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Protective Effect of Ebselen against Hydrogen Peroxide-induced Cytotoxicity and DNA Damage in $HepG_2$ Cells

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ABSTRACT. The protective effect of ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one), a selenoorganic compound, against hydrogen peroxide (H_2O_2)-induced cytotoxicity and DNA damage was investigated in a human hepatoma cell line, $HepG_2$. The inhibitory effect of H_2O_2 on cell growth was determined using the tetrazolium dye colorimetric test (MTT test), and the cytotoxicity and lipid peroxidation were estimated by lactate dehydrogenase (LDH) leakage and malondialdehyde (MDA) formation, respectively. DNA damage was detected using single cell gel electrophoresis (comet assay), and intracellular reactive oxygen species (ROS) formation was measured using a fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA). The results showed that H_2O_2 suppressed the growth of $HepG_2$ cells and the addition of ebselen significantly reduced the suppression. Furthermore, ebselen also displayed a dose-dependent reduction of LDH leakage and MDA formation in H_2O_2-treated cells. The results also demonstrate that ebselen was able to reduce the ROS formation and DNA damaging effect caused by H_2O_2 in a dose-dependent manner. These findings suggest that ebselen has a strong protective ability against the cytotoxicity and DNA damaging effect caused by reactive oxygen species. BIOCHEM PHARMACOL **57**;3:273–279, 1999. © 1998 Elsevier Science Inc.

KEY WORDS. ebselen; hydrogen peroxide; cytotoxicity; DNA damage; $HepG_2$ cells

The role of ROS† has been implicated in many human degenerative diseases of aging, antioxidants having been found to have some preventive and therapeutic effects on these diseases [1]. Therefore, substantial efforts have been made in recent years to identify both natural and synthetic antioxidants. Ebselen, 2-phenyl-1,2-benzoisoseleazol-3(H)-one, a synthetic seleno-organic compound, is a novel anti-inflammatory agent [2]. The glutathione peroxidase-like activity of ebselen was first described in 1984 [3], and since then its antioxidative property has attracted much attention. The preventive effect of this compound against transition metal ion-catalyzed lipoprotein oxidation has been demonstrated through removal of hydroperoxides [4]. The protective effect against iron–ADP-induced lipid peroxidation in hepatic microsomes has also been studied [5]. In addition to its hydroperoxide-reducing effect, ebselen also exhibits direct peroxyl radical scavenging ability [6, 7] and efficient scavenging of peroxynitrite [8]. However, some authors recently reported that ebselen did not prevent lipid peroxidation during homogenization of porcine heart tissue [9], and the addition of ebselen even increased copper-induced low density lipoprotein oxidation [10]. These controversies suggest that the antioxidative function of ebselen remains to be further explored. In contrast, relatively little was known about ebselen's effect on ROS-induced cytotoxicity and DNA damage in intact cells.

H_2O_2, one of the main ROS, is known to cause lipid peroxidation and DNA damage in cells [11, 12]. The present study was thus designed to investigate whether ebselen is capable of reducing the hydrogen peroxide-induced cytotoxicity and DNA damage in a human hepatoma cell line, $HepG_2$.

MATERIALS AND METHODS

Cell and Chemicals

The human hepatoma cell line ($HepG_2$) was purchased from American Type Culture Collection. H_2O_2 and TBA were from Merck; MEM and low melting and normal melting point agarose were from GIBCO; and FBS was from Cytosystems. Ebselen (2-pheyl-1,2-benzoisoselenazol-3(H)-one), *N*,*N*-dimethylformamide, MTT, SDS, penicillin, streptomycin and ethidium bromide were all from Sigma; DCFH-DA was purchased from Molecular Probes.

