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Induction of Cellular Necrosis by the Glutathione Peroxidase Mimetic Ebselen

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Abstract The selenium-based compound ebselen is a powerful antioxidant, a potent anti-inflammatory agent and a potential neuroprotective compound. Several studies have demonstrated that part of the biological effect of ebselen is the result of the inhibition of apoptosis. We show in this report that ebselen induced the necrotic cell death of Sp2/0-Ag14 hybridoma cells. This process was rapid, with over 90% of the cells being dead after a 2 h exposure to 50 μM ebselen. The toxic effect of ebselen could not be prevented by the caspase inhibitor Z-VAD-fmk but could be blocked with thiol-containing compounds. Interestingly, ebselen addition completely prevented caspase activation in cycloheximide-treated Sp2/O-Ag14 cells, indicating that this antioxidant interferes with the apoptotic machinery. Our results indicate that some cell types are acutely sensitive to the toxic effect of ebselen, and that ebselen-induced cell death interferes with apoptotic processes. These observations are of particular importance since ebselen is currently used in clinical trials for possible use as therapeutic agent for stroke. J. Cell. Biochem. 89: 203–211, 2003.

Key words: ebselen; antioxidant; apoptosis; necrosis; hybridoma; neuroprotection

The seleno-organic compound ebselen (2-phenyl-1,2-benzisoselenazol-3[2H]-one) is a lipid-soluble antioxidant with glutathione peroxidase activity [Schewe, 1995]. As a glutathione peroxidase mimetic, ebselen inhibits both non-enzymatic and enzymatic membrane lipid peroxidation in cells [Schewe, 1995]. By directly reacting with protein thiol groups, ebselen also inhibits several enzymes involved in the inflammatory process, including lipoxygenases, nitric oxide synthases, NADPH oxidase, and protein kinase C [Schewe, 1995]. The chemical properties of ebselen, combined with its low toxicity to animals and humans [Wendel et al., 1984; Terlinden et al., 1988] have generated an interest in its use as a potential anti-inflammatory agent. Animal studies demonstrated that ebselen exhibited anti-inflammatory properties and protected against the toxic effects of cisplatin [Rybak et al., 2000] and paracematol [Li et al., 1994], as well as against alcohol-induced injury [Kono et al., 2001] and oxidative damage following ischemia [Maulik and Yoshida, 2000; Imai et al., 2001].

Recent studies have demonstrated that part of the biological effect of ebselen is the result of the inhibition a cell death process called apoptosis [Ramakrishnan et al., 1996; Holl et al., 2000; Kotamraju et al., 2000; Maulik and Yoshida, 2000; Yerushalmi et al., 2001]. Apoptotic cells undergo a series of morphological changes including cell shrinkage, membrane blebbing, chromatin condensation and fragmentation, the formation of apoptotic bodies and phagocytosis by neighboring cells [Trump et al., 1997]. The mitochondrion is at the heart of this process, being the host of several pro-apoptotic factors (e.g., caspases, cytochrome c, apoptosis inducing factor) as well as the target of a number of apoptotic regulatory proteins (e.g., Bcl-2, Bax) [Hengartner, 2000]. Mitochondrial disruption is a hallmark event in

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apoptosis, resulting in membrane permeability transition and the release of mitochondrial apoptogenic factors [Hengartner, 2000]. Ebselen has been shown to modulate the expression and/or activity of several proteins involved in the regulation of cell survival, including HSP 70, JNK, and NF-kB [Hoshida et al., 1997; Shimohashi et al., 2000], in addition to protecting the integrity of the mitochondria [Boireau et al., 1999, 2000].

Interestingly, there have been reports that ebselen can also provoke cell death [Engman et al., 1997; Powis et al., 1997; Yang et al., 2000a,b]. Ebselen inhibited colony formation in several cancer cell lines, raising the possibility that it could potentially be used as an antitumor agent [Engman et al., 1997; Powis et al., 1997]. When exposed to ebselen, $HepG_2$ cells die by apoptosis through a mechanism that involves intracellular thiol depletion and mitochondrial permeability transition [Yang et al., 2000a,b]. However, ebselen does not induce caspase activation in these cells [Yang et al., 2000b], raising doubt as to whether this antioxidant actually triggers apoptosis and not an alternative, caspase-independent death pathway, such as necrosis.

