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Ebselen Is a Dehydroascorbate Reductase Mimic, Facilitating the Recycling of Ascorbate via Mammalian Thioredoxin Systems

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ABSTRACT

Ebselen is a selanazal drug recently revealed as a highly efficient peroxiredoxin mimic catalyzing the hydroperoxide reduction by the mammalian thioredoxin system [thioredoxin (Trx), thioredoxin reductase (TrxR), and NADPH]. The mammalian Trx system is a dehydroascorbic acid reductase recycling ascorbic acid essential for cell functions. Here we report that ebselen strongly facilitated the recycling of ascorbic acid by the TrxR both with and without Trx present. Reduction of dehydroascorbic acid by TrxR has a pH optimum of 6.4, and only ~55% of this activity at a physiological pH of 7.4. Ebselen at 6 μ*M* enhances this reaction three-fold and with the same pH optimum of 6.4. The mechanism of the ebselen effect is suggested to involve reduction of dehydroascorbic acid by the ebselen selenol, a highly efficient two-electron reductant. Thus, ebselen acts as an antioxidant to lower the peroxide tone inside cells and to facilitate the recycling of dehydroascorbic acid to ascorbic acid, so as to increase the radical scavenging capacity of ascorbic acid directly or indirectly via vitamin E. The high ascorbic acid recycling efficiency of ebselen at pH 6.4 may play a major role in oxidatively stressed cells, where cytosol acidosis may trigger various responses, including apoptosis. *Antioxid. Redox Signal.* 6, 99–104.

INTRODUCTION

EBSELEN [2-phenyl-1,2 benzisoselenazol-3(2H)-one], a seleno-organic compound classically considered as a glutathione peroxidase mimic, has recently been shown to be a peroxiredoxin mimic, catalyzing the hydroperoxide reduction with the thioredoxin (Trx) system as the predominant effecter (38, 40). With its low toxicity (27) and excellent antiinflammatory, antiatherosclerotic, and cytoprotective properties (20, 26, 28), ebselen has recently been used in clinical trials against delayed neurological deficits after aneurysmal subarachnoid hemorrhage (25) and acute ischemic stroke (21, 36).

The mechanism and kinetics of ebselen antioxidant action using mammalian thioredoxin reductase (TrxR), a selenoprotein, and Trx as electron donors have been studied in detail (38). Ebselen is rapidly reduced by both TrxR and Trx in the presence of NADPH to form the ebselen selenol, the active form of ebselen, reacting with hydrogen peroxide with a high rate (38). Ebselen also reacts rapidly with its selenol to form the ebselen diselenide, a relatively insoluble compound that also acts as a substrate of the mammalian Trx system, slowly forming the active selenol as a final product (38).

Ascorbic acid (vitamin C; ASA), a water-soluble vitamin, is an essential nutrient in man, monkey, and guinea pigs, animals that do not have the ability to synthesize the compound (4). ASA has diverse functions in the body, including classical roles as a cofactor in the enzymatic biosynthesis of collagen, carnitine, and catecholamine and peptide neurohormones (4, 12, 30, 33). ASA also is a fascinating antioxidant, reducing reactive oxygen and nitrogen species to stable molecules and breaking the radical propagation chain in lipid

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peroxidations (2). In all these functions of ASA, dehydroascorbic acid (DHA) is formed either directly or via the disproportionation of the ascorbyl radicals (2). Mammalian cells efficiently transport and reduce DHA to ASA, and thereby recycle this essential coenzyme and accumulate it in tissues as much as 50-fold compared with plasma (31, 33). DHA enters cells via Na^+-independent glucose transporters and is directly converted to ASA either by direct chemical reaction with glutathione (GSH) (34) or by NADHPH-dependent enzymes, *e.g.,* TrxR, GSH-dependent DHA reductase (14, 17, 32, 35).

In this article, we report that both ebselen and ebselen diselenide largely facilitate the reduction of DHA by the mammalian Trx system. The pH dependencies of the reaction were also studied. Thus, the antioxidant action of ebselen together with the Trx system is not only a peroxiredoxin mimic, catalzying the hydrogen peroxide reduction, but also a DHA reductase mimic, stimulating the recycling of ASA, important for human cell survival, especially in oxidative stress conditions.

