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Ebselen protection against hydrogen peroxide-induced cytotoxicity and DNA damage in HL-60 cells

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KEY WORDS organoselenium compounds；hydrogen peroxide；antioxidants；cultured cells；DNA damage；lipid peroxidation

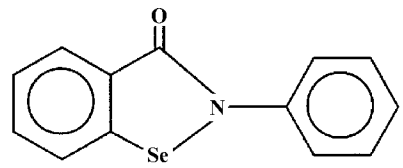
ABSTRACT

AIM：To study the protective effect of ebselen (Ebs) on hydrogen peroxide (H_2O_2)-induced cytotoxicity and DNA damage in human leukemia cell line HL-60. **METHODS**：The inhibitory effect of H_2O_2 on cell growth was determined using the tetrazolium dye colorimetric test，and the lipid peroxidation was estimated by malondialdehyde (MDA) formation. DNA damage was detected using single cell gel electrophoresis，and intracellular reactive oxygen species (ROS) formation was measured using a fluorescent probe，2′，7′-dichlorofluorescin diacetate (DCFH-DA). **RESULTS**：H_2O_2 (100 μmol·L^{-1}) suppressed the growth of HL-60 cells and the addition of Ebs (1－20 μmol·L^{-1}) reduced the suppression in a concentration-dependent manner. Furthermore，Ebs also displayed a concentration-dependent reduction of MDA formation in H_2O_2-treated cells，at the concentration of 20 μmol·L^{-1} the inhibitory rate was 56.4 %. Ebs was able to reduce the ROS formation and DNA damage caused by H_2O_2 in a concentration-dependent manner. **CONCLUSION**：Ebs has a strong protective ability against the cytotoxicity and DNA damage caused by reactive oxygen species (ROS).

INTRODUCTION

The role of reactive oxygen species (ROS) has been implicated in many age-related degenerative diseases，and antioxidants have been found to have some preventive and therapeutic effects in these diseases[1]. Therefore，substantial efforts have been made in recent years to identify both natural and synthetic antioxidants. Ebselen (Ebs)，2-phenyl-1，2-benzisoselenazol-3 (2H)-one，a synthetic seleno-organic compound，is a novel anti-inflammatory agent[2]. The preventive effect of this compound on transition metal ion-catalyzed lipoprotein oxidation has been demonstrated through scavenging of hydroperoxides[3]. In addition，Ebs also exhibits direct peroxyl radical and peroxy nitrite scavenging activity[4,5]. However，relatively little is known about Ebs's effect on ROS-induced cytotoxicity and DNA damage in intact cells.

Hydrogen peroxide (H_2O_2) is known to initiate lipid peroxidation and results in DNA damage in cells[6]. The present study was thus designed to investigate whether Ebs was able to reduce the hydrogen peroxide-induced cytotoxicity and DNA damage in a human leukemia cell line，HL-60.



Ebselen

MATERIAL AND METHODS

Cell and chemicals The human leukemia cell HL-60 was obtained from Shanghai Cell Biology Institute，Chinese Academy of Sciences. Ebselen (2-phenyl-1，2-benzisoselenazol-3 (2H)-one)，*N*，*N*′-dimethylformamide，3-(4，5-dimethylthiazol-2-yl) 2，5-diphenyl-tetrazolium bromide (MTT)，H_2O_2，thiobarbituric acid (TBA)，and ethidium bromide (EB) were all purchased from Sigma. 2，7-Dichlorofluorescin diacetate (DCFH-DA) was purchased from Molecular Probes. Other chemicals were of AR.

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Cell culture and treatment HL-60 cells were cultured in RPMI-1640 medium (containing 10 % FBS, benzyl penicillin 1×10^5 U·L^{-1}, streptomycin 100 mg·L^{-1}, pH 7.2) at 37 ℃ in 5 % CO_2. The effect of Ebs on H_2O_2-induced cytotoxicity and lipid peroxidation was tested by treating cells with different concentrations of Ebs and H_2O_2 100 μmol·L^{-1} in FBS-free medium for 24 h. H_2O_2-induced DNA damage was estimated by incubating cells with H_2O_2 100 μmol·L^{-1} for 2 h. The inhibitory effect of Ebs on intracellular ROS formation was determined by incubating cells with different concentrations of Ebs and H_2O_2 100 μmol·L^{-1} for 4 h.

