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Dual effect of ebselen on mitochondrial permeability transition

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Abstract

This study reports an investigation on the effect of the seleno-organic compound ebselen on rat liver mitochondria. We show that low concentrations of ebselen induced an increase in rat liver mitochondrial membrane permeability, resulting in swelling and loss of membrane potential. These effects were mediated by the opening of the permeability transition pore. They required Ca^{2+}, were independent of pyridine nucleotide oxidation, and involved the oxidation of thiol groups. Ebselen pore induction is apparently promoted by the glutathione peroxidase mimicking activity of the drug. Opposite effects, that is, inhibition of both pore opening and thiol oxidation, were observed when concentrations higher than 20 μM were used. These data demonstrate that ebselen is able to modulate the opening of the permeability transition pore and that it might be a critical event for both the proapoptotic and cytoprotective activities of the drug.

Keywords: Ebselen; Mitochondrial membrane permeability; Membrane potential; Ca^{2+}; Thiol groups; Oxidation

1. Introduction

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one), is a nontoxic seleno-organic drug with anti-inflammatory, anti-atherosclerotic and cytoprotective properties. It has been shown to be a potent neuroprotective compound in stroke in humans [1] and in rodents [2,3] and also prevents tissue injuries during heart [4], liver [5] and gastric [6] ischemia-reperfusion. Its mechanism of action involves an effect on the anti-oxidant defensive system. It was shown to mimic the catalytic activities of phospholipid hydroperoxide GSH peroxidase [7], producing an increase in ROS trapping. Recent data suggest that the cytoprotective effect of ebselen may be due in part to its anti-oxidant properties at the mitochondrial level.

It has been demonstrated that ebselen protects liver mitochondria from lipid peroxidation induced by iron/ascorbate and iron/citrate [8,9]. This effect was associated with inhibition of release of the apoptogenic factor cytochrome *c* [10], which might contribute to the beneficial effect of the drug. This is in agreement with other studies indicating that ebselen possesses anti-apoptotic properties linked to its capacity to scavenge, or to inhibit formation of hydrogen peroxide [11].

Conversely, it was recently shown that ebselen was able to trigger apoptosis [12] and to inhibit the growth of human cancer cells in culture [13]. This effect was thought to be due to a mitochondrial action of the drug and, especially, to the induction of the PTP [14]. This hypothesis is particularly relevant since this phenomenon is now considered to be a critical event in the induction of the apoptotic process [15].

The present study was designed to investigate the interaction of ebselen with isolated liver mitochondria. We demonstrate that ebselen is capable of inducing the generation of the PTP in isolated liver mitochondria and that this effect is due to mitochondrial membrane thiol depletion.

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Abbreviations: GSH, glutathione; ROS, radical oxygen species; PTP, permeability transition pore; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TBARS, thiobarbituric acid-reactive substances; CsA, cyclosporin A; NEM, *N*-ethylmaleimide.

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2. Materials and methods

2.1. Isolation of mitochondria

Male Wistar rats weighing approximately 250–300 g were decapitated. The livers were excised rapidly and placed in buffer containing 250 mM sucrose, 10 mM Tris and 1 mM EGTA, pH 7.2 at 4°. The tissue was minced and homogenized on ice using a Teflon Potter homogenizer. The homogenate was centrifuged at 600 *g* for 10 min (Sorvall® RC 28 S). The supernatant was centrifuged for 5 min at 15,000 *g* to obtain the mitochondrial pellet which was washed in the same buffer and centrifuged at 15,000 *g* for 5 min.

The mitochondrial pellet was washed in the same buffer minus EGTA and centrifuged for 5 min at 15,000 *g* resulting in a final pellet containing approximately 50 mg of protein/mL. When mitochondria were isolated from rat heart, hearts were promptly excised, minced and homogenized in 5 mM MOPS, 300 mM sucrose, 1 mM EGTA, 5 mM KH_2PO_4, 0.1% bovine serum albumin, pH = 7.4 at 4°. The homogenate was centrifuged at 2800 *g* for 10 min. The supernatant was centrifuged at 8900 *g* for 10 min. The resulting pellet was washed twice in this buffer. The final pellet representing the heart mitochondria fraction contained approximately 9 mg of protein/mL.

2.2. Mitochondrial swelling measurement

Mitochondrial swelling was assessed in mitochondria energized with 6 mM succinate by measuring the change in absorbance of their suspension at 520 nm by using a Hitachi® model U-3000 spectrophotometer as described by Elimadi *et al.* [16]. Mitochondria (4 mg) were preincubated in the presence or absence of different inhibitors for 3 min in 3.6 mL of a phosphate buffer (250 mM sucrose, 5 mM KH_2PO_4, 6 mM succinate, pH 7.2 at 25°) and 1.8 mL of this suspension was added to both sample and reference cuvettes. After 1 min of incubation at 25°, the swelling was initiated by the introduction of either Ca^{2+} or ebselen to the sample cuvette only and the A_{520} scanning was started.