We show in this report that ebselen induced the rapid necrotic cell death of Sp2/0-Ag14 hybridoma cells. Necrosis caused by ebselen could not be prevented by the pan-caspase inhibitor Z-VAD-fmk, while the thiol-containing compound *N*-acetyl-L-cysteine (NAC) was a potent inhibitor. Interestingly, the presence of ebselen completely inhibited caspase activation induced by cycloheximide (CHX) treatment, indicating that the cell death mechanisms triggered by ebselen interfere with the apoptotic death machinery.

MATERIALS AND METHODS

Reagents

Unless specified otherwise, all reagents were obtained from Sigma-Aldrich (Oakville, ON).

Cell Culture

The murine hybridoma cell line Sp2/0-Ag14 (Sp2/0; ATCC # CRL1581) was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in Iscove's modified Dulbecco's media (Media Preparation Lab, Princess Margaret Hospital, Toronto, ON), supplemented with 5% (v/v) Fetalclone I (Hyclone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin (both from Life Technologies, Burlington, ON). All cell cultures were performed at 37°C under a humidified atmosphere of 5% CO_2.

Ebselen Treatment

Stock solutions of ebselen (5 mM) were prepared in ethanol. Cells were incubated in Iscove's media (with 5% FetalClone and antibiotics) containing the indicated amount of ebselen at 37°C in an atmosphere of 5% CO_2. An amount of ethanol equivalent to that delivered with ebselen was added to the cells incubated in the absence of ebselen. *N*-acetyl-L-cysteine (NAC; 15 mM in phosphate-buffered saline [PBS; 9.1 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 150 mM NaCl, pH 7.4]), glutathione (GSH; 15 mM in PBS), dithiothreitol (DTT; 15 mM in PBS), and the pan-caspase inhibitor Z-VAD-fmk (12 μM in DMSO, Biovision, Inc., Mountain View, CA) were all added to the cells at the same time as ebselen. When NAC, GSH, DTT, or Z-VAD-fmk were used, the control also contained the appropriate amount of vehicle.

Fluorescence Microscopy

All fluorescence microscopy experiments were performed at the Northeastern Ontario Regional Cancer Center (Sudbury) using a Carl Zeiss Axiovert 100 fluorescence microscope (Carlzeiss, Thornwood, NY). Photographs were taken with the Optronics 3 CCD cooled color camera and analyzed with the Northern Eclipse software (Empix emission, Mississauga, ON).

Acridine orange/ethidium bromide staining. Cells (2.5×10^5) were incubated for 2 h with ebselen or ethanol. Alternatively, cells were cultured in the presence of 25 μg/ml cycloheximide (CHX, dissolved in PBS) for 2 h. At the end of the incubation period, the cells were put on ice for 10 min and washed once in PBS. Cells were then resuspended in PBS containing 4 μg/ml each of acridine orange (AO) and ethidium bromide (EB) and incubated at room temperature for 1 min before examination by fluorescence microscopy.

Annexin V/propidium iodide assay. Five million cells were treated for 2 hours with ebselen or ethanol, incubated on ice for 10 min and washed once with PBS. Annexin V–propidium iodide (AV–PI) staining was then carried out using the AV–FITC Apoptosis Detection Kit (Biovision, Inc.). Briefly, the cells were

resuspended in AV Binding Buffer at a density of 2×10^4 cells/μl. AV and PI (2.5 μl each) were added, the samples were incubated at room temperature for 5 min and visualized by fluorescence microscopy.

