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Thioredoxin Reductase Reduces Lipid Hydroperoxides and Spares α-Tocopherol

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We investigated whether and how rat liver thioredoxin reductase spares α-tocopherol in biomembranes. Purified hydroperoxides of β-linoleoyl-γ-palmitoylphosphatidylcholine were decreased 35% by treatment with thioredoxin reductase and 54% by thioredoxin reductase plus *E. coli* thioredoxin. Thioredoxin reductase also halved the amount of hydroperoxides that had been formed during photoperoxidation of liposomes composed of β-linoleoyl-γ-palmitoylphosphatidylcholine, and of emulsions of both cholesterol and cholesteryl linolenate. In erythrocyte ghosts, thioredoxin reductase spared α-tocopherol from oxidation by both soybean lipoxygenase and ferricyanide. Thioredoxin reductase also decreased F_2-isoprostanes in ghosts oxidized by ferricyanide, suggesting that its ability to spare α-tocopherol relates to reduction of lipid hydroperoxides. © 2002 Elsevier Science (USA)

Key Words: **lipid hydroperoxides; selenium; thioredoxin reductase; thioredoxin; ferricyanide; α-tocopherol; human erythrocytes.**

Free radical attack on the unsaturated lipids of biomembranes generates lipid hydroperoxyl radicals, which then propagate a chain reaction leading to the formation of lipid hydroperoxides and ultimately to membrane disruption (1). As the primary lipid-soluble antioxidant in the cell membrane, α-tocopherol breaks this chain of propagation by reducing lipid hydroperoxyl radicals to hydroperoxides (2, 3). However, in so doing the vitamin contributes to accumulation of hydroperoxides (4, 5). The latter can in turn decompose to other reactive species and perpetuate the peroxidative chain reaction. Detoxification of lipid hydroperoxides is thus an important mechanism to halt further lipid peroxidation. This may occur through the action of phospholipases that remove fatty acids containing the hydroperoxides from phospholipids in the membrane (6). Such free fatty acid hydroperoxides are substrates for cytosolic glutathione peroxidase (GPx), which converts them to alcohols (6). Alternatively, phospholipid hydroperoxide GSH peroxidase (PHGPx) can directly reduce hydroperoxides of both cholesterol and intact phospholipids (7, 8). It has also been shown that another selenoenzyme, thioredoxin reductase (TR), can reduce free fatty acid hydroperoxides to alcohols in solution, an effect that was not enhanced by thioredoxin (Trx) (9). However, it is not known whether such reduction by TR extends to fatty acids present in phospholipids or to cholesterol, which are the primary constituents of biomembranes. Therefore, in this work we examined whether TR can decrease lipid peroxidation in biomembranes, and whether this correlates with sparing of α-tocopherol.

Abbreviations used: GSHPx, glutathione peroxidase; HPLC, high-performance liquid chromatography; LPPC, L-α-β-linoleoyl-γ-palmitoylphosphatidylcholine; PBS, phosphate-buffered saline; PHGPx, phospholipid hydroperoxide glutathione peroxidase; TR, thioredoxin reductase; Trx, thioredoxin.

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MATERIALS AND METHODS

Materials. Trx from *E. coli* (specific activity provided by the manufacturer: ΔA_{650} = 62 μmol/min, using insulin), L-α-β-linoleoyl-γ-palmitoylphosphatidylcholine (LPPC), cholesteryl linolenate, soybean phospholipid (type II-S soybean lecithin), and Type V lipoxygenase were purchased from Sigma Chemical Co. (St. Louis, MO). TR was purified from rat liver by the method of Luthman and Holmgren (10) and stored at −80°C. This specific activity of this preparation, as previously described (11), was 90 ± 6 A_{412} units · (mg protein)$^{-1}$ in the insulin assay.

Preparation of liposomes and emulsions. Small unilamellar liposomes were prepared from LPPC as previously described (12). Briefly, the phospholipid was dissolved in a minimal volume of chloroform, dried under nitrogen to a thin film, reconstituted in 1 ml of phosphate-buffer saline (PBS) by vigorous mixing, and sonicated to an opalescent solution. Sonication was performed for 1 min on ice using the full microtip power of an Ultrasonics Model W-220F sonicator. PBS was composed of 140 mM NaCl and 12.5 mM sodium phosphate, pH 7.4. Micelles of cholesterol and cholesteryl linolenate were prepared by sonication as described for phospholipids.

