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# OXIDATIVE DAMAGE TO MITOCHONDRIA AND PROTECTION BY EBSELEN AND OTHER ANTIOXIDANTS

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**Abstract**—Iron/ascorbate induced lipid peroxidation in liver mitochondria isolated from normal and glutathione-depleted rats was monitored by low-level chemiluminescence and by accumulation of thiobarbituric acid-reactive substances (TBARS). Antioxidant capacity was assessed by the duration of the lag phase preceding the onset of active peroxidation. The lag phases in state 4 and in the presence of uncouplers were similar, but shorter in the presence of ADP (state 3). In glutathione-depleted rats the lag periods were less than those in normal mitochondria. A biphasic pattern of loss of membrane α-tocopherol was typical in state 4 with about 55% remaining after 40 min, while in presence of ADP there was a steady and rapid loss to about 30% of the initial level. Synthetic antioxidants such as ebselen or its glutathione adduct protected mitochondrial membranes against peroxidative reactions. There was a 5-fold increase in the lag phase with 1 μM ebselen in state 4 (lag doubling concentration, 0.4 μM) and a significantly lower rate of loss of α-tocopherol with about 90% of the initial level still remaining after 40 min. Likewise, the lag doubling concentrations were 0.04 μM for diethyldithiocarbamate, 0.3 μM for 5-hydroxyindole, 10 μM for dihydroxyphenylalanine and serotonin, and about 40 μM for epinephrine and norepinephrine.

Mitochondria have been recognized as a major physiological source of reactive oxygen species which arise as a consequence of oxygen reduction, the rates of production of these species being influenced by the respiratory state (for review see Ref. 1). Iron was found to cause more peroxidative damage in state 4 than in state 3 in isolated hepatic mitochondria [2]. Lipid peroxidation damages the structural integrity of the mitochondria; large amplitude swelling, increased permeability to cations, decreased membrane potential and damage to electron transfer activities have been reported (for review see Ref. 3). Protection against such oxidative damage is provided by antioxidants like vitamin E [4] or glutathione [5].

Here we investigate lipid peroxidation induced non-enzymatically in mitochondria. The antioxidant effect of ebselen and its derivatives, as well as serotonin, 5-hydroxyindole and 2,3-dihydroxyphenylalanine (DOPA) was evaluated and compared with their effect in microsomal peroxidation as previously established [6, 7]. Low-level chemiluminescence and accumulation of thiobarbituric acid-reactive substances were taken as indices of lipid peroxidation. Further, the loss in the contents of α-tocopherol in the mitochondrial membranes was followed.

## MATERIALS AND METHODS

*Chemicals*. Ebselen and derivatives were kind gifts from Dr Erich Graf, A. Nattermann Co./Rhône-Poulenc (Cologne, F.R.G.). Other chemicals used were either from the Sigma Chemical Co. (Munich, F.R.G.) or from Boehringer (Mannheim, F.R.G.).

*Preparation of mitochondria*. Mitochondria were prepared from liver of male Wistar rats according to Klingenberg and Slenczka [8] and were suspended in 0.25 M sucrose, 10 mM triethanolamine, 5 mM potassium phosphate, 20 mM potassium chloride, 2 mM EDTA and 5 mM magnesium chloride, pH 7.2. The respiratory control ratio [9] with 5 mM succinate as substrate was around 8.

*Depletion of hepatic glutathione*. Hepatic glutathione was depleted by treatment of rats with phorone (250 mg/kg body weight, i.p., 25% in olive oil) 2 hr before being killed. The level of mitochondrial GSH was measured by an enzymatic method using glutathione *S*-transferase and 1-chloro-2,4-dinitrobenzene [10] and was found to be 2.41 ± 0.09 nmol/mg protein in normal rats and 0.21 ± 0.01 nmol/mg protein in phorone-pretreated rats.

