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S-Thiolation and Oxidation of Glycogen Phosphorylase b and Peroxidation of Liposome Initiated by Free Radical Species

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Abstract: The relationship of S-thiolation and oxidation of glycogen phosphorylase b and peroxidation of phosphatidyl choline liposome by xanthine oxidase (XOD), 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH), and 2,2'-azobis(dimethylvaleronitrile) (AMVN)-generated free radicals was investigated. Glycogen phosphorylase b was S-thiolated in the presence of glutathione and oxidized in the absence of it by XOD, AAPH and AMVN. In XOD-initiated reaction, the rates of S-thiolation and oxidation of phosphorylase were very similar and addition of liposome to the reaction mixture showed little inhibition of the modifications. In AAPH-initiated reaction, the rate of oxidation was higher than that of S-thiolation and addition of liposome increased oxidation of the protein but had no effect on S-thiolation. In AMVN-initiated reaction, S-thiolation was higher than oxidation and addition of liposome increased S-thiolation remarkably but showed no effect on oxidation. The effect of liposome on modifications of protein in AAPH and AMVN reaction seemed to be caused by certain reactive degradation products or intermediates of liposome by free radical attack. Peroxidation of liposome was not observed in XOD-initiated reaction. Liposome was gradually peroxidized by AAPH reaction. The peroxidation was inhibited by addition of GSH and phosphorylase. Peroxidation of liposome by AMVN was extreamly fast, and was not affected by GSH and phosphorylase.
Key words: glycogen phosphorylase b, lipid peroxidation, liposome, oxidation, protein S-thiolation.

Proteins are oxidized when treated with oxidants or free radical species (Rivett, 1986; Davies and Goldberg, 1987). And they are S-thiolated when low-molecular-weight thiols are present along with radical species (Armstrong and Buchanan, 1978). The mixed-disulfide formation between protein thiols and low-molecular-weight thiols is called S-thiolation. Oxidation of protein is usually irreversible, on the other hand, S-thiolated proteins can be reduced by dethiolating systems in cells (Park and Thomas, 1989). It was also shown that S-thiolated proteins were resistant to oxidation (Coan *et al.*, 1992). Therefore, protein S-thiolation might be considered as a mechanism which renders protection to the cells under oxidative stress (Thomas and Sies, 1992). Glutathione is present in most types of cells in millimolar concentrations (Halliwell and Gutteridge, 1985), while cysteine and cysteamine are much less abundant (Ida *et al.*, 1984; Garcia *et al.*, 1984). Therefore, S-thiolation of protein with glutathione may be assumed to be the most important form. Many proteins were found to be S-thiolated in cultured cells by treatment with oxidants (Grimm *et al.*, 1985) or free radical generators (Rokutan *et al.*, 1989; Chai *et al.*, 1994). Proteins can be S-thiolated by thiol-disulfide exchange mechanism in which low-molecular-weight disulfides react with protein thiols (Ziegler, 1984; Gilbert, 1984). However, thiol-disulfide exchange mechanism is not satisfactory to account for S-thiolation of proteins which occurred when proteins and reduced glutathione are incubated with radical species (Park and Thomas, 1988). And it was found that proteins were rapidly S-thiolated in stimulated neutrophils without increase of cellular glutathione disulfide (Chai *et al.*, 1994). Therefore, an alternative mechanism of S-thiolation in which protein sulfhydryls are activated by direct reaction with free radical species and then activated protein sulfhydryls react with reduced form of glutathione seems more feasible (Park and Thomas, 1988).

Lipids containing polyunsaturated fatty acids undergo peroxidation by a free radical reaction mechanism when oxidants strong enough to abstract hydrogen from unsaturated fatty acid producing lipid radicals are present (Tappel, 1975; Tien and Aust, 1982; Porter,

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1984). Peroxidation of membrane phospholipids results in progressive degeneration of membrane structure and loss of cellular activity.