Solutions of the 13-hydroperoxide of LPPC were prepared exactly as previously described (13) by oxidation with Type V soybean lipoxygenase (14). These were extracted in methylene chloride:

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methanol (2:1, by volume) and taken to dryness under a stream of nitrogen. The hydroperoxide product of LPPC was purified by reverse-phase HPLC on a C-18 column with methanol:acetonitrile: water (90:6:4), containing 20 mM choline chloride (14). Purity of the hydroperoxide peak was confirmed by UV spectroscopy (impurities absorbing at 280 nm were <2% of the major conjugated diene chromophore at 234 nm).

Preparation of white erythrocyte ghosts. Erythrocyte ghosts were prepared from blood drawn from normal volunteers as described previously (11). The cells were lysed in 40 volumes of hypotonic 5 mM sodium phosphate buffer, pH 8.0, and leaky or white ghosts were prepared as described by Steck and Kant (15). Ghosts were stored at −20°C until use within 1–2 weeks.

Lipid photoperoxidation and assay of hydroperoxide reduction. Lipids were photoperoxidized essentially as described previously (8) by irradiating sonicated suspensions of LPPC (6.4 mM), cholesteryl linolenate (2.2 mM), or cholesterol (13 mM) in PBS that contained 5 μM rose bengal. Irradiation was carried out for 1 h using a 150 W tungsten lamp that was placed 1.5–2 cm from the stirred suspensions with a 400-nm cutoff filter between the lamp and the suspensions. Incubations were carried out in 24-well culture plates that were cooled on ice. After photoperoxidation, aliquots of the lipids were diluted five-fold into PBS that contained 0.4 mM NADPH and, where indicated, TR and Trx. Incubation was carried out for 30 min at 37°C, and terminated by a 10-fold dilution into the methanolic Fox-2 reagent for assay of lipid hydroperoxides (see below). Duplicate aliquots were assayed, with correction for readings in paired samples that were not exposed to light or rose bengal. In separate experiments, 0.1 μM rose bengal was without effect on the activity of TR or TR + Trx in standard the insulin assay (16).

Other assays. The content of α-tocopherol in erythrocyte ghosts or in liposomes to which α-tocopherol had been added was measured by high performance liquid chromatography (HPLC) using electrochemical detection (17). In this assay, suspensions of ghosts or liposomes (100 μl) were mixed with 20 μl of a 5 mg/ml solution of pyrogallol in ethanol, followed by addition of 0.3 ml of reagent alcohol (95:5 ethanol:methanol, v/v), and lastly by 0.4 ml of heptane. The solution was mixed vigorously for 1 min and was centrifuged for 2–3 min at 600*g* to separate the phases. An aliquot of the upper heptane phase was removed, dried under nitrogen, and the residue was dissolved in a minimal volume of methanol for HPLC analysis. The HPLC assay of α-tocopherol was carried out as previously described using a mobile phase of 95% methanol that contained 20 mM sodium perchlorate, with electrochemical detection of tocopherols (17).

Lipid hydroperoxides in ghosts, liposomes, and emulsions were measured using the ferrous oxidation of xylenol orange (Fox-2) assay without modification (18). This assay detects the color change that occurs when the dye xylenol orange binds ferric iron. The latter is generated in the assay following oxidation of ferrous iron by lipid hydroperoxides. Specificity for hydroperoxides was established in paired samples that were incubated with triphenylphosphine (19). F_2-isoprostanes were measured in erythrocyte ghost membranes as previously described (20). Protein was measured by the BCA method (Pierce Chemical Co.). Phospholipids were determined by their phosphorus content (21).

Data analysis. Data are shown as mean ± SE. Statistical significance was assessed by analysis of variance with appropriate post-hoc testing using the statistical software package Sigmastat 2.0 (Jandel Scientific, St. Louis, MO).

RESULTS

The ability of TR to decrease pre-formed hydroperoxides in purified LPPC is shown in the first row of Table 1. In these experiments, soybean lipoxygenase-oxidized LPPC was purified by HPLC as described under Materials and Methods, then incubated for 30 min at 37°C with 0.4 mM NADPH in the presence or absence of TR and Trx. The failure to obtain a molar equivalent of hydroperoxides to LPPC is probably due to loss of some hydroperoxides during the 30 min incubation. Hydroperoxides were decreased 35% with the inclusion of 0.1 μM TR, and 54% by 0.1 μM TR plus 5 μM Trx. In these experiments paired samples of oxidized LPPC were incubated with 1 mM triphenylphosphine in methanol, which decreased hydroperoxides to less than 3% of the control value. The ability of triphenylphosphine to ablate the signal in the Fox-2 lipid hydroperoxide assay confirms the presence of hydroperoxides, and that the observed decreases due to TR and TR plus Trx were due to reduction of hydroperoxides.