*Mitochondrial incubations*. The incubation mixture consisted of 125 mM potassium chloride, 1 mM magnesium chloride, 5 mM glutamate and mitochondria (1 mg protein/mL) in 10 mM potassium phosphate buffer, pH 7.2, 37° (state 4). State 3 conditions were initiated by addition of 1 mM ADP, and state 4 incubations were also occasionally performed in the presence of 0.2 μM rotenone, added in 10 μL ethanol. Uncouplers such as 2,4-dinitrophenol (DNP) and pentachlorophenol (PCP) were used at 5 μM concentration. In experiments with antioxidants preincubation was for 2 min. Ebselen and derivatives were dissolved in DMSO (10 μL in 6.5 mL final volume of assay); control experiments had 10 μL DMSO.

*Peroxidation measurements*. Lipid peroxidation was initiated by addition of 12 μM ferrous sulfate and 0.5 mM ascorbate. Chemiluminescence was used as an index of lipid peroxidation and was measured with a single-photon counting system equipped with a red-sensitive photomultiplier (EMI 9658 AM) [11].

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Table 1. Lipid peroxidation in hepatic mitochondria as measured by chemiluminescence under different respiratory states in normal and glutathione-depleted rats

| Respiratory state | Chemiluminescence parameters | | |
|---|---|---|---|
| | Lag phase (min) | Slope (counts/s/min) | Maximum (counts/s) |
| Normal rats | | | |
| State 4 | 6.0 ± 0.6 | 610 ± 22 | 4100 ± 200 |
| + Rotenone | 8.0 ± 0.3 | 596 ± 30 | 3470 ± 260 |
| State 3 | 1.5 ± 0.2 | 890 ± 20 | 11,570 ± 350 |
| Uncoupled | 8.0 ± 0.6 | 480 ± 27 | 2470 ± 145 |
| Phorone-pretreated rats | | | |
| State 4 | 4.6 ± 0.3 | 715 ± 21 | 4750 ± 144 |
| + Rotenone | 3.7 ± 0.3 | 650 ± 26 | 3750 ± 115 |
| State 3 | 0.6 ± 0.07 | 930 ± 10 | 11,300 ± 435 |
| Uncoupled | 3.7 ± 0.3 | 570 ± 12 | 2800 ± 150 |

Incubation mixture consisted of 125 mM KCl, 1 mM $MgCl_2$, mitochondria (1 mg protein/mL) and 5 mM glutamate in 10 mM potassium phosphate buffer, pH 7.2, 37°; state 3 with 1 mM ADP, uncoupled state with 5 μM DNP; rotenone, 0.2 μM was added in 10 μL ethanol. Lipid peroxidation was induced by 12 μM ferrous sulfate and 0.5 mM ascorbate; Values represent means ± SE of three independent experiments.

Peroxidation was also assessed by measuring the level of thiobarbituric acid-reactive substances (TBARS) formed as malondialdehyde equivalents [12] at defined time intervals after initiation.

*Measurement of α-tocopherol.* Loss of α-tocopherol in the mitochondrial membrane upon iron/ascorbate-induced lipid peroxidation was followed by HPLC as described [13] using an ESA model 5100A Coulochem electrochemical detector with the analytical cell potential set at +0.3 V. β-Tocopherol (1 nmol) was used as an internal standard.

*Microsomal experiments.* Microsomes were prepared as described [14]. Microsomal fractions (0.5 mg protein/mL) were incubated under oxygenation at 37° in 0.1 M potassium phosphate buffer pH 7.2, containing 16 μM ferrous sulfate and 2 mM ADP. Peroxidation was initiated by addition of 0.5 mM ascorbate as reductant.

## RESULTS

### *Lipid peroxidation in normal and glutathione-depleted mitochondria*

Lipid peroxidation in mitochondria was followed by monitoring the low-level chemiluminescence in normal and phorone-pretreated rats (Table 1). In mitochondria from normal rats peroxidation proceeded rapidly in state 3, with glutamate as substrate, at a rate of about 900 counts/s/min with a lag time of 1.5 min. In state 4 a lag phase of 6.0 min was observed. With the uncoupler DNP (or PCP, not shown in Table 1) the time course was similar to that observed in state 4. Similarly, the presence of 0.2 μM rotenone in state 4 did not cause significant alterations on the lag phase, slope and maximum of the chemiluminescence. In glutathione-depleted mitochondria the lag times were shorter than in control mitochondria.