2.3. Determination of mitochondrial membrane potential and NAD(P)H level

Mitochondrial membrane potential was evaluated by uptake of rhodamine 123, which accumulates electrophoretically into energized mitochondria in response to their negative-inside membrane potential. 1.8 mL of phosphate buffer (250 mM sucrose, 5 mM KH_2PO_4, 1 μM rotenone, pH 7.2 at 25°), and 0.3 μM rhodamine 123 were added to the cuvette, and the fluorescence scanning of rhodamine 123 was monitored using a Perkin-Elmer SA LS 50B fluorescence spectrometer at the excitation and emission wavelengths of 503 and 527 nm, respectively. After 30 s, mitochondria (0.5 mg/mL) were added and 60 s latter mitochondrial respiration was induced by the addition of 6 mM succinate. Relative changes of membrane potential were expressed in arbitrary fluorescence units and were not converted in potential values.

Mitochondrial pyridine nucleotides (NAD(P)H) were monitored by measuring their autofluorescence at excitation and emission wavelenghts of 360 and 450 nm, respectively, in a Perkin-Elmer SA LS 50B fluorescence spectrometer, according to the procedure described by Minezaki *et al.* [17].

2.4. Measurement of membrane lipid peroxidation and protein thiol content

Lipid peroxidation was assayed as the generation of thiobarbituric acid-reactive substances according to Zini *et al.* [18]. Protein thiol content was measured according to Morin *et al.* [19] with some modifications. Briefly, mitochondria (1 mg/mL) were incubated in phosphate buffer in the presence of ebselen or other agents in a total volume of 1 mL for 15 min at 25°. After this time, 100 μL of mitochondrial solution were added to 700 μL of methanol and 200 μL of Tris–EDTA (250/20 mM, pH = 8.2 at 20°). Twenty microliters of Ellman's reagent (10 mM) were then added and the reaction mixture was incubated for 15 min at room temperature. The amount of thiol groups was estimated using the difference in absorbance at 410 nm before and after addition of Ellman's reagent corrected for the absorbance of Ellman's reagent. In order to estimate protein thiol concentration, GSH in a concentration range of 10–500 μM was assayed under the same conditions.

2.5. H_2O_2 production

H_2O_2 production was estimated by the scopoletin/horseradish peroxidase method as previously described [20]. Briefly, rat heart mitochondria (0.3 mg protein/mL) were preincubated for 1 min in a medium containing 0.25 M sucrose, 6 mM succinate, 1 mM EGTA, 10 mM KCl, and 10 mM MOPS (pH = 7.3 at 25°) supplemented with 1.2 μM scopoletin in the absence or presence of ebselen. After addition of 10 units horseradish peroxidase, the production of H_2O_2 was induced by the addition of 10 μM antimycin A and monitored using a Perkin-Elmer SA LS 50B fluorescence spectrometer at the excitation and emission wavelengths of 366 and 460 nm, respectively. The decrease in fluorescence corresponds to the oxidation of scopoletin by H_2O_2, via horseradish peroxidase. The calibration of the assay was performed by adding known amounts of H_2O_2.

2.6. Depletion and measurement of mitochondrial GSH

GSH was depleted *in vitro* by preincubation of mitochondria with 100 μM 1-chloro-2,4-dinitrobenzene for

3 min at 30° followed by removal of the agent through washing as previously described [21]. This procedure leads to a severe depletion of mitchondrial GSH without alteration of mitochondrial functions [21]. For *in vivo* depletion, the protocol of Gerard-Monnier *et al.* [22] was used. Rats were injected intraperitonealy with 4 mmol/kg diethylmaleate, killed 60 min later, and liver mitochondria were isolated as described above.

The measurement of GSH was carried out according to Barhoumu *et al.* [23] using 4-chloro-1-methyl-7-trifluoromethyl quinolinium methylsulfate as reagent. This compound was synthesized by Synthe-Pharma. Briefly, mitochondria (1 mg) were precipitated in 5% metaphosphoric acid (500 μL) and sonicated for 15 s before centrifugation at 12,000 *g* for 3 min. Three hundred microliters of supernatant were mixed with 600 μL of potassium phosphate buffer (100 mM, pH = 7.8 at 25°) containing diethylenetriamine pentaacetic acid (1 mM) and Triton X-100 (0.025%). Then, 50 μL of 4-chloro-1-methyl-7-trifluoromethyl quinolinium methylsulfate (10 mM) were added and the solution was vigorously stirred (30 s). Afterwards 50 μL of sodium hydroxyde (30%) were then added and the mixture was again vortexed. The final solution was incubated 10 min at 25° in the dark and the absorbance was read at 400 nm. In parallel, a GSH standard curve (5–100 μM) was carried out.

