Ebselen Alters Mitochondrial Physiology and Reduces Viability of Rat Hippocampal Astrocytes

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The seleno-organic compound and radical scavenger ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) have been extensively employed as an anti-inflammatory and neuroprotective compound. However, its glutathione peroxidase activity at the expense of cellular thiols groups could underlie certain deleterious actions of the compound on cell physiology. In this study, we have analyzed the effect of ebselen on rat hippocampal astrocytes in culture. Cellular viability, the intracellular free- Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$), the mitochondrial free- Ca^{2+} concentration ($[\text{Ca}^{2+}]_m$), and mitochondrial membrane potential (ψ_m) were analyzed. The caspase-3 activity was also assayed. Our results show that cell viability was reduced by treatment of cells with ebselen, depending on the concentration employed. In the presence of ebselen, we observed an initial transient increase in $[\text{Ca}^{2+}]_c$ that was then followed by a progressive increase to an elevated plateau. We also observed a transient increase in $[\text{Ca}^{2+}]_m$ in the presence of ebselen that returned toward a value over the prestimulation level. The compound induced depolarization of ψ_m and altered the permeability of the mitochondrial membrane. Additionally, a disruption of the mitochondrial network was observed. Finally, we did not detect changes in caspase-3 activation in response to ebselen treatment. Collectively, these data support the likelihood of ebselen, depending on the concentration employed, reduces viability of rat hippocampal astrocytes via its action on the mitochondrial activity. These may be early effects that do not involve caspase-3 activation. We conclude that, depending on the concentration used, ebselen might exert deleterious actions on astrocyte physiology that could compromise cell function.

Introduction

ASTROCYTES REPRESENT THE major brain cell population in the mammalian central nervous system (CNS) and are intimately associated with neurons. This cell type was initially considered to form a substrate with supportive and metabolic roles in the CNS. However, nowadays, it is known that astrocytes provide more than a mere structural and trophic support for the neurons. Astrocytes respond with an increase in the intracellular free- Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) after stimulation with a variety of neurotransmitters, neuromodulators, and hormones. They play an important role in the developmental guidance of migrating neurons, in the regulation of neurotransmitter and ion levels, in the nutrition of neurons, and in the production of neurotrophic factors (Eysseric *et al.*, 2000). Furthermore, astrocytes communicate with other surrounding astrocytes and neurons, and thereby regulate the neuronal activity and synaptic neurotransmission (Araque *et al.*, 2001). Thus, impairment of astrocyte physiology may compromise brain function.

The seleno-organic compound 2-phenyl-1,2-benzisoselenazol-3(2H)-one (ebselen) is a substance with the radical-scavenging activity (Fujisawa and Kadoma, 2005). The ebselen

main effect involves attenuation of a free radical generation and detoxification of free radicals and the toxic products of their reactions. Its beneficial effects are mediated through its antioxidant and glutathione peroxidase-mimetic properties. Therefore, it has been extensively employed as an anti-inflammatory and neuroprotective compound.

Ebselen acts as an antiaging agent by attenuating oxidative stress in *Caenorhabditis elegans* (Avila *et al.*, 2012), protects neurons from ischemic damage via control of the expressions of GABA shunt enzymes (Seo *et al.*, 2009), has a marked inhibitory effect on neuronal damage during stroke (Yamagata *et al.*, 2008), protects against excitotoxicity induced by glutamate in isolated chick retina (Centurião *et al.*, 2005), and prevents ischemia-induced cytotoxicity and apoptosis (Gabryel and Malecki, 2006) and reactive oxygen species (ROS) production (Pérez-Ortiz *et al.*, 2004) in astrocytes.

Epidemiological evidence suggests that overexposure to selenium can facilitate the appearance of chronic degenerative diseases. Under the ebselen's activity, as an enzyme mimic, the reaction catalyzed is that of a glutathione peroxidase. Substrates for the reaction vary from hydrogen peroxide and smaller organic hydroperoxides to membrane-bound phospholipids and cholesterol hydroperoxides, in

addition to other suitable thiol compounds (Sies, 1993). Many cellular proteins contain cysteine residues, are central to cellular homeostasis, and are sensitive to redox status (Murphy, 2012a). Thus, agents that block protein surface thiols can disrupt the activity of enzymes and transporters, by means of changing their redox status (Antony and Bayse, 2011). Indeed, it has been found that concentrations of 20, 50, and 100 μ M ebselen induce cell death (Yang *et al.*, 2000; Brown *et al.*, 2009), for which the mechanism remains unclear.