The rate of loss of α-tocopherol in the mitochondrial membranes was assessed (Fig. 1, panel A). In state 3, a steady and rapid loss of α-tocopherol, 18 pmol/mg protein/min was observed, depleting the content to about 30% of the initial level within 40 min. In state 4, an initial slow rate of loss of α-tocopherol, 4 pmol/mg protein/min which lasted for about 15 min, was followed by a rapid phase, 13 pmol/mg protein/min. About 55% of the initial level of α-tocopherol remained after 40 min. These observations indicate the occurrence of active peroxidation in state 3 and a relatively slower process in state 4 corroborating the chemiluminescence data (Table 1). Simultaneous accumulation of TBARS (Fig. 1, panel B) was observed at rates of 0.37 and 0.26 nmol MDA/mg protein/min in states 3 and 4, respectively, with lag phases similar to those found in chemiluminescence measurements. The rate of loss of α-tocopherol in glutathione-depleted mitochondria was essentially similar to that found in the control group.

The rates of lipid peroxidation in states 3 and 4 were correspondingly similar with glutamate, β-hydroxybutyrate or α-ketoacids as substrates (data not shown). However, with succinate there was a significant increase in lag period to 37 ± 3 min in presence of ADP. Succinate also elicited a moderate inhibitory effect on the accumulation of TBARS after 50 min, the level being about 75% compared to that with glutamate as substrate.

### *Antioxidant activity of selenoorganic compounds*

Table 2 and Fig. 2 show the extent of protection afforded by ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one; PZ 51) by prolonging the lag phase before the onset of peroxidation. In state 3, the lag phase of 1.5 min in control experiments was prolonged 10–15-fold in the presence of 0.6 μM ebselen, while in state 4, there was a 5-fold increase by 1 μM

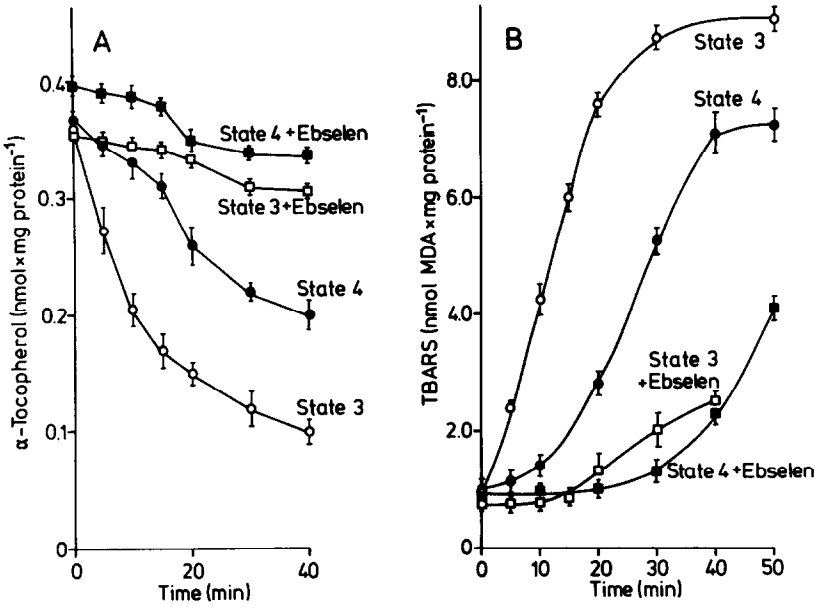


Fig. 1. Loss of α-tocopherol and accumulation of TBARS during mitochondrial lipid peroxidation. Panel A: loss of α-tocopherol. Panel B: accumulation of TBARS. Incubation mixture and conditions as described in Table 1. State 3 (open symbols) and state 4 (solid symbols), in presence (□, ■) or absence (○, ●) of 1 μM ebselen. Values are means ± SE (N = 4).

ebselen (Fig. 2A). Ebselen, (1 μM) decreased the maximal level of TBARS accumulated after 50 min to about 55%, and also the slope of the rapid phase of TBARS accumulation (Fig. 2B). The protection afforded by ebselen was further seen in the rate of loss of α-tocopherol (Fig. 1, Table 2). Ebselen decreased the rate of loss of α-tocopherol, with nearly 90% of the initial level still remaining after 40 min. The antioxidant effect of ebselen in delaying the onset of the rapid phase of peroxidation was not diminished in mitochondria from glutathione-depleted rats.