TABLE 1

Reduction of Lipid Hydroperoxides by TR[a]

Lipids	Control	TR (0.12 μM)	TR + Trx (5 μM)	*N*
		Lipoxygenase-treated and purified		
LPPC	370 ± 40	240 ± 10[b]	160 ± 30[b]	3
		Photoperoxidized		
LPPC	19 ± 5.3	14 ± 4.7[b]	10 ± 3.6[b,c]	6
Cholesteryl linolenate	111 ± 18	78 ± 16[b]	57 ± 14[b]	4
Cholesterol	0.53 ± 0.07	0.34 ± 0.06[b]	0.13 ± 0.05[b,c]	6

[a] In experiments shown in the top row, purified LPPC was prepared and treated with lipoxygenase as described under Materials and Methods. In the photoperoxidation studies, sonicated liposomes/emulsions of the indicated lipids were photoperoxidized and incubated as described under Materials and Methods in the presence or absence of TR or TR plus Trx as indicated. Units are nmol of lipid hydroperoxide per μmol lipid. Statistical differences in the "*N*" experiments are as follows: [b] $P < 0.05$ compared to control and [c] $P < 0.05$ compared to the TR-treated sample by one-way analysis of variance.

To determine whether TR or TR plus Trx can reduce hydroperoxides in liposomes of LPPC, in micelles of unsaturated fatty acids esterified with cholesterol, and in micelles of cholesterol, sonicated suspensions of these lipids were photoperoxidized by singlet oxygen that was generated using visible light and rose bengal (8). In lipids not exposed to light, no hydroperoxides were detected. As shown in the last three rows of Table 1, photoperoxidation increased the hydroperoxide content of the different lipids, although cholesterol itself was much more resistant than were unsaturated fatty acids esterified with phosphatidylcholine or with cholesterol. Whereas the extent of photoperoxidation observed was variable from experiment-to-experiment, the relative changes induced by TR and TR + Trx were consistent. Incubation with TR decreased lipid hy-

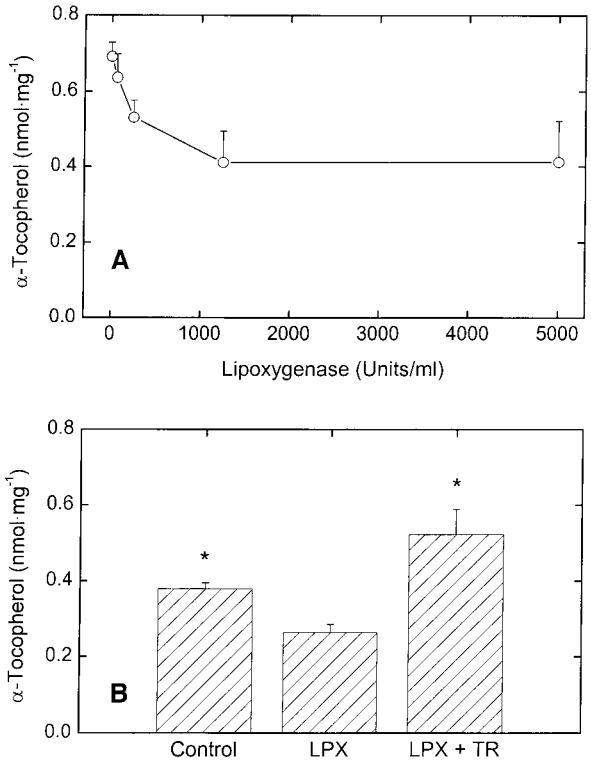

FIG. 1. Oxidation of α-tocopherol in erythrocyte ghosts by soybean lipoxygenase. (A) Erythrocyte ghosts (1–2 mg/ml) were incubated for 3 h at 37°C in PBS in the presence of the indicated concentration of soybean lipoxygenase before extraction and assay of α-tocopherol. (B) Conditions were the same as in A, except that ghosts were incubated for 3 h at 37°C in PBS containing 400 μM NADPH with 1) no further additions (Control), 2) 2500 units/ml lipoxygenase (LPX), or 3) lipoxygenase + 0.12 μM TR (LPX + TR), before assay of α-tocopherol. Data are shown from 7 experiments, with an "*" indicating $P < 0.05$ compared to lipoxygenase treatment by one-way analysis of variance.

droperoxides by 30–35% in each lipid fraction, and TR plus Trx resulted in total decreases of 50–75%.