We have previously shown that ebselen induced Ca^{2+} release from the endoplasmic reticulum (ER). In addition, other signs were observed; those signs could be the basis of astroglial reactivity, which might evolve into impairment of astroglial physiology (Salazar *et al.*, 2008). In spite of the great number of investigations carried out to demonstrate the actions of ebselen against established pathological events, the hypothesis that ebselen might exert side effects on normal cell physiology requires further attention.

Here we show that ebselen, beyond its putative protective role against harmful radicals, depicts a dark side on astroglial physiology. Ebselen could significantly compromise astroglial function, in such a way that it might lead to detrimental consequences in healthy cells.

Materials and Methods

Subjects

Newborn (24 h) Wistar rats were used for this study. Animals were obtained from the animal house of the University of Extremadura. Animal procedures were approved by the institutional Bioethics Committee.

Materials

Cell culture reagents, the Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salts (HBSS), and Trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) were obtained from Gibco BRL (Invitrogen, Barcelona, Spain). MITO+ (concentrated serum extender) and rat-tail collagen were obtained from BD Biosciences (Bedford, MA). Fetal bovine serum (FBS) was purchased from HyClone (Thermo Scientific, Erembodegen, Belgium). Penicillin/streptomycin was obtained from BioWhittaker (Lonza, Basel, Switzerland). Papain was obtained from Worthington Biochemical Corp. (Lakewood, NJ). AlamarBlue[®] was purchased from AbD serotec (bioNova Científica, Madrid, Spain). Ebselen, 1,2-Bis(2-amino-5-methylphenoxy) ethane- N,N,N',N' -tetraacetic acid tetrakis(acetoxymethyl) ester (Dimethyl BAPTA), ethylene glycol-bis(2-aminoethylether)- N,N,N',N' -tetraacetic acid (EGTA), N -acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (ACDEVD-AMC), and thapsigargin were obtained from Sigma Chemicals Co. (Madrid, Spain). Fura-2 acetoxymethyl ester (fura-2/AM), MitoTracker[™] Green FM, rhod-2 acetoxymethyl ester (rhod-2/AM), tetramethylrhodamine methyl ester perchlorate (TMRM), and trypsin-EDTA were purchased from Invitrogen (Barcelona, Spain). All other reagents were of analytical grade.

Preparation of cell cultures

Subcultures of astrocytes were prepared following previously established methods (González *et al.*, 2006). Briefly, following extraction of the brain, the hippocampus was

dissected and subjected to digestion with papain during 1 h at 37°C. After enzymatic digestion the tissue was washed, resuspended in a culture medium consisting of DMEM supplemented with FBS (10%), penicillin (20,000 IU), streptomycin (0.1 mg/mL), and MITO+ (0.1%), and mechanically disaggregated by gently pipetting the tissue through tips of small diameter. Cells were then plated in 75-cm² flasks and allowed to grow in a culture medium at 37°C in a humidified incubator (5% CO_2); cultures were confluent 10–14 days after seeding.

Thereafter, the culture medium was removed and replaced by the HBSS medium containing trypsin-EDTA. Cells were detached by 5-min incubation in this medium. The trypsinization step was stopped by adding a medium consisting of the culture medium supplemented with 0.25% bovine serum albumin and 0.25% trypsin inhibitor. Cells were centrifuged (50–60 g, 5 min) and resuspended in the culture medium. Finally, the cells were reseeded again in 75-cm² flasks and grown in the culture medium in a humidified incubator at 37°C and 5% CO_2 . Cells reached confluence 10–12 days after reseeding. On the day of use, cells were detached by trypsinization, centrifuged (50–60 g, 5 min), and resuspended in a Na- N -(2-hydroxyethyl) piperazine- N' -(2-ethanesulfonic acid (HEPES) buffer containing (in mM) 140 NaCl, 4.7 KCl, 1.3 CaCl_2 , 1 MgCl_2 , 10 HEPES, 10 glucose, and 0.2% bovine serum albumin (pH adjusted to 7.4). The purity of astrocytes culture obtained with this technique has been previously assessed by GFAP immunostaining in our laboratory. In our conditions, we can assume that over 95% of the cells were astrocytes (González *et al.*, 2006).

