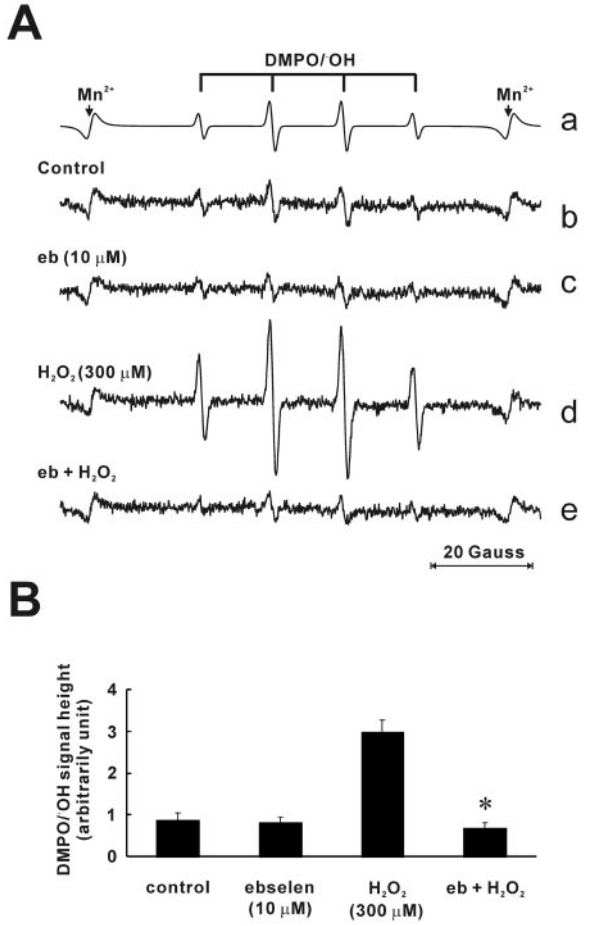

Figure 4 EPR spectra of DMPO/$^{\cdot}$OH adduct from PC12 cells by H_2O_2 stimulation and its dismutation by ebselen. (A) a: computer simulation of DMPO/$^{\cdot}$OH adduct EPR spectra; b: PC12 cells (1×10^6 cells ml^{-1}) with 0.9 M DMPO; c: same as b, but with ebselen (10 μM); d: same as b, but with H_2O_2 (300 μM) and e: same as b, but with ebselen (10 μM) pretreatment for 5 min and following H_2O_2 (300 μM) stimulation. To avoid interference from the extracellular EPR signal of H_2O_2, an excess amount of catalase (100 units) was added to the incubation medium immediately before measurements. (B) Relative signal intensity was defined as a relative peak height of the second EPR signal of the DMPO/$^{\cdot}$OH adduct with the first EPR signal of the external standard, MnO. Values are the means$\pm$s.d. of five separate experiments done in duplicate. Two-way ANOVA detected significant differences among groups ($F=148.879$, $P<0.0001$, d.f.$=3$). *$P<0.05$ when compared with H_2O_2-treated groups (ANOVA followed by Scheffe's test).

H_2O_2-induced JNK activation regulates DNA binding activity of AP-1, which was inhibited by ebselen

It was reported that JNK activation leads to c-Jun phosphorylation, which constitutes a transcription factor AP-1 complex, composed of c-Fos and c-Jun homo/heterodimers (Karin *et al*., 1997). Activated AP-1 has been reported to induce various gene expressions, such as c-*jun* itself and Fas ligand, which are believed to be involved in neuronal cell death including apoptosis (Ham *et al*., 2000). As shown in Figure 5A, DNA binding activity of AP-1 from the nuclear extracts of PC12 cells increased at 2 h treatment with 300 μM H_2O_2, and was sustained up to 24 h. The bands were confirmed to be a result of specific binding for AP-1, because the addition of unlabelled AP-1 consensus oligonucleotide resulted in abolishment of the band intensity. Furthermore, the addition of pan-c-Jun antibody to the binding reaction produced supershifted complexes. Ebselen pretreatment of the cells inhibited the increase in AP-1 DNA binding activity induced by H_2O_2 in a concentration-dependent manner similarly to the findings in Figure 3 (Figure 5B).