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† *Abbreviations:* DCF, dichlorofluorescein; DCFH, dichlorofluorescin; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; FBS, fetal bovine serum; H_2O_2, hydrogen peroxide; LDH, lactate dehydrogenase; MDA, malondialdehyde; MEM, minimum essential medium; MTT, 3,(4,5-dimethylthiazol-2-yl)2,5-diphenyll-tetrazolium bromide; ROS, reactive oxygen species; and TBA, thiobarbituric acid.

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Cell Culture and Treatment

$HepG_2$ cells were cultured in complete MEM (containing 10% FBS, 100 units/mL of penicillin, 100 mg/mL of streptomycin, pH 7.4) at 37° in 5% CO_2. The effect of ebselen on H_2O_2-induced cytotoxicity and lipid peroxidation was tested by treating cells with different concentrations of ebselen and 100 μM H_2O_2 in FBS-free MEM for 24 hours. H_2O_2-induced DNA damage was estimated by incubating cells with 50 μM H_2O_2 for 2 hr. The inhibitory effect of ebselen on intracellular ROS formation was determined by incubating cells with different concentrations of ebselen and 100 μM H_2O_2 for up to 4 hr.

Effect of Ebselen and H_2O_2 on $HepG_2$ Cell Growth (MTT Test)

The tetrazolium dye colorimetric test (MTT test) is used to monitor cell growth indirectly as indicated by the conversion of the tetrazolium salt to the colored product, formazan, the concentration of which can be measured spectrophotometrically [13]. Briefly, $HepG_2$ cells were first cultured in 96-well microplates (1×10^4 cells/well in 100 μL of complete MEM) for 12 hr. Cells were then washed with PBS and coincubated with ebselen (1–50 μM) and/or H_2O_2 (50 μM) in FBS-free MEM for 24 hr. At the end of incubation, 25 μL of MTT (5 mg/mL) was added to each well and incubation was allowed to continue for a further 2 hr. Finally, 100 μL of lysing buffer (50% DMF and 20% SDS, pH 4.6) was added to each well and incubated for another 2 hr. The plate was read using a microplate reader (BIO-RAD Model 3550) at a wavelength of 595 nm.

Determination of Cytotoxicity: LDH Leakage

LDH activity was determined with an Abbott VP Biochemical Analyzer using a commercial test kit (Abbott Laboratories) [14]. At the end of the incubation, one aliquot of medium (0.2 mL) was taken out for extracellular LDH activity analysis. The total LDH activity was determined after cells were disrupted thoroughly by sonication. The percentage of LDH leakage was then calculated to reflect the cytotoxicity.

Measurement of Lipid Peroxidation: MDA Formation

MDA, an end product of lipid peroxidation, was measured to estimate the extent of lipid peroxidation in $HepG_2$ cells. MDA concentration in cell homogenate was determined using the TBA method [15]. Briefly, at the end of the incubation, cells were collected using a cell scraper and washed with PBS. Cell homogenate (prepared in 0.5 mL of PBS with 1% SDS) was mixed with 3 mL of 1% phosphoric acid and 1 mL of 0.67% TBA and heated in boiling water for 60 min. After cooling, 1.5 mL of *n*-butanol was added and mixed vigorously. After centrifugation, the absorbance of the butanol phase was read at 535 nm and 520 nm, respectively. The difference between 535 nm and 520 nm was used to calculate the MDA concentration, which was expressed as nanomoles per milligram protein.

Analysis of DNA Damage (Comet Assay)