Cell viability assay

Analysis of cell proliferation and cytotoxicity under the different treatments applied was analyzed using Alamar-Blue. This is a proven cell viability indicator that uses the natural reducing power of living cells. The active ingredient of AlamarBlue (resazurin) is a nontoxic, cell permeable compound that is blue in color and virtually nonfluorescent. Upon entering cells, resazurin is reduced to resorufin, which produces very bright red fluorescence. Viable cells continuously convert resazurin to resorufin, thereby generating a quantitative measure of viability and cytotoxicity.

To evaluate cell survival, once isolated, cells were centrifuged and transferred to a culture medium of the following composition: DMEM supplemented with FBS (10%), penicillin (20,000 IU), streptomycin (0.1 mg/mL), and MITO+ (0.1%). This medium was prepared under sterile conditions. Then, the cells were seeded onto independent Petri dishes (35-mm diameter) at a cell density of 30,000 per dish and incubated in the culture medium in a humidified incubator at 37°C and 5% CO_2 . Twenty-four hours after isolation, cells were washed twice with standard phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4 ; pH adjusted to 7.4) to remove death cells, and a fresh culture medium was added to the dishes. At this point, AlamarBlue was added to the dishes at the desired concentration and the cells were challenged with the stimuli. Determinations of cell viability were performed according to the manufacturer's directions.

Data of cell viability show the mean reduction of AlamarBlue expressed in percentage $\pm$ S.E.M. (n) with respect to

control (nonstimulated) cells, where n is the number of independent experiments.

Determination of intracellular free- Ca^{2+} concentration

Changes in $[\text{Ca}^{2+}]_c$ were monitored following changes in fura-2-derived fluorescence. For dye loading of the cells, the culture medium was replaced by a physiological solution containing 130 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl_2 , 1 mM MgCl_2 , 1.2 mM KH_2PO_4 , 10 mM glucose, 10 mM HEPES, and 0.2% bovine serum albumin (pH=7.4 adjusted with NaOH). Cultured cells were then loaded with the fluorescent ratio-metric Ca^{2+} indicator fura-2 by incubation with fura-2/AM (4 μM) at room temperature (23°C–25°C) for 40 min as previously described (González *et al.*, 2002). Changes in fura-2-derived fluorescence closely report changes in $[\text{Ca}^{2+}]_c$ (Gryniewicz *et al.*, 1985).

For monitorization of Ca^{2+} -dependent fluorescence signals, the coverslip with cultured astrocytes was mounted on an experimental perfusion chamber and placed on the stage of an epifluorescence inverted microscope (Nikon Diaphot T200, Melville, NY). The cells were continuously superfused with a control Na-HEPES buffer containing (in mM) 140 NaCl, 4.7 KCl, 2 CaCl_2 , 2 MgCl_2 , 10 HEPES, and 10 glucose (pH adjusted to 7.4).

For fura-2 fluorescence changes determination, an image acquisition and analysis system for video microscopy was employed (Hamamatsu Photonics, Hamamatsu, Japan). Cells were alternatively excited with light from a xenon arc lamp passed through a high-speed monochromator (Polychrome IV, Photonics, Hamamatsu, Japan) at 340/380 nm. Fluorescence emission at 505 nm was detected using a cooled digital CCD camera (Hiscam CCD C-6790, Hamamatsu, Japan) and recorded using dedicated software (Aquacosmos 2.5, Hamamatsu Photonics, Hamamatsu, Japan). All fluorescence measurements were made from areas considered individual cells.

All stimuli were dissolved in the extracellular Na-HEPES buffer, and applied directly to the cells in the perfusion chamber. When Ca^{2+} free conditions were applied, the Na-HEPES buffer contained no added Ca^{2+} and was supplemented with 0.5 mM EGTA. In a set of experiments, cells were loaded with dimethyl 1,2-bis(2-aminophenoxy)ethane- N,N,N',N' -tetra acetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) (10 μM for 30 min), and then challenged with stimuli in the absence of extracellular Ca^{2+} (medium supplemented with 0.5 mM EGTA).

Experiments were performed at room temperature (23°C–25°C) and results are expressed as the mean ratio of fluorescence emitted at both excitation wavelengths previously normalized to the basal (resting) fluorescence $\pm$ SEM, n (where n is the number of independent experiments/number of analyzed cells of each treatment).