H_2O_2-induced PC12 cell death was inhibited by ebselen

Next we examined the effect of ebselen on H_2O_2-induced PC12 cell death by measuring MTT reduction. As shown in Figure 6, application of 300 μM H_2O_2 for 24 h resulted in PC12 cell death of about 45%. Ebselen pretreatment inhibited H_2O_2-induced cell death in a concentration-dependent manner, although ebselen itself had no effect on the cell viability. These findings are similar to those of ebselen inhibition of H_2O_2-induced JNK activation (Figure 3) and AP-1 activation (Figure 5). However, the preventing effect of ebselen on H_2O_2-induced PC12 cell death was incomplete and

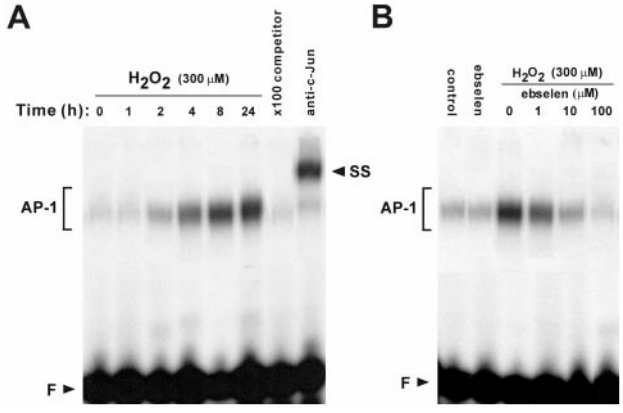

Figure 5 Time course for the activation of AP-1 DNA binding from PC12 cell nuclear extracts stimulated with 300 μM H_2O_2 (A) and its inhibition by ebselen (B). (A) The bracket in the panel indicates PC12 cell nuclear extract AP-1 DNA binding complexes induced by H_2O_2 (300 μM) stimulation at the indicated time points. A competition assay for AP-1 was carried out in the presence of a 100 fold molar excess of unlabelled AP-1 oligonucleotide (competitor). Supershift analysis was performed with specific pan-c-Jun antibody (SS). F, free probe. (B) Effects of ebselen on H_2O_2-induced increase in AP-1 DNA binding activity from PC12 cell nuclear extracts. For the experiments of ebselen against H_2O_2-induced increase in AP-1 DNA binding activity, different concentrations of ebselen were added to the incubation medium 30 min prior to H_2O_2 (300 μM) stimulation for 4 h. Representative blots from three separate experiments are shown.

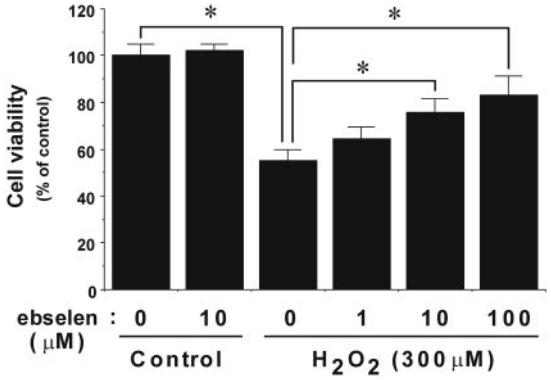

Figure 6 H_2O_2-induced PC12 cell death and its inhibition by ebselen. Cell viability was evaluated with MTT reduction as described in Methods. Cells were treated with or without 300 μM H_2O_2 for 24 h, then assayed. Ebselen at the indicated concentrations was added to the incubation medium 30 min prior to H_2O_2 stimulation. Values are the means$\pm$s.d. of five experiments preformed in triplicate. Two-way ANOVA detected significant differences among groups ($F=46.247$, $P<0.0001$, d.f.$=5$). The asterisks represent significant differences between the groups ($^*P<0.05$, ANOVA followed by Scheffe's test).

