



Ebselen attenuates oxidative stress in ischemic astrocytes depleted of glutathione. Comparison with glutathione precursors

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Abstract:

In this study, we investigated the protective effect of ebselen, a seleno-organic compound with antioxidant activity, towards astrocyte degeneration caused by exposure to simulated *in vitro* ischemic conditions and simultaneous depletion of glutathione (GSH). Depletion of GSH was induced by 24 h pretreatment with L-buthionine-(S,R)-sulfoximine (BSO). In this experimental paradigm, we examined the effects of ebselen (1–40 μ M) on apoptosis, mitochondrial function, reactive oxygen species (ROS) production, intracellular GSH level and mitochondrial transmembrane potential (MTP). In addition, we also compared the antioxidant potential of ebselen with cystine and methionine as precursors of GSH synthesis as well as with GSH ethyl ester. Our study demonstrated that toxicity of simulated ischemia conditions was enhanced when intracellular GSH was depleted. Treatment with ebselen, especially at concentrations of 20 and 40 μ M prevented ischemia-induced cytotoxicity. Our study has shown that antiapoptotic effect of ebselen is associated with its strong antioxidant properties, preservation of MTP and possibly conservation of mitochondrial GSH during cytoplasmic GSH depletion caused by oxidative damage. Also, promoting GSH synthesis by the delivery of its substrates, like cystine or inhibition of the efflux by methionine may be a powerful strategy to minimize cell damage in the nervous tissue after ischemia.

Key words:

apoptosis, astrocytes, cystine, ebselen, glutathione, ischemia, methionine

Abbreviations: BSO – L-buthionine-(S, R)-sulfoximine, DCF – 2',7'-dichlorofluorescein, DIV – day *in vitro*, DMEM – Dulbecco's modified Eagle's medium, FBS – fetal bovine serum, GPx – glutathione peroxidase, GSH – glutathione, GSSG – glutathione disulfide, JC-1 – 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolyl-carbocyanine iodide, MCB – monochlorobimane, MPT – mitochondrial permeability transition, MPTP – mitochondrial permeability transition pore, MTP – mitochondrial transmembrane potential, ROS – reactive oxygen species, TSA – thiol-specific antioxidant enzyme

Introduction

Generation of large amount of reactive oxygen species (ROS) is considered to be a causative factor of

apoptotic or necrotic death of the central nervous system (CNS) cells and to be involved in the pathogenesis of neurological disorders, such as Alzheimer's disease, Parkinson's disease and stroke [6, 20]. ROS induce damage of DNA, enzymes, structural proteins, lipid peroxidation as well as disturb intracellular redox processes, diminish antioxidant levels (particularly glutathione, GSH) and oxidize other intracellular thiols [21]. Glial cells play a significant role in the antioxidant defense mechanisms operating in the brain due to high intracellular concentrations of antioxidants (e.g., GSH) and antioxidant enzymes, such as glutathione peroxidase (GPx), glutathione reductase, superoxide dismutase and catalase [4, 57].

The presence of GSH, a tripeptide composed of cysteine, glutamate and glycine, is necessary during

cell division, in intracellular metabolism regulation and apoptosis. Moreover, results of recent studies indicate the localization of GSH receptors on astrocytes and activation of a second messenger system by GSH. It could suggest that GSH may be also an important regulatory neuropeptide in the CNS [19].

There is considerable evidence of the involvement of GSH in astrocyte-induced neuroprotection. An increased efflux of GSH from astrocytes and decrease in intracellular GSH were demonstrated in ischemic brain and may cause oxidative stress by altering the reducing capacity of cells [26, 56]. Astrocytes also supply substrates like cysteine and dipeptide cysteinyl-glycine (CysGly) for GSH synthesis in neighboring neurons [2, 8, 10, 48]. Moreover, neurons may undergo degenerative changes when astrocytes, damaged by oxidative stress do not generate sufficient quantity of neuropeptides and nerve growth factors [27]. For this reason, a decrease in GSH in astrocytes would indirectly induce oxidative stress in neurons by depletion of essential GSH precursors [25]. Thus, various antioxidant compounds, including ebselen, are potential therapeutic agents for stroke and other neurological disturbances connected with escalated ROS production [41, 42, 60].

Ebselen [2-phenyl-1,2-benziselenazol-3(2H)-one] is a seleno-organic compound which mimics the activity of the endogenous GPx and phospholipid hydroperoxide GPx [31, 40]. The drug acts also as an oxidant at redox modulatory sites within several ligand-gated ion channels e.g. the nicotinic acetylcholine receptor and the NMDA subtype of glutamate receptor [22]. Protective effect of ebselen has been reported in various experimental *in vivo* and *in vitro* models of ischemia, as well as in human ischemic brain [30, 60]. Recently, a new ability of ebselen to inhibit apoptosis has been demonstrated in several paradigms of this type of cell death [49, 58, 61].