Determination of mitochondrial free- Ca^{2+} concentration

Determining changes in mitochondrial Ca^{2+} signals ($[\text{Ca}^{2+}]_m$) was performed following previously established methods (González *et al.*, 2000). Briefly, cells were loaded with rhod-2/AM (8 μM) at 4°C for 15 min. After incubation with rhod-2/AM, the extracellular medium was replaced by a fresh Na-HEPES buffer, and cells were incubated at room temperature (23°C–25°C) for 30 min. At the concentration used, rhod-2 accumulates within mitochondria. An increase

in rhod-2 fluorescence reflects an increase in $[\text{Ca}^{2+}]_m$. For monitoring fluorescence signals, coverslips with dye-loaded cells were placed in a perfusion chamber on the stage of the above-mentioned confocal system. Using a $\times 60$ oil immersion objective fluorescence, images of 256×256 pixels with a resolution of 0.287 $\mu\text{m}/\text{pixel}$ were recorded every 4 s. Excitation light at 543 nm from a 25-mW helium–neon laser was employed. Emitted fluorescence was collected through a 605/32-nm band-pass filter, employing different photomultipliers as previously described (González *et al.*, 2003). Neutral density filters had to be employed to reduce laser intensity to 1%–3% and diminish photobleaching. Results are expressed as the absolute values of fluorescence emission at the excitation wavelength employed. Data were normalized previously to the basal (resting) fluorescence values.

Mitochondrial membrane potential determination

Changes in ψ_m were recorded using the dye TMRM as described previously (González *et al.*, 2003). Cells were incubated during 30 min in the presence of 50 nM TMRM at 37°C. At the concentration used in our conditions, TMRM accumulates within mitochondria driven by the membrane potential, but autoquenching is negligible. A decrease in TMRM fluorescence reflects depolarization of ψ_m , because of diffusion of the dye to the cytosol. No TMRM was added to the medium after the initial loading period.

Fluorescence images from cells loaded with TMRM were obtained using the laser scanning confocal microscope mentioned earlier. Using a $\times 60$ oil-immersion objective, fluorescence images of 256×256 pixels with a resolution of 0.287 $\mu\text{m}/\text{pixel}$ were recorded every 4 s. TMRM was excited by the 543-nm line from a 25-mW helium–neon laser, and emission was collected through a 605/32-nm band-pass filter. Neutral density filters had to be employed to reduce laser intensity to 1%–3% and diminish photobleaching. Results are expressed as the absolute values of fluorescence emission at the excitation wavelength employed. Data were normalized previously to the basal (resting) fluorescence values.

Localization of mitochondria

Localization of mitochondria was assayed by incubation of astrocytes in the presence of MitoTracker Green FM. Loading of cells with MitoTracker Green FM (100 nM) was performed at room temperature (23°C–25°C) for 30 min. After incubation, the extracellular medium was replaced by the fresh Na-HEPES buffer. This dye has been employed as a mitochondrial marker (González *et al.*, 2003).

For monitoring fluorescence signals, coverslips with dye-loaded cells were placed in a perfusion chamber on the stage of a confocal Nikon Eclipse TE300 microscope (Nikon Instruments, Inc.) and continuously superfused with the Na-HEPES buffer. Fluorescence images from cells loaded with Mito Tracker Green FM were obtained employing a confocal laser scanning system Bio-Rad MRC 1024 (American Laser Corp., Salt Lake City, UT). Using a $\times 60$ oil immersion objective fluorescence, images of 256×256 pixels with a resolution of 0.287 $\mu\text{m}/\text{pixel}$ were recorded every 4 s.

Excitation light at 488 nm from a 100-mW argon laser was employed. Emitted fluorescence was collected through a 522/35-nm band-pass filter employing a photomultiplier, as previously described (González *et al.*, 2000). Laser intensity

was reduced to 1%–3% with neutral density filters to reduce photobleaching. The software used for the imaging was Laser Sharp MRC-1024 Version 3.2 (Bio-Rad, Deisenhofen, Germany).

Caspase activity assay

To determine the caspase-3 activity, stimulated or resting cells were sonicated and cell lysates were incubated with 2 mL of a substrate solution containing 20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM dithiothreitol, and 8.25 μ M of caspase-3 substrate, for 2 h at 37°C.