Detection of reactive oxygen species (ROS) formation using dihydroethidium. Dihydroethidium (DHE) was prepared as a 315 μM solution in a 50% (v/v) DMSO. Cells (2.5×10^5/ml) were treated with ethanol or 50 μM ebselen for 2 h. DHE was added to the cells (final concentration of 5 μM) 15 min before the end of the ebselen treatment. The cells were then incubated on ice for 10 min, washed once, resuspended in PBS, and examined by fluorescence microscopy.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) analysis was performed at the Electron Microscopy Laboratory, University of New Brunswick (Fredericton, NB). Cells (1×10^7) were prefixed in 0.5% (v/v) glutaraldehyde, fixed in 3% (v/v) glutaraldehyde, and post-fixed in 1% (v/v) OsO_4. Dehydration was done through a series of acetone washes. Samples were infiltrated with an Epon-Araldite resin/acetone mixture and embedded in resin in gelatin capsules and flat embedding moulds. Sections (100 nm) were collected onto uncoated copper grids, double stained with uranyl acetate and Reynolds lead citrate, and observed under a Philips 400T transmission electron microscope. Images were exposed onto negative film (Kodak Electron Microscope film 4489).

Western Blot Analysis

RIPA cell extracts were prepared as described previously [Charbonneau and Gauthier, 2000]. The protein content was determined using the DC protein assay from Bio-Rad (Mississauga, ON), and the extracts were stored at −80°C. Proteins were fractionated on a PAGE–SDS gel and transferred onto a Hybond-P membrane (Amersham Bioscience, Baie d'Urfé, Qc). Western analysis was performed with a rabbit polyclonal anti-PARP (BioMol, Plymouth Meeting, PA) primary antibody and a goat anti-rabbit IgG secondary antibody coupled to horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Detection was carried out using the ChemiGlow reagent (Canberra Packard Canada, Mississauga, ON) and the Fluorchem 8000 Imaging System (Alpha Innotech, San Leandro, CA).

DNA Fragmentation Analysis

Agarose gel electrophoresis of DNA fragmentation was performed as described previously [Charbonneau and Gauthier, 2000]. Briefly, 5×10^5 cells were sampled and washed once in PBS. The cell pellet was resuspended in 50 μl of lysis buffer (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8.0, 0.5% *N*-lauroyl sarcosine, 0.25 mg/ml proteinase K, and 0.02 mg/ml RNAse A) and the mixture was incubated for 5 min at 50°C. Fifty microliters of sample buffer (40% sucrose, 0.08% bromophenol blue) was then added, and 30 μl of the sample was loaded into the wells of a 2% agarose gel. Electrophoresis was then performed in TBE Buffer (0.089 M Tris base, 0.089 M boric acid, 0.002 M EDTA pH 8.0), and visualization done under UV transillumination after staining with EB (0.7 μg/ml, 5 min). Image capture was done with the Fluorchem 8000 Imaging System.

Statistical Analysis

Data were analyzed by one-way analysis of variance and Scheffe's post-hoc test.

RESULTS

Ebselen Induces Sp2/0 Cell Death in a Caspase-Independent Manner

The viability of Sp2/0 murine hybridoma cells treated with ebselen was determined by fluorescence microscopy using the AO–EB staining procedure. In this assay, live cells are stained with AO, while only cells that have lost membrane integrity (e.g., dead cells) take up EB. Over 90% of Sp2/0 cells were permeable to EB after 2 h of ebselen exposure, indicating that ebselen caused cell death (Fig. 1). The toxic effect of ebselen was concentration-dependent, and doses as low as 15 μM caused over 60% of Sp2/0 cells to be permeable to EB after a 2 h incubation period (Fig. 1).

Apoptosis is characterized by the activation of caspase enzymes, which catalyze the limited proteolysis of a variety of cellular proteins, leading to the cell's demise. To determine whether ebselen-induced cell death required caspase activity, Sp2/0 cells were treated simultaneously with ebselen and the pan-caspase inhibitor Z-VAD-fmk. As a control, Sp2/0 cells were induced to undergo apoptosis by the

