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Ebselen Induced C6 Glioma Cell Death in Oxygen and Glucose Deprivation

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Abstract

Studies have shown that ebselen is an anti-inflammatory and antioxidative agent. Its protective effect has been investigated in oxidative stress related diseases such as cerebral ischemia in recent years. However, experimental evidence also shows that ebselen causes cell death in several different cell types. Whether ebselen will have beneficial or detrimental effect on cells under ischemic condition is not known. Herein, we studied the effect of ebselen on C6 glioma cell under oxygen and glucose deprivation (OGD), an *in vitro* ischemic model. We found that ebselen significantly enhanced cell death after three-hour OGD as observed by lactate dehydrogenase (LDH) release and cellular morphological changes. Further studies revealed that depletion of cellular glutathione level by the combined action of ebselen and OGD played a role in enhanced cell death as demonstrated by the following evidence: 1) Cellular GSH was significantly depleted by combined effort of ebselen and OGD, compared to ebselen or OGD insult alone; 2) Exogenous addition of N-acetyl cysteine completely diminished the cell damage induced by ebselen and OGD; 3) Supplement of glucose, which provides cellular reducing agents and thus maintains cellular GSH level, to the OGD medium diminished C6 cell damage induced by ebselen. We conclude that depleting cellular glutathione plays an important role in ebselen-induced cell death with OGD. Our results suggest that ebselen can have beneficial or toxic effect, depending on the availability of GSH.

Introduction

Experimental evidence has demonstrated that ebselen (2-phenyl-1, 2-benzisoselenazol-3 (2*H*)-one, see scheme 1 for its chemical structure), a synthetic selenium-containing heterocyclic compound, possesses antioxidative activities because it can act as a glutathione peroxidase (GPx)¹ mimic in reducing hydrogen peroxide (H₂O₂) and lipid hydroperoxides (1,2), scavenge the highly reactive species peroxynitrite (3), and inactivate free radical generating enzymes such as lipoxygenase (4,5), cyclo-oxygenase (5), and NADPH-oxidase (6). Additionally, ebselen has potent anti-inflammatory ability (6,7). Oxidative stress results from the imbalance in the neutralization of reactive oxygen species such as superoxide radical anion, H₂O₂, and hydroxyl radical, which are normally scavenged by the antioxidant enzymes superoxide dismutase, GPx, and catalase. Oxidative stress is an integral part of the cascade of events leading to cell death in many pathophysiological conditions such as ischemia (8-10). Because of its antioxidative and anti-inflammatory properties, researchers have been exploring ebselen as a protective agent in cerebral ischemia. Although Salom et al (11) recently reported

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¹Abbreviations: C6, C6 glioma cells; DMEM, Dulbecco's Modified Eagle's Medium; H₂O₂, hydrogen peroxide; LDH, lactate dehydrogenase; NAC, N-acetyl cysteine; OGD, oxygen and glucose deprivation; PBS, phosphate buffered saline (pH 7.4).

that ebselen did not provide neuroprotection to rats subjected to severe focal cerebral ischemia, others have observed its beneficial effects in animal models of cerebral ischemia (12-16) and clinical stroke trials (17,18).

However, in contrast to its reported anti-oxidative and anti-inflammatory effects, ebselen can be also toxic to cells. For example, Engman et al. (19) reported that ebselen inhibited MCF-7 cell growth. Yang et al. (20,21) observed that ebselen induced apoptosis of HepG₂ cells. Guerin and Gauthier (22) reported that ebselen induced cell death in Sp2/O-Ag14 hybridoma cells. In addition, a recent *in vivo* study showed that ebselen presented pro-oxidative effect on livers of suckling rat pups (23). The mechanism underlying the toxicity induced by ebselen is not completely understood, but it has been observed that ebselen reduces cellular glutathione (GSH) (20,21). Among small molecular antioxidants, GSH is considered vital for cell survival. With the activity of GPx and glutathione reductase, GSH serves to detoxify H₂O₂. It helps maintain the reduced state of the cysteinyl-thiol groups of proteins and rescues cells from apoptosis by buffering an endogenously induced oxidative stress (24,25). Decreased level of GSH has been regarded as a marker for increased susceptibility to oxidant injury. Brain damage associated with oxidative stress has been reported following GSH depletion (26-29).