Effect of Ebs and H_2O_2 on HL-60 cell growth[7] Briefly, HL-60 cells were first cultured in 96-well microplates (10^4 cells/well in 100 μL medium) for 12 h. Cells were then washed with PBS and coincubated with Ebs (1 – 20 μmol·L^{-1}) and/or H_2O_2 (100 μmol·L^{-1}) in FBS-free RPMI-1640 for 24 h. At the end of incubation, MTT 10 μL (5 g·L^{-1}) was added to each well and incubation was allowed to continue for 4 h. The formazan product was solubilized by the addition of 100 μL Me_2SO. The absorbance of each well was measured using Bio-Rad Model 3550 plate reader at 595 nm with reference at 655 nm.

Measurement of lipid peroxidation Malondialdehyde (MDA), a terminal product of lipid peroxidation, was measured to estimate the extent of lipid peroxidation in HL-60 cells. MDA concentration in cell homogenate was determined using the thiobarbituric acid (TBA) method[8]. 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 0.67 % TBA 1 mL and heated in boiling water for 1 h. After cooling, *n*-butanol 1.5 mL 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 μmol·g^{-1} protein.

Analysis of DNA damage H_2O_2-induced DNA damage was estimated using single cell gel electrophoresis (comet assay)[9]. Briefly, an aliquot 100 μL of the cell suspension was suspended in 1 mL melted 0.75 % low gelling temperature agarose in PBS at 33 ℃ giving a final concentration of about 10^7 cells·L^{-1}. The mixture was dispersed on a microscope slide pretreated with low concentration (0.1 %) agarose in order to improve the adhesion of the 0.75 % agarose onto the slide. After gel formation at 0 ℃, the slides were treated for 15 – 60 min in a lysing solution containing 2.5 % SDS and edetic acid 0.025 mmol·L^{-1}, pH 9.5. An electric field of about 5 V/cm was applied for 5 min and the slides were then washed with distilled water for 5 min, and 1 h followed by drying. The agarose layer was now very thin. The slides were stored at – 18 ℃ for further processing. After a brief short wash in distilled water the DNA was stained by immersing the slide in a solution of ethidium bromide 2 mg·L^{-1} and cells were observed through an Olympus fluorescent microscope. 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: Grade 0, no damage (< 5 %); Grade 1, low level damage (5 – 20 %); Grade 2, moderate level damage (20 – 40 %); Grade 3, high level damage (40 – 90 %); Grade 4, total damage (> 95 %).

Measurement of intracellular ROS Intracellular ROS was estimated by using a fluorescent probe, DCFH-DA[10]. 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. 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 Ebs and H_2O_2 100 μmol·L^{-1} were added to the cells simultaneously with DCFH-DA (5 μmol·L^{-1}), and incubated at 37 ℃ up to 4 h. DCF fluorescence intensity was detected at different time intervals using a luminescence spectrometer (Hitachi M850) with excitation wavelength at 485 nm and emission wavelength at 530 nm.

Statistical analysis Data were shown as $\bar{x} \pm s$ and compared by *t*-test.

RESULTS

Effect of Ebs on H_2O_2-induced cell growth suppression Cell growth was greatly suppressed in the H_2O_2-treated group (Tab 1). The treatment of Ebs (1 – 20 μmol·L^{-1}, as more than 20 μmol·L^{-1} was cytotoxic, data not shown) inhibited the suppression and increased cell growth in a concentration-dependent manner. The growth suppression was completely removed with the

addition of Ebs 10 and 20 $\mu mol \cdot L^{-1}$.

Tab 1. Effect of Ebs on H_2O_2-induced cell growth suppression. $n = 3$. $\bar{x} \pm s$. $^bP < 0.05$ *vs* the control group. $^eP < 0.05$ *vs* the group treated with H_2O_2 alone.

