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Studies on Protection by Glutathione against Lipid Peroxidation in Rat Liver Microsomes. Effect of Bromosulfophthalein

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The effect of bromosulfophthalein (BSP), an inhibitor of cytosolic glutathione (GSH) S-transferases, on GSH-dependent protection against lipid peroxidation in rat liver microsomes was studied. Microsomal lipid peroxidation induced by ferrous-reduced nicotinamide adenine dinucleotide phosphate (NADPH-Fe^{2+}) or ascorbate was prevented by GSH, and addition of BSP abolished the protective effect of GSH in both peroxidation systems. The effect of BSP seemed to occur at concentrations which inhibited the activity of GSH S-transferase in microsomes.

The liberation of free fatty acid hydroperoxides from microsomes during lipid peroxidation occurred at pH 7.5—8.0. The rate of NADPH oxidation in the system containing peroxidized microsomes, NADPH, GSH, and GSH reductase was significantly higher than that in the system containing normal microsomes, and was inhibited dramatically by BSP and moderately by phospholipase A_2 inhibitors.

The above findings suggest that microsomal GSH S-transferase may be responsible for GSH-dependent protection against peroxidation, probably *via* radical scavenging and the cooperative action of microsomal phospholipase A_2 and GSH peroxidase activity, which is associated with GSH S-transferase.

Keywords——lipid peroxidation; glutathione; liver microsome; glutathione S-transferase; bromosulfophthalein; mepacrine

Introduction

Free radical-initiated lipid peroxidation in biomembranes has been proposed as one of the mechanisms of cellular injury.[1,2] Many cellular antioxidants, superoxide dismutase, catalase, and other anti-peroxidative enzymes, are known to act as cellular defense factors against oxidative stress. Glutathione (GSH) is considered to be an important inhibitor of lipid peroxidation. The inhibitory effect of GSH on lipid peroxidation has been demonstrated in microsomes,[3-7] mitochondria,[3,8] and cytosol[8-13] of rat liver. The inhibitory action in microsomes is assumed to be due to prevention of radical formation[4] or scavenging of free radicals generated during lipid peroxidation.[6]

Another protective action may be reduction of lipid peroxides *via* GSH peroxidase activity. Sevanian *et al.*[14] and Tan *et al.*[15,16] suggested that the microsomal phospholipase A_2 activity was responsible for the elimination of peroxides from microsomal membranes and the inhibition by the soluble supernatant fraction[14] or purified GSH S-transferase B[15,16] of microsomal lipid peroxidation. Morgenstern *et al.*[17] reported the occurrence of GSH S-transferase activity in rat liver microsomes. Reddy *et al.*[18] have demonstrated that selenium-independent GSH peroxidase activity, which is associated with GSH S-transferase, occurs in microsomes, but selenium-dependent GSH peroxidase activity does not.

Thus, microsomal GSH S-transferase might be involved in the protective action of GSH against lipid peroxidation. We have examined the effect of bromosulfophthalein (BSP), which is known to be an inhibitor of cytosolic GSH S-transferase,[19] on the protection by GSH.

Experimental

Chemicals——Reduced nicotinamide adenine dinucleotdide phosphate (NADPH) and GSH reductase were purchased from Oriental Yeast Co., Ltd. BSP, mercaptosuccinic acid (MSA), and catalase (Type III) were purchased from Sigma Chemical Company (St. Louis, Mo.), and mepacrine and *p*-bromophenacyl bromide (*p*-BPB) was obtained from Nakarai Chemicals Ltd. Partial purification of the selenium-dependent GSH peroxidase from rat liver cytosol was accomplished by taking the $105000 \times g$ supernatant from the liver and applying it to a Sephadex G-150 column by the method of Lawrence and Burk.[20] GSH peroxidase was eluted with 0.05 M Tris (pH 8.3) containing 0.1 M K_2HPO_4 without ethylenediaminetetraacetic acid (EDTA) at a flow rate of 60 ml/h. Fractions of peak I,[20] containing selenium-dependent GSH peroxidase, were collected. All other chemicals employed were of commercial reagent-grade quality.