When the cells are challenged by free radical species, the components damaged and the types of modification may differ according to the nature and the location of the radical species. We attempted to determine the relationship between modification of protein, i.e., protein S-thiolation and oxidation, and peroxidation of lipid by reacting phosphorylase b and liposome with various free radical generating species in a mixture. In order to gain insight about the importance of the two processes in the toxicity of oxidative stress in many tissues, glycogen phosphorylase b was used since it was shown to be a major protein to be S-thiolated in cultured cells under oxidative stress (Collison and Thomas, 1987). Xanthine oxidase (XOD), 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH) and 2,2′-azobis(dimethylvaleronitrile) (AMVN) were used as free radical generators. Xanthine oxidase produces superoxide anion and hydrogen peroxide. AAPH and AMVN produce carbon-centered free radicals by thermal decomposition which may subsequently react with O_2 to yield reactive oxygen species.

Materials and Methods

Materials

Glycogen phosphorylase b, xanthine oxidase (XOD), superoxide dismutase (SOD), glutathione (GSH), soybean phosphatidyl choline, ascorbate, α-tocopherol, and thiobarbituric acid (TBA) were obtained from Sigma Chemical Company (St. Louis, USA). Ampholytes, Gel-Bond PAG film were from Phamacia LKB (Piscataway, USA). 2,2′-Azobis(2-amidinopropane) hydrochloride (AAPH) and 2,2′-azobis(dimethylvaleronitrile) (AMVN) were from Polyscience (Warington, USA).

Treatment of glycogen phosphorylase b and xanthine oxidase

Glycogen phosphorylase b was treated with 10 mM dithiothreitol, 2 mM EDTA, 20 mM β-glycerophosphate, pH 7.0 for 30 min at 30℃ and dialyzed against 20 mM β-glycerophosphate, pH 7.0. Xanthine oxidase was treated with 2 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM EDTA overnight at 4℃ to remove contaminating protease activity and dialyzed against 20 mM β-glycerophosphate, pH 7.0 (Park and Thomas, 1988).

Preparation of liposome

Chloroform solution (48%) of soybean phosphatidyl choline was dried by rotary evaporator. The resulting thin lipid film was suspended in 50 mM NaCl, 50 mM Tris-HCl, pH 7.0 by brief bath sonication and then further sonicated with 0.5 inch probe for 8×15 sec with 45 sec intervals (Liebler *et al.*, 1986).

Analysis of S-thiolation and oxidation of phosphorylase b

S-thiolation and oxidation of protein was analyzed by isoelectric focusing (Thomas and Beidler, 1986). Protein samples were alkylated with 40 mM iodoacetamide for 15 min before focusing on polyacrylamide gels with 4% T-2.6% C containing 2% of a mixture of pH 4~6 and preblended pH 5~8 Ampholytes at 1 to 5 ratio. Gels were prefocused for 10 min, samples were applied in sample wells approximately 2.0 cm from anode, and then focused for 50 min at 4℃ with limiting voltage, 1500 V, and limiting current, 2.8 mA/cm gel width. Gels were stained with coomassie blue R. The protein concentration was determined by the method of Lowry *et al.* (1951).

Analysis of peroxidation of lipid

Lipid peroxidation was analyzed by measuring malondialdehyde by thiobarbituric acid assay (Buege and Aust, 1978). 1 ml of sample was combined with 2 ml of stock thiobarbituric acid reagent (0.25 N HCl, 15% trichloroacetic acid, 0.375% TBA). The solution was heated for 15 min in a boiling water bath, and then cooled down to room temperature. After centrifugation at 1000×*g* for 10 min, the absorbance of the supernatant was determined at 535 nm.

Analysis of glutathione

Glutathione was analyzed by a modification of the HPLC method by Reed *et al.* (1980). 5% perchloric acid was added to the samples to precipitate proteins. Iodoacetic acid (24 mg/ml) was added and powder of $KHCO_3$ was added to neutralyze samples. The samples were derivatized with 1-fluoro-2,4-dinitrobenzene (FDNB) by adding an equal volume of 3% FDNB in absolute ethanol and were separated on 3-amino-propyl bonded reverse-phase ion-exchange column. A second aliquot of each sample was reacted with 20 mM N-ethylmaleimide before treatment to correct for the oxidation of glutathione (GSH) to glutathione disulfide (GSSG) which might occur during sample preparation (Collison *et al.*, 1986).