2.7. Chemicals and reagents

Acetoacetate, antimycin A, L-buthionine sulfoximine, CCCP, catalase, diethylmaleate, 1-chloro-2,4-dinitrobenzene, dithiothreitol, ebselen, Ellman's reagent, horseradish peroxidase, β-hydroxybutyrate, monobromobimane, rhodamine 123, ruthenium red, scopoletin, *tert*-butylhydroperoxide, ubiquinone 0 were purchased from Sigma. Diethylenetriamine pentaacetic acid was obtained from Aldrich and NEM from Merck. CsA was kindly offered by Novartis Laboratories. Ebselen was solubilized in dimethylformamid at 0.02 M and then diluted in water.

3. Results and discussion

3.1. Dual effect of ebselen on mitochondrial swelling

Recent findings suggest that PTP could be a critical event in ebselen-induced apoptosis. In order to verify this hypothesis and to study the mechanism by which ebselen can induce PTP, isolated liver mitochondria energized by succinate were incubated in a phosphate buffer in the presence of increasing concentrations of ebselen. Fig. 1A shows that ebselen-induced mitochondrial swelling as revealed by the large decrease in the A_{520} of the suspension. This effect was concentration-dependent and a maximal effect was obtained around 10–15 μM. A parallel dissipation of the mitochondrial membrane potential was observed (Fig. 1B).

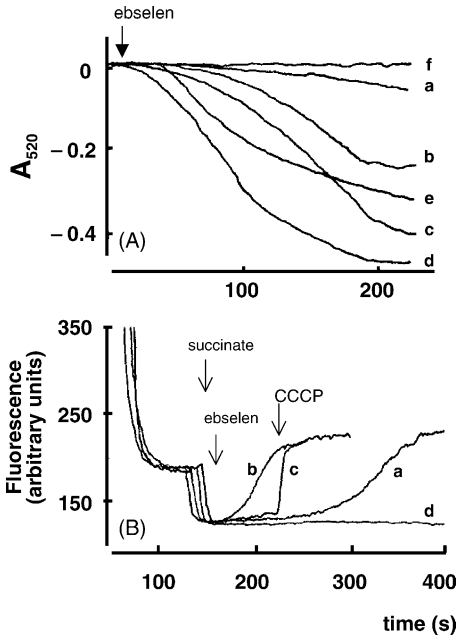

Fig. 1. Induction of mitochondrial swelling and dissipation of mitochondrial potential by ebselen. Panel (A): swelling was assessed by measuring the change in absorbance of the mitochondrial suspension (1 mg/mL) at 520 nm (A_{520}) and was induced by increasing concentrations of ebselen, 1 μM (line a), 2.5 μM (line b), 5 μM (line c), 10 μM (line d), 25 μM (line e) and 50 μM (line f). Panel (B): rhodamine 123 was incubated at 25° in 1.8 mL of phosphate buffer and 30 s latter mitochondria (0.5 mg/mL) were added (not shown). Mitochondrial membrane potential was measured after the addition of 6 mM succinate, followed by either 10 μM ebselen (line b), 1 μM CCCP (line c) or 10 μM ebselen + 1 μM CsA (line d). Line (a): no addition of ebselen (control mitochondria). The exact time when the compounds are added to the medium is indicated by the arrows. CsA (line d) was added just after mitochondria.

The addition of the protonophore CCCP shows that Δψ dissipation is complete (Fig. 1B). Moreover, the effects of ebselen on mitochondrial swelling were completely prevented in the presence of 1 μM CsA or 50 μM ubiquinone 0 (Fig. 2), two specific inhibitors of mitochondrial permeability transition [24,25]. This demonstrates that mitochondrial swelling is due to PTP opening.

If Ca^{2+} was eliminated from the incubation medium using the Ca^{2+} chelator EGTA, swelling was also completely prevented (Fig. 2). In the same way, if Ca^{2+} entrance into mitochondria was prevented by the presence of 1 μM ruthenium red, a well-known inhibitor of the Ca^{2+} uniporter [26], mitochondrial swelling was also inhibited (Fig. 2). Conversely, the addition of 25 μM Ca^{2+} to the medium enhanced both the rate and the extent of the swelling induced by ebselen (Fig. 2). As PTP opening is highly dependent on the intramitochondrial Ca^{2+} concentration [27], these experiments reinforce the involvement of this channel in ebselen-induced mitochondrial swelling.