As ischemic insults decrease cellular GSH levels and result in oxidative stress (30), we hypothesized that cells under ischemic conditions may be more sensitive and vulnerable to ebselen exposure, due to the combined action of both factors. In the current study, using oxygen and glucose deprivation (OGD) as an *in vitro* ischemic model (31), we studied effect of ebselen on C6 glioma cells. We focused our study towards addressing the following specific questions: 1) Is ebselen toxic to cells under ischemic (OGD) condition? and 2) What is the role of GSH in ebselen-induced cell death under ischemia?

Experimental Procedures

Materials

Ebselen, glucose, GSH, N-acetyl cysteine (NAC), Dulbecco's Modified Eagle's Medium (DMEM), DMEM without glucose, HO33258, penicillin, and streptomycin were purchased from Sigma Chemicals Co. (St. Louis, MO). All of the reagents were of the highest grade available.

Ebselen Concentration

The concentration range of ebselen (0.5–20 μ M) was selected to mimic doses used *in vivo*. For example, ebselen had been administered at a dose range of 100 – 400 mg/day to patients (17). Based on the assumption that the average body weight of the patients was 75 kg, the dose range used in patients was 1.33–5.33 mg/kg, which approximately corresponds to 4.9–19.4 μ M. Higher dose of ebselen ranging from 36 to 109 μ M had been used in a rodent model (13), which induced severe cellular damage to C6 cells based on our preliminary data.

Cell culture

C6 glioma cell line (C6) was obtained from Dr. Lee-Anna Cunningham's laboratory at University of New Mexico Department of Neurosciences. The cells were cultured in DMEM containing 10% heat inactivated fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μ g/ml) in a humidified 5% CO₂ and 95% air environment at 37 δ C. The medium was changed every 3 days. For each experiment, C6 were plated to 12- or 6-well plates in standard DMEM culture media at a specified density so that cells grew to confluence on the next day when experiments were performed.

Oxygen and glucose deprivation (OGD)

On the day of the experiment, the culture medium was removed, the cells were washed with warm phosphate buffered saline (PBS, pH 7.4), and then the experimental medium was added. For experiments at normal culture conditions (21% oxygen and with glucose), the experimental medium was DMEM (glucose concentration at 5.5 mM). To achieve OGD, a technique similar to that described by Newcomb-Fernandez et al. (32) was used. Briefly, the OGD experimental medium (DMEM without glucose) was previously gassed with nitrogen for 30 min, and was added to cell culture wells, which had been washed three times with PBS. OGD was induced by incubating cells in a humidified airtight chamber (Billups-Rothberg Inc., Del Mar, CA) equipped with an air lock and continuously flushed with 95% N₂/5% CO₂ for 15 min under 37 °C. The airtight chamber was then sealed, and kept in a 37 °C incubator for 3 h. Oxygen concentration was below 0.2%, as monitored by an oxygen analyzer (Sable Systems, Las Vegas, NV). Ebselen or vehicle (DMSO) was added 5 min before the start of OGD, and the DMSO concentration in the experimental medium was less than 1%.

Assay of lactate dehydrogenase (LDH) release

As a marker of cytotoxicity, LDH was measured in the supernatant of C6 cells submitted to the different experimental conditions. LDH was measured as absorbance at 490 nm spectrophotometrically using a LDH cytotoxicity detection kit (Takara Bio Inc., Shiga, Japan). Extract from cells lysed with 2% Triton x-100 was used as 100% cell death. Five sets of experiments were carried out, unless otherwise specified.

Morphological change

Cell morphology was observed with a phase-contrast microscopy. The changes in nuclear morphology were investigated by labeling the cells with the nuclear stain HO33258 and examined under a fluorescent microscopy (Nikon 70, Tokyo, Japan). Briefly, the C6 cells pre-plated in 12-well plates were treated with ebselen at different concentrations under normal condition or OGD for 3 h. After OGD, the cells were washed with cold PBS, fixed, stained with HO33258 (5 µg/ml), and observed under the fluorescence microscopy.