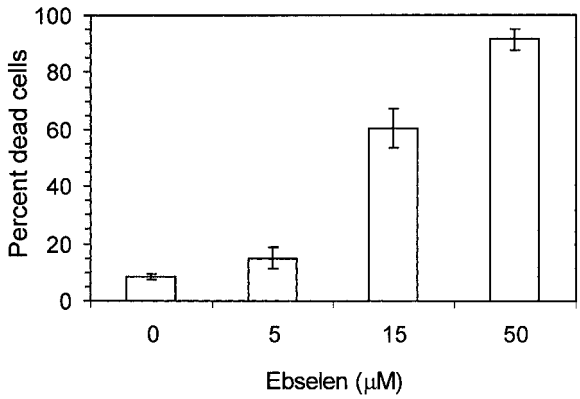

Fig. 1. Ebselen induces Sp2/0 hybridoma cell death. Sp2/0 cells were incubated for 2 h in the presence of increasing amounts of ebselen. Cell viability was then assessed by the AO/EB staining procedure. At least 300 cells were enumerated. Results are the average ± SD of three independent experiments.

addition of CHX. While Z-VAD-fmk potently inhibited the induction of apoptosis by CHX, it was completely ineffective in protecting Sp2/0 cells against ebselen-induced cell death (Fig. 2). Thus, ebselen induced cell death in a caspase-independent manner in Sp2/0 cells.

Ebselen Induces Sp2/0 Cell Necrosis

While ebselen has been shown by others to induce cell death by apoptosis [Yang et al., 2000a,b], caspase-independent cell death is characteristic of cells undergoing necrosis [Hirsch et al., 1997; Kitanaka and Kuchino, 1999]. We therefore examined the apoptotic/necrotic status of ebselen-treated Sp2/0 cells using the PI–AV method. In this assay, AV labels apoptotic cells by binding to phosphatidylserine which has been externalized to the cell surface, an early biochemical event of apoptosis. Necrotic cells will also be AV positive due to access to the inner leaflet of the plasma membrane following cell rupture. On the other hand, PI uptake is indicative of plasma membrane permeabilization, a phenomenon which occurs in late apoptotic cells or during necrosis. Therefore, a cell population undergoing apoptosis will be typically AV-positive and PI-negative, while necrotic cells will be both AV and PI positive. Upon incubation with 50 μM ebselen, Sp2/0 cells strongly stained positive for both PI and AV (Fig. 3), suggesting they underwent a necrotic, and not apoptotic, mode of cell death.

To confirm this observation, we examined the morphology of ebselen-treated Sp2/0 cells by way of electron microscopy. Control Sp2/0 cells exhibited a normal morphology, with a large nucleus and numerous mitochondria (Fig. 4A). Ebselen treatment dramatically altered the cellular ultrastructure: cells appeared disintegrated, and most organelles were swollen or

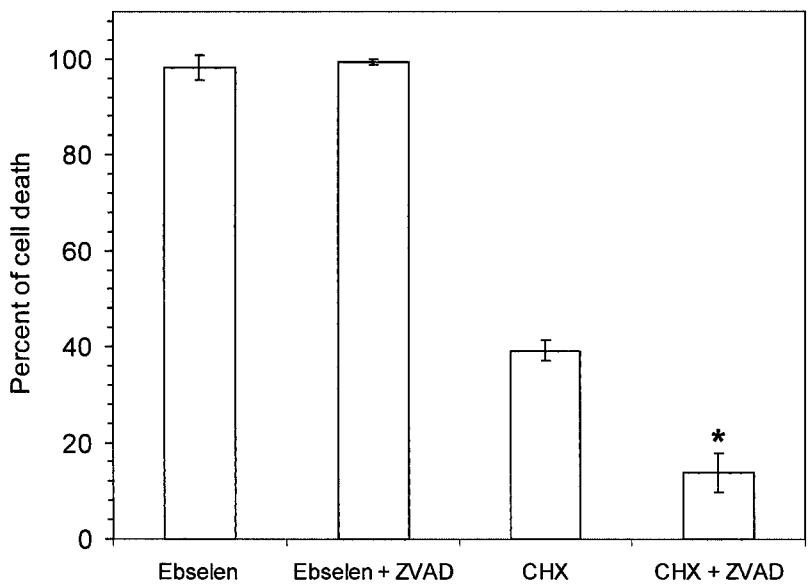

Fig. 2. The caspase inhibitor Z-VAD-fmk does not prevent ebselen-induced cell death. Sp2/0 cells were incubated for 2 h with ebselen (50 μM) or CHX (25 μg/ml), in the presence or not of 12 μM Z-VAD-fmk. The cells were then processed for AO/EB analysis. At least 300 cells were enumerated. Results are the average ± SD of three independent experiments. *$P < 0.01$ vs. CHX-treated cells.

