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BROMOSULFOPHTHALEIN ABOLISHES GLUTATHIONE-DEPENDENT PROTECTION AGAINST LIPID PEROXIDATION IN RAT LIVER MITOCHONDRIA

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Abstract—The effect of bromosulfophthalein (BSP) on GSH-dependent protection against lipid peroxidation in rat liver mitochondria was examined. Mitochondrial lipid peroxidation induced by ascorbate-Fe^{2+} was prevented by GSH, and addition of BSP abolished the protective effect of GSH. The effect of BSP was apparently not due to causing disappearance of GSH from the reaction mixture by interacting directly with GSH. BSP strongly inhibited the mitochondrial GSH *S*-transferase activity rather than the GSH peroxidase activity. Ascorbate-Fe^{2+}-induced lipid peroxidation in mitochondria without addition of GSH was also stimulated to some extent by BSP, and the stimulation seems likely to be due to abolition of the inhibitory effect of endogenous GSH. GSH could not be replaced as an inhibitor of lipid peroxidation by cysteine, β-mercaptoethanol, or dithiothreitol. The inhibitory effect of GSH on lipid peroxidation was not observed in vitamin E-deficient mitochondria. No inhibitory effect of exogenous vitamin E was demonstrated either in vitamin E-deficient mitochondria or in vitamin E-sufficient mitochondria in the presence of BSP, whether GSH was added or not. These results indicate that a mitochondrial GSH-dependent factor which inhibits lipid peroxidation requires vitamin E to exert its function. It is suggested that mitochondrial GSH *S*-transferase(s) may be responsible for GSH-dependent inhibition of lipid peroxidation in mitochondria, probably by scavenging lipid radicals.

It has been demonstrated that lipid peroxidation induced by ADP-iron in the presence of NADH or NADPH, or by CHP† in mitochondria is inhibited by respiratory substrates such as succinate, and the inhibition is brought about by a potent antioxidant, reduced ubiquinone [1–4]. Further protection against lipid peroxidation is provided by GSH. GSH-dependent protection has been demonstrated in mitochondria [5, 6], as well as in microsomes [5, 7–9] or cytosol [10–13] of rat liver. Flohé and Zimmermann [14] suggested that GSH peroxidase is the essential factor in preventing accumulation of lipid peroxides and lysis of membranes in mitochondria. McCay *et al.* [6] proposed that GSH-dependent antiperoxidative activity may not be due to reducing lipid hydroperoxides generated during peroxidation by a peroxidase activity, but may possibly be based on prevention of free radical attack on membrane lipids. On the other hand, the occurrence in microsomes of a GSH-dependent protein which inhibits lipid peroxidation and probably functions by scavenging free radicals has been reported [8, 15, 16].

A broad picture of GSH *S*-transferases and GSH peroxidases in subcellular fractions of the liver has been known. The GSH *S*-transferases are enzymes that catalyze the conjugation of GSH with a broad spectrum of hydrophobic compounds bearing an electrophilic center [17]. In addition, some of the enzymes exhibit non-selenium-dependent GSH peroxidase activity toward organic hydroperoxides as the substrate, but do not exhibit toward hydrogen peroxide, which is reduced by selenium-dependent GSH peroxidase [18, 19]. We have recently suggested that microsomal GSH *S*-transferase may be responsible for the GSH dependent-protection, since BSP, which inhibits the GSH *S*-transferase activity, abolished the protective effect of GSH [20]. GSH *S*-transferases also occur in the hepatic mitochondria [21, 22]. In order to investigate the mechanism of GSH-dependent protection against lipid peroxidation in mitochondria, the effect of BSP on the protection has been examined.

MATERIALS AND METHODS

Chemicals. GSH was purchased from Wako Pure Chemicals Ind. Ltd., and BSP (bromosulfophthalein disodium salt) and DL-α-tocopherol were obtained from Sigma Chemical Company (St. Louis, MO) and E. Merck (Darmstadt), respectively. Microsomal phospholipids were prepared as follows. Total microsomal lipids were extracted from rat liver microsomes by the method of Svingen *et al.* [23]. All solvents were purged with nitrogen and all operations were performed under nitrogen to minimize autoxidation of unsaturated lipids. Extracted lipids were subjected

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† Abbreviations used: GSH, reduced glutathione; BSP, bromosulfophthalein; MDA; malondialdehyde; TBARS, thiobarbituric acid-reactive substances; CHP, cumene hydroperoxide; CDNB, 1-chloro-2,4-dinitrobenzene.

to silicic acid column chromatography. Phospholipids were eluted with mixtures of chloroform and methanol, and identified by silica gel thin-layer chromatography [24]. Lipid phosphorus was measured according to the method described by Ames *et al.* [25]. All other chemicals employed were of commercial reagent-grade quality.