When ebselen concentrations higher than 15 μM were used, a concentration-dependent decrease in swelling was observed; at 50 μM no swelling occurred (Fig. 1A). This inhibition was confirmed by the experiments depicted in Fig. 3 showing that ebselen 25 and 50 μM was able to

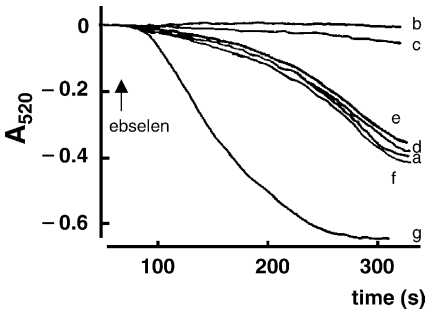

Fig. 2. Inhibition of the mitochondrial swelling induced by ebselen. Swelling was assessed by measuring the change in absorbance of the mitochondrial suspension (1 mg/mL) at 520 nm (A_{520}) and was induced by 15 μM ebselen (line a). Preincubation (3 min) of mitochondria with 1 μM CsA before swelling induction completely prevented the effect of ebselen (line b). The same result was obtained when mitochondria were preincubated with either 100 μM EGTA, 1 μM ruthenium red or 50 μM ubiquinone 0. To enhance clarity, the curves were not represented (almost identical to line b). Preincubation (3 min) of mitochondria with 5 mM GSH (line c) also inhibited mitochondrial swelling whereas 100 μM α-tocopherol (line d), dimethylsulfoxide (e) or mannitol (f) were without effect. Conversely, preincubation with 25 μM Ca^{2+} enhanced the extent of the swelling induced by ebselen (line g).

counteract the swelling induced by 25 μM Ca^{2+} in energized mitochondria. Together, these data reveal a dual effect of ebselen on PTP opening. At low concentrations, ebselen acts as an inducer of PTP opening; at higher concentrations, it acts as an inhibitor.

3.2. Effect of ebselen on mitochondrial redox signalling

Fig. 4 shows that a high concentration of catalase was able to prevent PTP opening. The protection confered by catalase was dose-dependent and catalase inactivated by boiling for 10 min did not cause inhibition of mitochondrial swelling (Fig. 4). This suggested the involvement of H_2O_2 in the process. At the reverse, α-tocopherol, a scavenger of peroxyl radicals and, dimethylsulfoxide and mannitol, two well-known scavengers of hydroxyl radicals ($HO^{\bullet}$), were ineffective, eliminating a possible role of $HO^{\bullet}$ radicals. This hypothesis was strengthened by the fact that ebselen itself did not induce lipid peroxidation (data not shown) as measured by the production of malondialdehyde by liver membranes. This rules out the possibility that ebselen is increasing membrane permeabilization by activating $HO^{\bullet}$ generation. Ebselen also failed to inhibit lipid peroxidation induced by the same reaction. Therefore, we investigated the effect of ebselen on H_2O_2 production.

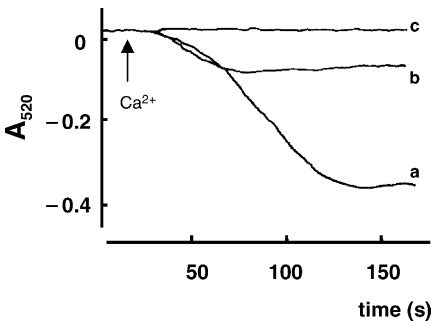

Fig. 3. Ebselen counteracts Ca^{2+}-induced mitochondrial swelling. Swelling was induced by 25 μM Ca^{2+} in the absence (line a) or in the presence of 25 μM (line b) and 50 μM (line c) ebselen.

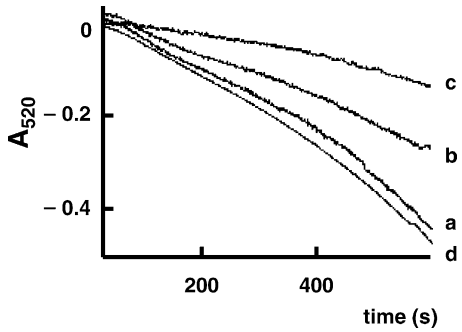

Fig. 4. Effect of catalase on mitochondrial swelling induced by ebselen. Swelling was induced by 10 μM ebselen in the presence of: 0 unit/mL (line a), 5000 units/mL (line b) or 10,000 units/mL (line c) of catalase. Line (d): effect of catalase (10,000 units/mL) inactivated by boiling.

Under normal conditions, 2–4% of the oxygen consumed is essentially released at the level of complex III in the mitochondrial matrix as a superoxide radical ($O_2^{\bullet -}$) which, following dismutation, can generate H_2O_2 [28].

However, we were unable to measure H_2O_2 production in liver mitochondria energized with succinate. This was likely due to the rapid trapping of H_2O_2 by high concentrations of anti-oxidant enzymes in the liver [29,30]. Therefore, we used heart mitochondria to elucidate the possible role of H_2O_2 in ebselen-induced PTP opening, since several recent studies reported successful measurement of H_2O_2 in these mitochondria [20,31]. We used the scopoletin/horseradish peroxidase method described by Korshunov *et al.* [20]. The production of H_2O_2 was stimulated by antimycin A, a complex III inhibitor. Fig. 5 shows that ebselen decreased H_2O_2 production; the maximal effect was obtained around 15–20 μM, a concentration where a maximal swelling effect was observed (Fig. 1). This suggests that the effect of ebselen was not mediated by an overproduction of H_2O_2 but rather an interaction with H_2O_2 released by the electron transfer chain stimulated by succinate.