In the present study, we evaluated the ebselen's capacity to protect astrocytes against degeneration caused by exposure to simulated *in vitro* ischemic conditions and simultaneous depletion of GSH. Reduction in intracellular GSH level may be accomplished experimentally by pretreatment with L-buthionine-(S, R)-sulfoximine (BSO), which selectively inhibits γ -glutamylcysteine synthetase, the rate-limiting enzyme in GSH synthesis and allows for characterization of antioxidant defense mechanisms [15]. In this experimental paradigm, we examined the effects of

ebselen on apoptosis, mitochondrial function, ROS production, intracellular GSH content and mitochondrial transmembrane potential (MTP). In addition, we also compared the antioxidant potential of ebselen with cystine and methionine as precursors of GSH synthesis as well as with GSH ethyl ester.

Materials and Methods

Cell cultures

Astrocytes were isolated from one-day-old Wistar rats and cultured essentially according to the method of Hertz et al. [23]. The study was approved by the Local Ethics Committee for the Animal Experimentation. Briefly, hemispheres of newborn rats were removed aseptically from the skulls, freed of the meninges, minced and mechanically disrupted by vortexing in Dulbecco's modified Eagle's medium (DMEM) containing penicillin (100 U/ml) and streptomycin (100 mg/ml). The suspension was filtered through sterile nylon screening cloth with pore sizes 70 μ m (first sieving) and 10 μ m (second sieving). Subsequently, cultures were incubated at 37°C in 95% air and 5% CO₂ with 95% relative humidity (CO₂-Incubator, Kebo-Assab, Sweden). The cells were counted in a Coulter Z1 counter (Coulter Counter, UK). The concentration of cells in suspension was adjusted to 1×10^6 cells/ml. For MTT conversion, GSH determination, DCF and JC-1 fluorescence measurements 0.1 ml was aliquoted into each well of 96-well Nunc tissue culture plate. For Hoechst 33342 staining, astrocytes were grown on coverslips covered with poly-D-lysine (100 μ g/ml) at a density 3×10^5 cell/dish. The culture medium initially contained 20% of FBS and after 4 days was replaced with medium containing 10% FBS. The total volume of culture medium was changed twice a week. The cells were cultured for two weeks until confluence. On 14th day *in vitro* (DIV) astrocyte cultures were deprived of microglia by shaking for 5 h and incubating with 5 mM L-leucine methyl ester [54]. Under these conditions, microglial cells were detached from the layer of astrocytes and treatment with L-leucine methyl ester reduced microglial cell contamination because of destroying microglia but not astrocytes [5]. To identify astrocytes, cultures were stained immunocytochemically for glial fibril-

lary acidic protein (GFAP) (Sigma-Aldrich, St. Louis, USA), a specific marker for astrocytes. Analysis of the cultures has shown that 90–95% of cells were GFAP-positive. About 1–2% of cells in cultures reacted with *Ricinus communis* agglutinin-1, a lectin that binds to surface glycoproteins on microglia (Vector, Burlingame, USA). No neurons, as confirmed by an immunocytochemical staining method using monoclonal antibodies against MAP-2 (Promega, Madison, USA), were detected. All experiments were performed on 21-day-old cultures.

Treatment of astrocyte cultures

Prior to the experiment, the cells were incubated overnight with fresh medium. At the 21st DIV, cultures of astrocytes were placed in the medium deprived of glucose and serum, and incubated for 24 h in the ischemia simulating conditions: 92% N₂, 5% CO₂ and 3% O₂ at 37°C [11]. Osmolarity of the medium was measured and adjusted to 319 mOsm with mannitol. Ebselen, cystine, L-methionine and glutathione ethyl ester (GSH) were purchased from Sigma-Aldrich (St. Louis, USA). Astrocytes were exposed to ebselen (1, 10, 20 and 40 µM) for 24 h of simulated ischemia *in vitro*. Some astrocyte cultures were exposed for 24 h only to normoxic or ischemic conditions. In the same experiments, cultured astrocytes were also treated with cystine, L-methionine or GSH (100 µM each).

Depletion of GSH in astrocytes

To decrease GSH levels, astrocytes were pretreated for 24 h with 100 µg/ml of BSO (Sigma-Aldrich, St. Louis, USA), a potent and selective inhibitor of γ -glutamylcysteine synthetase [17]. Then, cells were washed twice with serum-free DMEM, and treated with the studied compounds.

Hoechst 33342 staining

Apoptosis of astrocytes was determined by Hoechst 33342 (Sigma-Aldrich, St. Louis, USA) staining, which allows to determine and quantify the cells with fragmented and condensed chromatin. After washing with PBS astrocytes cultured on coverslips were fixed for 10 min with a 4% paraformaldehyde at room temperature. Subsequently, after being washed twice with PBS the samples were dehydrated first in 70% ethanol and then in absolute ethanol. The samples were kept

at –20°C until they were stained with Hoechst 33342 (5 µg/ml in PBS) for 5 min at room temperature. Then, the cells were washed again with PBS. Cell nuclei analysis was conducted with the fluorescence imaging MiraCal Pro III workstation (Life Science Resources Ltd., Cambridge, UK) combined with inverted microscope Eclipse TE200 (Nikon GmbH, Düsseldorf, Germany) (ex/em 340/510 nm) with 20 × lenses. The number of apoptotic nuclei was determined on at least six randomly selected areas from three coverslips of every experimental group, each containing approximately 200 cells. The results were expressed as a percentage of apoptotic cells relative to the total number of cells.