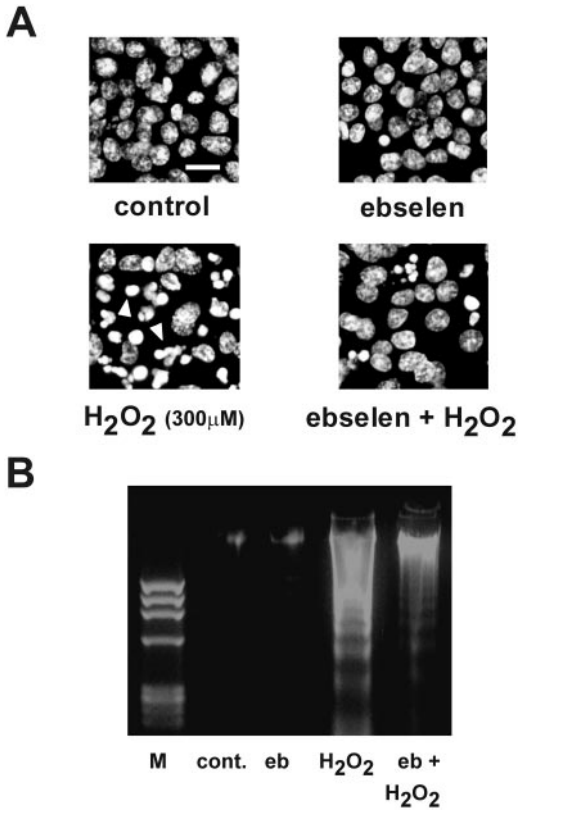

Figure 7 Ebselen inhibits H_2O_2-induced PC12 cell apoptosis assessed by apoptotic nuclei staining with Hoechst 33248 dye (A) and agarose gel electrophoretic analysis of DNA fragmentation (B) as described in Methods. Arrowheads indicate the typical apoptotic nuclei and the bar represents 20 μm in (A). Cells were treated with or without 300 μM H_2O_2 for 24 h. Ebselen (10 μM) was added to the incubation medium 30 min prior to H_2O_2 stimulation. M, marker (B). Representative photographs from three separate experiments are shown in both (A) and (B).

cell viability did not return to the control level by ebselen pretreatment.

Ebselen inhibits H_2O_2-induced PC12 cell apoptosis

To determine whether H_2O_2-induced MTT reduction is due to apoptosis, we examined apoptotic nuclei staining and DNA fragmentation in H_2O_2 administered PC12 cells with or without ebselen pretreatment. As shown in Figure 7A, apoptotic nuclei staining with Hoechst 33248 revealed that 33% of cells showed apoptosis by treatment with 300 μM H_2O_2 for 24 h. Ebselen pretreatment inhibited H_2O_2-induced apoptosis to 16% of total cells although the inhibition was incomplete. These findings are consistent with those of DNA fragmentation analysis with agarose gel electrophoresis (Figure 7B) and suggest that ebselen attenuates H_2O_2-induced PC12 cell death including apoptosis.

Discussion

The major findings of the present study were as follows: (1) H_2O_2 stimulates rapid and significant activation of three MAP kinase members; ERK1/2, JNK and p38 kinase in PC12 cells and JNK was more sensitive to H_2O_2 than the other two kinases; (2) H_2O_2-induced JNK activation was inhibited by ebselen in a concentration-dependent manner, whereas ERK1/2 and p38 activation by H_2O_2 was not affected by ebselen; (3) EPR measurements of PC12 cells revealed that ebselen pretreatment almost abolished $^{\cdot}$OH generation caused by H_2O_2 stimulation of the cells; (4) The H_2O_2-induced increase in DNA binding activity of AP-1, a downstream transcription factor of JNK, was also inhibited by ebselen by experiments with gel mobility shift analysis and (5) Ebselen attenuated H_2O_2-induced PC12 cell death including apoptosis by measuring MTT reduction, apoptotic nuclei staining and DNA fragmentation.