3.3. Effect of ebselen on NAD(P)H oxidation

The mechanism(s) by which an oxidant may induce PTP opening has been the subject of many studies (for a recent review see Ref. [32]). Lehninger *et al.* [33] were the first to identify pyridine nucleotide oxidation as a critical step of mitochondrial membrane permeability transition, and recently it was suggested that ebselen promotes Ca^{2+} release from intact mitochondria via a NAD(P)H-dependent oxidation mechanism [34]. This suggestion was based on an indirect observation showing that the complex I inhibitor rotenone stimulates Ca^{2+} release. In our experi-

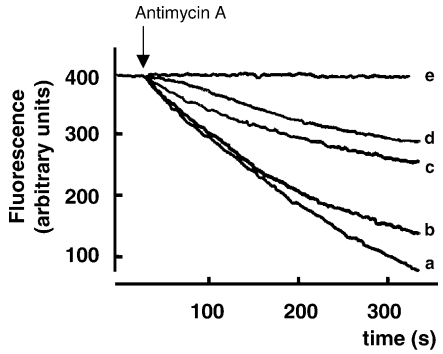

Fig. 5. Effect of ebselen on H_2O_2 production by rat heart mitochondria. After the addition of 10 units horseradish peroxidase, the production of H_2O_2 was induced by the addition of 10 μM antimycin A to the mitochondrial suspension (0.3 mg protein/mL). Line (a): no ebselen. Lines (b–e): ebselen 2.5, 10, 15 and 20 μM, respectively. The calibration of the assay was performed by adding known amounts of H_2O_2: 30 arbitrary units correspond to 0.1 μM H_2O_2.

ments, no clear evidence of a rotenone effect on ebselen-induced PTP opening could be obtained. Therefore, to address the potential role of pyridine nucleotides in PTP opening, we carried out a direct determination of pyridine nucleotide oxidation.

In the first series of experiments, deenergized mitochondria were incubated in the presence of rotenone, and NAD(P)H oxidation was monitored by fluorimetry. The effect of ebselen was compared to that of Ca^{2+}, acetoacetate (a well-known inducer of pyridine nucleotides oxidation which is a substrate of 3-hydroxybutyrate deshydrogenase), and the protonophore CCCP. Ebselen, acetoacetate and CCCP caused a significant and similar oxidation of pyridine nucleotide whereas Ca^{2+} was ineffective. β-Hydroxybutyrate inhibited oxidation or reestablished pyridine nucleotide levels when ebselen or acetoacetate were used as inducer (Fig. 6A). The absence of rotenone did not modify the oxidation process. Preincubation of mitochondria with 1 μM CsA or 100 μM EGTA did not prevent pyridine nucleotide oxidation, indicating that NAD(P)H oxidation was not induced by PTP opening. The same effect was observed in a Tris buffer (in the absence of phosphate) confirming that this oxidation process is not related to PTP. Interestingly, the thiol substitution compound, NEM, completely prevented the ebselen effect but, contrary to 3-hydroxybutyrate, NEM was unable to reestablish reduced pyridine nucleotide levels when pyridine nucleotides were fully oxidized. In addition, NEM did not alter the effects of acetoacetate. These data demonstrated that ebselen does not act on 3-hydroxybutyrate deshydrogenase and suggested that thiol groups might be implicated in the mechanism of action of the drug.

In a second step, the same set of experiments was performed with mitochondria energized with succinate to ascertain whether pyridine nucleotide oxidation induced by ebselen correlated with an inducing effect against PTP opening. Fig. 6B shows that ebselen, acetoacetate and Ca^{2+}