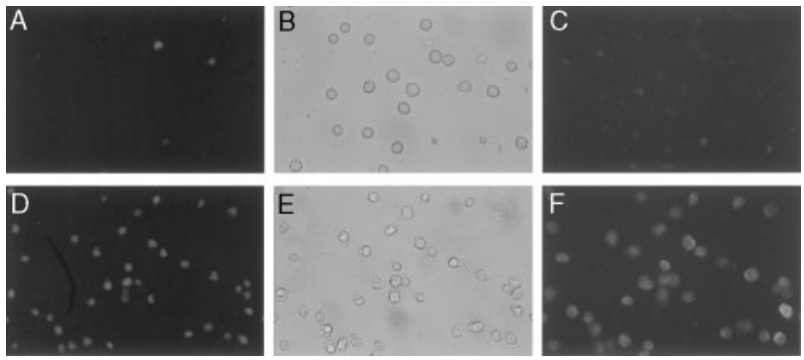

Fig. 3. Ebselen induces necrosis in Sp2/0 cells. Sp2/0 cells were incubated with 50 μM ebselen for 2 h, and then processed for the AV/PI staining procedure. **A**–**C**: Cells treated with ethanol. **D**–**F**: Cells treated with ebselen. A and D: PI staining. B and E: Brightfield microscopy. C and F: AV staining. Original magnification: 400 ×.

absent (Fig. 4B). Several perforations of the plasma membrane could be observed. The nucleus remained largely defined but showed signs of karyolysis (Fig. 4B). These ultrastructural features are characteristic of necrotic cells [Trump et al., 1997], and confirm that ebselen induced Sp2/0 cell death by necrosis.

Thiol-Containing Compounds Inhibit Ebselen-Induced Necrosis

Ebselen is known to react with thiol groups, a reaction, which is part of the mechanism of action of ebselen [Schewe, 1995]. We reasoned that an excess of a thiol-containing reagent could prevent the toxic effect of ebselen on Sp2/0 cells. To test this hypothesis, Sp2/0 cells were co-incubated for 2 h with NAC and ebselen, after which time cell morphology was examined. NAC completely prevented the ebselen-induced necrosis (Fig. 5I), an observation which was confirmed by electron microscopy (Fig. 4C). Similar results were obtained when GSH and DTT were used (not shown). Ebselen treatment also lead to the formation of superoxide anion in Sp2/0 cells (Fig. 5D), which is likely the result of a disruption in mitochondrial integrity. Confirming their protective role against ebselen-induced necrosis, NAC, GSH, and DTT (Fig. 5G; not shown) were also all effective in blocking superoxide anion generation.

Ebselen Inhibits Apoptotic Sp2/0 Cell Death

Ebselen has been shown by various groups to inhibit the induction of apoptosis, a phenomenon which is thought to form the basis of the neuroprotective action of this antioxidant. To investigate the possible effect of ebselen on apoptotic processes in our model system, Sp2/0 cells were co-incubated with ebselen and CHX for 2 h and processed for DNA fragmentation and PARP cleavage analysis, two biochemical events of apoptosis which require prior caspase activation. As expected by its ability to induce apoptosis in Sp2/0 cells, CHX triggered both