Measurement of cellular GSH

Cellular GSH concentration was determined by HPLC coupled with electrochemical detection according to Lakritz et al. (33). Briefly, cells were cultured in 6-well plates and exposed to OGD and/or ebselen for 3 h. Cells were scraped and collected from plates, washed with cold PBS, and lysed with 30 mM sulfonic acid and 30% methanol. The supernatants were subjected to HPLC analysis after the lysates were centrifuged at 12,000 g. HPLC chromatography was performed with an ESA 560 system (ESA Inc., Chelmsford, MA) with a C18 column (4.6 × 250 mm, ZORBAX, Agilent). Mobile phase contained 50 mM NaH₂PO₄, 0.05 mM octane sulfonic acid, and 2% acetonitrile at pH 2.7 with phosphoric acid. Flow rate was at 1.0 ml/min. Analytical channel potential was at +880 mV.

Statistical analysis

Results are expressed as mean ± S.D. A Two-tailed Student's t-test was conducted. Difference was considered significant at p<0.05.

Results

Effect of ebselen on C6 cell viability under OGD condition

To examine the effect of ebselen on cell viability of C6 under ischemic condition, experiments were carried out under both normal culture condition and OGD condition. Three different methods were used to determine cell viability, namely, LDH release, cellular morphological

change, and nuclear staining. The viability results based on LDH release are shown in Figure 1. Under normal condition, ebselen showed no effect on C6 cell viability at concentrations lower than 5.0 μM while it was toxic to C6 cells at concentration of 5.0 μM or higher after 3 h treatment. It induced 6, 8, and 60% cell death at 5.0, 10.0, and 20.0 μM , respectively. This was in accordance with the observation on HepG2 cells, which were vulnerable to exposure of ebselen at high concentration under normal culture condition (21).

Figure 1 also shows that ebselen exposure markedly augmented cell death of C6 under OGD treatment. Three hour OGD exposure alone caused 29% cell death. In the presence of ebselen, greater percentage of cell death was observed. For example, ebselen at 5 μM caused 61% cell death after 3 h OGD treatment. This observed outcome is much greater than the sum of cell death induced by OGD or ebselen alone.

Based on the above result, we selected 5 μM and 10 μM as concentration points to observe the effect of ebselen on cellular morphological and nuclear morphological changes. Representative phase contrast photomicrographs of C6 cells are shown in Fig. 2. Under normal condition, ebselen caused some observable morphological change at the concentrations of 5 μM and 10 μM (Fig. 2B and 2C). OGD for 3 h alone also caused morphological change in some cells (Fig. 2D). Under OGD, ebselen triggered significantly morphological change at the concentration of 5 μM (Fig. 2E). Severe damage was observed under OGD with 10 μM ebselen (Fig. 2F). This observation is consistent with the result of cell viability measured by LDH release (Fig. 1) that combined treatment of ebselen and OGD caused more cell death than OGD or ebselen alone.

The nuclear stain HO33258 was used to indicate the change of nuclear morphology. Examination of HO33258 staining revealed that ebselen and OGD treatments induced cellular nuclear morphologic changes. As shown in Fig. 3B, chromatin condensation and margination, as indicated by arrows, could be seen after incubation with 5 μM ebselen for 3 h under normal condition. OGD alone also induced nuclear condensation and fragmentation of cell nucleus (Fig. 3 C). The formation of apoptotic bodies was observed in some cells, as indicated by arrows. In the presence of 5 μM ebselen under OGD, nuclear condensation was observed in more cells, compared to that under normal condition with ebselen (Fig. 3D and 3B). In control cells at normal condition in the absence of ebselen, the nucleus remained intact after 3 h incubation (Fig. 3A). This observation provides further evidence that ebselen exacerbate cell damage under OGD condition.