H_2O_2-induced DNA damage was estimated using single cell gel electrophoresis (SCGE or comet assay) [16]. Briefly, fully frosted slides were covered with 0.65% of NMA as the first layer, a mixture of cell suspension and 0.65% of LMA as the second layer, and finally with 0.65% of LMA (without cell) as the third layer. After solidification at 4°, all slides were immersed in the lysing buffer (2.5 M of NaCl, 100 mM of EDTA, pH 10, with freshly added 1% Triton X-100 and 10% DMSO) at 4° for 1 hr, and the slides were then placed in a horizontal electrophoresis tank (BIO-RAD). The tank was filled with freshly prepared electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH 13, 4°), and the slides were left in the solution for 20 min to allow DNA unwinding and expression of alkali labile damage before electrophoresis. Electrophoresis was then conducted at 4° for 20 min using 25 V and 0.3 A. After electrophoresis, the slides were neutralized in neutralization buffer, stained with ethidium bromide, and kept in a humidified airtight container and examined using a fluorescence microscope (Nikon). Images of 100 randomly selected cells from each slide were analyzed. The degree of DNA damage was graded visually into 5 categories according to the amounts of DNA in the tail [17]: Grade 0, no damage, $< 5\%$; Grade 1, low level damage, 5–20 %; Grade 2, medium level damage, 20–40 %; Grade 3, high level damage, 40–95 %; Grade 4, total damage, >95 %.

Measurement of Intracellular ROS

Intracellular ROS was estimated by using a fluorescent probe, DCFH-DA [18]. DCFH-DA diffuses through the cell membrane readily and is enzymatically hydrolyzed by intracellular esterases to nonfluorescent DCFH, which is then rapidly oxidized to highly fluorescent DCF in the presence of ROS. The DCF fluorescence intensity is believed to be parallel to the amount of ROS formed intracellularly [19]. Cells were collected using a cell scraper and washed twice with PBS. Each fluorescence cuvette contained 2×10^5 cells in 3 mL of PBS. Various concentrations of ebselen and 100 μM H_2O_2 were added to the cells simultaneously with DCFH-DA (final concentration 5 μM), and incubated at 37° up to 4 hr. DCF fluorescence intensity was detected at different time intervals using a luminescence spectrometer (Perkin-Elmer LS-5B) with excitation wavelength at 485 nm and emission wavelength at 530 nm.

Statistical Analysis

Data are presented as means ± standard deviation (SD) and analyzed using one-way ANOVA with Scheffe's test. The result of the comet assay is presented as percentage of