Recently, the role of MAP kinases, i.e. ERK1/2, JNK and p38 kinase, in the fate of neuronal cells as well as PC12 cells, such as differentiation, proliferation, and apoptosis after ischaemia and reperfusion injury has been investigated (Hu *et al*., 2000; Ozawa *et al*., 1999; Xia *et al*., 1995). These studies suggested that activation of JNK and p38 and concurrent inhibition of ERK in PC12 cells are critical for induction of apoptosis caused by NGF withdrawal. Another report also showed that the ERK signalling pathway contributes to neuroprotection, whereas JNK signalling leads to neuronal cell death in transient ischaemia of the hippocampus (Tsuji *et al*., 2000). In addition, differential activation and the distinct role of ERK and p38 in neuronal protection and damage after focal cerebral ischaemia have been observed (Irving *et al*., 2000). Specific links of ROS to JNK activation have also been shown (Fei *et al*., 2000; Lo *et al*., 1996; Mansat-de Mas *et al*., 1999). The findings of these studies suggested that ERK1/2 activation works as a cell survival factor against oxidative stress, whereas activation of JNK and p38 contributes to cell death machinery. Conversely, it was reported that inhibition of ERK with its inhibitors resulted in neuroprotection against oxidative stress (Satoh *et al*., 2000). Therefore, the precise roles of these three major

MAP kinases in ischaemic brain damage are still controversial and remain to be elucidated.

In the present study, we found that these three MAP kinases were rapidly and significantly activated by oxidative stress in cultured PC12 cells (Figures 1 and 2). We utilized H_2O_2 as an oxidative stress to the cells because the examined concentrations of H_2O_2 were in the range of those reached under pathophysiological conditions, such as during transient cerebral ischaemia (Hyslop *et al*., 1995). However, the concentration-dependency and the time course were different among the three kinases. We found in this study that H_2O_2-induced ERK1/2 and p38 activation was transient while JNK activation was sustained for at least 60 min in PC12 cells. The sustained activation of JNK by oxidative stress in the present study was consistent with the findings of Herdegen *et al*. (1998) where sustained JNK activation was observed after ischaemia and reperfusion in the rat brain. In addition, our findings that H_2O_2-induced ERK1/2 and p38 activation was observed at concentrations higher than 300 μM, whereas JNK was activated at lower concentrations from 30 μM of H_2O_2 are similar to those of Bhat & Zhang (1999) who reported higher sensitivities of JNK to H_2O_2 than the other two kinases in oligodendrocytes. We also reported that ERK1/2, JNK and p38 were activated by H_2O_2 in cultured fibroblasts and vascular smooth muscle cells (Yoshizumi *et al*., 2000). However, ERK1/2 was more sensitive to oxidative stress than JNK and p38 in these cells. It is difficult to explain the discrepancies regarding the sensitivities of three MAP kinases to oxidative stress, it may be cell type specific. However, it was found that JNK is more sensitive to oxidative stress than ERK1/2 and p38 in PC12 cells.

Ebselen has been shown to cause protective actions on neurons, including a glutathione peroxidase activity, antioxidative effect, and inhibitory effect on lipid peroxidation (Muller *et al*., 1984; Safayhi *et al*., 1985; Wendel *et al*., 1984). It was reported that ebselen has a potential to protect ischaemia-induced brain damage probably due to ROS scavenging actions (Matsui *et al*., 1990). Other reports showed that ebselen reduced the cerebral infarct size in experimental rat models as well as in clinical trials (Dawson *et al*., 1995; Yamaguchi *et al*., 1998). However, no evidence has been reported concerning the direct effect of ebselen on MAP kinase activity in neuronal cells including PC12 cells. We observed for the first time that ebselen specifically inhibited H_2O_2-induced JNK activation, but not ERK1/2 and p38 activation in PC12 cells. To our knowledge, only one study has described that ebselen inhibits JNK activation, but not p38 activation induced by lipopolysaccharide in rat Kupffer cells (Shimohashi *et al*., 2000).