MATERIALS AND METHODS

L-Ascorbic acid (ASA), NADPH, EDTA potassium phosphate, and bromine were from Sigma–Aldrich. DHA was prepared by bromine oxidation of the ASA. This method obviously has the advantage that ASA is a weak acid and dissolves easily in water, forming a solution with pH below 5. Its oxidation by bromine quickly forms the DHA and two equivalents of hydrogen bromide, which will even lower the pH of the solution. This acidic solution thus can stabilize the DHA, which is very unstable at physiological pH and irreversibly decomposes (8).

TrxR from calf thymus was purified to homogeneity (25 μmol of NADPH oxidized/min/mg) essentially as described for the rat liver enzyme (1, 13). Trx from *E. coli,* a homogeneous preparation, and recombinant human Trx and the mutant C61S/C72S prepared as described by Ren *et al.* (24) were from IMCO Ltd. (Stockholm, Sweden; www.imcocorp.se). As the Cys61 and Cys72 in human Trx form disulfides upon storage, which produce a lag phase in the reduction assay, the double-mutant C61S/C72S was used instead to save the dithiothreitol reduction preparation step as noted (7). The sources of other materials have been described in previous publications (3, 11, 42). Ebselen and ebselen diselenide were products of Daiichi and were dissolved in dimethyl sulfoxide before addition into the acqueous solvents. Concentrations of dimethyl sulfoxide were <2% of the solvent buffer and shown to dissolve the drug effectively.

The activity of enzymes was determined at room temperature using an Ultrospec 3000 UV/visible spectrophotometer (Amersham Pharmacia).

The DHA reductase activities of enzymes and ebselen were measured as described by May (17) with minor modifications. In brief, to potassium phosphate buffers prepared with the desired pH containing 2 m*M* EDTA and 200 μ*M* NADPH, DHA, Trx, and/or ebselen were added. The reactions were measured by following either the consumption of NADPH by the absorption at 340 nm ($\Delta\epsilon$ = 6,200 M^{-1} cm^{-1}) or the formation of ASA by the absorption increase at 265 nm ($\Delta\epsilon$ = 14,800 M^{-1} cm^{-1}). With these two sets of experiments, similar turnover rates were obtained. Initial rates were obtained during the first 3 min following addition of the enzyme.

RESULTS AND DISCUSSION

In 1997, May *et al.* first reported that mammalian TrxR and Trx catalyzed reduction of DHA to ASA (18). We have seen the same phenomenon. Figure 1 shows that at pH 6.4, 50 n*M* calf-liver TrxR catalyzed reduction of 1 m*M* DHA with a turnover rate of 57 min^{-1}. Addition of 10 μ*M* human Trx increased the reduction rate to a turnover of 73 min^{-1}. If instead 6 μ*M* ebselen was added, the DHA reduction rate became 162 min^{-1}, *i.e.,* an increase of 2.8-fold. In the presence of both 10 μ*M* Trx and 6 μ*M* ebselen, the rate of turnover was further increased to 180 min^{-1}. Figure 1 also shows that ebselen diselenide (3 μ*M*) catalyzed the reduction of 1 m*M* DHA by mammalian TrxR, giving a turnover rate of DHA of 130 min^{-1}, and that this molecule also is a DHA reductase catalyst.

The concentration dependency of ebselen as a DHA reductase catalyst via mammalian TrxR is shown in Fig. 2. We see that ebselen stimulated DHA reductase activity of mammalian TrxR up to 10 μ*M*, with an apparent K_m value of 2 μ*M*. This is a range far below cytotoxic doses of 50–100 μ*M*, where ebselen may induce apoptosis (37). Ebselen is a substrate of mammalian TrxR with a K_m value of 2.5 μ*M* and a turnover rate (k_{cat}) of 588 min^{-1}. This makes ebselen a highly efficient substrate of mammalian TrxR. The activity relationships shown in Fig. 2 resembled the substrate saturation curve