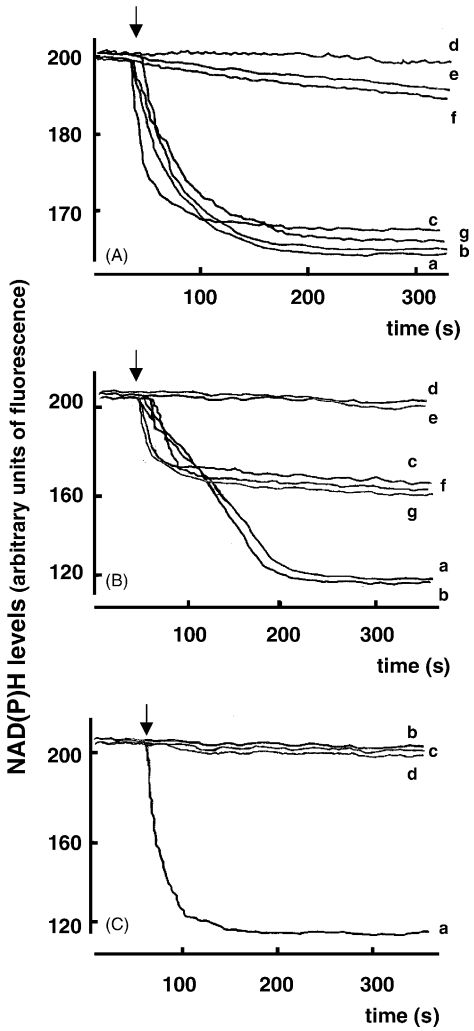

Fig. 6. Oxidation of mitochondrial NAD(P)H. (A) Deenergized conditions. Liver mitochondria (0.5 mg/mL) were incubated in a buffer containing 0.25 M sucrose, 5 mM KH_2PO_4, pH = 7.2 at 25°. NAD(P)H oxidation was induced (arrow) by either 10 μM ebselen (line a), 2 mM acetoacetate (line b), 1 μM CCCP (line c) or 25 μM Ca^{2+} (line d). Preincubation (2 min) of mitochondria with either 50 μM NEM (line e) or 10 mM β-hydroxybutyrate (line f) counteracted the effect of ebselen whereas 1 μM CsA or 100 μM EGTA did not (line g). (B) Energized conditions. Liver mitochondria (0.5 mg/mL) were incubated in a buffer containing 0.25 M sucrose, 5 mM KH_2PO_4, 6 mM succinate and 2 μM rotenone, pH = 7.2 at 25°. NAD(P)H oxidation was induced (arrow) by either 10 μM ebselen (line a), 2 mM acetoacetate (line b) or 1 μM CCCP (line c). Preincubation (2 min) of mitochondria with either 50 μM NEM (line d) or 10 mM β-hydroxybutyrate (line e) prevented the effect of ebselen. Preincubation (2 min) of mitochondria with 1 μM CsA inhibited partially the effect of either ebselen (line f) or acetoacetate (line g). (C) Energized conditions. Liver mitochondria (0.5 mg/mL) were incubated in a buffer containing 0.25 M sucrose, 5 mM KH_2PO_4, 6 mM succinate and 2 μM rotenone, pH = 7.2 at 25°. NAD(P)H oxidation was induced (arrow) by 25 μM Ca^{2+} (line a). This effect was prevented by 1 μM CsA (line b), 100 μM EGTA (c) or 50 μM ubiquinone 0 (d).

caused a rapid oxidation of pyridine nucleotides. These effects were much more pronounced than those obtained in the absence of succinate and were not inhibited in the presence of rotenone. This eliminates the possibility that pyridine nucleotide oxidation occurred at the level of

complex I. On the other hand, the effect of CCCP (lower than that observed with the other "oxidants," Fig. 6B, line c) was blocked by rotenone but not by CsA and thus resulted from the increased rate of respiration mediated by this protonophoric agent.

The oxidation induced by Ca^{2+} was totally inhibited by the specific inhibitors of PTP (CsA and ubiquinone 0) as well as EGTA (Fig. 6C). Partial CsA inhibition was observed when oxidation was induced by ebselen or acetoacetate (Fig. 6B), and the extent of the remaining oxidation corresponded to the value observed with deenergized mitochondria (Fig. 6A). It should be noted that NEM completely prevented the effect of ebselen.

Taken together, the data demonstrate that ebselen is able to promote pyridine nucleotide oxidation by two different processes. The first observed in deenergized mitochondria, is related to NAD(P)H oxidation, and involves thiol groups. This hypothesis is supported by the experiments showing that the effect of ebselen is similar to that produced by acetoacetate and is inhibited by 3-hydroxybutyrate and NEM. The second was due to PTP opening which collapsed the pH gradient, resulting in a complete oxidation of NAD(P)H [35]. Based on the present results, we conclude that the effect of ebselen on NAD(P)H oxidation is a consequence rather than a primary cause of PTP opening, in agreement with the recent results of Brunner *et al.* [36].

3.4. Ebselen promoted thiol oxidation

The results obtained with NEM suggested that thiol functions might be involved in the mechanism of action of ebselen. Indeed, it is now well-established that thiol oxidation plays a major role in PTP opening [32,37,38]. More precisely, oxidation and cross-linkage of inner membrane protein thiol groups appear to be responsible for membrane permeabilization, which was observed with ROS generated by the respiratory chain in the presence of oxidizing or of cross-linker agents [39]. Thiol substitution or disulfide reduction prevented this effect. Therefore, we investigated the effect of thiol reagents on ebselen-induced mitochondrial swelling.