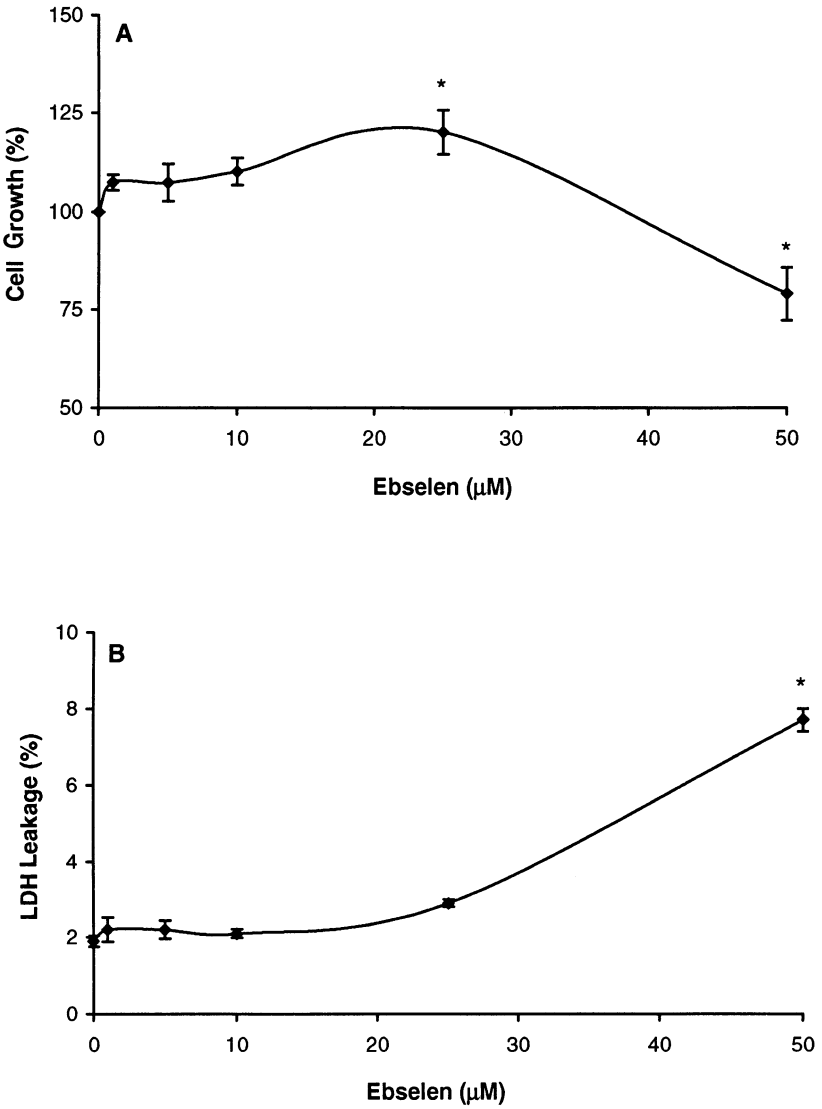

FIG. 1. Effect of ebselen on cell growth (MTT test) (A) and LDH leakage (B). For the MTT test, $HepG_2$ cells were precultured in 96-well microplates (1×10^4 cells per well in 100 μL of MEM) for 12 hr and then incubated with ebselen (1–50 μM) for 24 hr. For the analysis of LDH leakage, $HepG_2$ cells were cultured in 75 cm^2 flasks and incubated with ebselen (1–50 μM) for 24 hr. Data are presented as means ± SD (N = 5–6). The percentage of cell growth in the control group was treated as 100%. *P < 0.05 compared to the control (one-way ANOVA with Scheffe's test).

grade and analyzed using the chi-square test. A *P* value of less than 0.05 was considered as statistically significant.

RESULTS

The Cytotoxicity of Ebselen in $HepG_2$ Cells

The cytotoxicity of ebselen in $HepG_2$ cells was evaluated based on its effect on cell growth (MTT test) (Fig. 1A) and LDH leakage (Fig. 1B). Results in Fig. 1A show that, at concentrations ranging from 1 to 25 μM, ebselen was able to stimulate cell growth. A significant difference was noted at 25 μM compared to that of the untreated cells. At a higher concentration of 50 μM, ebselen significantly inhibited the growth of $HepG_2$ cells. Parallel with the results of the MTT test, cells treated with ebselen at concentrations of 1–25 μM for 24 hr did not show an obvious increase in LDH leakage (Fig. 1B). However, a significant increase in LDH activity in the medium was observed in cells treated with 50 μM ebselen. This finding suggests that the toxicity of ebselen increases after 25 μM. We thus decided to use 1–25 μM for all subsequent experiments.

Effect of Ebselen on H_2O_2-induced Cell Growth Suppression

Figure 2 shows that cell growth was significantly suppressed in the H_2O_2-treated group (Group B). The treatment of ebselen (1–25 μM) inhibited the suppression and increased cell growth in a dose-dependent manner (Groups C to F). The growth suppression was completely removed with the addition of 10 and 25 μM of ebselen (Groups E and F).

Effect of Ebselen on H_2O_2-induced LDH Leakage and Lipid Peroxidation

Figure 3A shows that H_2O_2 induced significant LDH leakage (Group B). An obvious dose-dependent inhibitory effect of ebselen on H_2O_2-induced LDH leakage was noted in $HepG_2$ cells. After 24-hr incubation, the treatment with 1–25 μM ebselen significantly reduced H_2O_2-induced LDH leakage (Groups C to F).