Effect of ebselen and OGD on cellular GSH level

It has been suggested that reduced GSH may be responsible for ebselen-toxicity in HepG2 cells under normal condition (21). Meanwhile, ischemia can also contribute to cellular GSH decrease (30), which results in oxidative stress. We therefore hypothesized that ebselen and OGD causes cell death through their combined effect in reducing cellular GSH. As shown in Figure 4, in the absence of ebselen cellular GSH level was decreased to 80% after 3 h OGD. Under normal conditions, the GSH level was decreased significantly to 67% after the cells were incubated with 5 μM ebselen for 3 h. Combined treatment of OGD with 5 μM ebselen further reduced GSH levels in C6 cells to 33%. These results indicate that ebselen and OGD may act together to diminish cellular GSH.

N-acetyl cysteine (NAC) protected C6 cells from damage induced by ebselen and OGD

To investigate the role of GSH in ebselen-induced cell damage, we pretreated C6 cells with a GSH precursor, NAC, followed by concomitant ebselen and OGD exposure. The presence of 2 mM NAC markedly protected the cells against ebselen-induced cell damage not only under normal condition but also under OGD. NAC brought the levels of cell damage under combined

ebesen and OGD exposure to near those seen in the OGD only groups (Figure 5). These results are in accordance with the notion that cell damage induced by ebesen is mediated by the decrease of cellular GSH level.

Glucose ameliorated the damage of C6 Cells induced by ebesen and OGD

Glucose not only provides energy but also sustains a cellular reducing environment by generating reducing agents, such as NADPH, through pentose phosphate pathway (34-36). This pathway has been suggested as the major source of NADPH production for the maintenance of cellular GSH, which is critical in maintaining the cellular milieu in a reduced state (34-39). The finding that GSH depletion is likely the major cause of cell death in the OGD and ebesen exposure experiments (Figures 4 and 5) prompted us to explore the role of glucose in the cell death. As shown in Figure 6, the addition of glucose to the OGD medium completely inhibited cell death caused by ebesen at various concentrations (0.5–10 μ M) under OGD. This result confirmed that an insufficient GSH supply was indeed responsible for cell death induced by ebesen under OGD.

Discussion

In this study, we provide the evidence that ebesen is toxic to C6 glioma cells not only at normal culture condition, but also significantly more so under ischemic insult (OGD). The results show that ebesen and ischemic insult induces cell death together. Furthermore, GSH depletion may explain the effect of ebesen and OGD on cell viability based on three observations: 1) combined effect of OGD and ebesen on cellular GSH depletion, 2) protective effect of NAC on cell damage induced by ebesen and OGD, and 3) diminished cell damage caused by OGD and ebesen in the presence of glucose.

One of ebesen's chemical characteristics is its strong affinity to thiols (40-42). Ebesen can form a seleno-sulfide linkage with thiols. It reacts with GSH in chemical systems in less than 15 sec, with 1 mol GSH consuming 1 mol ebesen and the major product being the GSH-selenenyl sulfide of ebesen (40). It also binds SH group on proteins such as lipoate (40) and albumin (41,42). It has been suggested that ebesen inhibits enzyme functions, such as thioredoxin reductase (19), inducible nitric oxide synthase (43), lipoxygenase (4), cyclooxygenase (5) and NADPH oxidase (6), by chemically modifying SH-groups on the enzymes, forming selenosulfide complexes. Furthermore, experiments showed that proteins thiols in rat liver mitochondria and microsome membranes were decreased to about 65% and 20% of the control, respectively, when the membrane particles were incubated with 0.2 mM ebesen (44), suggesting the binding of ebesen with thiols of membrane proteins. These observations suggest that ebesen binds thiols not only in chemical systems but also in biological systems. The affinity of ebesen for thiols may contribute to its two distinctive actions. First, the reaction between ebesen and GSH or other thiols is required in order for ebesen to gain the ability to scavenge oxidants, such as hydrogen peroxide. Haenen et al (40) has proposed the mechanism of glutathione peroxidase activity of ebesen: it reacts with GSH to form selenenyl sulfide, which is further reduced by GSH to form a selenol intermediate. The selenol reacts with a selenenyl sulfide to form a diselenide, which decomposes hydrogen peroxide. Thus, GSH is regarded as the cofactor of the peroxidase activity of ebesen (40,45). Second, the reaction of ebesen with thiols consumes cellular GSH and other thiols. It has been reported that ebesen at certain concentrations depletes cellular GSH in 15 min (20,21). Besides its affinity to thiols, ebesen may also decrease NADPH, a major substrate for recycling GSH, in the presence of the thioredoxin system composing of thioredoxin, thioredoxin reductase, and NADPH (46). Together, these may lead to significant reduction of concentration of cellular thiols, resulting in inhibition of cell growth and induction of cell death, as observed in this study, as well as in previous reports (20-22). In addition, it is noteworthy to note that there is