Preincubation of energized mitochondria with 5 mM GSH completely prevented the swelling induced by 15 μM ebselen (Fig. 2). The same results were obtained with increasing concentrations of NEM and of the reducing agent dithiothreitol (Fig. 7). In the same experiment, monobromobimane, which, like NEM, forms adducts with GSH, also inhibited mitochondrial swelling (Fig. 7). This is in agreement with the results of Costantini *et al.* [40] who suggested that PTP opening might be modulated at two separate oxidation sites in equilibrium with both the GSH and the pyridine nucleotide pools. Since oxidation of pyridine nucleotide did not appear to be the cause of the ebselen effect (see above), these data led to the conclusion that oxidation of the GSH pool and/or of membrane protein thiol groups is a prerequisite to the modulation of PTP

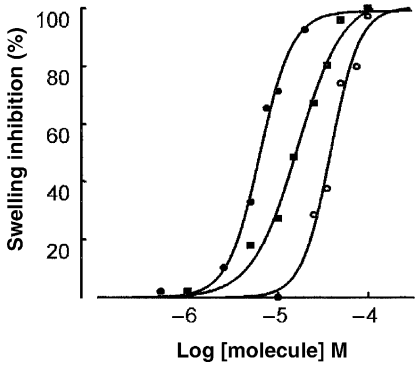

Fig. 7. Typical experiments showing the inhibitory effects of NEM, MBM and DTT on the initial rate of mitochondrial swelling induced by 15 μM ebselen. Mitochondria (1 mg/mL) were incubated at 25° in the presence of increasing concentrations of either NEM (●), MBM (■), or DTT (✪). After 3 min, swelling was induced by the addition of 15 μM ebselen. IC_{50} values were 5.06 ± 2.76, 17.9 ± 1.87 and 34.5 ± 6.15 μM for NEM, MBM and DTT, respectively.

opening by ebselen. This is in agreement with recent data suggesting that ebselen is able to induce a rapid depletion of intracellular thiols [12].

Therefore, we investigated the effect of ebselen on protein thiol groups and GSH. Mitochondria were incubated in the presence of increasing concentrations of ebselen, mitochondrial matrix and membrane proteins were extracted and the content of GSH and of protein thiol groups were determined. Table 1 shows that ebselen induced a concentration-dependent depletion of GSH and a U-shape effect on thiol proteins. At low concentrations (<15 μM) a decrease in free thiols was observed as seen with *tert*-butylhydroperoxide used as a control. Ca^{2+} was not necessary for this process since addition of EGTA had

Table 1
Ebselen-induced oxidation of membrane protein thiols

Conditions	Thiol groups (%)	GSH
Control	100.0 ± 1.6	100.0 ± 0.35
t-BH 1 mM	47.1 ± 3.6**	–
Ebselen		
1 μM	85.2 ± 5.3*	102.2 ± 2.9
2.5 μM	77.3 ± 2.7**	95.7 ± 2.5*
5 μM	67.2 ± 3.3**	91.7 ± 2.7**
10 μM	64.8 ± 4.4**	79.1 ± 3.1**
15 μM	58.3 ± 2.8**	73.0 ± 2.8**
20 μM	64.3 ± 4.1**	70.8 ± 1.7**
30 μM	82.1 ± 3.1**	68.6 ± 1.0**
50 μM	94.3 ± 3.0	64.6 ± 1.5**
Ebselen 20 μM + EGTA 100 μM	60.1 ± 1.9**,NS	–
Ebselen 20 μM + α-tocopherol 100 μM	64.9 ± 1.8**,NS	–

Control values, expressed as 100%, corresponds to 240 nmol of thiol groups/mg proteins and 5.65 nmol of GSH/mg proteins. Values represent the means ± SD of five experiments in triplicate. *$P < 0.05$; **$P < 0.01$ compared to the control value.

NS, values compared to ebselen 20 μM alone.

t-BH: *tert*-butylhydroperoxide.

no effect. This indicates that Ca^{2+} was not required to promote thiol group oxidation, whereas it was absolutely necessary to induce mitochondrial swelling. This effect was reversed when ebselen concentrations increased. Interestingly, this U-shape profile was similar to that obtained in swelling experiments, and reinforced the belief for a role of protein thiol groups in the mechanism of action of ebselen. These results also indicate that ebselen consumed GSH during PTP opening and that the decrease in swelling observed for concentrations higher than 15 μM cannot be explained by the restoration of GSH to normal values (Table 1). In order to analyse a possible role for GSH in the mechanism of action of ebselen, experiments were performed in mitochondria depleted of GSH. In a first approach GSH was depleted *in vivo*, through acute administration of diethylmaleate (4 mmol/kg) which reacts with GSH to form a stable conjugate, leading to GSH depletion [22,41]. Diethylmaleate was preferred to the commonly used depleting agent L-buthionine sulfoximine [42] because in our hands it resulted in a higher mitochondrial GSH depletion (−50% compared to −25% for L-buthionine sulfoximine). Ebselen was able to induce swelling in GSH depleted mitochondria (Fig. 8B) but the extent of the swelling was decreased and this effect was obtained with lower ebselen concentrations, compared to control mitochondria (Fig. 8A). The maximal effect was observed at 5 μM (Fig. 8B). Thus, when GSH was decreased 2-fold, a lower concentration of ebselen was required to induce swelling. These results indicated that GSH plays a major role in ebselen-induced swelling and that the phenomenon was dependent on the GSH/ebselen ratio. These data were confirmed by *in vitro* depletion experiments. Incubation of rat liver mitochondria with 1-chloro-2,4-dinitrobenzene, a favored substrate for GSH transferase [21] resulted in a more severe decrease in mitochondria GSH (−75%) without alterations of other thiols and impairement of oxidative phosphorylation in accordance with previous results [21]. *In vitro* depletion caused a more important inhibition of the swelling than *in vivo* depletion, since no swelling could be observed at 20 μM ebselen (Fig. 8C).