MTT conversion

Cell viability of astrocytes treated with the studied compounds was evaluated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion method [39]. The cells ability to convert MTT indicates mitochondrial integrity and activity, which might in turn indicate cell viability. Tetrazoline ring in MTT is mainly cleaved by the mitochondrial succinate dehydrogenase and this reaction depends on the activity of the respiratory chain and the redox state of the mitochondria [39, 51].

MTT (Sigma-Aldrich, St. Louis, USA) was added to the medium at final concentration of 0.25 mg/ml three hours before the scheduled end of the experiment and then the cultures were incubated at 37°C under proper conditions. At the end of the experiment, after being washed twice with PBS, cells were lysed in 100 µl of dimethyl sulfoxide which enabled the release of the blue reaction product – formazan. Absorbance at the wavelength of 570 nm was read on a microplate reader and results were expressed as a percentage of absorbance measured in control cells (normoxia).

Cellular oxidative stress [2', 7'-dichlorofluorescein (DCF) fluorescence]

Cellular oxidative stress was determined on the basis of reactive oxygen species (ROS)-mediated conversion on 2', 7'-dichlorofluorescein diacetate (DCF-DA) into fluorescent DCF. This assay allows the measurement of cellular oxidation in viable cells [35]. The cultured astrocytes were loaded with 100 µM DCF-DA (Molecular Probes, Leiden, Netherlands) by

incubating for 50 min. Cells were washed three times with HBSS and DCF fluorescence was quantified using Fluoroscan microplate reader (Labsystems, Finland). The excitation wavelength of the dye was 485 nm, and emission was filtered using a 538 nm barrier filter. All fluorescent measurements were corrected for autofluorescence of cells not loaded with DCF-DA; the value was constant throughout the experiment. ROS production was expressed as a percentage of control (normoxia).

Glutathione level

The cell-permeable monochlorobimane (MCB) has been extensively used for quantitating GSH levels in living cells. GSH is specifically conjugated with MCB to form a fluorescent bimane-GSH adduct, in a reaction catalyzed by glutathione S-transferase (GST) [52]. MCB stock (100 mM) was made in dimethyl sulfoxide (DMSO) and diluted to 100 μ M before experiment. Cultured astrocytes were loaded with 100 μ M MCB (Molecular Probes, Leiden, Netherlands) by incubating for 30 min. Cells were washed three times with PBS and bimane-GSH conjugate fluorescence was quantified using Fluoroscan microplate reader (Labsystems, Helsinki, Finland). The excitation wavelength of the dye was 395 nm, and emission was filtered using a 460 nm barrier filter. All fluorescence measurements were corrected for autofluorescence of cells not loaded with MCB; the value was constant throughout the experiment. GSH level was expressed as a percentage of control (normoxia).

Mitochondrial transmembrane potential [JC-1 fluorescence]

5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) is a sensitive fluorescent dye used to determine the mitochondrial transmembrane potential (MTP) [46]. The uptake of JC-1 dye is directly related to MTP across the mitochondrial inner membrane [35]. The cultured astrocytes were loaded with 10 μ M JC-1 (Molecular Probes, Leiden, Netherlands) for 20 min by incubation at 37°C. Cells were washed three times with HBSS and depolarization of inner mitochondrial membrane was assessed by JC-1 fluorescence measurement using Fluoroscan microplate reader (Labsystems, Helsinki, Finland). The excitation wavelength of the dye was 485 nm, and emission was filtered using 590 nm barrier filter (J-

aggregates). During the measurements, cells were maintained at 37°C and protected from light. Fluorescence intensity was measured for < 2 s to minimize photobleaching. All fluorescent measurements were corrected for autofluorescence of cells not loaded with JC-1; the value was constant throughout the experiment. In control study, no photobleaching was observed during fluorescence assay. JC-1 red fluorescence intensity was expressed as a percentage of control (normoxia).

Statistical analysis

Data were analyzed using two-way analysis of variance (ANOVA) followed by the Dunnett's test. In all analyses, $p < 0.05$ was considered to be statistically significant. All data were expressed as the mean \pm SD.

Results

Effect of ebselen and GSH precursors on apoptosis

In the initial experiment, we investigated the effect of ebselen and GSH precursors on the apoptotic death of astrocytes depleted of GSH and exposed to simulated ischemia conditions *in vitro* (Fig. 1). Quantitative results were obtained by counting the number of apoptotic cells stained with the Hoechst 33342. We have found that simulated ischemia significantly increased the number of apoptotic nuclei in comparison with the normoxic conditions. Pretreatment of the cell cultures in normoxia with BSO had no effect on the escalation of apoptotic process. The apoptosis rate after pretreatment of the cells with BSO during ischemia was 74.6%. In subsequent experiments, the cells were maintained for 24 h in simulated ischemic conditions in the presence of ebselen, methionine, cystine and GSH ethyl ester. Ebselen at all used concentrations (1–40 μ M) provided dose-dependent protection against ischemia-induced cell death. The protective effect of ebselen reached a maximum at 40 μ M, i.e. 10.8% apoptotic cells in cultures exposed to ischemia was observed (Fig. 1). Treatment with methionine, cystine and GSH ethyl ester during 24 h ischemic conditions turned out to be the effective method of the prevention of astrocyte apoptosis.