Preparation of mitochondria. 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. In some experiments, vitamin E-deficient rats were used; these were rats which (weanling males) were reared on diet deficient in vitamin E (vitamin E 8 mg/kg, Oriental Yeast Co. Ltd.) for 4 weeks. Rats were sacrificed by decapitation. The liver of each rat was perfused with 0.9% NaCl, and homogenized in 9 vol. of 0.25 M sucrose containing 1 mM Tris–HCl (pH 7.4). The homogenate was centrifuged at 600 *g* for 10 min and the resulting supernatant was centrifuged at 9000 *g* for 15 min. The pellet obtained was washed by resuspension in 50 mM Tris–KCl (0.14 M), pH 7.4, instead of 25 mM Tris–KCl (0.175 M) used by Hunter *et al.* [26] and centrifuged at 9000 *g* for 15 min. The washing procedure was repeated three times, and the third washing showed no detectable GSH peroxidase and GSH *S*-transferase activities. The washed pellet was resuspended in 50 mM Tris–HCl buffer, pH 7.4, and stored at −80°. The mitochondrial preparations were normally used within 5 days of preparation. The storage (at −76° for 5 days) in the frozen state of mitochondria has no effect on oxygen consumption, while the loss in phosphorylating activity would be produced [27].

Lipid peroxidation. The standard mixture contained 2 mg of mitochondrial protein, ascorbate–Fe^{2+} (0.25 mM–2 μM) and 50 mM Tris–HCl buffer, pH 7.4, in a total volume of 1.0 ml. Reactions were started by adding a freshly prepared $FeSO_4$ solution under nitrogen and were performed at 37° for 30 min unless otherwise specified.

The assay for lipid peroxidation of liposomes was performed in the following manner. An aliquot of microsomal phospholipid chloroform solution was transferred into a glass tube, the solvent was removed under a stream of nitrogen, nitrogen-saturated Tris buffer (10 mM), pH 7.4, was added, and the whole was mixed vigorously. The lipid suspension obtained was sonicated for 6 min in ice-cold water. The final concentration of lipid in the suspension was 0.75 μmole of lipid phosphorus per ml. Reaction mixtures (of 1.5 ml total volume) containing 10 mM Tris–HCl (pH 7.4), 1 ml of liposomal suspension, 0.1 mM ascorbate, and 5 μM Fe^{2+} were incubated for 10 min at 37°.

Lipid peroxidation was assayed by measuring TBARS according to a partial modification of the method described by Buege and Aust [28]. The assay mixture included 0.04% butylated hydroxytoluene to prevent the formation of TBARS during the assay procedure. The colored pigments, after being heated, were extracted with *n*-butanol, and the absorbance was measured at 535 nm. Peroxide formation was expressed in terms of nmole of MDA by using tetraethoxypropane as a standard.

Assay. GSH peroxidase activity was measured spectrophotometrically by a modification of the procedure described by Tappel [29]. The standard assay mixture contained 50 mM 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. GSH *S*-transferase activity was spectrophotometrically assayed by monitoring an increase in absorbance due to conjugate formation between GSH and CDNB at 340 nm according to the procedure of Habig *et al.* [30]. GSH [31] was determined with 5,5′-dithiobis(nitrobenzoic acid). Amounts of vitamin E in mitochondria were measured by the method of Taylor *et al.* [32]. Protein was determined by the method of Lowry *et al.* [33] using bovine serum albumin as a standard. All data represent the means ± standard deviation of three experiments.