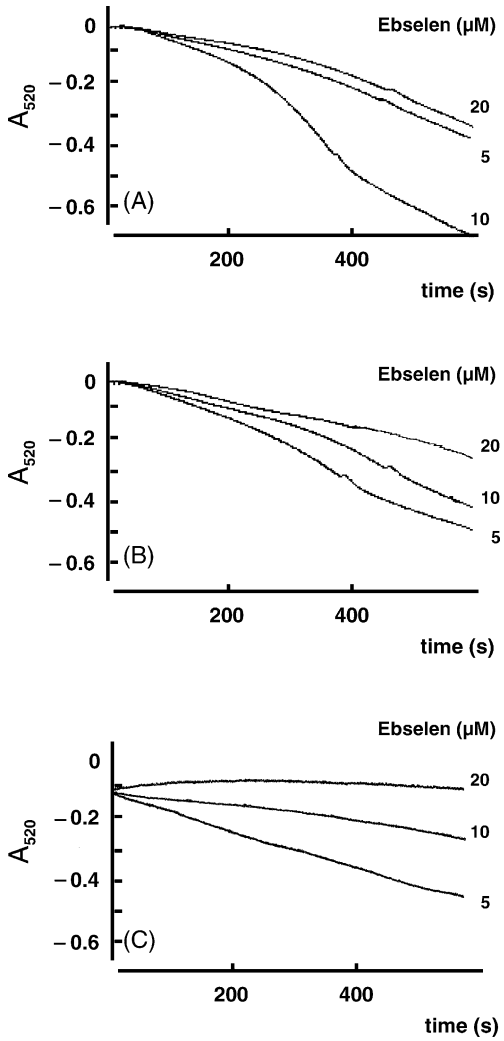

Fig. 8. Ebselen-induced mitochondrial swelling in GSH-depleted mitochondria (1 mg/mL). Swelling was induced by increasing concentrations of ebselen in control mitochondria (A), in mitochondria depleted *in vivo* by diethylmaleate (B) or in mitochondria depleted *in vitro* by 1-chloro-2,4-dinitrobenzene (C).

3.5. Mechanism(s) by which ebselen modulate(s) PTP

Ebselen modulates mitochondrial functions and, more particularly induces opening of the PTP in liver mitochondria. This is a paradoxical effect for a drug considered to be a potent anti-oxidant, anti-inflammatory and cytoprotective drug [43]. However, this effect could explain the proapoptotic and the anti-proliferative properties of this molecule observed in cell lines [12,13], since PTP is now considered as one of the key element of the apoptotic process.

Although ebselen is able to oxidize pyridine nucleotides, this effect seems to be a consequence rather than a cause of PTP opening. PTP opening most likely involves a depletion of thiol functions since it is accompanied by a potent oxidation of both GSH and membrane protein thiols and prevented by thiol substitution or disulfide reduction. We also demonstrate that H_2O_2 plays a key role in the ebselen effect. Taken together, these results suggest that the most plausible mechanism of action is the GSH peroxidase activity of ebselen. Indeed, when added to mitochondria, ebselen can reduce H_2O_2 which is formed during respiration and subsequently oxidize GSH. This cycle of reaction mimics the catalytic activity of GSH peroxidase, restoring ebselen [7] and exhausting mitochondria from GSH. Concomitantly, this cycle enhances oxidized GSH which in turn is able to oxidize vicinal thiol groups critical for PTP opening [44].

This scheme implies that under our experimental conditions, GSH reductase is able to detoxify the medium from oxidized GSH. β-Hydroxybutyrate, which stimulates NAD(P)H production and, thus, indirectly GSH reductase activity, partially inhibited ebselen-induced PTP opening (not shown).

The protective effect of ebselen toward PTP opening is more difficult to explain. However, the results presented above underline the similar behavior of NEM and ebselen when the latter is used at sufficiently high concentrations. The capacity of NEM to prevent PTP opening seems to be mediated through its properties to form adducts with thiol groups, thus preventing the oxidation of thiol groups, crucial for PTP opening [37]. Ebselen has also been shown to form adducts with thiol groups [45]. This constitutes a reasonable hypothesis to explain the protecting effect of the drug when used at concentrations higher than 20 μM.

In conclusion, we report a dual effect of ebselen towards PTP in liver mitochondria. The correlation between its preventing effect and its ability to induce PTP depends on the concentration of the drug, and could explain the opposite pharmacological effects observed with this drug in different models.

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