Preparation of Microsomes——Male rats (8—9 weeks old) of the Wistar strain, kept on a standard laboratory diet (rat chow MF; Oriental Yeast Co., Ltd.), were used. Rats were sacrificed by decapitation, and the livers were perfused with 0.9% NaCl, removed, and homogenized in 4 volumes of 0.05 M Tris–KCl (0.14 M) buffer, pH 7.4. Microsomal fractions were prepared by the method of Reddy *et al.*[18] The homogenates were centrifuged at $16000 \times g$ for 30 min and the resulting supernatants were centrifuged again at $16000 \times g$ for 30 min. The supernatants thus obtained were centrifuged at $105000 \times g$ for 60 min. The pellets were washed by resuspension (in the homogenizing buffer) and centrifuged at $105000 \times g$ for 30 min. The washing procedure was repeated three times, and the third washings of the microsomal pellets showed no detectable GSH peroxidase or GSH S-transferase activity. The washed pellets were resuspended (about 10 mg protein/ml) in 0.05 M Tris–HCl buffer, pH 7.4, and stored at −80 °C. The microsomal preparations were normally used within a week of preparation. Heated microsomes were prepared by heating a test tube containing fresh microsomes in a water bath at 90 °C for 10 min.

Lipid Peroxidation——The standard reaction mixture contained 1 mg of microsomal protein, ascorbate (0.25 mM) or NADPH–$FeSO_4$ (0.4 mM–5 μM), and 0.05 M Tris–HCl buffer, pH 7.4 in a total volume of 1.0 ml. Reactions were started by adding ascorbate or a freshly prepared $FeSO_4$ solution under N_2. Lipid peroxidation was assayed by measuring thiobarbituric acid-reactive substances (TBARS) according to a partial modification of the method described by Buege and Aust.[21] The assay mixture included 0.04% butyrated hydroxytoluene to prevent the formation of TBARS during the assay procedure. The colored pigments, after being heated, were extracted with *n*-butanol, and their absorbance was measured at 535 nm. Peroxide formation was expressed in terms of nmol of malonic dialdehyde (MDA) by using tetraethoxypropane as a standard.

To examine whether release of lipid peroxides from membranes during microsomal lipid peroxidation occurs, microsomes (2 mg protein/ml) were incubated at various pH values (5.5—8.0) in 0.05 M Tris–HCl buffer at 37 °C, with NADPH (0.4 mM) alone, NADPH–$FeSO_4$ (0.4 mM–5 μM), or NADPH (0.4 mM) and Fe^{2+}–oxalate (0.5 μM–1 mM). After 60 min, the reaction mixture was centrifuged at $105000 \times g$ for 30 min, and then TBARS and lipid hydroperoxides in both the supernatant and pellet were determined by the method of Buege and Aust.[21]

Preparation of Peroxidized Microsomes——Microsomes (2 mg protein/ml) suspended in 0.05 M Tris–HCl buffer (pH 7.4) were incubated in the presence of 60 μM or 120 μM cumene hydroperoxide (CHP) for 2 h at 0 °C. The reaction mixture was centrifuged at $105000 \times g$ for 30 min and the pellets were washed once with the same buffer, and resuspended in 0.05 M Tris–HCl buffer, pH 8.0. One milliliter of the suspension was mixed thoroughly with 5.0 ml of chloroform–methanol (2 : 1), followed by centrifugation at $1000 \times g$ for 5 min. The chloroform layer was recovered, and subjected to iodometric assay.[21] Under the conditions used, higher levels of lipid peroxides were retained in microsomal membranes as compared to those after incubation at 37 °C for 15 min. Lipid hydroperoxide levels were about 110 nmol per 2 mg of microsomal protein, when peroxidation was induced by addition of 60 μM CHP for 2 h at 0 °C.

Assay——GSH peroxidase activity was measured spectrophotometrically by a modification of the procedure described by Tappel.[22] The standard assay mixture contained 0.05 M Tris–HCl, pH 7.4, 1 mM GSH, 0.12 mM NADPH, 5 units of GSH reductase, and 0.25 mM H_2O_2 or 1.2 mM CHP. When peroxidized microsomes were used as a substrate, NADPH oxidation was determined by measuring the decrease in absorbance at 340 nm with a Hitachi 556 dual-wavelength, double beam spectrophotometer at 37 °C. The standard mixture consisted of normal or peroxidized microsomes (2 mg protein/ml), 1 mM GSH, 1.0 unit of GSH reductase, 133 units of catalase, 0.12 mM NADPH, and 0.05 M Tris–HCl buffer, pH 8.0, in a total volume of 1 ml. Catalase was added to prevent GSH peroxidase activity induced by H_2O_2 which may be generated.