A similar inhibitory effect of ebselen on H_2O_2-induced

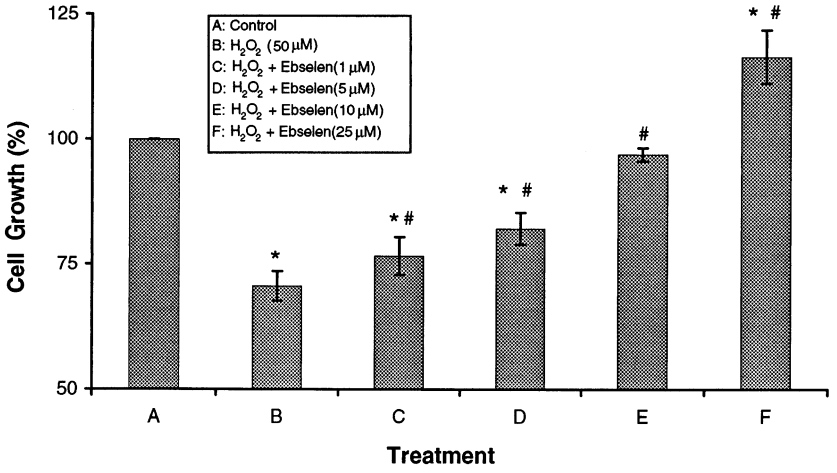

FIG. 2. Effect of ebselen on H_2O_2-induced cell growth suppression (MTT test). $HepG_2$ cells were precultured in 96-well microplates (1×10^4 cells per well in 100 μL of MEM) for 12 hr and then treated with H_2O_2 (50 μM) and/or ebselen (1–25 μM) for 24 hr. Data are presented as means ± SD (N = 6). The percentage of cell growth in the control group was treated as 100%. *$P < 0.05$ compared to the control group (Group A) and #$P < 0.05$ compared to the group treated with H_2O_2 only (Group B) (one-way ANOVA with Scheffe's test).

lipid peroxidation was also observed. As shown in Fig. 3B, treatment of cells with 1–25 μM ebselen significantly reduced the MDA concentration. Meanwhile, compared to cells without ebselen (Group A), ebselen did not cause obvious changes in MDA formation up to 25 μM (data not shown).

Effect of Ebselen on H_2O_2-induced DNA Damage

The effect of ebselen on H_2O_2-induced DNA damage is presented in Fig. 4. It was found that $HepG_2$ cells treated with 50 μM H_2O_2 resulted in serious DNA damage. The damage was mainly composed of Grades 3 and 4. In