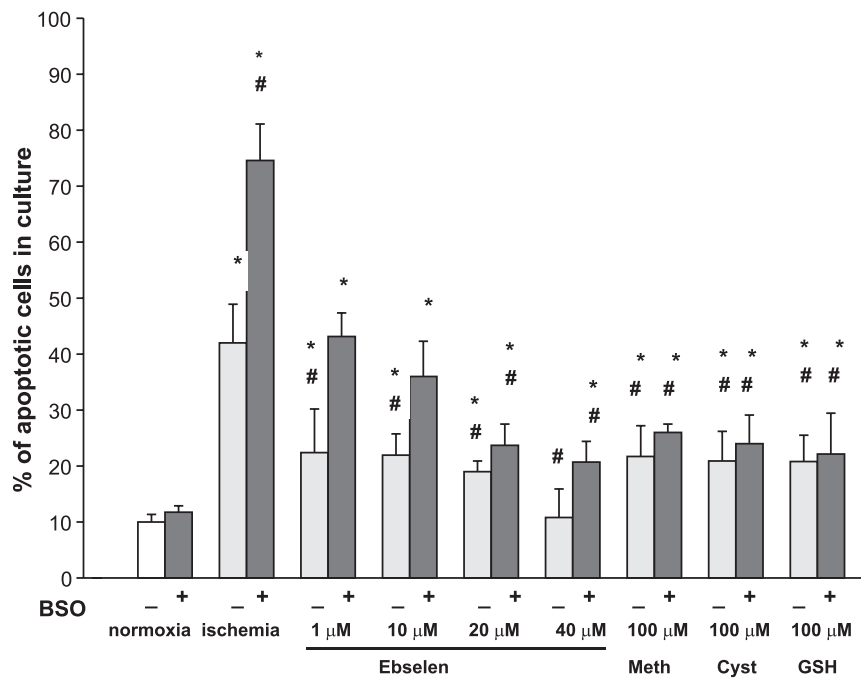


Fig. 1. Effect of ebselen, methionine (Meth), cystine (Cyst) and glutathione ethyl ester (GSH) on apoptosis of cultured rat astrocytes exposed to normoxia or simulated ischemia. To induce intracellular GSH depletion, some of the cell cultures were preincubated with 100 μM BSO for 24 h before the addition of the compounds. The cell nuclei were stained with Hoechst 33342 and then visualized with a fluorescent microscope. Astrocytes with fragmented or condensed DNA and apparently normal DNA were counted. The results are shown as a percentage of the apoptotic nuclei to the total amount of nuclei in the field and are the mean ± SD of the six randomly selected areas from three culture dishes; * p < 0.05 vs. normoxia; # p < 0.05 vs. ischemia

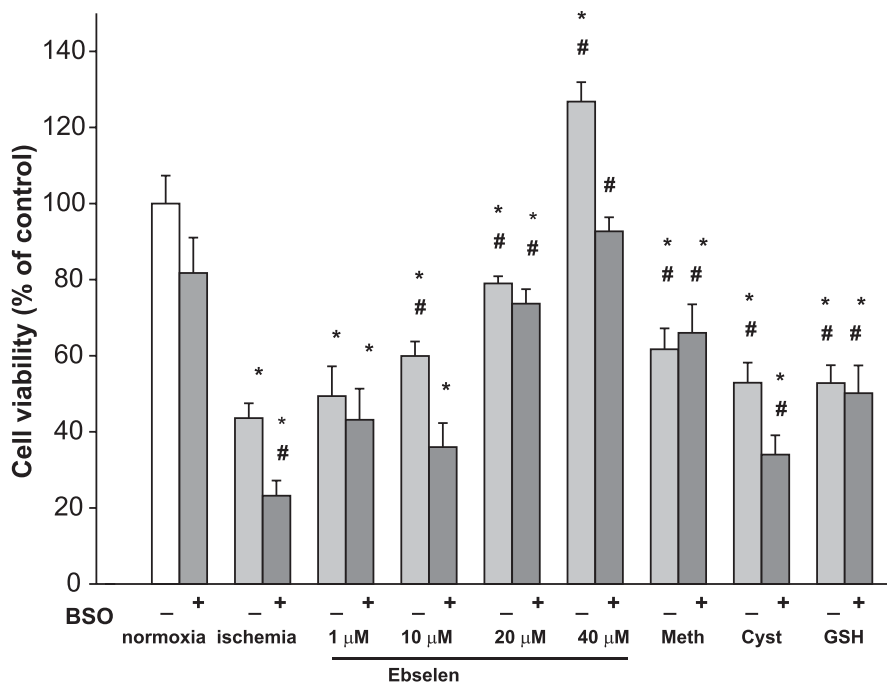


Fig. 2. Effect of ebselen, methionine (Meth), cystine (Cyst) and glutathione ethyl ester (GSH) on cell viability cultured rat astrocytes exposed to normoxia or simulated ischemia as measured by MTT conversion. To induce intracellular GSH depletion, some of the cell cultures were preincubated with 100 μM BSO for 24 h before the addition of the compounds. The results are presented as a percentage of the control value in normoxia. Each value is the mean ± SD of 12 wells in three separate experiments (n = 12); * p < 0.05 vs. normoxia; # p < 0.05 vs. ischemia

In cultures pretreated with BSO and then exposed to ebselen in ischemia, the number of apoptotic nuclei was increased in comparison with untreated cells. Furthermore, our results showed that preexposure to BSO did not affect the antiapoptotic effect of GSH precursors.