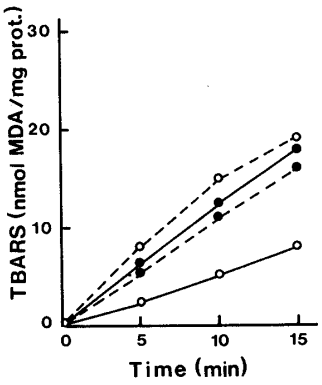
GSH S-transferase activity was assayed by monitoring the formation of the thioether between GSH and 1-chloro 2,4-dinitrobenzene (CDNB) according to the procedure of Habig *et al.*[23] GSH was determined with 5,5′-dithiobis-(2-nitrobenzoic acid).[24] Protein was determined by the method of Lowry *et al.*[25] using bovine serum albumin as a standard.

The means of at least three experiments are shown. The significance of differences between the means was determined by using Student's *t*-test.

Results

Effect of BSP on Lipid Peroxidation in Presence of GSH

Lipid peroxidation induced by NADPH-Fe^{2+} or ascorbate in rat liver microsomes was prevented by GSH, and addition of 0.05 mM BSP abolished the protective effect of GSH in both peroxidation systems, though BSP itself had no effect on peroxidation without GSH (Fig. 1). The protective effect of GSH on microsomal lipid peroxidation was not observed in microsomes heated for 10 min at 90 °C (data not shown). GSH consumption during the ascorbate-induced lipid peroxidation was less than a few percent after 30 min of incubation in both cases (GSH alone and GSH together with BSP), although it was slightly enhanced in the latter case (data not shown). The effect of BSP on lipid peroxidation induced by NADPH-Fe^{2+} was examined at GSH concentrations from 0.1 mM to 5 mM (Fig. 2).



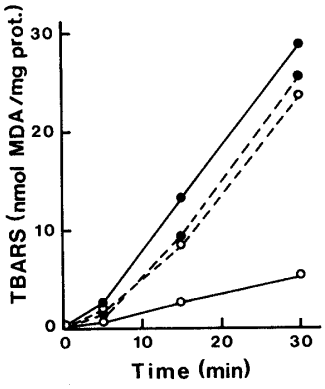


Fig. 1. Effects of GSH and/or BSP on Microsomal Lipid Peroxidation

Lipid peroxidation was induced by NADPH-Fe^{2+} (left) and ascorbate (right). The incubations were done: ○---○, with no GSH and no BSP; ○—○, with 0.5 mM GSH; ●---●, with 0.05 mM BSP; ●—●, with 0.5 mM GSH and 0.05 mM BSP.

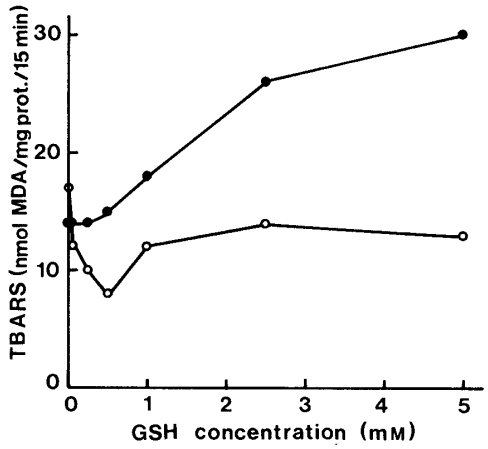


Fig. 2. Effect of BSP on Microsomal Lipid Peroxidation Induced by NADPH-Fe^{2+} in the Presence of GSH at Various Concentrations

The incubations were done: ○—○, with no BSP; ●—●, with 0.2 mM BSP.