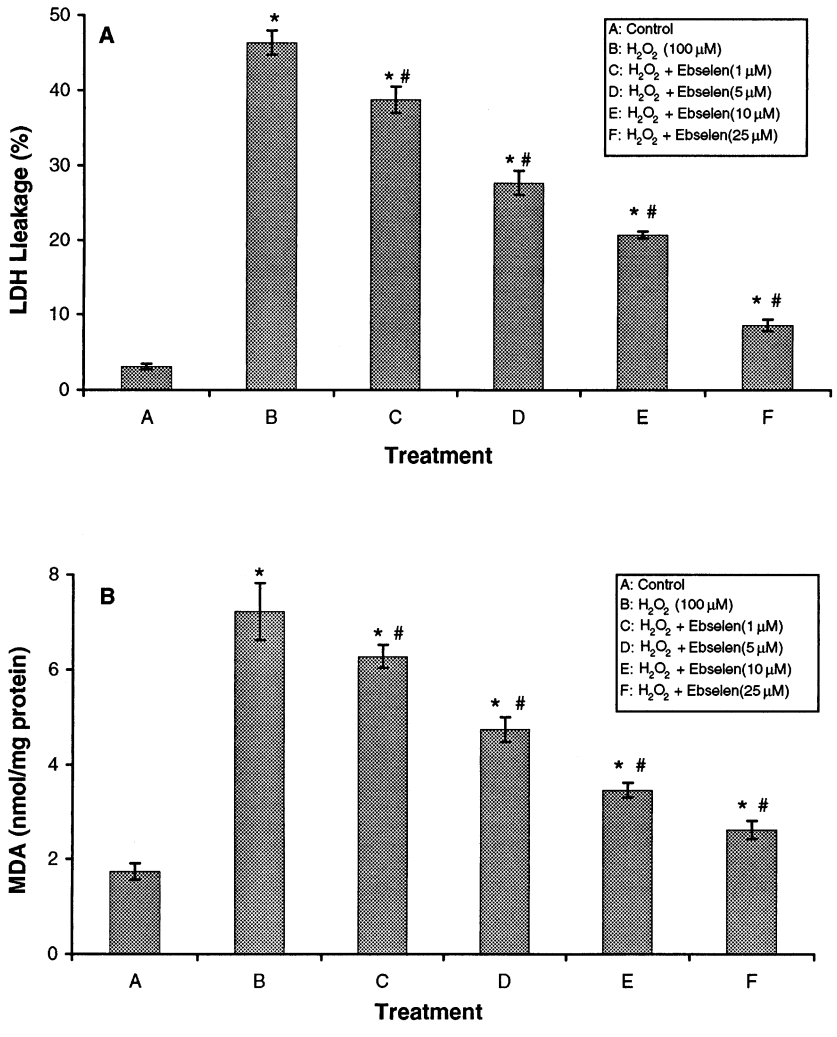

FIG. 3. Effect of ebselen on H_2O_2-induced LDH leakage (A) and MDA formation (B). $HepG_2$ Cells were precultured in 75 cm^2 flasks for 12 hr prior to H_2O_2 (100 μM) and/or ebselen (1–25 μM) treatment for 24 hr. Data are presented as means ± SD (N = 5). *$P < 0.05$ compared to the control group (Group A) and #$P < 0.05$ compared to the group treated with H_2O_2 only (Group B) (one-way ANOVA with Scheffe's test).

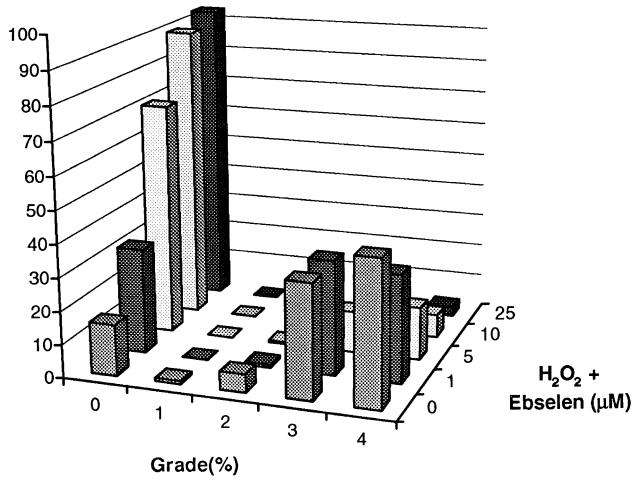

FIG. 4. Effect of ebselen on H_2O_2-induced DNA damage in $HepG_2$ cells estimated with the comet assay. Cells were treated with H_2O_2 (50 μM) and/or ebselen (1–25 μM) for 2 hr. Data are presented as percentage of grade (N = 4) and analyzed using the chi-square test.

contrast, DNA damage was significantly reduced in cells treated with ebselen. Treatment with 10 and 25 μM ebselen almost completely inhibited H_2O_2-induced DNA damage (Fig. 4). The results also indicated that treatment with ebselen up to 25 μM did not cause obvious DNA damage in $HepG_2$ cells (data not shown).

Reductive Effect of Ebselen on Intracellular ROS Level in H_2O_2-treated Cells

The concentration of intracellular ROS was evaluated by the changes in DCF fluorescence intensity. Figure 5A shows an obvious dose-dependent reduction of ebselen on the intracellular ROS level in H_2O_2-treated cells. DCF fluorescence intensity dropped significantly from 260.2 ± 22.1 in cells treated with H_2O_2 only to values between 115.6 ± 13.1 and 61.1 ± 6.22 in cells to which 1–25 μM ebselen was added (Fig. 5A).