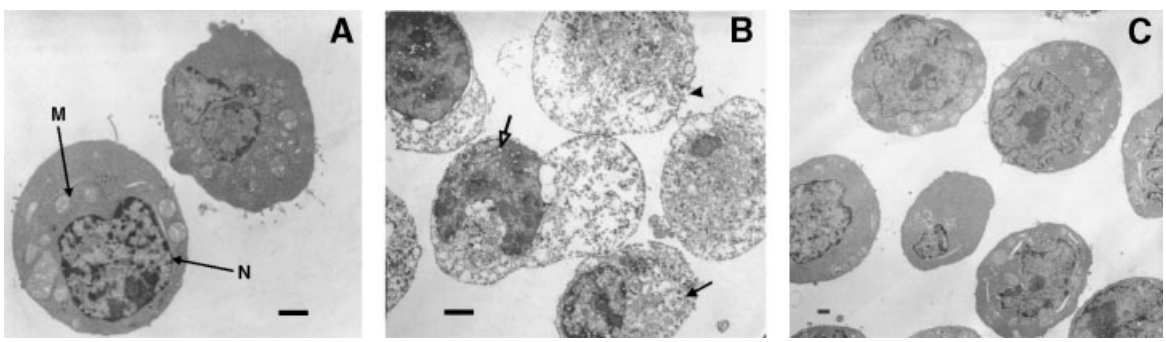

Fig. 4. Electron microscopy of ebselen-treated Sp2/0 cells. Sp2/0 cells were treated for 2 h with ethanol (**A**), 50 μM ebselen (**B**), or 50 μM ebselen and 15 mM *N*-acetyl-L-cysteine. Cells were then processed for electron microscopic analysis. M, mitochondria; N, Nucleus; Open arrow, nuclear karyolysis; Closed arrow, damaged mitochondria; Arrowhead, perforation of the plasma membrane; Bar, 1 μm.

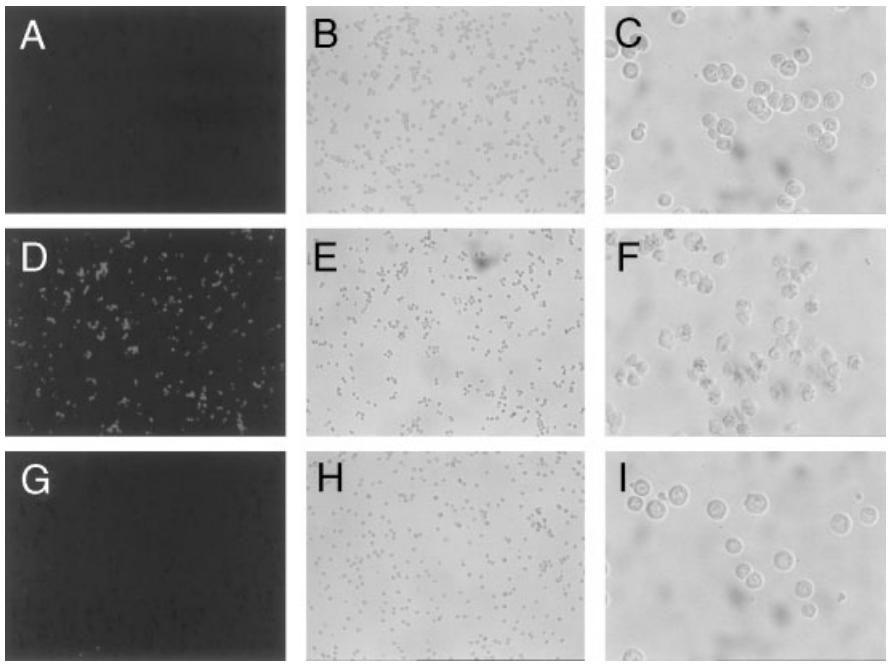

Fig. 5. NAC inhibits both ebselen-induced necrosis and ROS generation. Sp2/0 cells were incubated for 2 h in the presence of ethanol (**A**–**C**), 50 μM ebselen (**D**–**F**), or 50 μM ebselen and 15 mM NAC (**G**–**I**). Cells were then processed for the detection of ROS generation using the DHE assay (A, D, and G). B, C, E, F, H, I: Brightfield microscopy. Original magnification: 100 × (A, B, D, E, G, H) and 400 × (C, F, I).

PARP cleavage (Fig. 6A) and DNA laddering (Fig. 6B). On the other hand, ebselen treatment did not cause DNA fragmentation on its own, confirming the lack of caspase activation. Importantly, cells incubated in the presence of ebselen and CHX showed no DNA fragmentation and PARP proteolysis, indicating that ebselen interfered with the apoptotic pathways triggered by CHX. Similar results were obtained when L-glutamine starvation, anisomycin, puromycin, or actinomycin D were used to trigger apoptosis (not shown).