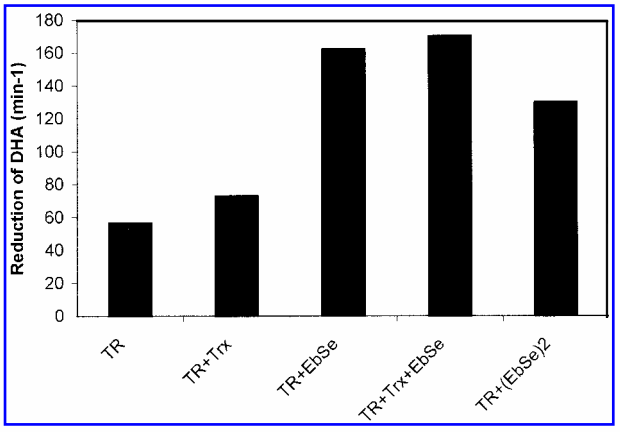


FIG. 1. Ebselen and ebselen diselenide largely increase the rate of reduction of DHA by mammalian TrxR and Trx. Reduction of 1 m*M* DHA by 50 n*M* mammalian TrxR (TR) was stimulated by 6 μ*M* C61S/C72S double-mutated human Trx (Trx), by 6 μ*M* ebselen (EbSe), in the presence of both 6 μ*M* ebselen and 6 μ*M* C61S/C72S double-mutated human Trx, and by 3 μ*M* ebselen diselenide [$(EbSe)_2$]. To potassium phosphate buffers, pH 6.4, with 2 m*M* EDTA and 200 μ*M* NADPH, DHA, Trx, ebselen, and diselenide were added as indicated. The reactions were started with addition of TrxR, and the A_{265} was followed against the A_{265} at zero time.

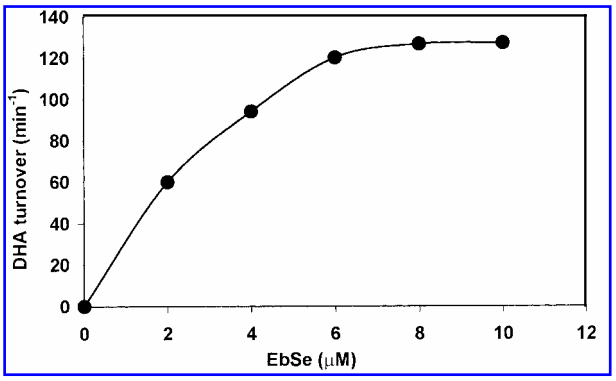
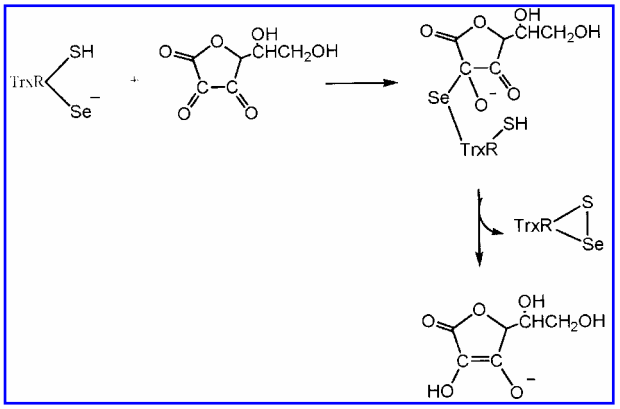


FIG. 2. Concentration dependency of ebselen as DHA reductase mimic via mammalian TrxR. To potassium phosphate buffers, pH 6.4, with 2 m*M* EDTA, 200 μ*M* NADPH, and 1 m*M* DHA, the indicated concentrations of ebselen (EbSe) were added. The reactions were started with addition of 50 n*M* TrxR, and the A_{265} was followed against the A_{265} at zero time. The turnover number is relative to the sample without ebselen.