The time-course of the reductive effect of ebselen on H_2O_2-induced DCF fluorescence intensity is presented in Fig. 5B. DCF fluorescence intensity was significantly reduced at as little as 30 min incubation with ebselen. The results also showed that with extension of incubation time, the reductive effect of ebselen on H_2O_2-induced DCF fluorescence intensity was further enhanced. Meanwhile, a significant reductive effect of ebselen on spon-

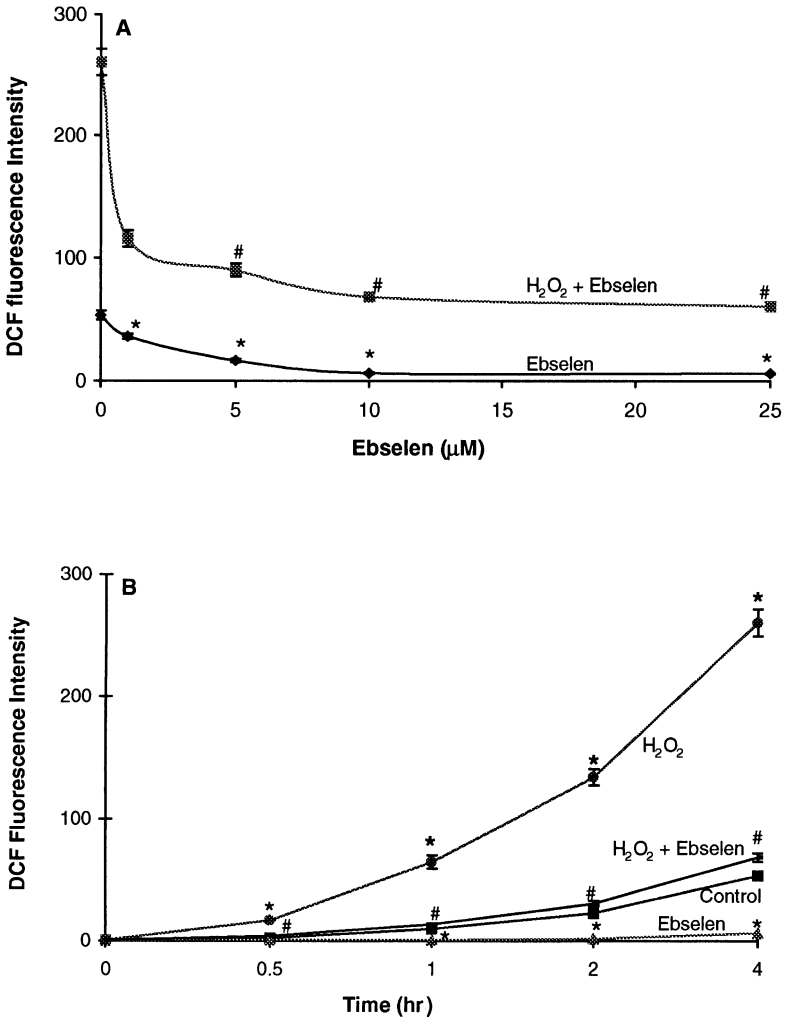

FIG. 5. Reductive effect of ebselen on intracellular ROS level in H_2O_2-treated $HepG_2$ cells as shown by dose-response (A) and time-course (B). The reaction took place with 2×10^5 cells and 5 μM DCFH-DA in 3 mL of PBS. In the dose-response study, cells were incubated with H_2O_2 (100 μM) and/or ebselen (1–25 μM) for 4 hr. In the time-course study, cells were incubated with H_2O_2 (100 μM) and/or ebselen (10 μM) for 4 hr. Data are presented as means ± SD (N = 5). *$P < 0.05$ compared to the control and #$P < 0.05$ compared to the group treated with H_2O_2 only (one-way ANOVA with Scheffe's test).

taneous DCF fluorescence intensity was also observed (Fig. 5, A and B).