Drug/$\mu mol \cdot L^{-1}$	Cell viability/%
Control	100
H_2O_2 (100)	49 ± 7^b
H_2O_2 (100) + Ebs (1)	58 ± 7^b
H_2O_2 (100) + Ebs (5)	75 ± 8^{be}
H_2O_2 (100) + Ebs (10)	96 ± 9^e
H_2O_2 (100) + Ebs (20)	101 ± 12^e

Effect of Ebs on H_2O_2-induced lipid peroxidation Generation of lipid peroxidation in HL-60 cells was stimulated by H_2O_2 (Tab 2). An obvious concentration-dependent inhibitory effect of Ebs on H_2O_2-induced lipid peroxidation was noted in HL-60 cells. Treatment of cells with Ebs 1 – 20 $\mu mol \cdot L^{-1}$ reduced the MDA concentration (Tab 2). Meanwhile, compared to control, Ebs alone did not cause obvious changes in MDA formation up to 20 $\mu mol \cdot L^{-1}$ (data not shown).

Effect of Ebs on H_2O_2-induced DNA damage The effect of Ebs on H_2O_2-induced DNA damage is presented in Tab 3. It was found that HL-60 cells treated with H_2O_2 100 $\mu mol \cdot L^{-1}$ resulted in serious DNA damage. The damage mainly comprised Grade 3 and 4 categories. In contrast, DNA damage was reduced in cells treated with Ebs. Treatment with Ebs 10 and 20 $\mu mol \cdot L^{-1}$ almost completely inhibited H_2O_2-induced DNA damage (Tab 3). And treatment with Ebs alone upto 20 $\mu mol \cdot L^{-1}$ did not cause obvious DNA damage in HL-60 cells (data not shown).

Tab 2. Effect of Ebs on H_2O_2-induced MDA formation. $n = 4$. $\bar{x} \pm s$. $^bP < 0.05$ *vs* control. $^eP < 0.05$ *vs* the group treated with H_2O_2 only.

Drug/$\mu mol \cdot L^{-1}$	MDA/$\mu mol \cdot g^{-1}$ protein
Control	1.6 ± 0.2
H_2O_2 (100)	7.8 ± 0.8^b
H_2O_2 (100) + Ebs (1)	7.5 ± 0.6^b
H_2O_2 (100) + Ebs (5)	5.8 ± 0.6^{be}
H_2O_2 (100) + Ebs (10)	4.2 ± 0.3^{be}
H_2O_2 (100) + Ebs (20)	3.4 ± 0.4^{be}

Reducing effect of Ebs on intracellular ROS level in H_2O_2-treated cells The concentration of intracellular ROS was evaluated by the changes in DCF fluorescence intensity. A significant concentration-dependent reducing effect of Ebs on the intracellular ROS level in H_2O_2-treated cells was observed. DCF fluorescence intensity dropped from 258 ± 22 in cells treated with H_2O_2 alone to values between 111 ± 12 and 58 ± 7 in cells treated with H_2O_2 and Ebs 1 – 20 $\mu mol \cdot L^{-1}$. The time-course of the reducing effect of Ebs on H_2O_2-induced DCF fluorescence intensity is presented in Fig 1. DCF fluorescence intensity was reduced at as little as 30 min incubation with Ebs. The results also showed that with an increase in incubation time, the reducing effect of Ebs on H_2O_2-induced DCF fluorescence intensity was further enhanced (Fig 1).

DISCUSSION

This study presents evidence that Ebs possesses a strong protective effect against H_2O_2-induced oxidative damage, including DNA damage, in HL-60 cells. H_2O_2 is a major component of ROS produced intracellularly during many physiopathological processes, and causes oxidative damage[6]. In the present study, the cytotoxic effects of H_2O_2 on HL-60 cells were demonstrated by its strong inhibitory action on the cell growth and on increase in MDA formation. The present results also demonstrate

Tab 3. Effect of Ebs on H_2O_2-induced DNA damage in HL-60 cells estimated by the comet assay. $n = 3$. $\bar{x} \pm s$.