Having shown that the TR system can decrease lipid hydroperoxides in the components of lipid bilayers, its effects on oxidant-induced loss of α-tocopherol in human erythrocyte ghost membranes was studied. As shown in Fig. 1A, incubation of ghosts with increasing amounts of soybean lipoxygenase caused depletion of about 40% of membrane α-tocopherol over 3 h of incubation at 37°C. No lipid hydroperoxides were detected in these membranes by the FOX-2 assay. When erythrocyte ghosts prepared from different volunteers were incubated with 2500 units/ml of soybean lipoxygenase, α-tocopherol concentrations decreased by 32%, an effect that was completely prevented by TR + Trx (Fig. 1B). In fact, ghosts treated with the TR system had α-tocopherol concentrations that were significantly higher than observed in control membranes following this prolonged incubation. Although these results could indicate that TR spares α-tocopherol directly, we were unable to demonstrate sparing of α-tocopherol in emulsions in Tween 80 (results not shown).

To obtain a more substantial decrease in membrane α-tocopherol, the effects of ferricyanide were studied. As shown in Fig. 2, incubation of erythrocyte ghosts with 1 mM ferricyanide caused a rapid oxidation of endogenous α-tocopherol in the membranes, about 20–30% of which appeared as α-tocopherolquinone in the HPLC assay of α-tocopherol (data not shown). When TR and Trx were added to the incubation, loss of α-tocopherol was significantly delayed throughout the time course. When 10 μM aurothioglucose was present during the incubation, the protective effect of TR plus Trx was inhibited after the 5 min time point, such that this curve differed from both control and the TR plus Trx curve. Aurothioglucose is a potent and relatively selective inhibitor of TR (22).

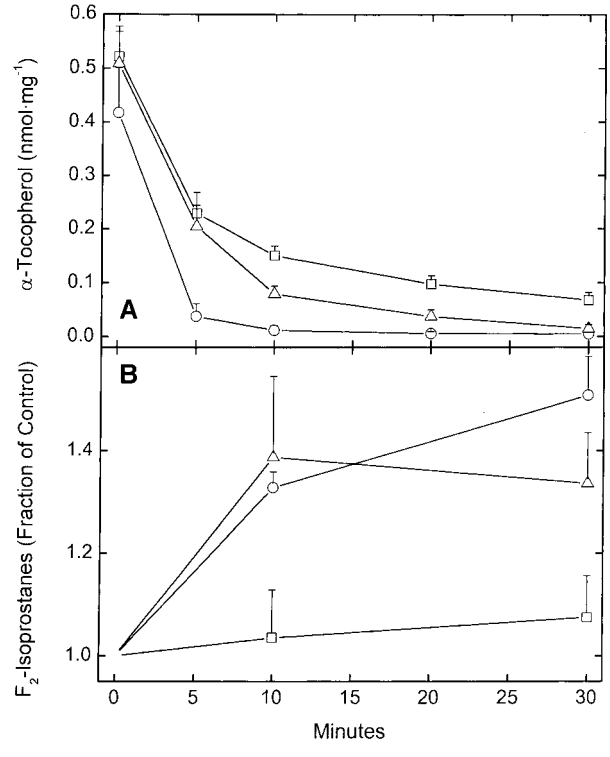

FIG. 2. Ferricyanide oxidation of α-tocopherol in erythrocyte ghosts. (A) Leaky erythrocyte ghosts were incubated in PBS at 37°C with 400 μM NADPH, 1 mM ferricyanide, and one of the following: no further additions (circles), 0.1 μM TR plus 5 μM *E. coli* Trx (squares), or TR plus Trx and 10 μM aurothioglucose (triangles). At the indicated times, ghosts were washed twice in PBS and taken for assay of α-tocopherol (A) or F_2-isoprostanes (B). Data are from 4 experiments in A, and all three curves differed from one another by three-way analysis of variance. Data are from 4 experiments in B and are shown as a fraction of the time zero value. In B, treatment with TR plus Trx (squares) was significantly different than both control (circles) and treatment with TR plus Trx and aurothioglucose (triangles) at both the 10 and 30 min times by three-way analysis of variance. Initial F_2-isoprostane values (ng/mg protein) were: Control, 0.16 ± 0.09; TR + Trx, 0.12 ± 0.04; TR + Trx + aurothioglucose, 0.1 ± 0.03.