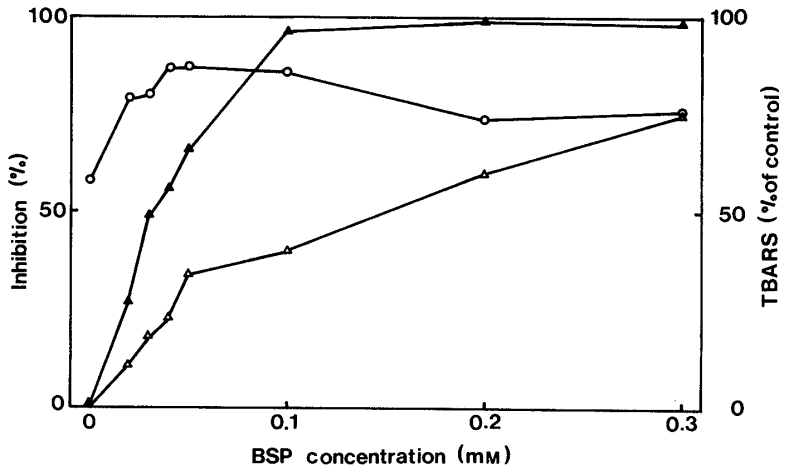


Fig. 3. Inhibition of the Activities of GSH S-Transferase and GSH Peroxidase by BSP and Effect of BSP Concentration on GSH Protection against Microsomal Lipid Peroxidation Induced by NADPH-Fe^{2+}

GSH peroxidase activity was assayed by using CHP as a substrate. Measurements shown are: ○—○, lipid peroxidation in the presence of 0.5 mM GSH; △—△, GSH peroxidase; ▲—▲, GSH S-transferase.

BSP abolished the protection by GSH at all GSH concentrations tested. The maximum protective effect of GSH was observed at 0.5 mM, while lipid peroxidation was promoted to higher levels than the control by addition of BSP when GSH concentrations were increased above 1 mM. The effect of BSP at various concentrations on GSH-dependent protection against lipid peroxidation and the activities of GSH S-transferase and GSH peroxidase in microsomes are shown in Fig. 3. The eliminating effect of BSP on protection by GSH was maximum at BSP concentrations in the range of 0.04—0.1 mM. In the presence of 0.05 mM BSP the activity of GSH S-transferase and GSH peroxidase in microsomes was inhibited by approximately 70% and 35%, respectively. The GSH S-transferase activity was inhibited almost completely at BSP concentrations higher than 0.1 mM, while the inhibition of GSH peroxidase activity increased gradually with increase in concentration of BSP.

No increase in absorbance at 330 nm, according to the method of Habig *et al.*,[23] was observed in the reaction mixtures which contained GSH (0.5 or 5 mM), BSP (0.05 or 0.2 mM), and microsomes (0.5 or 1 mg protein/ml) (data not shown), indicating that BSP was not conjugated with GSH by microsomal GSH S-transferase.

Elimination and Reduction of Lipid Hydroperoxides of Microsomal Membranes

Microsomal lipid peroxidation was induced by addition of NADPH alone at pH 5.5—7.0 and NADPH with Fe^{2+} or Fe^{2+}–oxalate at pH 7.5 and pH 8.0, and the reaction mixtures after incubation were centrifuged at $105000 \times g$. As shown in Fig. 4, TBARS values in the supernatant were higher than those in the microsomes at all pH ranges studied, whereas lipid hydroperoxide levels were high in the microsomes and very low in the supernatant under the conditions of pH 5.5—7.0. However, at pH 7.5—8.0, an appreciable amount of lipid hydroperoxides was found in the supernatant, and at pH 8.0, which is optimal for phospholipase A_2 activity,[26] lipid hydroperoxide levels in the supernatant were higher than those in the microsomes. The result indicates that liberation of free fatty acid hydroperoxides from microsomal membranes into the medium occurs during incubation at pH 7.5—8.0, presumably with the involvement of phospholipase A_2 originating from microsomes.

Removal of lipid hydroperoxides present in previously CHP-peroxidized microsomes was examined by monitoring the rate of NADPH oxidation (GSH peroxidase activity) in the