a threshold of concentration for ebselen to cause its cellular toxicity, as shown in this report and previous reports (20-22). In other words, when ebselen concentration is high enough, it lowers cellular GSH to a level that is harmful to cells.

Oxygen and glucose deprivation mimics tissue ischemia. It's generally regarded that ischemic insults result in oxidative stress. Decreased GSH level has been reported in cells after OGD treatment (30). Although the exact mechanisms of OGD-induced GSH depletion is not completely known, there are two main factors in which glucose is involved in the explanation. First, cellular synthesis of GSH requires ATP. Cellular energy supply is minimal under OGD because of lack of both oxygen and glucose. Thus, GSH supply through the synthesis pathway is hindered. Second, a key factor for maintaining GSH levels in cells is glucose metabolism through the pentose-phosphate pathway, the activity of which provides NADPH to regenerate GSH from GSSG (34-36). In the absence of glucose as under OGD conditions, the cellular system can't regenerate GSH. In addition, free radical generation may also contribute to GSH depletion during OGD. These notions are supported by our experimental results that cell damage caused by ebselen and OGD vanished in the presence of glucose (Figure 6). The effect of glucose in protecting cells from damage induced by ebselen and OGD again is evident in that GSH depletion plays a role in the observed cell death.

Intracellular GSH plays an important role in determining cellular vulnerability (34,47,48). Under ebselen and OGD exposure, both factors contribute to cellular GSH depletion. Pre-treatment with NAC, a thiol and a precursor of GSH, diminished ebselen-induced cell damage under OGD. The result confirms that GSH depletion is responsible for the cell damage caused by ebselen and OGD. Based on the above discussion, we propose the mechanism of ebselen-increased cell death under OGD as illustrated in Scheme 2. In the absence of glucose, cells lack both the reducing power to regenerate GSH and the energy required to synthesize GSH. Meanwhile, ebselen consumes GSH by binding to it. The depleted GSH results in various molecular signaling changes, which eventually causes an increase in cell death.

It is evident from reported studies that ebselen is a neuroprotective agent in ischemic/reperfusion because of its suggested antioxidant properties (13,15,16,18). It is worth noting that all the beneficial effects of ebselen have been observed in either animal models or human subjects. In the present study, we observed different effects of ebselen in a cellular model. As discussed above, ebselen can act beneficially as a peroxidase mimic, or detrimentally through the depletion of GSH. We suggest that in a system with a sufficient GSH supply, ebselen will primarily be beneficial, and that in a system with a limited GSH supply, the harmful effects of ebselen, i.e. depleting GSH, may dominate. The cellular model used in the present study differs from the animal models and human subjects to the extent that the available amount of GSH in the presence of ebselen may be different in the two systems. This may account for the discrepancies observed in the outcomes. In addition, various thiols can bind with ebselen to form selenenyl sulfide intermediate, whose stability affects the ability of ebselen as a peroxidase mimic. For example, it has been suggested the reaction between NAC and ebselen form a stable selenenyl sulfide intermediate (49), which causes a relatively poor activity of ebselen towards the reduction of hydroperoxides. In other words, thiol cosubstrates with chemical properties that enhance conversion of the selenenyl sulfide intermediate to the selenol form enhance the catalytic activity of ebselen. Therefore, the protective effects of ebselen in whole animal models may also be the result of a greater variety of thiol cosubstrates available that enhance the formation of the catalytically active form of ebselen.