Effect of ebselen and GSH precursors on MTT conversion

Figure 2 presents the effect of ebselen and GSH precursors on MTT conversion into formazan dye in the cultures of rat astrocytes compared to normoxic and ischemic controls. Pretreatment of normoxic cultures

depleted of GSH with ebselen also attenuated the effect of BSO. GSH precursors intensified intracellular MTT conversion as well, however, treatment with cystine did not change this parameter in ischemic cells previously depleted of GSH.

Effect of ebselen and GSH precursors on ROS production

Figure 3 shows the effects of ebselen and GSH precursors on cellular oxidative stress compared to normoxic and ischemic controls. Simulated *in vitro* ischemia increased DCF fluorescence, indicating that ischemic conditions stimulated ROS production. Ad-

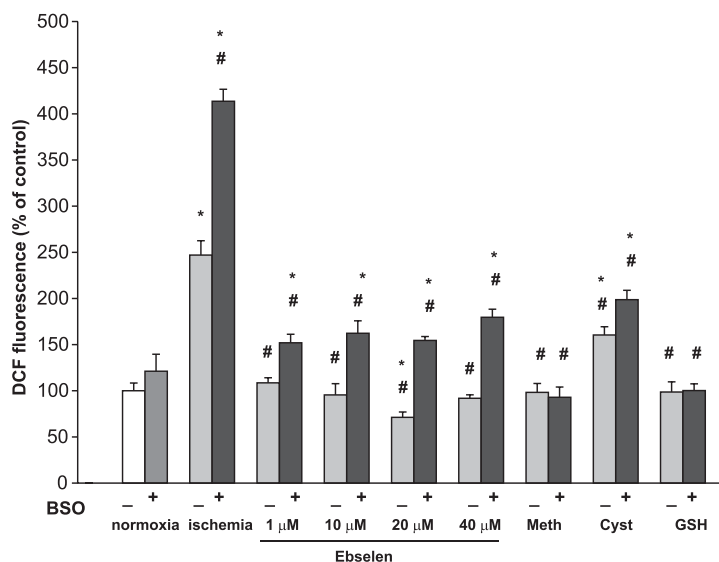


Fig. 3. Effect of ebselen, methionine (Meth), cystine (Cyst) and glutathione ethyl ester (GSH) on DCF fluorescence (as measure of ROS production) in cultured rat astrocytes exposed to normoxia or simulated ischemia. To induce intracellular GSH depletion, some of the cell cultures were preincubated with 100 mM BSO for 24 h before the addition of the compounds. The results are presented as a percentage of the control value in normoxia. Each value is the mean \pm SD of 12 wells in three separate experiments ($n = 12$); * $p < 0.05$ vs. normoxia; # $p < 0.05$ vs. ischemia

with BSO did not change cell viability in comparison with untreated cultures. The 24 h exposure of astrocytes to simulated ischemia *in vitro* resulted in attenuation of MTT conversion by 56.4% in comparison with control. Further significant decrease to the level of 23.2% of normoxic control in cell viability was observed after an additional exposure of astrocytes to BSO under ischemic conditions. A substantial ebselen (1–40 μ M)-induced increase in MTT conversion in ischemia indicated a significant restoration of mitochondrial activity. Treatment of the ischemic cul-

ditional exposure of astrocytes to BSO, which preceded ischemia for 24 h, significantly intensified DCF fluorescence. Figure 4 depicts a significant attenuation of cellular oxidative stress after treatment with ebselen observed in ischemic astrocytes. The most potent antioxidative effect was observed after administration of 20 μ M of ebselen. The effects of all ebselen concentrations as well as cystine were weaker but still significant in cultures pretreated with BSO. Treatment with methionine, cystine and GSH ethyl ester decreased the DCF fluorescence intensity after

24 h of ischemia both in BSO-treated and untreated cultures. We have observed no differences between experimental groups pretreated or not with BSO and then exposed to ischemia in the presence of methionine or GSH ethyl ester in culture medium in comparison with normoxic control group.

Effects of ebselen and GSH precursors on intracellular GSH level

Figure 4 presents the effects of ebselen and GSH precursors on intracellular glutathione level in astrocyte cultures exposed to ischemic conditions *in vitro*. Exposure of astrocytes to simulated ischemia for 24 h decreased the GSH level by about 60%.

The treatment with ebselen caused only a moderate and not significant increase in intracellular GSH level in ischemic cultures. Cystine, L-methionine and GSH ethyl ester prevented ischemia-induced depletion in GSH level. Pretreatment of cells with BSO before ischemia significantly decreased cellular GSH synthesis to 19.5%. Ebselen prevented further reduction of GSH caused by pretreatment with BSO securing GSH content at the level similar to that observed in group exposed only to ischemia. Methionine, cystine and GSH ethyl ester increased intracellular GSH concentration in ischemic astrocyte cultures. From all studied GSH precursors only cystine did not increase GSH level in cells pretreated with BSO and then exposed to ischemic condition.