Although the potential targets of ebselen leading to JNK inhibition remain to be clarified, it is likely that ebselen inhibits ROS generation in PC12 cells. In the present study, we confirmed directly with EPR that ebselen inhibited $^{\cdot}OH$ generation in PC12 cells evoked by H_2O_2 stimulation. Since it was reported that ebselen has a scavenging activity for superoxide radical ($O_2^{\cdot -}$) as well as an inhibitory action on NADH/NADPH oxidase enzyme (Ichikawa *et al*., 1987), it is conceivable that the inhibitory effect of ebselen on JNK activation may be attributable to its radical scavenging effect on ROS or an inhibitory action on NADH/NADPH oxidase. It was also reported that doxorubicin-induced ROS generation, probably through NADH/NADPH oxidase, was inhibited by ebselen in bovine aortic endothelial cells (Kotamraju *et al*., 2000) in which JNK was activated by ROS (Wang *et al*., 1999). However, further studies are needed to define the target molecule(s) of ebselen that links signals from ROS to JNK activation in neuronal cells.

JNK, when activated, can phosphorylate c-Jun, which is a component of the transcription factor complex AP-1 that binds to a specific DNA sequence, the AP-1 site (5′-TGACTCA-3′) (Angel & Karin, 1991). In the present study, gel mobility shift analysis revealed that H_2O_2 caused significant and sustained increases in the DNA binding activity of AP-1 in PC12 cells. These findings are consistent with those of Herdegen *et al*. (1998) that lasting c-Jun phosphorylation was observed in the nuclei of axotomized neurons. Moreover, we observed that H_2O_2-induced increase in AP-1 binding was inhibited by ebselen in a concentration-dependent manner. In PC12 cells, JNK activation and c-Jun N-terminal phosphorylation were suggested to be critical components in the pathway leading to apoptosis after either expression of MEKK1, a potent upstream activator of the JNK cascade (Eilers *et al*., 1998), or deprivation of NGF from differentiated PC12 neurons (Xia *et al*., 1995). Another report that microinjection of a c-Jun dominant-negative mutant into rat sympathetic neurons protected these cells from apoptosis may support this notion (Ham *et al*., 1995). Using MTT assay, we found in the present study that 300 μM H_2O_2 treatment for 24 h resulted in 45% cell death which was recovered to 17% by ebselen pretreatment. In addition, H_2O_2-induced apoptosis in PC12 cells was also prevented by ebselen, as evaluated by apoptotic nuclei staining and DNA fragmentation analysis. These findings are consistent with previous observations that ebselen inhibited doxorubicin-induced apoptosis in endothelial cells and cardiomyocytes through its ROS scavenging effects (Kotamraju *et al*., 2000). Considering the above findings, it may be reasonable to conclude that ebselen attenuates H_2O_2-induced cell death including apoptosis through the inhibition of the ROS-mediated JNK and AP-1 signalling pathway in PC12 cells. Recent observations that ebselen reduced cytochrome *c* release and DNA fragmentation after focal cerebral ischaemia in mice may support our findings (Namura *et al*., 2001). However, since ebselen inhibition of PC12 cell death as well as apoptosis was incomplete, involvement of pathways other than JNK and AP-1 cannot be denied in H_2O_2-induced insults. In addition, we did not define the downstream effector of AP-1 that leads to apoptosis in the present study. One likely function of N-terminally phosphorylated c-Jun is to induce Fas-ligand expression *via* several AP-1 sites in the *fas-ligand* promoter (Kasibhatla *et al*., 1998). Further studies are needed to define the entire mechanism of ebselen inhibition for neuronal cell damage caused by ROS.

In conclusion, we showed for the first time that ebselen specifically inhibited H_2O_2-induced JNK activation, but not ERK1/2 and p38 activation in PC12 cells. Ebselen also attenuated H_2O_2-induced PC12 cell death including apoptosis. Although the mechanisms of the beneficial effect of ebselen against ROS-induced cellular injury remain to be elucidated, it was suggested in part that ebselen affects the ROS-mediated JNK and AP-1 signalling pathway in PC12 cells. The findings of the present study may shed light on the pharmacological basis for the clinical application of selenoorganic compounds in cerebral ischaemia and/or reperfusion injury.

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