Substrate cleavage was measured at an excitation wavelength of 360 nm and emission at 460 nm. An ELISA spectrofluorimeter (Tecan Infinite M200, Grödig, Austria) was employed for the determinations. The activity of caspase was calculated from the cleavage of the specific fluorogenic substrate (AC-DEVDAMC). The data were calculated as fluorescence units per milligram of protein, and were presented as percentage $\pm$ S.E.M. (n) with respect to control (nonstimulated) cells, where n is the number of independent experiments.

Statistical analysis

Statistical analysis of data was performed by one-way analysis of variance followed by the Tukey *post hoc* test, and only p -values < 0.05 were considered statistically significant. For individual comparisons and statistics between individual treatments, we employed the Student's *t*-test and only p -values < 0.05 were considered statistically significant.

Results

Effect of ebselen on cell viability

In a former work, we proposed that astroglial physiology could be altered by ebselen (Salazar *et al.*, 2008). To investigate whether the antioxidant has any effect on cell viability, different batches of astrocytes were incubated for 24–96 h in the presence of no stimulus (control cells), ebselen (1, 10, and 100 μ M), or 100 μ M H_2O_2 . Viability of astrocytes did not change significantly along the incubation period in the presence of 1 μ M ebselen compared to control (unstimulated) cells, considered 100% (Fig. 1A). However, cell survival was reduced following incubation of cells in the presence of 10 and 100 μ M ebselen, compared to control cells. The reduction of cell survival was dependent on the time of incubation and on the concentration of ebselen used (Fig. 1A). As a control, we incubated astrocytes in the presence of 100 μ M H_2O_2 , an oxidant with negative effects on cell viability. Under this treatment, cell survival also dropped with the time of incubation (Fig. 1B). Throughout the following experiments, we used 100 μ M ebselen, because it exhibited the highest effect on cell viability.

Effect of ebselen on $[Ca^{2+}]_c$

Perfusion of astrocytes with ebselen (100 μ M), in the presence of Ca^{2+} in the extracellular medium, led to an increase in $[Ca^{2+}]_c$, consisting of an initial transient increase that was then followed by a progressive increase to an elevated value over the prestimulation level (152 cells of 152 total cells studied, $n = 4$ experiments; Fig. 2A).

On the other hand, stimulation of astrocytes with ebselen (100 μ M) in the absence of Ca^{2+} in the extracellular medium (medium containing 0.5 mM EGTA) induced a transient increase in $[Ca^{2+}]_c$ that was later followed by a slow and progressive increase toward a plateau over the prestimulation level (208 cells of 208 total cells studied, $n = 8$ experiments; Fig. 2B). The plateau value of $[Ca^{2+}]_c$ reached under these conditions was lower compared to that observed in the presence of extracellular Ca^{2+} . These results reflect a release of the ion from intracellular stores.

A phenomenon characterized by appearance of irregular bulges, blebbing, at the plasma membrane was observed following the increase in $[Ca^{2+}]_c$, when the cells were stimulated with ebselen (100 μ M) in the presence of Ca^{2+} in the extracellular medium. When astrocytes were stimulated with the antioxidant in the absence of extracellular Ca^{2+} (medium containing 0.5 mM EGTA), membrane blebbing was delayed in time, compared to the experiments performed in the presence of extracellular Ca^{2+} . Similar results were obtained when the cells were challenged with ebselen (100 μ M) in the

FIG. 1. Analysis of viability of rat hippocampal astrocytes. Cell proliferation and cytotoxicity under the different treatments applied were analyzed studying AlamarBlue[®] reduction, as described in Materials and Methods. (A) Incubation of cells in the presence of 1 μ M ebselen did not alter cell viability significantly (continuous line—open circles). However, incubation of cells with 10 μ M (dashed line—full circles) or 100 μ M ebselen (dotted line—open squares) led to a decrease in cell viability compared to unstimulated cells. (B) Incubation of cells in the presence of 100 μ M H_2O_2 led to a decrease in cell viability compared to unstimulated cells. Graphics are representative of four independent experiments (Ebs, ebselen; *** $p < 0.001$ for treatments vs. unstimulated cells). Ebselen, 2-phenyl-1,2-benzisoselenazol-3(2H)-one.