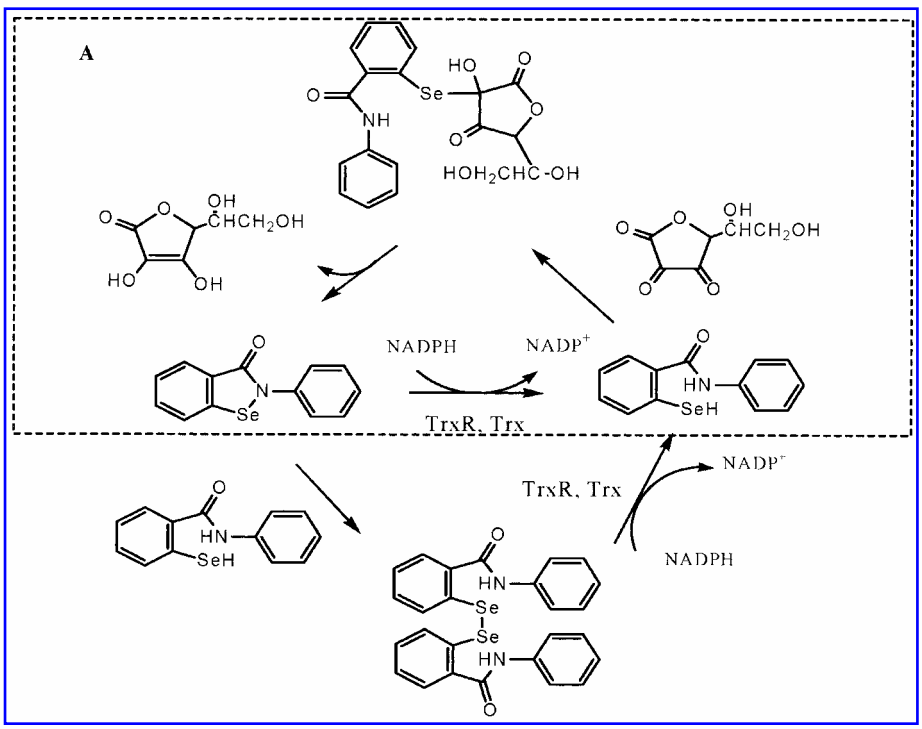


SCHEME 1. Reduction mechanism of dehydroascorbate by mammalian TrxR.

of ebselen by the mammalian TrxR in the presence of hydrogen peroxide (40).

Reduction of DHA by the mammalian TrxR, which has a selenol thiol in its active site (41, 42), is presumably via a mechanism in which the active-site selenolate undergoes nucleophilic addition to form the 3′-selenohemiketal intermediate, which reacts with the active-site partner thiol, and releases the ASA and oxidized enzyme for another catalytic cycle (Scheme 1). In the presence of ebselen, the ebselen selenol is rapidly formed by the mammalian TrxR and reduces the DHA more efficiently due to the higher nucleophilicity and better leaving character of the arylselenolate moiety (Scheme 2, part A) (23).

The ebselen diselenide is also a substrate of mammalian. TrxR forming the ebselen selenol with a K_M value of 40 μ*M* and a turnover of 79 min^{-1} (38). In its role as a hydroperoxide reductase, ebselen diselenide was reduced to the ebselen selenol, and the latter reacts with the hydroperoxide to form the ebselen for another catalytic cycle. As ebselen and ebselen selenol react to form the ebselen diselenide with a second-order rate constant of 350 M^{-1} s^{-1}, ebselen diselenide thus acts as a storage form of ebselen, which can be activated by the mammalian Trx system. Correspondingly, the catalytic activity of ebselen diselenide toward DHA reduction can be explained as illustrated in Scheme 2.



SCHEME 2. Mechanism that ebselen and its diselenide catalyze the recycling of ASA via mammalian Trx system.