DISCUSSION

We show in this report that the glutathione peroxidase mimetic compound ebselen potently induces necrosis in the Sp2/0-Ag14 murine hybridoma cell line. This conclusion is supported by fluorescence and electron microscopy data. Our results are in line with previous work showing that ebselen can trigger death mechanisms in mammalian cells [Engman et al., 1997; Powis et al., 1997; Yang et al., 2000a,b]. Importantly, our study extends these findings by demonstrating that ebselen can not only induce apoptosis, as indicated by Yang et al. [2000a,b] but that it can also lead to necrosis.

The induction of Sp2/0 cell necrosis by ebselen could not be inhibited by Z-VAD-fmk. Additionally, ebselen treatment did not result in DNA laddering or in nuclear chromatin condensation or fragmentation, indicating that caspases were not activated. These results suggest that ebselen-induced Sp2/0 cell death occurred in a caspase-independent manner, which is consistent with a necrotic mode of cell death [Hirsch et al., 1997; Kitanaka and Kuchino, 1999]. Yang and coworkers [Yang et al., 2000b], using the human hepatoma cell line $HepG_2$, similarly noted that ebselen induced apoptosis in a process which was not inhibited by Z-VAD-fmk, did not lead to PARP cleavage, and did not result in caspase 3 activation. In both cell lines, increased ROS production was observed upon ebselen treatment, likely because of mitochondrial disruption [Yang et al., 2000b]. Therefore, our results and those of Yang and coworkers suggest that common pathways are activated by ebselen in Sp2/0 and $HepG_2$ cells which lead to a loss of mitochondrial integrity and caspase-independent cell death. Interestingly, ebselen was shown recently to bind to thioredoxin [Zhao et al., 2002], a ubiquitous protein system involved in the regulation of mitochondrial function and cell death [Chen et al., 2002;

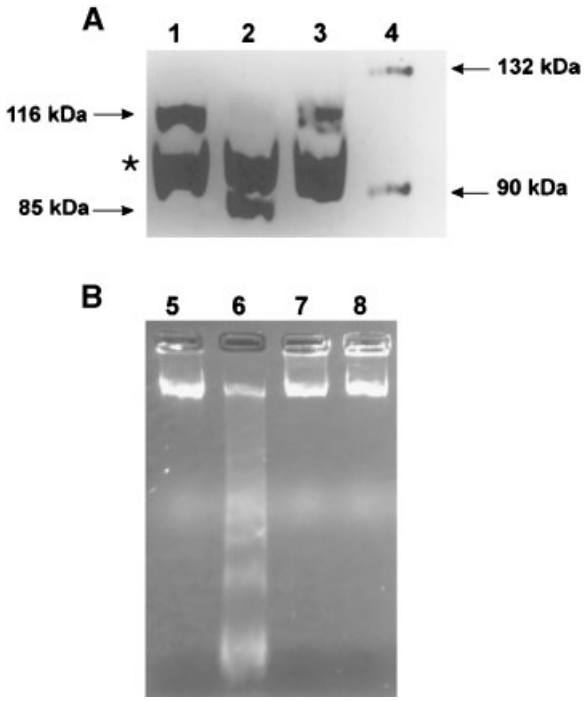

Fig. 6. Ebselen-induced cell death prevents PARP cleavage and DNA fragmentation induced by CHX in Sp2/0 cells. Sp2/0 cells were treated for 2 h with ethanol (**lanes 1** and **5**), 25 μg/ml CHX (**lanes 2** and **6**), 50 μM ebselen (**lane 7**), or 50 μM ebselen and 25 μg/ml CHX (**lanes 3** and **8**). **Lane 4**: Cruz marker. **A**: PARP cleavage. The star indicates the presence of a protein cross-reacting with the PARP antibody. Both the 116 kDa uncleaved PARP protein and its 85 kDa caspase-mediated degradation product are shown. **B**: Agarose electrophoresis analysis of DNA fragmentation.

Damdimopoulos et al., 2002]. Whether this interaction is relevant to the induction of cell death in ebselen-treated Sp2/0 and $HepG_2$ cells remain to be demonstrated.