In addition, several observations *in vivo* have suggested that high dose of ebselen is not as efficient as lower dose ebselen. For example, Takasago et al. reported that ebselen at 10 mg/kg was more efficient than 30mg/kg in a rodent model of permanent middle cerebral artery occlusion (13). Yamaguchi et al observed that the ebselen at doses of 300 mg/d achieved a

better outcome than those of 400 mg/d (17). These results indicate that high dose ebselen reduces the beneficial effect resulted from a lower and optimal dose. However, the mechanism is not clear. The findings from our *in vitro* study may provide a plausible explanation on the mechanism of reduced efficacy of ebselen at higher doses.

In conclusion, this work demonstrates that ebselen significantly increased C6 glioma cell damage under ischemic condition. The overall results suggest that GSH depletion may play a role in the cell damage observed under OGD and ebselen treatment. Future studies examining the effect of ebselen on GSH level and cell death with neural and animal model may further define the interaction of ebselen and GSH in cerebral ischemia.

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Figure 1.

Effect of ebselen and oxygen and glucose deprivation (OGD) on C6 cell viability. Cell death was assessed by measuring the release of the cytosolic marker LDH. Data are expressed as means $\pm$ SD, n=5. Normal: 21% oxygen with glucose. *p<0.05 v.s. normal without ebselen; ** p<0.01 v.s. normal with 10 μ M ebselen; #p<0.05 v.s. OGD without ebselen; ##p<0.001 v.s. OGD without ebselen.

Figure 2.

Effect of ebselen and OGD on C6 cell viability observed by a phase-contrast microscopy. A) The vehicle (DMSO)-treated C6 cells under normal culture condition; B) Cells treated with 5 μ M ebselen under normal condition for 3 h; C) Cells treated with 10 μ M ebselen under normal condition for 3 h; D) Cells treated under OGD without ebselen for 3 h; E) Cells treated with OGD and 5 μ M ebselen for 3 h; F) Cells treated with OGD and 10 μ M ebselen for 3 h. (bar = 5 μ m)

Figure 3.

Fluorescence microscopy of C6 cells 3 h after cultured with ebselen under normal condition or OGD. A) The vehicle (DMSO)-treated culture shows healthy C6 cells under normal culture condition (with glucose and oxygen). B) Cells were cultured under normal condition 3 h after addition of 5 μ M ebselen. C) Cells were cultured for 3 h under OGD condition without ebselen. D) Cells were cultured for 3 h under OGD with 5 μ M ebselen. Arrows indicate chromatin condensation and margination. (bar = 5 μ m)

Figure 4.

Effect of ebselen and OGD on GSH levels in C6 cells. Cells were cultured under normal condition or OGD for 3 h in the presence or absence of 5 μM ebselen. The results are mean $\pm$ SD, $n=4-5$. * $p<0.001$ v.s. normal without ebselen; ** $p<0.05$ v.s. normal with ebselen; # $p<0.05$ v.s. normal without ebselen.

Figure 5.

Effect of N-acetyl cysteine (NAC) on ebselen and OGD induced cell damage. Cells were cultured under normal condition or OGD for 3 h in the presence or absence of 5 μM ebselen. NAC (2 mM) was pre-incubated with cells 10 min before the addition of ebselen. The results are mean ± SD, n=5. *p>0.05 v.s. no ebselen group; #p<0.05 v.s. OGD without ebselen; ##p<0.05 v.s. normal without ebselen.

Figure 6.

Effect of glucose on ebselen induced cell damage. Cells were cultured under normal condition (21% oxygen with glucose) OGD or OGD supplemented with 5.5 mM glucose for 3 h in the presence or absence of ebselen. The results are mean $\pm$ SD, n=3–5.

Scheme 1.
Chemical structure of ebselen.

Scheme 2.

Mechanism of ebselen-induced cell death under oxygen and glucose deprivation.