RESULTS

Effect of GSH and BSP on lipid peroxidation

Lipid peroxidation induced by ascorbate–Fe^{2+} in rat liver mitochondria was inhibited by the addition of 0.5 mM GSH, and 0.05mM BSP abolished the inhibitory effect of GSH (Fig. 1A). Thiols such as cysteine, β-mercaptoethanol, and dithiothreitol were also treated, all at a concentration of 0.5 mM, to determine whether they inhibited the mitochondrial peroxidation, but they had no effect (data not shown). GSH was significantly consumed during lipid peroxidation in the presence of both mitochondria and ascorbate–Fe^{2+}, though a lag was observed for 10 min (Fig. 1B). The amount of GSH that disappeared during incubation for 40 min was 20%, while in the absence of ascorbate–Fe^{2+} or mitochondria little or no GSH was consumed. GSH consumption was greatest in the presence of GSH and BSP. Therefore, whether BSP can be conjugated with GSH in mitochondria was tested (Table 1). Mitochondria did not show the significant activity of GSH *S*-transferase toward BSP. The enzyme activity in rat cytosol was decreased to 64% in the presence of mitochondria. The GSH *S*-transferase activity in cytosol toward CDNB as the substrate was 590 nmole CDNB/mg protein/min (N = 3), and the activity was 21 times as the activity (28 nmole CDNB/mg protein/min, N = 3) in mitochondria. Thus, the specific activity toward BSP even in either cytosol or mitochondria was much lower than that toward CDNB. From the points, even if the conjugation of GSH with BSP occurs in mitochondria, the levels of GSH

Table 1. The GSH-conjugating ability toward BSP in mitochondria

Fraction	Activity nmole CDNB/mg protein/min (%)
Mitochondria	< 0.1*
Cytosol	2.55 ± 0.67 (100)
Mitochondria + Cytosol	1.63 ± 0.51 (64)

* Detection limit.

All BSP concentrations used were 0.05 mM, and 2 mg of mitochondrial or cytosolic protein per ml of incubations were added. Change in absorbance at 330 nm was monitored at 25°, according to the method of Habig *et al.* [30].

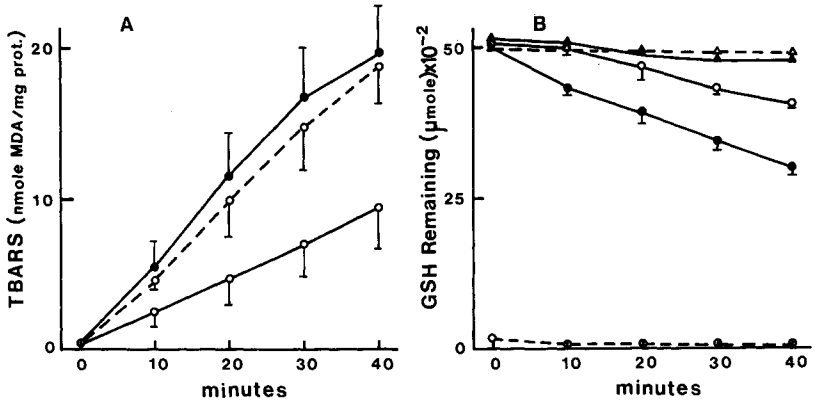

Fig. 1. Inhibition of mitochondrial lipid peroxidation by GSH, the effect of BSP, and the GSH consumption. Mitochondria (2 mg protein/ml) were incubated with 0.25 mM ascorbate and 2 μM Fe^{2+} at 37° in 50 mM Tris–HCl buffer (pH 7.4). The amount of TBARS present and the GSH concentration were measured at various incubation times. The additions were as indicated below. (A) Lipid peroxidation: no addition (○---○); 0.5 mM GSH (○—○), 0.5 mM GSH and 0.05 mM BSP (●—●). (B) Time course of GSH concentration: no addition (○---○); 0.5 mM GSH (○—○); 0.5 mM GSH and 0.1 mM BSP (●—●); 0.5 mM GSH, minus mitochondria (△---△); 0.5 mM GSH, minus ascorbate–Fe^{2+} (▲—▲).