FIG. 2. Monitorization of the effect of ebselen on $[Ca^{2+}]_c$. **(A)** Time-course of changes in $[Ca^{2+}]_c$ in fura-2-loaded hippocampal rat astrocytes stimulated with 100 μ M ebselen, in the presence of Ca^{2+} in the extracellular medium. The horizontal bar indicates the time during which ebselen was applied to the cells. **(B)** Time course of the effect of 100 μ M ebselen on $[Ca^{2+}]_c$ in the absence of extracellular Ca^{2+} (medium containing 0.5 mM EGTA). The horizontal bar indicates the time during which the Ca^{2+} free medium and ebselen were applied to the cells. Traces are representative of 152–208 cells analyzed in four to eight independent experiments. $[Ca^{2+}]_c$, cytosolic free- Ca^{2+} concentration.

additional presence of 10 μ M of the intracellular Ca^{2+} chelator dimethyl-BAPTA (data not shown).

Effect of ebselen on mitochondria activity

In our experiments, we employed different fluorescent dyes that selectively accumulate into mitochondria, and report changes in physiological parameters within these organelles. Mitochondria are organelles with a high ability to sequester Ca^{2+} when it is released from the ER. Accumulated Ca^{2+} into mitochondria could mean a stimulatory signal that serves to modify the organelle activity (González *et al.*, 2000; González *et al.*, 2003). Ca^{2+} released by ebselen could then accumulate into mitochondria. As expected, when rhod-2-loaded cells were stimulated with 100 μ M ebselen, we observed a transient increase in fluorescence, related to increase in $[Ca^{2+}]_m$. Thereafter, $[Ca^{2+}]_m$ returned

toward a plateau value over the prestimulation level ($n=4$ exp/18 cell/90 mitochondrial areas) (Fig. 3).

On the other hand, the mitochondrial electron transport chain provides the energy that drives the diffusion of protons into the matrix promoting ATP synthesis, and the function of mitochondria depends on the maintenance of a ψ_m . In relation to a change in $[Ca^{2+}]_c$, depolarization of ψ_m occurs, which depends on Ca^{2+} uptake by mitochondria (González *et al.*, 2000; González *et al.*, 2003). Thus, it could be possible that ebselen had an effect on ψ_m . Therefore, we performed a series of experiments in which astrocytes were loaded with the mitochondrion-specific voltage-sensitive dye TMRM. As expected, when cells were perfused with 100 μ M ebselen which, as we have shown, induces an increase in both $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$, it could be observed a depolarization of ψ_m ($n=5$ exp/38 cell/190 mitochondrial areas). Under our experimental conditions, there was no recovery of ψ_m (Fig. 4).

The described effects for treatment of cells with 100 μ M ebselen on $[Ca^{2+}]_m$ and ψ_m were also observed when astrocytes were incubated in the presence of 20 or 50 μ M ebselen (data not shown).

Because prolonged depolarization of mitochondria could be the basis of a mitochondrial stress that could interfere with its functional network, we were also interested in evaluating the distribution of mitochondria within the cells. Localization of mitochondria was assayed by incubation of cells in the presence of MitoTracker Green FM, a dye that selectively accumulates inside these organelles (González *et al.*, 2003). Figure 5A shows a representative fluorescence image of control (unstimulated) astrocytes labeled with MitoTracker Green FM. We could observe a consistent network of mitochondria, which appeared distributed all through the cytosol. Following 5 min-incubation of astrocytes in the presence of 100 μ M ebselen, a disruption of the mitochondrial network was observed (Fig. 5B).

In addition, we observed an increase of MitoTracker Green FM-derived fluorescence in the cytosol, following treatment of cells with ebselen (Fig. 5C, continuous line). This was accompanied by a decrease in mitochondrial fluorescence (Fig. 5C, dashed line). This reflects an increase in

FIG. 3. Effect of ebselen on $[Ca^{2+}]_m$ in rat hippocampal astrocytes. Time course of changes in $[Ca^{2+}]_m$ in response to 100 μ M ebselen. A horizontal bar shows the time during which the stimulus was applied to the cells. The graphs are representative of four independent experiments. $[Ca^{2+}]_m$, mitochondrial free- Ca^{2+} concentration.