Drug/$\mu mol \cdot L^{-1}$	Grade/%				
	0	1	2	3	4
H_2O_2 (100)	12.3 ± 1.8	1.6 ± 0.3	2.3 ± 0.2	39 ± 4	45 ± 5
H_2O_2 (100) + Ebs (1)	36 ± 4	1.7 ± 0.2	2.5 ± 0.4	30.5 ± 2.8	30 ± 4
H_2O_2 (100) + Ebs (5)	66 ± 7	1.5 ± 0.2	2.1 ± 0.5	14.2 ± 1.6	15.7 ± 1.9
H_2O_2 (100) + Ebs (10)	91 ± 8	1.4 ± 0.3	1.8 ± 0.4	4.2 ± 0.6	2.0 ± 0.1
H_2O_2 (100) + Ebs (20)	94 ± 8	1.4 ± 0.1	1.3 ± 0.2	2.2 ± 0.1	1.3 ± 0.1

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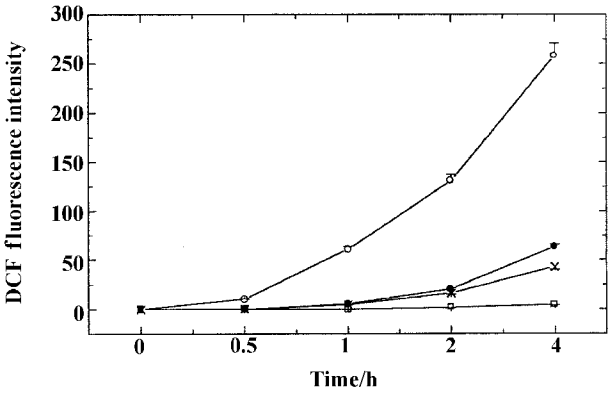

Fig 1. Reducting effect of Ebs on intracellular ROS level in H_2O_2-treated HL-60 cells. Cells were incubated with H_2O_2 (100 μmol·L^{-1}) and/or Ebs (10 μmol·L^{-1}) for 4 h. $n = 3$. $\bar{x} \pm s$. (×) Control; (○) H_2O_2; (●) H_2O_2 + Ebs; (□) Ebs.

that Ebs reduce these effects in a concentration-dependent manner. These findings suggest that Ebs may be 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[6]. 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 breakage at the individual cell level. Consistent with protective effect on H_2O_2 induced cytotoxicity, Ebs displayed a significant protective capability against H_2O_2-induced DNA damage.

Although the antioxidative effect of Ebs has been demonstrated in several non-cell studies, its mechanism has not yet been fully elucidated. To further explore the mechanism of the protective effect of Ebs on H_2O_2-induced oxidative damage in HL-60 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[11]. The results showed that Ebs treated cells displayed concentration-dependent reducing effect on H_2O_2-induced DCF fluorescence intensity, which suggests that Ebs suppressed 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 hydroxyl radical (·OH) in the prescence of transition metal ions or via other mechanisms[1]. The formation of ·OH initiates lipid peroxidation and causes damage, including the DNA damage. Oxidative base modifications typically produced by ·OH are formed under oxidative stress, and it would be expected that strand breakage were also simultaneously formed under these conditions. The decrease in H_2O_2 concentration helped to reduce ·OH formation and therefore reduced the oxidative damage. It is thus believed that the protective effect of Ebs against H_2O_2-induced cytotoxicity and DNA damage observed in the present study is due to its reducing effect on intracellular H_2O_2 concentration.

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Ebselen 拮抗 H_2O_2 对 HL-60 细胞的毒性及 DNA 损伤作用

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关键词 有机硒化合物；过氧化氢；抗氧化剂；培养的细胞；DNA 损伤；膜脂过氧化作用

目的：以 H_2O_2 造成人白血病 HL-60 细胞的氧化损伤，研究含硒化合物 Ebselen (Ebs)对氧化性损伤的影响. 方法：MTT 比色法测定细胞增殖，TBA 法检测膜脂过氧化水平，单细胞电泳法确定 DNA 损伤程度，并用荧光探针 DCFH-DA 检测细胞内活性氧自由基(ROS)水平的变化. 结果：H_2O_2 (100 μmol·L^{-1})可显著抑制 HL-60 细胞增殖，引起膜脂过氧化水平升高，Ebs 对此表现出浓度依赖性的抑制效应，Ebs 20 μmol·L^{-1}对 H_2O_2 100 μmol·L^{-1}引起的膜脂过氧化水平抑制率达 56.4 %；同样，Ebs 对 H_2O_2 100 μmol·L^{-1}造成的 DNA 损伤和细胞内活性氧水平的升高均有拮抗效应，也呈明显量效关系. 结论：含硒化合物 Ebs 对由活性氧诱发的细胞毒性和 DNA 损伤有强的防护效应.

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