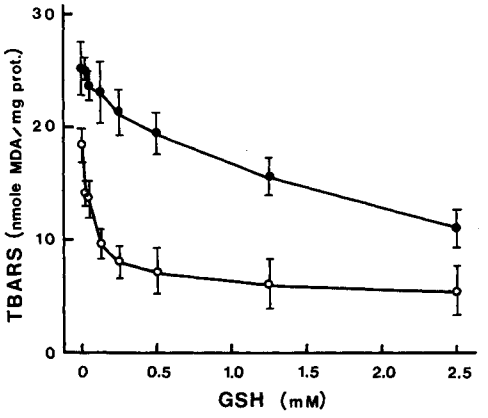

Fig. 2. Effect of GSH concentration on lipid peroxidation in the absence or the presence of BSP. Lipid peroxidation was induced as described in Fig. 1 and the incubation time was 30 min. Open symbols represent incubations with no BSP, and closed symbols represent incubations with 0.05 mM BSP.

(500 nmoles added initially to the peroxidation systems per ml) may be hardly affected.

The effect of various concentrations of GSH on lipid peroxidation in the absence or the presence of BSP was examined (Fig. 2). A precipitous decrease of lipid peroxidation was produced by GSH added at lower concentrations than 0.25 mM. The inhibition was observed even at the concentration of 0.025 mM GSH. Without the addition of GSH, lipid peroxidation was stimulated by BSP. In this case, the endogenous GSH level in mitochondria was 6.9 nmoles/mg protein, in close agreement with 8.8 nmoles/mg protein of GSH concentration in mitochondrial matrix, which are correspond to 10.7 mM GSH in mitochondrial space [21]. The peroxidation in the presence of BSP (0.05 mM) gradually decreased with an increase of added amounts of GSH. Lipid peroxidation in mitochondria was stimulated with increase of BSP concentrations (up to 0.3 mM) regardless of the absence or the presence of GSH (Fig. 3A). BSP itself, however, had no stimulatory effect on

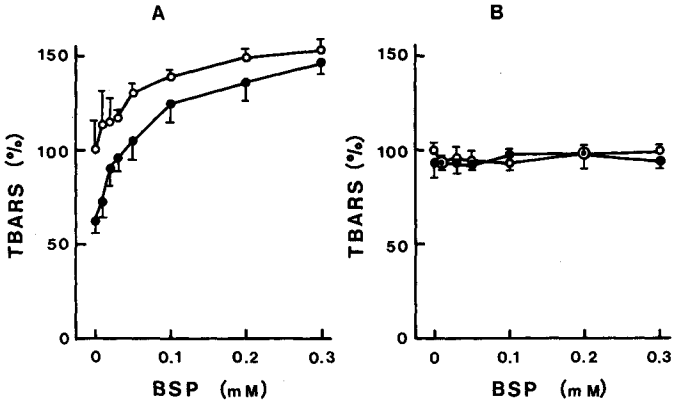

Fig. 3. Effect of BSP concentration on ascorbate–Fe^{2+}-induced lipid peroxidation in the absence or the presence of GSH in mitochondria and in liposomes. Mitochondrial lipid peroxidation was induced as described in Fig. 1. Liposomes (0.5 μmole of inorganic phosphate/ml of 50 mM Tris–HCl buffer, pH 7.4) were prepared with microsomal phospholipids and incubated with 0.1 mM ascorbate and 5 μM Fe^{2+} for 10 min at 37°. (A) Lipid peroxidation in mitochondria and (B) lipid peroxidation in liposomes. Open symbols represent incubations with no GSH and closed symbols represent incubations with 0.5 mM GSH.

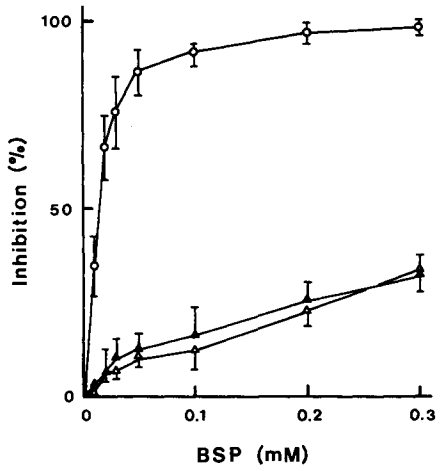

Fig. 4. Inhibition of the GSH-dependent enzyme activities in mitochondria by BSP. The GSH *S*-transferase activity was measured with CDNB as a substrate, and the GSH peroxidase activity was estimated with H_2O_2 or CHP as a substrate. Inhibition curves shown are: GSH *S*-transferase (○—○); GSH peroxidase, H_2O_2 (△—△) or CHP (▲—▲).

lipid peroxidation induced by ascorbate–Fe^{2+} in liposomes, and GSH (0.5 mM) did not inhibit the liposomal lipid peroxidation (Fig. 3B). These observations indicate that stimulation of lipid peroxidation by BSP without adding GSH in mitochondria may be due to abolition of the inhibitory effect of endogenous GSH.