The sulfur analogue of ebselen, PZ 25, afforded less protection compared to ebselen, requiring approximately a 5–10-fold higher concentration to produce similar effects, as observed previously with microsomes [6]. At 5 μM PZ 25, the maximal level of TBARS accumulated decreased to about 60% of the control values in state 4 (Figs. 2A and B). The glutathione adduct of ebselen, ebselen-SG, elicited a pronounced antioxidant capacity comparable to that of ebselen (Fig. 2A). In state 4 this effect was particularly pronounced. In glutathione-depleted mitochondria a 4-fold higher concentration of ebselen-SG was required to produce lag phases comparable to those in the normal group.

*Effect of thiols*

Glutathione had an attenuating effect on iron/ascorbate-induced peroxidative damage in mitochondria (Fig. 3). In normal mitochondria, 40 μM GSH extended the lag phase in state 3 about 6-fold. In state 4, added GSH did not prolong the lag phase. However, a pronounced decrease in the slope and maximum of chemiluminescence were noted. In state 3, 0.12 mM GSH diminished the slope of the rapid phase of chemiluminescence to about 2% of control values, and in state 4, 0.05 mM GSH decreased the slope to less than 3% of control values. In mitochondria from phorone-pretreated rats, increasing GSH resulted in prolongation of the lag phase: 0.04 mM glutathione extended the lag period 4-fold in state 4. However, there was a less pronounced decrease in the slope and maximal level of chemiluminescence as compared to that observed in normal mitochondria.

Diethyldithiocarbamate exerted the highest protection against peroxidation in the mitochondria (Table 3). The antioxidant capacity is expressed as the concentration of compound required to double the lag phase in state 4 over that of controls; for diethyldithiocarbamate, it was 0.04 μM.

A much higher concentration, 2.5 mM, was required for dihydrolipoate to double the lag phase, and lipoate did not elicit any protective effect but even accelerated the process (data not shown). The lag doubling concentrations for dithiothreitol and β-mercaptoethanol were also high, 2.8 and 9 mM, respectively. *N*-Acetylcysteine and cysteine even decreased the lag phase to values lower than control and accelerated the rapid phase of chemiluminescence (data not shown).

Among some hydroxylated compounds, 5-hydroxyindole had the highest protective capacity, with a lag doubling concentration of 0.3 μM. It also decreased the slope of accelerating chemiluminescence to about 15% of control values, and diminished the maximum. DOPA and serotonin had lag doubling concentrations of 10 μM. On the other hand, 5-hydroxytryptophan did not show any effect up to 10 mM concentration in state 4.