Effect of ebselen and GSH precursors on MTP

We examined whether protective effects of ebselen or GSH precursors were associated with control of thiol-dependent ion channels, i.e. the mitochondrial permeability transition pore (MPTP) and prevention of mitochondrial membranes depolarization (Fig. 5).

Severe depolarization of the mitochondrial inner membrane, as indicated by the loss of the red fluorescence (J-aggregates) was observed in ischemic astrocytes both pretreated or not with BSO (51.8 and 68.7%, respectively) (Fig. 5). Ebselen under ischemic conditions increased JC-1 fluorescence in a range from 2 to almost 7 times at 1 μ M and 40 μ M concentrations, respectively. Administration of ebselen during 24 h ischemia significantly increased the red JC-1 fluorescence, indicating that the compound prevented mitochondrial depolarization. Moreover, the treatment of the cells in which intracellular GSH was de-

pleted by BSO with ebselen also significantly reduced 24 h ischemia-induced depolarization as shown by the increase in the red JC-1 fluorescence intensity. Furthermore, the administration of GSH precursors in both experimental variants did not prevent mitochondrial membranes depolarization in comparison with the normoxic control suggesting that mitochondrial membranes were permeabilized.

Discussion

Ebselen is a selenium-containing heterocyclic compound exhibiting neuroprotective potential. Because of sequestered selenium, ebselen, in contrast to dietary selenium is not cytotoxic [55]. Ebselen can induce anti-inflammatory and immunomodulatory activities as well as reduce lipid hydroperoxides [45, 50, 53]. The main protective effect of ebselen has been linked to its ability to mimic GPx activity [53], especially activity of hydroperoxide GPx [55]. In the cultured spinal cord neurons, Małeckı et al. [33] observed that exposure to ebselen increased cellular GSH and this effect was responsible for ebselen-mediated prevention against 4-hydroxynonenal-induced oxidative stress. Furthermore, it was shown that ebselen could inhibit activities of several potentially prooxidative enzymes, including lipoxygenases, NADPH-oxidase, protein kinase C and nitric oxide synthase [50]. In clinical practice, neuroprotective effects of ebselen were demonstrated in patients with aneurysmal subarachnoid hemorrhage [47] or acute ischemic stroke [44, 60].

The purpose of the present study was to determine whether the antioxidative potential of ebselen is involved in its protective effects against simulated *in vitro* ischemia-induced apoptosis in primary astrocyte cell cultures depleted of intracellular GSH. Previously, in this experimental model we have shown that ischemic conditions *in vitro* induced chromatin condensation, caspase-3 activation, decrease in efficiency of antioxidative mechanisms, disturbances in energy metabolism and cell death [12]. We also have shown that exposure of GSH-depleted astrocytes (through pretreatment with BSO) to ischemia resulted in an increased cell sensitivity to oxidative stress and apoptosis by mitochondrial cell death pathways [13].

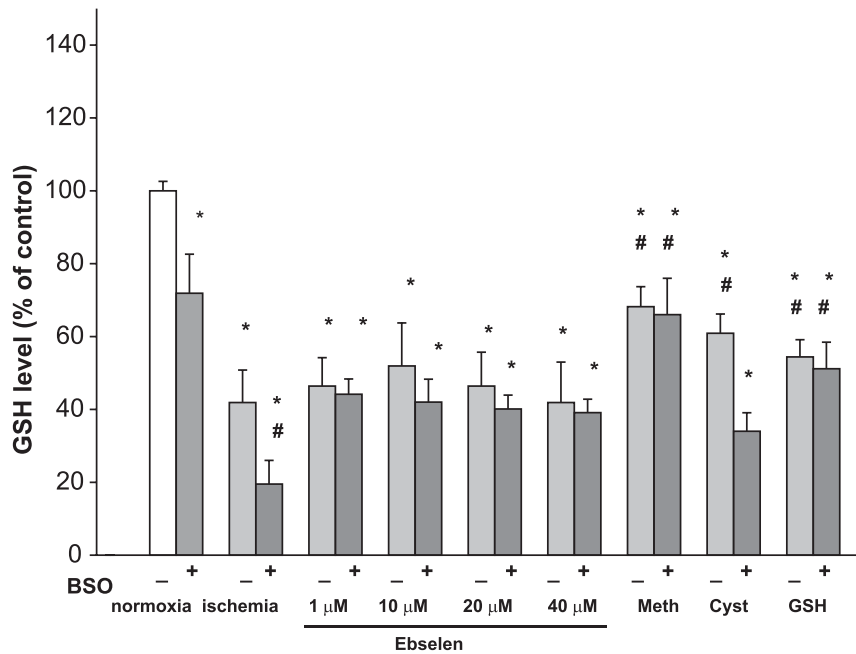


Fig. 4. Effect of ebselen, methionine (Meth), cystine (Cyst) and glutathione ethyl ester (GSH) on intracellular GSH level in cultured rat astrocytes exposed to normoxia or simulated ischemia. To induce intracellular GSH depletion, some of the cell cultures were preincubated with 100 μ M BSO for 24 h before the addition of the compounds. The results are presented as a percentage of the control value in normoxia. Each value is the mean \pm SD of 12 wells in three separate experiments (n = 12) * p < 0.05 vs. normoxia; # p < 0.05 vs. ischemia