Effect of BSP on the activities of GSH S-*transferase and GSH peroxidase*

BSP strongly inhibited the GSH *S*-transferase activity, and slightly inhibited the GSH peroxidase activity with either CHP or H_2O_2 as a substrate (Fig. 4). Inhibition curves of mitochondrial GSH *S*-transferase by BSP added in two different environments, by using Tris buffer, pH 7.4 (peroxidation condition) and 0.1 M phosphate buffer, pH 6.5 (assay condition of GSH *S*-transferase activity according to Habig *et al.* [30]) was examined, and consequently the similar results between the two environments were obtained (data not shown). In the presence of 0.05 mM BSP the inhibitions of the GSH *S*-transferase and GSH peroxidase activities were approximately 86% and 10%, respectively.

Effect of vitamin E on mitochondrial lipid peroxidation

Exogenous vitamin E inhibited lipid peroxidation in vitamin E-sufficient mitochondria, but the effect of vitamin E was not significant in the presence of BSP, whether GSH was present or not (Fig. 5A). The inhibitory effect of GSH or exogenous vitamin E on lipid peroxidation was little observed in vitamin E-deficient mitochondria. The addition of BSP did not cause any change of endogenous vitamin E levels in mitochondria from either vitamin E-sufficient rats or vitamin E-deficient rats, and the uptake of vitamin E for the mitochondria, to which 40% or less of added amounts of vitamin E were retained (Table 2).

DISCUSSION

GSH-dependent protection and abolition by BSP

A mitochondrial factor which exhibits inhibitory action on lipid peroxidation in the presence of GSH is heat-labile [5, 6]. The present experiments demonstrate that GSH cannot be replaced by cysteine, β-mercaptoethanol, or dithiothreitol in preventing lipid peroxidation by ascorbate–Fe^{2+} in mitochondria, and also show that the inhibitory effect of GSH on lipid peroxidation is abolished by BSP. Moreover, it is suggested that BSP probably may compete with GSH, because the peroxidation in the presence of BSP decreased with an increase of added amount of GSH (Fig. 2). The effect of BSP was probably not caused by disappearance of GSH from the reaction

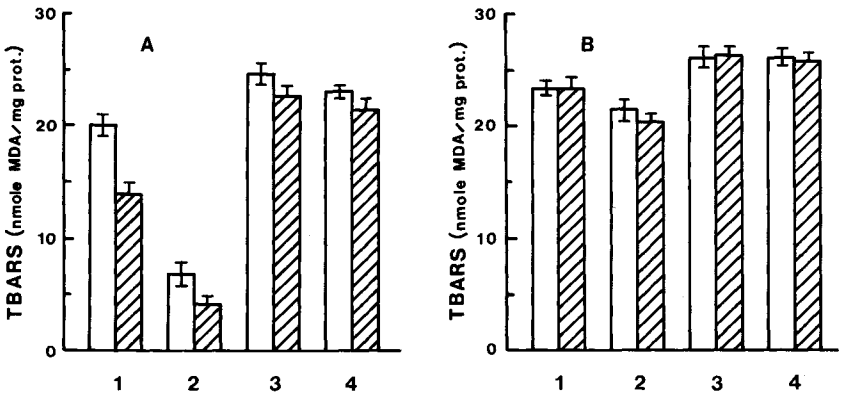

Fig. 5. Effect of endogenous and exogenous vitamin E on lipid peroxidation in mitochondria. Mitochondria from vitamin E-sufficient, control (A) and vitamin E-deficient (B) rat liver were incubated with the ascorbate–Fe^{2+} lipid peroxidation system without or with exogenous vitamin E, and the effect of GSH and/or BSP was tested. Additions were as follows: 1, no addition; 2, 0.5 mM GSH; 3, 0.1 mM BSP; 4, 0.5 mM GSH and 0.1 mM BSP. Open bars represent incubations with no exogenous vitamin E and shaded bars represent incubations with 16 μM vitamin E (5 μl of ethanol solution was added to 1.5 ml of incubation medium). Endogenous vitamin E levels in mitochondria from control and vitamin E-deficient rats were 0.246 μg/mg protein and 0.022 μg/mg protein, respectively.