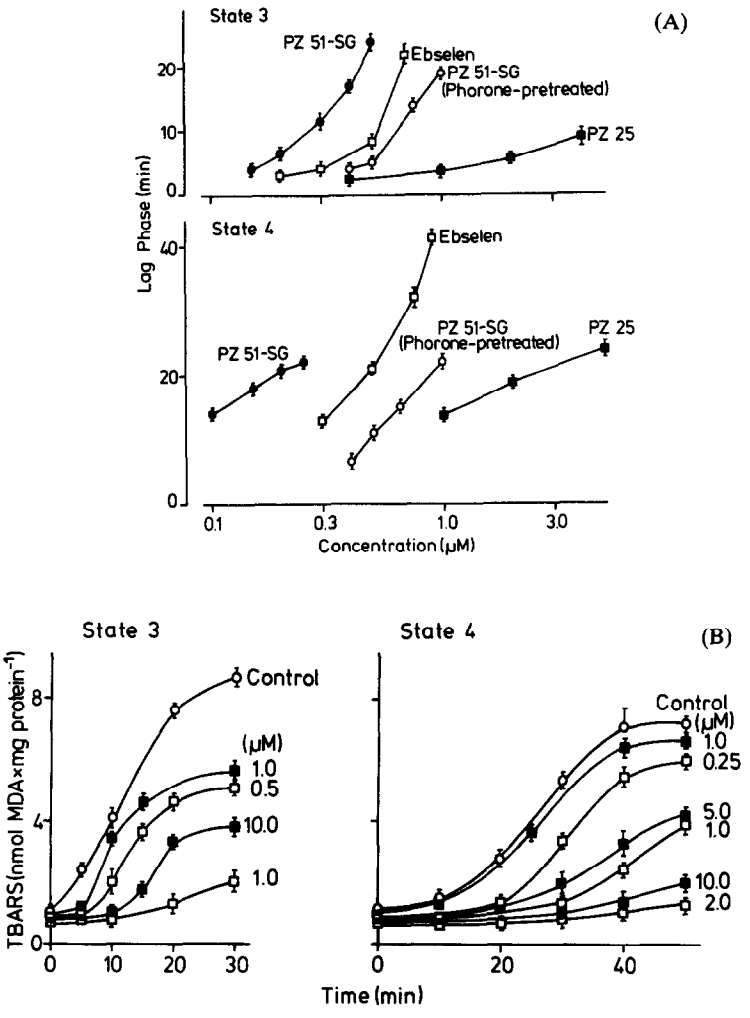


Fig. 2. (A) Dependence of lag phase of chemiluminescence on concentration of ebselen, PZ 25 and ebselen-SG (PZ 51-SG) during mitochondrial lipid peroxidation. (B) Dependence of extent of TBARS accumulation on concentration of ebselen and PZ 25. Conditions as in Table 1. Control (○); ebselen (□) and PZ 25 (■). Values are means ± SE from three sets of experiments.

Table 2. Effect of ebselen on mitochondrial lipid peroxidation. Summary of peroxidation parameters

| Parameter | Control | Ebselen (1 μM) |
|---|---|---|
| Chemiluminescence | | |
| Lag time (min) | 8.0 ± 0.3 | 54.0 ± 2.4 |
| Slope (counts/s/min) | 596 ± 30 | 100 ± 8.0 |
| Maximum (counts/s) | 3470 ± 260 | 1000 ± 98 |
| α-Tocopherol loss | | |
| Slow rate (pmol/mg/min) | 4.2 ± 0.1 | 1.2 ± 0.17 |
| Fast rate (pmol/mg/min) | 13.2 ± 0.3 | 5.2 ± 0.55 |
| Level after 40 min (% of initial) | 54 ± 1.5 | 86 ± 0.7 |
| TBARS accumulation | | |
| Lag time (min) | 12.0 ± 0.6 | 30.0 ± 1.5 |
| Slope (nmol MDA/mg/min) | 0.26 ± 0.01 | 0.11 ± 0.006 |
| Maximum (nmol MDA/mg) | 7.2 ± 0.11 | 4.1 ± 0.23 |

Mitochondrial incubations were performed in state 4 as described in Table 1 and Figs 1 and 2.