Even though Sp2/0 and $HepG_2$ cells both undergo cell death following ebselen treatment, significant differences exist between these two cellular systems. Firstly, Sp2/0 cells rapidly underwent necrosis upon ebselen exposure, whereas the effect on $HepG_2$ cells occurred after a much longer time and with higher concentrations of ebselen [Yang et al., 2000a]. While the biochemical basis for this difference remains unknown, it is interesting to note that Sp2/0 cells are acutely sensitive to the induction of apoptosis by several insults, including CHX, actinomycin D, and amino acid deprivation [Perreault and Lemieux, 1993a,b; Gauthier et al., 1996; Charbonneau and Gauthier, 2000]. This raises the interesting possibility that the high susceptibility to apoptosis and ebselen-induced necrosis in Sp2/0 cells might be mechanistically linked. Secondly, ebselen-treated $HepG_2$ cells clearly had a morphology characteristic of apoptotic cells and they also exhibited DNA laddering [Yang et al., 2000a]. This indicated that at least part of the $HepG_2$ apoptotic machinery is still functional in the presence of ebselen, despite the lack of caspase activity. It is possible that, in $HepG_2$ cells, mitochondrial apoptogenic factors such as endonuclease G (which leads to DNA degradation in a caspase-independent fashion [Li et al., 2001]) are released following ebselen exposure. Supporting this possibility, cytochrome c release from the mitochondria was indeed observed in ebselen-treated $HepG_2$ cells [Yang et al., 2000b]. All together, these observations indicate that, while Sp2/0 and $HepG_2$ cells are both sensitive to the toxic effect of the ebselen, cell-specific differences exist in the death pathways that are triggered by this antioxidant.

We show in this report that, even though it triggered necrotic cell death, ebselen potently blocked Sp2/0 cell apoptosis induced by CHX. Similarly, Yang et al. [2000b] observed that ebselen, when added to $HepG_2$ cells, prevented caspase activation triggered by an apoptotic inducer, in their case TNF-α. Therefore, the cell death process triggered by ebselen is incompatible with the activation of caspases and, in the case of Sp2/0 cells, with the induction of apoptosis. In both Sp2/0 and $HepG_2$ cells, ebselen switched the mode of cell death from caspase-dependent to caspase-independent. One likely explanation for these observations is that the alternative death pathway triggered by ebselen prevents apoptosis. Necrosis has been shown to occur when cellular ATP levels are depleted [Eguchi et al., 1997; Leist et al., 1997], a situation which results in the inhibition of several energy-dependent apoptotic processes [Eguchi et al., 1997, 1999]. By disrupting the mitochondria, ebselen could lead to an uncoupling of oxidative phosphorylation, ATP depletion, and necrosis. An alternative possibility is that ebselen itself participates in preventing apoptosis while, at the same time, activating cell death through mitochondrial damage. Considering that ebselen reacts readily with protein thiol groups [Schewe, 1995], ebselen could block apoptosis by forming inhibitory complexes with proteins which are part of the apoptotic machinery. Interestingly, the activation of cell death pathways with the simultaneous inhibition of

caspases has been shown, in several systems, to lead to a switch from apoptosis to necrosis [Vercammen et al., 1998; Samali et al., 1999; Sane and Bertrand, 1999].

Sp2/0 cells undergo necrosis in the presence of ebselen concentrations which are similar to the therapeutic plasma levels of ebselen (10 μM) [Ullrich et al., 1996; Imai et al., 2001]. This suggests that ebselen could, in principle, trigger a necrotic type of cell death in animal cells or tissues. In addition, our data suggest that some of the previous observations of apoptosis inhibition by ebselen may, in fact, not have been the result of a pro-survival function of the antioxidant, but rather the consequence of the induction of an alternative death pathway. Because the inhibition of cell death is thought to be central to the physiological action of ebselen, our results clearly indicate that a better understanding of the effect of this antioxidant on cell behavior is required. This is of particular importance considering that ebselen is currently used in clinical trials for possible use as a neuroprotective agent for the treatment of stroke.

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