Table 2. The effect of BSP on vitamin E status or uptake of vitamin E in mitochondria

Rats	Addition	Vitamin E Levels (g/mg protein) Pellet	Suspension	Uptake (%)
Vitamin E-sufficient	None	0.217 ± 0.008		
	BSP	0.213 ± 0.017		
	Vitamin E	1.313 ± 0.070	3.375 ± 0.253	41.6
	Vitamin E + BSP	1.317 ± 0.080	3.348 ± 0.290	41.7
Vitamin E-deficient	None	0.028 ± 0.015		
	BSP	0.024 ± 0.014		
	Vitamin E	1.059 ± 0.064	2.979 ± 0.045	34.9
	Vitamin E + BSP	1.053 ± 0.076	3.035 ± 3.337	34.1

BSP (0.1 mM) and/or vitamin E (16 μM) were added to mitochondrial suspension (2 mg protein/mg), and the mixtures were incubated for 15 min on ice. After centrifugation at 9000 *g* for 15 min, the pellets and suspensions (prior to centrifugation) were subjected to vitamin E determination. All data are mean ± SD for four rats.

mixture. Therefore, the increase of GSH consumption in the presence of BSP would be presumably due to oxidizing substances generated during the enhanced peroxidation.

Ascorbate–Fe^{2+}-induced lipid peroxidation without addition of GSH in mitochondria was stimulated by BSP, but BSP had no effect on lipid peroxidation induced by ascorbate–Fe^{2+} in liposomes. Thus, in stimulation of mitochondrial peroxidation by BSP without addition of GSH seems likely to be due to abolition of the inhibitory effect of endogenous GSH.

GSH-dependent factor in mitochondria

Concerning GSH *S*-transferases and GSH peroxidases in mitochondria, microsomes, and cytosol of rat livers, distribution of the activity, localization, properties, and possible identity are summarized in Table 3. Selenium-dependent GSH peroxidases [19, 34–36] and GSH peroxidase in inter membrane

Table 3. Summarized profile of GSH *S*-transferases and GSH peroxidases in the rat liver

	GSH *S*-transferase Substrate (CDNB)	GSH peroxidase Substrate	GSH peroxidase
Mitochondria*	W Distribution of total activity (Cyt. 90%, Mit. 7%) [21].	CHP	Distribution of total activity (Cyt. 60%, Mit. 28% Mic. 7%) [34].
	M Isolation of three enzymes [22].	tBH	Se-independent, 5% activity of Se-dependent Mit. activity [19].
		CHP and tBH H_2O_2	Purification of Se-dependent [36].
	It	CHP and H_2O_2	Se-independent, available both substrates [37].†
	Ot *N*-ethylmaleimide-activatable, immunochemical identity with microsomal enzyme [40].		
Microsomes	*N*-ethylmaleimide-activatable, purification, distinct from cytosolic enzymes [41–43]. Distribution of specific activity (Cyt. 91%, Mic. 9%) [44]. No activity toward BSP [42], inhibition by BSP [20].	CHP	*N*-ethylmaleimide-activatable, purification, associated with GSH *S*-transferase activity [39].
Cytosol	Presence of at least seven enzy. purification [17, 45, 46]. Inhibition by BSP [47].	CHP and H_2O_2	Presence of Se-dependent (CHP and H_2O_2) and Se-independent (CHP) [18].
		CHP and tBH	Identity of GSH *S*-transferase B and Se-independent, and inhibition by BSP [48].
		CHP	35% of Se-independent activity [49].