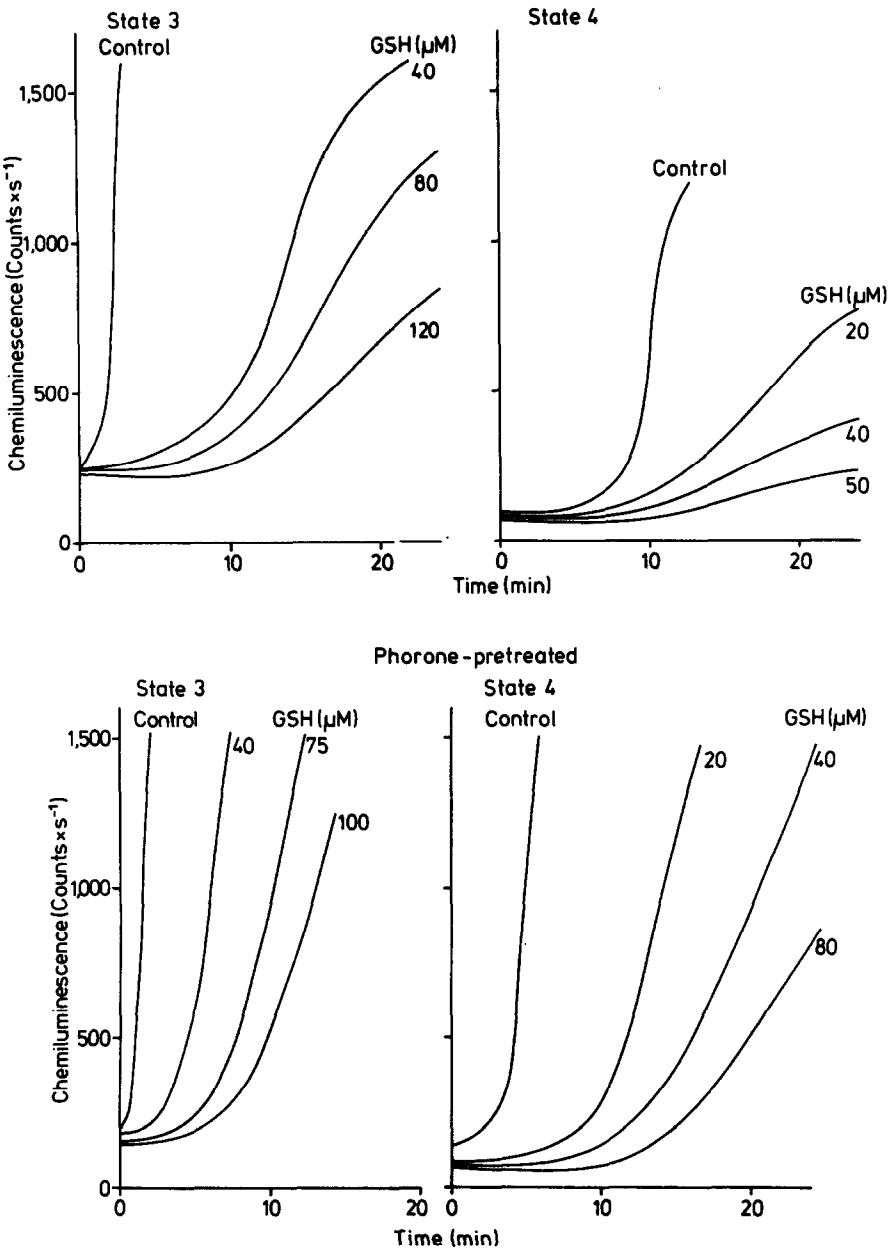


Fig. 3. Effect of glutathione on chemiluminescence parameters during lipid peroxidation in mitochondria from normal (top) or phorone-pretreated rats (bottom) in states 3 and 4. Conditions as in Table 1. Values are representative of three independent experiments.

Epinephrine and norepinephrine showed lag doubling concentrations between 40 and 50 μM.

## DISCUSSION

The rates of lipid peroxidation in rat liver mitochondria were similar in state 4 and in the presence of the uncouplers DNP and PCP, while in the presence of ADP there was a significant acceleration of the peroxidative process. This effect has been attributed to the involvement of ADP-chelated iron [15]. A protection by succinate in state 3 has been demonstrated previously in hydroperoxide-induced oxidative damage in rat liver mitochondria [16, 17], possibly explained by reduction of the mitochondrial ubiquinones. Iron-induced damage to mitochondria has also been shown to be dependent on the steady state oxidation level of the respiratory chain components [2], while uncouplers such as DNP or CCCP have been shown to afford considerable protection against peroxidation by decreasing the level of TBARS.