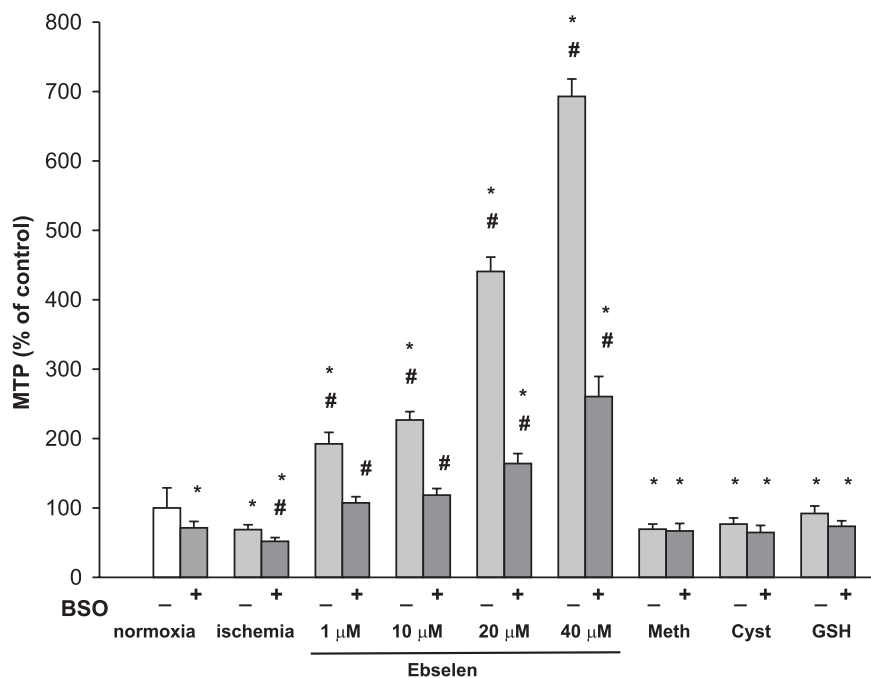


Fig. 5. Effect of ebselen, methionine (Meth), cystine (Cyst) and glutathione ethyl ester (GSH) on mitochondrial transmembrane potential (MTP) in cultured rat astrocytes exposed to normoxia or simulated ischemia. To induce intracellular GSH depletion, some of the cell cultures were preincubated with 100 μ M BSO for 24 h before the addition of the compounds. Astrocytes were then loaded with JC-1 (10 μ M) for 20 min at 37°C. The results are presented as a percentage of the control value in normoxia. Each value is the mean fluorescence value obtained from 12 wells \pm SD in three separate experiments (n = 12); * p < 0.05 vs. normoxia; # p < 0.05 vs. ischemia

In this study, we have observed a decrease in intracellular GSH level in astroglial cell culture after 24 h of simulated ischemia (Fig. 4). Furthermore, we have observed that after 24 h ischemia, the total GSH level and mitochondrial function decreased in parallel (Fig. 2 and 4). These changes were also connected with an increase in ROS production and disturbances in MTP (Fig. 3 and 5). Our results on GSH depletion are similar to values obtained by Dringen et al. [9] during starvation of cultured glial cells. Additionally, pretreatment of cell culture with 100 μ M BSO almost completely depleted astrocytes of GSH (Fig. 4).

BSO, a relatively specific inhibitor of GSH biosynthesis has been frequently used to manipulate the level of GSH both *in vivo* and *in vitro* [13, 16, 36, 38]. Cerebral GSH deficiency induced in newborn rats by giving BSO was associated with mitochondrial swelling with vacuolization, rupture of cristae and mitochondrial membranes [24]. Furthermore, inhibition of GSH synthesis by BSO pretreatment has been described as a good model for oxidative injury [34]. Of the various antioxidant systems in the brain, the GSH system is particularly important and effective in controlling cellular redox states and removing of peroxide from the brain [7]. Protective activity of GSH is based on the oxidation of thiol group of its cysteine residue with the formation of glutathione disulfide (GSSG), which in turn is catalytically reduced to the thiol form (GSH) by glutathione reductase [37]. Upon oxidative stress, GSSG may either recycle to GSH or exit the cells, leading to overall GSH depletion [14]. Reversible conversion of GSH to GSSG occurs in both mitochondria and cytoplasm, but synthesis of the tripeptide occurs only in the latter compartment because mitochondria do not show the enzyme activities required for this process [16]. Therefore, mitochondrial GSH originates from the cytosol and is imported into these organelles by a system that contains a high-affinity transporter [24].

Mitochondria are the main generators of superoxide anion radicals and hydrogen peroxide under pathophysiological situations and for this reason GSH level has a strong impact on protecting cells against oxidative stress [32].

When GSH level is deeply decreased, a significant fraction of oxygen is converted, apparently through superoxide, to hydrogen peroxide, which produces extensive mitochondrial damage [23].