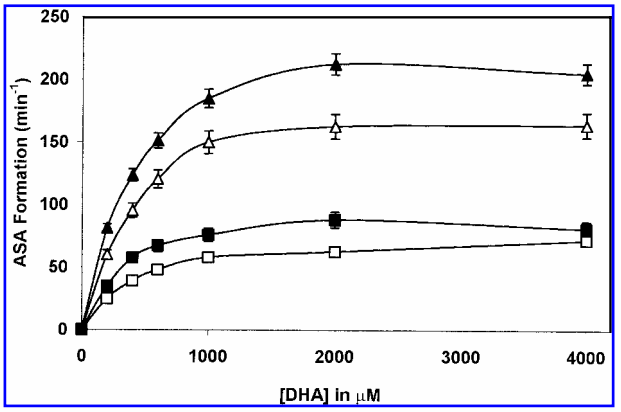


FIG. 3. Kinetic parameters for the DHA reductase activity of mammalian TrxR, and upon stimulation by human Trx and ebselen. Reduction of DHA by 50 n*M* mammalian TrxR (□) was stimulated by 6 μ*M* C61S/C72S double-mutated human Trx (■), by 6 μ*M* ebselen (△), and in the presence of both 6 μ*M* ebselen and 6 μ*M* C61S/C72S double-mutated human Trx (▲). To potassium phosphate buffers, pH 6.4, with 2 m*M* EDTA and 200 μ*M* NADPH, DHA, Trx, and ebselen were added as indicated. The reactions were started with addition of TrxR, and the A_{265} was followed against the A_{265} at zero time.

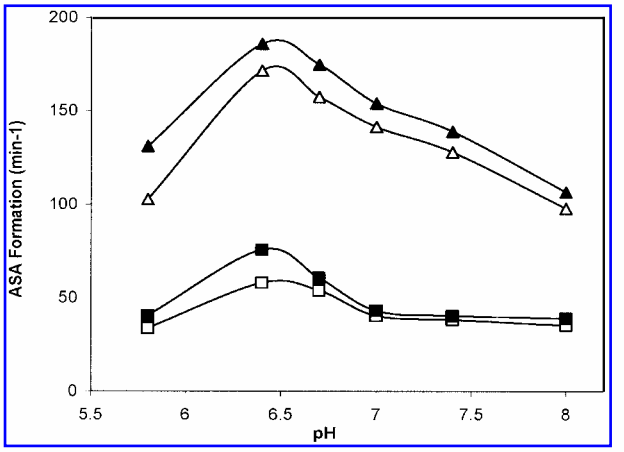


FIG. 4. pH profiles for the DHA reductase activity of mammalian TrxR, and upon stimulation by human Trx and ebselen. Reduction of DHA by 50 n*M* mammalian TrxR (□) was stimulated by 6 μ*M* C61S/C72S double-mutated human Trx (■), by 6 μ*M* ebselen (△), and in the presence of both 6 μ*M* ebselen and 6 μ*M* C61S/C72S double-mutated human Trx (▲). To potassium phosphate buffers, pH 6.4, with 2 m*M* EDTA and 200 μ*M* NADPH, DHA, Trx, and ebselen were added as indicated. The reactions were started with addition of TrxR, and the A_{265} was followed against the A_{265} at zero time.

The kinetic parameters of DHA reduction by mammalian TrxR, and in the presence of ebselen and Trx, were also determined as shown in Fig. 3. At pH 6.4, DHA is reduced by 50 *M* calf thymus TrxR with a K_M of 0.4 m*M*, and k_{cat} of ~70 min^{-1}, giving a k_{cat}/K_M of 2.9×10^3 M^{-1} s^{-1}. Addition of human Trx increased the reduction efficiency by lowering the K_M value slightly to 0.3 m*M*, an k_{cat} to 90 min^{-1}, thus giving a k_{cat}/K_M of 5×10^3 M^{-1} s^{-1}. Interestingly, we found that 6 μ*M* ebselen increased the reduction rate of DHA rate with a K_M of 0.3 m*M*, a turnover rate of 180 min^{-1}, and a calculated k_{cat}/K_M of 1×10^4 M^{-1} s^{-1}. This is an increase of efficiency of ~3.5-fold. In the presence of both 10 μ*M* Trx and 6 μ*M* ebselen, the K_M value remained at 0.3 m*M* and a k_{cat} of 212 min^{-1} was obtained. This gave a k_{cat}/K_M of 1.17×10^4 M^{-1} s^{-1} for the reduction reaction of DHA.