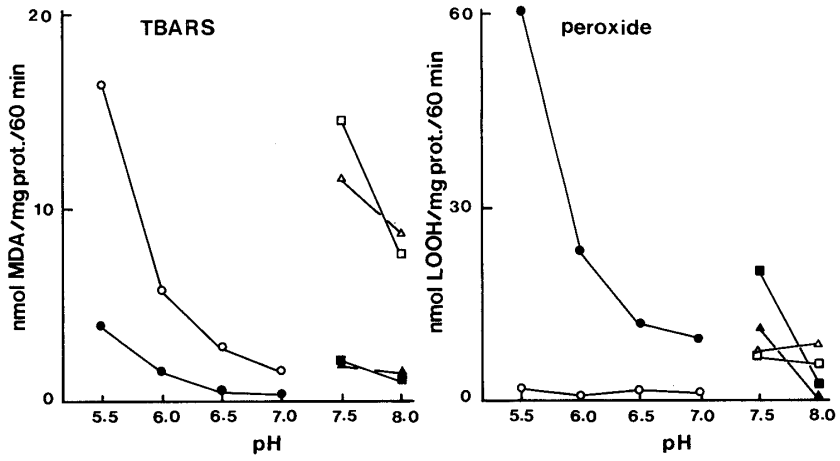


Fig. 4. pH Dependency on Liberation of Peroxidative Products from Microsomes during Lipid Peroxidation

Microsomal lipid peroxidation at various pH values was induced as described in Experimental, and after incubation, the reaction mixtures were centrifuged at $105000 \times g$ for 30 min. Fractions shown are: ○—○, supernatant (induced by NADPH); ●—●, microsomes (induced by NADPH); △—△, supernatant (induced by NADPH-Fe^{2+}); ▲—▲, microsomes (induced by NADPH-Fe^{2+}); □—□, supernatant (induced by NADPH and Fe^{2+}–oxalate); ■—■, microsomes (induced by NADPH and Fe^{2+}–oxalate).

TABLE I. Effects of Cytosolic GSH Peroxidase and Some Enzyme Inhibitors on NADPH Oxidation in Peroxidized Microsomes

Incubation systems		NADPH oxidation (nmol NADPH/ml/min)
Exp. A	Normal microsomes	
	Standard mixture	1.18±0.81
Exp. B	Peroxidized microsomes	
	Standard mixture	3.09±0.11
	+GSH peroxidase (0.03 unit/ml)	4.51±0.49[a]
	+GSH peroxidase (0.03 unit/ml)+*p*-BPB (0.25 mM)	2.05±0.24[b]
Exp. C	Peroxidized microsomes	
	Standard mixture	5.10±0.48
	+BSP (0.2 mM)	0.80±0.18[c]
	+mepacrine (0.25 mM)	2.94±0.13[a]
	+GSH peroxidase (0.03 unit/ml)	6.13±0.03
	+GSH peroxidase (0.03 unit/ml)+MSA (0.2 mM)	4.93±0.07[d]
Exp. D	Peroxidized microsomes	
	Standard mixture+GSH peroxidase (0.03 unit/ml)	4.93±0.46
	Standard mixture	
	+GSH peroxidase (0.03 unit/ml)+mepacrine (0.25 mM)	1.50±0.52[b]

Peroxidized microsomes were prepared as indicated in Experimental: Exp. B, induced by 120 μM CHP; exp. C and D, induced by 60 μM CHP. NADPH oxidation in the presence of both NADPH and microsomes with no other addition was subtracted from the data. The values represent the mean±standard deviation of three determinations. *a*) and *c*) are significantly different from the standard mixture, $p<0.05$, $p<0.001$, respectively. *b*) and *d*) are significantly different from the standard mixture plus GSH peroxidase, $p<0.01$, $p<0.001$, respectively.

system containing NADPH, GSH, and GSH reductase. The results are presented in Table I. The rate of NADPH oxidation in the systems containing peroxidized microsomes (exp. B and C) was significantly higher than that in normal microsomes (exp. A), and was inhibited dramatically by BSP and moderately by a phospholipase A_2 inhibitor, mepacrine[27] (exp. C). This appears to indicate that lipid hydroperoxides on microsomal membranes were hydrolyzed by phospholipase A_2, followed by reduction of lipid hydroperoxides by selenium-independent GSH peroxidase. On the other hand, the rate of NADPH oxidation was stimulated to some extent by the addition of cytosolic GSH peroxidase; the increment was prevented by MSA,[28] an inhibitor of selenium-dependent GSH peroxidase (exp. C). The increased rate of NADPH oxidation in the presence of GSH peroxidase was also inhibited by *p*-BPB,[29] a phospholipase A_2 inhibitor (exp. B) or mepacrine (exp. D). Thus, reduction of lipid hydroperoxides preformed on microsomes seems likely to be achieved by the cooperative action of microsomal phospholipase A_2 and GSH peroxidase present in microsomes as well as cytosol.[14,16]