Results and Discussion

S-thiolation and oxidation of phosphorylase b and peroxidation of liposome were initiated by xanthine oxidase. AAPH, which are water-soluble free radical gen-

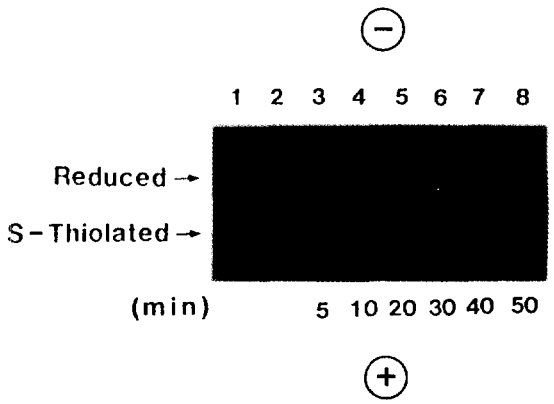

Fig. 1. S-thiolation of phosphorylase b by AAPH. Lane 1 is reduced glycogen phosphorylase alkylated with 40 mM iodoacetamide and lane 2 is fully S-thiolated phosphorylase. In lanes 3~8, phosphorylase b (3 μg/10 μl) was incubated at 37℃ with 20 mM AAPH at pH 7.0 in the presence of 0.2 mM GSH. At the indicated time, aliquots were alkylated with 40 mM iodoacetamide to stop the reaction.

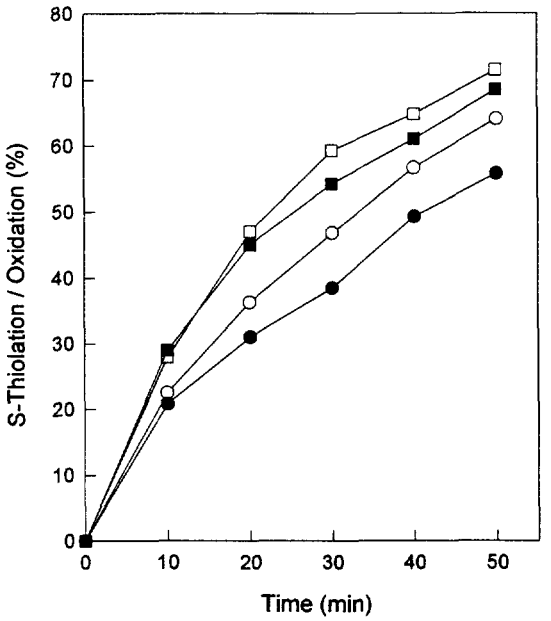

Fig. 2. S-thiolation and oxidation of phosphorylase b by XOD and the effect of liposome. Phosphorylase b (3 μg/10 μl) was incubated at 37℃ with 1.5 mM xanthine and 33 mU/ml xanthine oxidase at pH 7.0 in the presence of 0.2 mM GSH (for S-thiolation) or in the absence of GSH (for oxidation). Liposome (5 mM) was added to examine the effect on protein S-thiolation and oxidation. At the indicated time, aliquots were alkylated as in Fig. 1. □ : S-thiolation; ○ : oxidation; ■ : S-thiolation with liposome added; ● : oxidation with liposome added.

erators, or AMVN, which is a lipid-soluble radical generator.