DISCUSSION

As mentioned earlier, several non-cell studies have demonstrated that ebselen was capable of reducing the lipid peroxidation caused by ROS [6, 7]. However, relatively little was known about the effect of ebselen on oxidative damage induced by ROS in intact cells. Although some recent cell-free studies showed that ebselen did not prevent lipid peroxidation [9, 10], here we present evidence that ebselen possesses a strong protective effect against H_2O_2-induced oxidative damage, including DNA damage in $HepG_2$ cells.

H_2O_2 is a major component of ROS produced intracellularly during many physiological and pathological processes, and causes oxidative damage [11]. In the present study, the cytotoxic effects of H_2O_2 on $HepG_2$ cells were demonstrated by its strong inhibition on cell growth (Fig. 2), elevated LDH leakage (Fig. 3A), and MDA formation (Fig. 3B). The present results also demonstrate that ebselen reduced these effects in a dose-dependent manner. These findings suggest that ebselen is capable of reducing H_2O_2-induced cytotoxicity and lipid peroxidation.

H_2O_2 is also a well-known genotoxic agent able to induce oxidative DNA damage, including DNA strand breakage and base modification [12]. In this study, H_2O_2-induced DNA damage was assessed using the comet assay, a simple, sensitive and reliable method for detecting DNA strand breaks at the individual cell level. H_2O_2-induced alkali-labile lesions were revealed. Consistent with the protective effect on cytotoxicity caused by H_2O_2, ebselen displayed a significant protective capability against H_2O_2-induced DNA damage (Fig. 4). ROS-induced oxidative DNA damage has been implicated in mutagenesis and carcinogenesis and has attracted extensive attention in recent years [12]. Here, we present the first evidence that ebselen possesses strong protective capability against H_2O_2-induced DNA damage.

Although the antioxidative effect of ebselen has been demonstrated in several non-cell studies, its mechanism has not been fully elucidated. To further explore the mechanism of the protective effect of ebselen on H_2O_2-induced oxidative damage in $HepG_2$ cells, a fluorescent probe, DCFH-DA, was used to detect the intracellular ROS level. DCFH-DA is metabolized intracellularly to form DCFH. The nonfluorescent DCFH is then rapidly oxidized to a highly fluorescent DCF in the presence of ROS, mainly H_2O_2 [19]. DCF fluorescence intensity thus directly reflects intracellular H_2O_2 concentration. The results in Fig. 5 show that there was an elevated DCF fluorescence intensity in H_2O_2-treated cells. The data further show that ebselen-treated cells displayed a dose-dependent reductive effect on H_2O_2-induced DCF fluorescence intensity (Fig. 5A). This reductive effect appears quite early and is enhanced with time (Fig. 5B). It is also very interesting to note that ebselen is very effective in eliminating DCF fluorescence intensity in control cells. These results suggest that ebselen is capable of suppressing intracellular H_2O_2 formation.

It is believed that H_2O_2 itself is not highly reactive. The main mechanism of H_2O_2 toxicity in oxidative stress is the formation of a highly reactive species (hydroxyl radical) in the presence of transition metal ions or via other mechanisms [20]. The formation of hydroxyl radical and other ROS initiates lipid peroxidation and causes damage, including DNA damage. The decrease in H_2O_2 concentration could help to reduce hydroxyl radical formation and therefore reduce the effect caused by oxidative damage. It is thus believed that the protective effect of ebselen against H_2O_2-induced cytotoxicity and DNA damage observed in the present study is due to the reductive effect on intracellular H_2O_2 concentration. The exact mechanisms, however, are yet to be determined.

In summary, the present findings suggest that ebselen, a synthetic seleno-organic compound, is capable of protecting against H_2O_2-induced cytotoxicity and DNA damage in $HepG_2$ cells. It may be used as a potent antioxidant to protect against oxidative damage associated with elevated H_2O_2 production.

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