* W, whole; M, matrix, It, inter membrane space; Ot, outer membranes.
† Data in mice (Such activity is detectable in mitochondrial inter membrane space of rat livers; unpublished work).
tBH; t-butylhydroperoxide.

space [37] are present in mitochondria. In the current experiments, however, BSP strongly inhibited the mitochondrial GSH *S*-transferase activity rather than the GSH peroxidase activity. On the other hand, selenium-independent GSH peroxidase which is associated with GSH *S*-transferase is present in cytosol [18, 38] and microsomes [39] of rat liver, and it readily also reduces organic hydroperoxides. Sies and Moss [19] have reported that GSH peroxidase activity in the mitochondrial matrix from selenium-deficient rats was only about 5% of the control activity with *t*-butylhydroperoxide as a substrate. Thus, the selenium-independent GSH peroxidase activity which is possibly associated with the GSH *S*-transferases seems likely to be very low in mitochondria. Therefore, it is suggested that GSH peroxidase activity in mitochondria may be not related to inhibition of peroxidation, but mitochondrial GSH *S*-transferase(s) may be responsible for GSH-dependent inhibition of lipid peroxidation in mitochondria.

Kraus isolated three GSH *S*-transferases from the mitochondrial matrix [22]. On the other hand, Morgenstern *et al.* [40] have demonstrated immunochemically that approximately 5% of the total protein of mitochondrial outer membranes consist of microsomal GSH *S*-transferase. The enzyme is activated by *N*-ethylmaleimide in analogy with the microsomal enzyme. Therefore, it seems likely that the outer membrane-bound GSH *S*-transferase may be involved in GSH-dependent protection against lipid peroxidation in mitochondria, possibly by scavenging lipid radicals.

Role of vitamin E on GSH-dependent protection, In microsomes, the inhibitory action of GSH on lipid peroxidation requires vitamin E [7, 9], and it is proposed that this action may be exerted by preventing radical formation or scavenging free radicals. In the present experiments, little inhibitory effect of GSH on lipid peroxidation was observed in vitamin E-deficient mitochondria, and exogenous vitamin E was also ineffective in these mitochondria. Nevertheless, 35% of vitamin E added were retained in mitochondrial membranes. Furthermore, BSP had no effect on the vitamin E levels of mitochondria and uptake of vitamin E in mitochondria sufficient or deficient in vitamin E. These results indicate that a mitochondrial GSH-dependent factor which inhibits lipid peroxidation requires vitamin E to exert its function, and then in particular, its action is efficient in mitochondria nutritionally sufficient in vitamin E. In mitochondria from vitamin E-deficient rats, GSH-dependent antiperoxidative factor may be impaired. Thus, vitamin E may play an important role on defending the GSH dependent factor against oxidant species. Hill and Burk [16] have suggested that the GSH-dependent radical scavenging protein and α-tocopherol function independently to protect the microsomal membrane against free radical attack in microsomes.

On the other hand, Reddy *et al.* [7] have proposed that vitamin E is regenerated by a heat-labile inhibitory factor of microsomal lipid peroxidation in the presence of GSH. McCay *et al.* [50] have also proposed that vitamin E in microsomes functions as an electron shuttle by a heat-labile membrane protein which utilizes reducing equivalents from GSH. In vitamin E-sufficient mitochondria, no inhibitory effect of exogenous vitamin E was observed in the presence of BSP, even if GSH was added (Fig. 5). Therefore, it can be considered that vitamin E radical produced by scavenging lipid radicals during peroxidation is regenerated to vitamin E by mitochondrial GSH *S*-transferase(s) in the presence of GSH, as proposed to account for GSH-dependent protection against lipid peroxidation in microsomes [20]. With respect to a role of vitamin E on GSH-dependent protection against lipid peroxidation in mitochondria, additional work appears to be necessary.

Defense against oxidant stress in mitochondria. The deterioration of mitochondria under lipid peroxidation induced by ferrous ion or ascorbate results in swelling, the uncoupling of oxidative phosphorylation, and the inhibition of respiration [51]. When membrane thiols in mitochondria are exposed to a thiol-oxidizing agent, a critical decrease of the thiols results in stimulation of lipid peroxidation [52]. The amount of MDA accumulated during peroxidation correlates with SH oxidation in the membrane, and swelling is dependent on the disappearance of thiol groups [53]. The GSH-dependent defense system, as well as reduced ubiquinone [1–4], is likely to play an important role in the protection of mitochondrial membranes against oxidative stress.

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