FIG. 4. Effect of ebselen on mitochondrial membrane potential (ψ_m) in rat hippocampal astrocytes. Time course of changes in ψ_m in response to 100 μ M ebselen. A horizontal bar shows the time during which the stimulus was applied to the cells. The graphs are representative of six independent experiments.

mitochondrial membrane permeability that leads to appearance of the dye, which was initially trapped within mitochondria, in the cytosol ($n=5$ exp/17 cell).

Caspase activity assay

The effect of ebselen that we have observed could be a starting point for the process of apoptosis. Therefore, we examined the effect of the antioxidant on caspase-3 activation. For this purpose, rat hippocampal astrocytes were incubated during 15 min in the presence of 100 μ M ebselen. Under these conditions, we did not observe significant changes in caspase-3 activation ($1.45\% \pm 0.98\%$) compared to unstimulated cells. As a control, treatment of cells with 100 μ M H_2O_2 , a compound that promotes apoptosis (Kaufmann *et al.*, 2003), increased the caspase-3 activity by a $20.50\% \pm 2.25\%$ ($n=5$; $p<0.001$).

Discussion

Therapy with antioxidants has been extensively used in the treatment of illness in different tissues, including the brain. In this line, the seleno-organic compound ebselen, which was originally developed as an anti-inflammatory

FIG. 5. Confocal microscopy studies of rat hippocampal astrocytes loaded with the fluorescent probe Mito TrackerTM Green FM. Employing confocal laser scanning microscopy, bright fluorescent spots could be detected, being spread all through the cytosolic area. (A) A consistent network of organelles could be observed in unstimulated astrocytes. (B) Effect of 100 μ M ebselen on the mitochondrial network organization. (C) Time course of changes in Mito Tracker Green FM-derived fluorescence in the cytosol (continuous line) and in mitochondria (dashed line) of astrocytes incubated in the presence of 100 μ M ebselen. A horizontal bar shows the time during which the stimulus was applied to the cells. The data are representative of five independent experiments.

agent, has been shown to protect against damage caused by free radicals in the CNS (Pérez-Ortiz *et al.*, 2004; Green and Ashwood, 2005; Funchal *et al.*, 2006; Gabryel and Małecki, 2006; Ghisleni *et al.*, 2008). However, in spite of the positive effects of ebselen, certain signs were observed in response to selenium-containing compounds that could be the basis of potential damaging actions. In this respect, recent points of epidemiological evidence suggest that overexposure to selenium can facilitate the appearance of chronic degenerative diseases. However, the molecular mechanisms are not fully understood.

It has been shown that selenium-containing compounds are able to open Ca^{2+} release channels at the sarco ER membrane, leading to an increase in $[\text{Ca}^{2+}]_c$ (Xia *et al.*, 2004). Moreover, emptying of the Ca^{2+} content in the ER has been related to cell stress and impairment of cell physiology (Fradejas *et al.*, 2010; Kim *et al.*, 2010). In agreement to this, we have shown in a previous work that ebselen induced Ca^{2+} release from the ER (Salazar *et al.*, 2008).

On the other hand, a leading mechanism of selenium cytotoxicity is a change in the oxidative state of cellular systems, with a resultant production of ROS (Wallenberg *et al.*, 2010).

Mitochondria are organelles with a major function in the cellular oxidative metabolism. The mitochondrial matrix contains many of the central processes of metabolism (Duchen, 2004), depicting Ca^{2+} an important role in mitochondrial physiology (Duchen *et al.*, 2008).

Ebselen mimics glutathione peroxidase, changing therefore the cell redox status (Antony and Bayse, 2011). Selenium can also interact with endogenous -SH groups (Nogueira and Rocha, 2011). Moreover, a range of mitochondrial proteins containing cysteine residues is central to mitochondrial homeostasis, and is sensitive to redox status. And agents that block protein surface thiols can disrupt the activity of enzymes and transporters (Murphy, 2012b). Derived mitochondrial dysfunction has been implicated in cellular disorders (Dröse and Brandt, 2012).

Concentrations of ebselen, ranging from 1 to 40 μM , have been widely employed in actual and past studies on different tissues and cell types, where it shows a protective action (Yamagata *et al.*, 2008; Deng *et al.*, 2010; Hassan *et al.*, 2010; Yue *et al.*, 2011; Du *et al.*, 2012). However, as stated in the introduction, ebselen might induce cell death at certain concentrations.