The pH profiles upon the reduction of 1 m*M* DHA by the Trx system (50 n*M* TrxR and 10 μ*M* Trx) with and without 6 μ*M* ebselen were studied as well. As is seen in Fig. 4, the DHA reductase activity of the Trx system reached its optimum at pH 6.4 both with and without the presence of ebselen. Similar pH profiles were seen when different DHA concentrations (between 0.4 μ*M* and 2 m*M*) and ebselen concentrations (between 2.5 μ*M* and 8 μ*M*) were used (data not shown). At physiological pH 7.4, ebselen increased the reduction of DHA by the mammalian Trx system ~3.5-fold, which is similar to its action at pH 6.4. As the turnover rate for the DHA reduction by TrxR, with or without human Trx, was not different overall from that found for GSH-dependent DHA reduction by glutaredoxin or protein disulfide isomerase (18), the 3.5-fold increase in activities by ebselen is therefore rather significant.

Mammalian TrxR has an optimum pH of 6.7 toward its native substrate Trx, whereas its active-site SeCys498Cys mutant enzyme has a 100-fold lower activity and a pH optimum of 9 (41). This difference was attributed to the low pK_a value of the selenocysteine (5.3) as compared with the pK_a of a normal cysteine (8.23) (41). The pH profiles for the direct reduction of DHA by the mammalian TrxR and Trx reflect the normal pH preference of the enzymes. In the presence of ebselen, the ebselen selenol is formed and its reduction of DHA showed a wide increased efficiency with a maximum at pH 6.4. Ebselen selenol, an arylselenol, should have an even lower pK_a value compared with an alkylselenol, in parallel with their sulfur analogues (39). Thus, the pH profiles for the reduction of DHA by mammalian TrxR and Trx in the presence of ebselen are the outcome of the combination of pH preference of TrxR toward ebselen and the ebselen selenol toward DHA.

Ascorbate recycling in cells is accomplished by several mechanisms. At physiological pH of 7.5, direct chemical reaction of GSH with DHA is fast and is believed to be the major pathway for the ASA recycling. Ebselen was also reported to have DHA reductase activities catalyzing the DHA reduction by GSH (10). However, the rates of direct reduction of DHA by GSH fall substantially (~10^3-fold) following the fall of pH from 7.45 to 6.15 (34). Thus, the importance of alternative pathways for reduction of DHA becomes evident if cells are in oxidative stress situations where GSH concentrations are depleted (19), and intracellular pH values fall, which is characterized as acidosis (5, 6, 9, 16, 22). Several reports indicate that acidification of the cytosol occurs in mammalian cells undergoing apoptosis. The extent of the change in pH observed varies among reports, but typically represents a drop of ~0.3–0.4 pH units (15). Intracellular acidosis is also reported to be associated with the impairment in the conversion of DHA into ASA (6). Under pathological conditions characterized by oxidative stress, ASA is oxidized by reactive

oxygen species at rates that overwhelm the ability of cells to regenerate the vitamin. Thus, the Trx system in such cases can play a highly significant role with a well suited pH profile to recycle the ASA. Addition of micromolar concentrations of ebselen increases the recycling rate effectively with an even better pH profile. In ischemia, the cytosol of cardiomyocytes acidifies, and this is reversed upon reperfusion (22). Under hypoxia-induced acidosis during ischemia/reperfusion (15), ebselen may involve an antioxidant mechanism that enhances the recycling of ASA from DHA, and thus protects against the onset of cell death.

One of the major functions of ASA is to prevent lipid hydroperoxide formation in plasma lipoproteins (*e.g.,* low-density lipoprotein), and lipids in cell membranes, by reducing α-tocopherol radicals formed upon reactions with lipid peroxyl radicals (29). ASA is also an effective radical scavenger toward the vast majority of reactive radical species. The so-formed ascorbate radical anion is relatively stable and disproportionate to give an ASA and a DHA. Our understanding of the DHA reductase activity of ebselen via mammalian Trx thus adds to a new function of this antiinflammatory drug not only to reduce the peroxide tone inside cells, but also to facilitate the recycling of ASA, thus increasing the radical scavenging capacity of the cells directly or indirectly via vitamin E.

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ABBREVIATIONS

ASA, ascorbic acid; DHA, dehydroascorbic acid; GSH, glutathione; Trx, thioredoxin; TrxR, thioredoxin reductase.

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