Discussion

The microsomal factor which inhibits lipid peroxidation in the presence of GSH is inferred to be a heat-labile protein.[3,4,6] BSP was found to inhibit the microsomal membrane-bound GSH S-transferase activity as well as the selenium-independent GSH peroxidase activity. Fairhurst *et al.*[12] reported that the GSH-dependent cytosolic activity which inhibited lipid peroxidation in microsomes was inhibited by BSP. However, the effect of BSP might have been produced by its interaction with the microsomal protective factor in their experiments. We have demonstrated that the inhibitory effect of GSH on lipid peroxidation in

microsomes is abolished by BSP. This effect of BSP was not due to disappearance of reduced GSH from the reaction mixtures. The amount of GSH showed little change during incubation, and BSP was not conjugated with GSH. Some investigators[4,14,29,30] have reported that the inhibitory action of GSH on lipid peroxidation in microsomes requires vitamin E, and they suggested that its action might be exerted by preventing radical formation or by scavenging free radicals. The eliminating effect of BSP on inhibition of lipid peroxidation by GSH seemed to occur at BSP concentrations which inhibited the activity of GSH S-transferase. Thus, the effects of BSP indicate that microsomal GSH S-transferase may play an important role in the GSH-dependent inhibition of lipid peroxidation. Reddy *et al.*[31,32] have proposed that vitamin E is regenerated by a heat-labile inhibitory factor of microsomal lipid peroxidation in the presence of GSH. Similarly, it seems likely that vitamin E radical produced by scavenging lipid radicals during peroxidation is regenerated to vitamin E by microsomal GSH S-transferase in the presence of GSH.

Lipid peroxidation in the presence of BSP was rather stimulated by addition of GSH above 1 mM (Fig. 2). This result is in agreement with that reported by Tien *et al.*,[33] who confirmed the increase of lipid peroxidation with increase in GSH concentration in liposomes of extracted microsomal lipids incubated with GSH (1 – 5 mM) and adenosine diphosphate-chelated iron. The slight inhibition of lipid peroxidation observed at higher concentrations of GSH than 1 mM without BSP may reflect the balance of the two actions of GSH as a pro-oxidant and a peroxidation inhibitor.

Free fatty acid hydroperoxides are easily reduced by cytosolic GSH peroxidase, but peroxides esterified in phospholipids are resistant to the enzyme.[8,10,34] Sevanian *et al.*[26] pointed out that microsomal phospholipase A_2 displayed increased activity against epoxidized phosphatidylcholine as compared to its unoxidized counterpart. In the current experiments, the liberation of free fatty acid hydroperoxides from peroxidized microsomes was significant at pH 8.0, which is the optimum pH for phospholipase A_2 activity. NADPH oxidation in the systems containing peroxidized microsomes, GSH, and GSH reductase was inhibited considerably by mepacrine, and abolished by BSP. The results indicate that the elimination of lipid hydroperoxides on microsomal membranes is due to phospholipase A_2 activity, and then free hydroperoxides generated are reduced by the selenium-independent GSH peroxidase activity associated with GSH S-transferase. Further, cytosolic GSH peroxidase was also responsible for the reduction of free lipid hydroperoxides. This mechanism, however, may not contribute appreciably to the protection against lipid peroxidation, as GSH consumption during peroxidation was low.

Inhibition by BSP of the prevention by GSH of microsomal lipid peroxidation and inhibition of NADPH oxidation in previously peroxidized microsomes were demonstrated, suggesting that GSH S-transferase may be responsible for the protective action against lipid peroxidation, probably *via* two distinct pathways as mentioned above.

Tinberg and Barber[35] reported that inhibition of peroxidation by vitamin E was produced by its binding to structural protein derived from microsomal membranes. Whether or not the protective factor against lipid peroxidation in microsomes is identical with the structural protein has not been confirmed. However, since protection against peroxidation is dependent on GSH and is abolished by BSP, it seems unlikely that the protective factor is the structural protein. Ursini *et al.*[36] and Maiorino *et al.*[37] have reported that they isolated a GSH-dependent peroxidation-inhibiting protein, which can reduce lipid hydroperoxides in phospholipids, from cytosol of pig liver[36] and pig heart,[37] and they found that peroxidation inhibition by the protein was nullified by BSP. The occurrence of such a protein in microsomes has not been established, but its possible involvement cannot be excluded.

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