We have observed antiapoptotic effect of ebselen during ischemic injury of glial cells, which was sig-

nificantly dependent on the concentrations used (Fig. 1). Although the cellular mechanisms of the antiapoptotic effects of this compound are not completely elucidated, experimental data evidenced that protective effects of ebselen were associated with inhibition of cytochrome c release and caspase -3 activation, blockade of ASK1-p38, MAPK-p53 and JNK kinase phosphorylation or Erk1/2 kinase activation [49, 61]. The importance of antioxidant mechanism in ebselen protective effect was confirmed by the decrease in DCF fluorescence, which indicated a reduced ROS production (Fig. 3). Furthermore, we demonstrated that ebselen did not show significant influence on intracellular GSH content in cells exposed to ischemic conditions (Fig. 4).

It is known that an incorrect redox equilibrium may led to the miscontrol of thiol-dependent ion channels, i.e. the mitochondrial permeability transition pore (MPTP). MPTP is a multi-ion mitochondrial thiol-sensitive channel that seems to be responsible for the loss of mitochondrial membrane potential, an event that has recently been connected with the loss of glutathione during apoptosis [14, 62]. A disruption of the MTP and GSH depletion are closely associated with each other at the common stage of the apoptosis process. Numerous studies have shown that dysfunction of astrocytes observed *in vitro* after exposure to various proapoptotic factors was linked to the loss of MTP followed by cytochrome c release and caspase-3 activation [18, 57]. Our results of MTP measurement indirectly indicated that strong effect of ebselen on mitochondrial permeability transition might be involved in protective effect of the drug against ischemia in astrocytes. We have observed that ebselen significantly prevented mitochondrial depolarization in ischemic astrocytes as measured by JC-1 fluorescence (Fig. 5). Kowaltowski et al. [28] also described the inhibitory effect of ebselen on mitochondrial swelling and membrane protein thiol oxidation induced by Ca^{2+} and P_i at concentrations similar to thiol-specific antioxidant enzyme (TSA). This effect required the concomitant presence of GSH. The authors postulated that ebselen plus GSH, catalase and TSA remove mitochondria-generated H_2O_2 , which diffuses through the membrane and would bind to horseradish peroxidase. They proposed that the presence of these peroxidases, by decreasing significantly the concentrations of extramitochondrial H_2O_2 , would stimulate H_2O_2 diffusion through the membrane, resulting in lower intramitochondrial H_2O_2 concentrations. This would

decrease the intramitochondrial generation of the highly reactive hydroxyl radical, probably the main radical species responsible for oxidation leading to mitochondrial permeability transition (MPT) [28, 59].

In order to estimate the antioxidant properties of ebselen, we also conducted a series of studies testing cystine, methionine and GSH ethyl ester as the intracellular GSH precursors. It is worth noting that brain cells do not express the cystathionine- γ -lyase, an enzyme involved in the transsulfuration pathway in the process of GSH synthesis [3]. For this reason, to synthesize GSH both astrocytes and neurons are dependent on plasma cysteine derived primarily from the liver [25]. In plasma, cysteine is converted to the oxidized form, cystine and subsequently transported to the astrocytes for intracellular GSH synthesis [25]. In contrast to glial cells, neurons are unable to take up cystine, which seems to be the best donor of cysteine for astrocytes [29, 43, 48]. Thus, the availability of cystine determines intracellular GSH level and resistance of glial cells to oxidative stress. In our study, the GSH resynthesis in the presence of cystine was inhibited in ischemic astrocytes pretreated with BSO (Fig. 4).

Kranich et al. [29] have shown that methionine and homocysteine were not able to substitute for cysteine during resynthesis of glutathione in astroglial cultures due to lack of cystathionine- γ -lyase activity. On the other hand, it was proved that methionine in different cell lines could inhibit GSH extrusion and in this way protected the cells from various proapoptotic agents [14]. In our study, methionine increased the GSH level and reduced apoptosis when GSH neosynthesis was inhibited or not by BSO, implying that the mechanism of its protective action was probably mediated by inhibition of GSH extrusion (Fig. 4). The GSH level in ischemic astrocytes showed also a statistically significant increase when the cultures (both pretreated or not with BSO) were exposed to GSH ethyl ester, an esterified form of GSH, which is able to cross the cell membrane against the concentration gradient [1, 25]. In contrast to cystine, GSH restoration from methionine or GSH ethyl ester was not influenced by the inhibitor (Fig. 4).

In conclusion, exposure of GSH-depleted cultures of rat cortical astrocytes to simulated ischemia *in vitro* increased cellular oxidative stress level, disturbed mitochondrial function and decreased cell viability. Cytotoxicity was prevented by treatment of the cells with ebselen, especially at concentrations of 20 and 40 μ M.

Our study has shown that antiapoptotic effect of ebselen is associated with its strong antioxidative properties, preservation of the mitochondrial transmembrane potential and possibly conservation of mitochondrial GSH during cytoplasmatic GSH depletion caused by oxidative damage. Also, promoting GSH synthesis by the delivery of the substrates like cystine or inhibition of GSH efflux by methionine may be a powerful strategy to minimize cell damage in the nervous tissue after ischemia.

Acknowledgments:

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