Here we show that ebselen induces Ca^{2+} mobilization from intracellular stores and leads to an elevated $[\text{Ca}^{2+}]_c$, confirming our previous observations (Salazar *et al.*, 2008). The effect of ebselen on $[\text{Ca}^{2+}]_c$ could not be inhibited by incubation of astrocytes in the absence of extracellular Ca^{2+} , or by incubation of cells in the presence of the intracellular Ca^{2+} chelator dimethyl-BAPTA.

Taking into account the above-mentioned effects of selenium, we hypothesized that the Ca^{2+} -mobilizing action of ebselen could be injurious to the cell, and we investigated its effects on several mitochondrial parameters.

Our results show a transient accumulation of Ca^{2+} into mitochondria, following treatment of cells with ebselen. Thus, ebselen treatment might induce changes in mitochondrial function.

In addition to $[\text{Ca}^{2+}]_m$, in our study, we have also noticed changes in other mitochondrial parameters in the presence of ebselen. First, we have observed a depolarization of ψ_m .

In a former work, it has been shown that selenium can potentially influence the activity and function of oxidoreductases within the cell (Wallenberg *et al.*, 2010). These proteins carry out a central function in maintaining the redox balance within the cell, and their imbalance can contribute to selenium toxicity. Indeed, a recent work has shown that ebselen caused a statistically significant inhibition of the mitochondrial complexes I and II activity, an effect completely reversed by reduced glutathione (Puntel *et al.*, 2013). The authors suggest that ebselen-induced mitochondrial complexes I and II inhibition can be mediated by a thiol oxidation activity, that is, ebselen can oxidize critical thiol groups from mitochondrial complexes I and II. As a consequence, inhibition of mitochondrial respiratory chain complexes will lead to mitochondrial depolarization, as we have observed.

Second, we have also observed a disruption of mitochondrial network. Mitochondria form a dynamic network responsible for energy production, Ca^{2+} homeostasis, and cell signaling. Appropriate distribution of the mitochondrial network contributes to organelle function and is essential for cell survival (Frederick and Shaw, 2007). Additionally, redistribution of mitochondria within the cell can be a component of regulatory pathways (Murphy, 2012b). It has been proposed that fragmentation of the mitochondrial network is related neurodegeneration (Almajan *et al.*, 2012). In agreement to these observations, the effect of ebselen on mitochondrial network could be deleterious to astrocyte physiology.

And third, ebselen induced an increase in mitochondrial membrane permeability. A common process associated with oxidative stress and severe mitochondrial impairment is the increase in mitochondrial membrane permeability, as described in many neurodegenerative diseases (Eckmann *et al.*, 2013). The consequences of such an alteration of mitochondrial physiology are not uncommon. As expected, ebselen induces a decrease in cell viability.

In a previous work, we have shown that ebselen (1–10 μM) did not alter the integrity of the cellular membrane of astrocytes, indicated as absence of lactate dehydrogenase release to the extracellular medium. However, in the presence of 20 μM ebselen, it was detectable a slight, but significant decrease of astrocyte viability (Salazar *et al.*, 2008).

In the present study, we demonstrate that cell viability is decreased in the presence of ebselen, as reflected by the studies on the reducing power of living cells. The analysis employing the growth indicator AlamarBlue, are based on the detection of cellular metabolism. Its active compound resazurin is effectively reduced in mitochondria, making it useful also to assess the mitochondrial metabolic activity. Thus, in addition to its utility as a marker of diminished cell viability, the decreased reduction of AlamarBlue that we have observed is an index of decreased mitochondrial function. Therefore, our observations agree with a deleterious action of ebselen on cell survival, which might be a consequence of the evoked changes in mitochondrial physiology that we have observed.

On the other hand, we did not observe involvement of the caspase-3 activity in the actions of ebselen. In our hands, the effects of this compound could be early ones that might lead to an impairment of mitochondrial function that, in turn, could impair cellular homeostasis and induce cell damage without involving apoptosis.

In conclusion, our results confirm that ebselen induces changes in mitochondrial parameters that could compromise the function of the major energy synthesizing factory within the cell. If mitochondrial homeostasis is impaired, then astrocyte function and survival could be compromised. Causing mitochondrial dysfunction may be considered an important factor in the toxicity of ebselen.

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Disclosure Statement

The authors declare that there is no conflict of interests.

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