No lipid hydroperoxides were detected in ferricyanide-treated ghosts using the FOX-2 assay. Therefore, we measured time-dependent changes in F_2-isoprostanes in response to ferricyanide. Changes in erythrocyte membrane F_2-isoprostanes in response to ferricyanide oxidation were variable in experiments with ghosts prepared from different donors, so the results are expressed in Fig. 2B as a fraction of the initial value at time zero for each treatment. The increase in F_2-isoprostanes induced by ferricyanide was blunted by TR + Trx, and this effect was prevented by aurothioglucose. These results show that TR plus Trx can spare α-tocopherol from oxidative loss in erythrocyte ghosts and prevent lipid peroxidation to a similar extent. Since NADPH was present in all incubations, it was not the cause of the responses observed.

DISCUSSION

The primary function of mammalian TR is thought to be to regulate the activity of enzymes, receptors, and transcription factors by reducing protein disulfides, usually with the aid of Trx (16). However, TR has broad substrate specificity and has been shown to reduce diverse substrates, including 5,5′-dithiobis(2-nitrobenzoic acid) (23), lipoic acid (24), alloxan (25), and dehydroascorbic acid (11). The enzyme is also capable of reducing the 15-S hydroperoxide of arachidonic acid at rates comparable to its reduction of hydrogen peroxide (9). Our studies extend the previous results to show that both TR and the TR system (TR + Trx) can decrease preformed lipid hydroperoxides in emulsions of LPPC, although the addition of Trx did not consistently produce a further decrease. The TR system can also decrease lipid peroxides generated by photoperoxidation of linolenic acid esterified in phosphatidylcholine and cholesterol, and in cholesterol itself. These findings mirror previous results with PHGPX, which is known to reduce hydroperoxides present in the major unsaturated lipid components of membrane bilayers (7, 8).

Reduction of lipid hydroperoxides in membranes likely accounts for the ability of TR to spare α-tocopherol in human erythrocyte ghosts oxidized either with soybean lipoxygenase (Fig. 1) or ferricyanide (Fig. 2). This notion is supported by the finding that the TR system also caused a significant decrease in F_2-isoprostane formation in ghosts in response to ferricyanide (Fig. 2B). F_2-isoprostanes are a very sensitive marker of non-enzymatic lipid peroxidation (26), and we have previously reported that they can be detected in freshly prepared erythrocyte ghosts, and that they increase following treatment with ferricyanide (20). We interpret our results to support a mechanism of sparing of α-tocopherol in which small amounts of hydroperoxide are generated by ferricyanide, and that these are reduced by TR before they can decompose into free radical species capable of oxidizing α-tocopherol.

The ability of both GSH- and NADPH-dependent enzymes to reduce hydroperoxides in membrane lipids and to spare α-tocopherol from oxidative loss suggests redundant mechanisms to protect cell membranes from peroxidation. For example, the erythrocyte membrane encounters oxidant stress originating from superoxide and the hydroxyl radical generated by reaction of molecular oxygen with reduced hemoglobin (27). The membrane of the erythrocyte appears to be protected from peroxidative damage and lysis as long as α-tocopherol is not severely depleted (28). Loss of α-tocopherol is prevented by recycling of the tocopheroxyl free radical by NADH-dependent cytochrome b_5 reductase activity in the membrane or cytosol (29), or by cellular ascorbic acid (17). As pointed out by Maiorino *et al.* (30), reduction of lipid hydroperoxides by GPx or PHGPx prevents their decomposition to either hydroperoxyl or alkoxyl free radicals, which in turn will spare α-tocopherol. PHGPx has been suggested to be more effective in decreasing hydroperoxide accumulation in membranes than GPx and glutathione *S*-transferases, since it can directly reduce hydroperoxides present in both phospholipid and in cholesterol (8, 31). In this work we have shown a similar function for mammalian TR, which may provide another cellular defense against oxidative loss of α-tocopherol and membrane hydroperoxide formation.

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