The protective effect of ebselen against mitochondrial lipid peroxidation is concurrent with its known antioxidant role in microsomes. This effect may be attributed to the catalytic reduction of hydroperoxides by ebselen [6, 18] or to its ability to scavenge peroxyl radicals with rate constants [19]

Table 3. Lag doubling concentrations of some compounds in mitochondrial and microsomal lipid peroxidation as observed by low level chemiluminescence

| Antioxidant | Lag doubling concentration (μM) | | Ref.* |
|---|---|---|---|
| | Mitochondria | Microsomes | |
| Diethyldithiocarbamate | 0.04 | 0.30 | [24] |
| Ebselen-SG | 0.12 | 0.35 | [28] |
| Ebselen | 0.40 | 0.13 | [28] |
| PZ 25 | 1.40 | 2.20 | [28] |
| 5-Hydroxyindole | 0.30 | 8.0 | [7] |
| Serotonin | 10.0 | 30 | † |
| DOPA | 10.0 | 110 | [7] |
| Norepinephrine | 45.0 | 100 | † |
| Epinephrine | 40.0 | 130 | † |
| Dihydrolipoate | 2500 | 800 | [21] |
| Dithiothreitol | 2800 | 500 | † |
| β-Mercaptoethanol | 9000 | 1400 | † |

Conditions of assay as mentioned under Table 1 for mitochondrial lipid peroxidation in state 4. Ebselen and related derivatives were added in 10 μL DMSO. Conditions for microsomal experiments as described in Refs 7, 21 and 25. Values are representative of three individual experiments.

* References for microsomal data.

† Microsomal data from this paper.

comparable to those of α-tocopherol. Ebselen diminished the rate of loss of α-tocopherol during mitochondrial peroxidation, either by sparing α-tocopherol or by regenerating the reduced form of α-tocopherol. Substantial antioxidant property was also shown by ebselen-SG (Fig. 2A). Interestingly, this adduct was proposed as a probable intermediate during reduction of hydroperoxides by ebselen [18].

In general, when compared to microsomes a lower concentration of the compounds examined was required to double the lag phase in mitochondria (Table 3). However, ebselen, dihydrolipoate, β-mercaptoethanol and dithiothreitol were more effective in microsomes. In microsomes, dihydrolipoate was shown to provide protection during both enzymatically and non-enzymatically induced peroxidation in a manner dependent on vitamin E [21]. However, a dependence of dihydrolipoate on glutathione for protection against iron/ascorbate induced lipid peroxidation in microsomes was also reported [22]. *In situ*, the antioxidant property of dihydrolipoate would be of minor significance in view of its bound nature as lipoamide to the ketoacid dehydrogenases and due to the abundance of other thiols such as glutathione.

The marginal enhancement of the peroxidative process in GSH-depleted mitochondria is expected in view of reports on the enhanced rate and extent of lipid peroxidation upon oxidation of non-protein sulfhydryl groups in mitochondria by diamide [23]. The effect of added glutathione in suppressing the slope and maximum of chemiluminescence without prolongation of the lag period is similar to the behavior in microsomes, where it afforded protection at 0.1 mM, and higher levels did not enhance the protection [24].

The ability of micromolar concentrations of diethyldithiocarbamate to decrease the rate of alkane production and chemiluminescence during iron/ascorbate induced peroxidation in rat liver microsomes has been demonstrated [25]. Diethyldithiocarbamate is an efficient free radical scavenger as evidenced by its ability to be oxidized by radicals generated by 2,2'-azobis-(2-amidinopropane) [26].

A few phenolic derivatives such as 5-hydroxytryptophan, 5-hydroxyindole and DOPA have been shown to protect microsomes against lipid peroxidation in a vitamin E-dependent manner [7]. In the present study with mitochondria 5-hydroxyindole was found to exert substantial antioxidant activity and DOPA and serotonin were also effective. It is likely that the protection afforded by the catecholamines is due to their ability to scavenge radicals such as the hydroxyl radical [27]; however, their physiological functional relevance as antioxidants in mitochondria needs to be analysed in view of their susceptibility to oxidation by monoamine oxidases that are abundant in the outer mitochondrial membrane.

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