S-thiolation and oxidation of phosphorylase b was analyzed by isoelectric focusing (IEF) method. Fig. 1 shows an IEF gel of an experiment on S-thiolation of phosphorylase b with AAPH as a free radical generator. Alkylation with iodoacetamide ensured that reactive sulfhydryl groups did not ionize during focusing and thereby affect the protein analysis. Lane 1 shows reduced form of phosphorylase b (pI 6.1) and lane 2 shows fully S-thiolated phosphorylase b (pI 5.8). Fully S-thiolated phosphorylase b was prepared by reacting with 40 mM oxidized glutathione (GSSG). A protein gains a negative charge upon addition of a glutathione to the sulfhydryl group of protein. In lanes 3~8, bands of phosphorylase b moved toward anode with time indicating that phosphorylase was gradually S-thiolated. Dimeric glycogen phosphorylase b contains two reactive sulfhydryl groups per subunit. Therefore, five bands were observed during S-thiolation experiments. The bands from top to bottom indicate fully reduced, one, two, three, and four sulfhydryl groups modified phosphorylase b, respectively. Oxidation of phosphorylase by incubating with free radical generators in the absence of glutathione produced the pattern of five bands identical to that of S-thiolation. It suggests that oxidation also added a negative charge on each protein sulfhydryl group. However, oxidized phosphorylase b was not reduced by dithiothreitol (DTT), whereas S-thiolated phosphorylase b was fully reduced by treatment with DTT (Park and Thomas, 1988). The modification of phosphorylase b was quantitated by densitometry. The density of each protein band was multiplied by the charge change attributed to the position of the band, i.e., five bands from top to bottom were multiplied by 0, 1, 2, 3, and 4, respectively. The values were summed and divided by possible maximum charge change of four to obtain the percentage of modification.

Fig. 2 shows the time course of S-thiolation and oxidation of phosphorylase b by xanthine oxidase and the effect of added liposome. Glycogen phosphorylase b was gradually S-thiolated by reactive oxygen species produced by xanthine oxidase in the presence of GSH, and was oxidized in the absence of GSH. Rates of the two modification reactions were similar. Addition of liposome to the reaction mixture showed very limiting effect, i.e., almost no change on S-thiolation and a marginal inhibition on oxidation. In oxidation reaction, some of the protein showed broad spreading over the lane, and some precipitated at the origin. It indicates that the protein was denatured and degraded by oxidation. S-thiolation did not show such phenomena. The result suggests that proteins are protected by S-thiolation from degenerative oxidation in the situation of oxygen radical generation. Addition of liposome to the oxidation reaction mixture resulted some protection of phosphorylase from the degenerative effect possibly by trapping some of the oxygen free radicals.

Fig. 3 shows the time course of S-thiolation and oxidation of phosphorylase by AAPH and the effect of

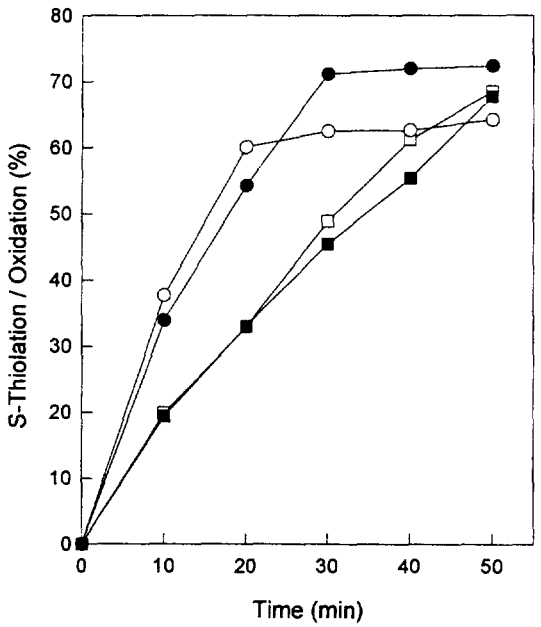

Fig. 3. S-thiolation and oxidation of phosphorylase b by AAPH and the effect of liposome. Phosphorylase b (3 μg/10 μl) was incubated at 37℃ with 20 mM AAPH at pH 7.0 in the presence of 0.2 mM GSH (for S-thiolation) or in the absence of GSH (for oxidation). Liposome (5 mM) was added to examine the effect on protein S-thiolation and oxidation. At the indicated time, aliquots were alkylated as in Fig. 1. □ : S-thiolation; ○ : oxidation; ■ : S-thiolation with liposome added; ● : oxidation with liposome added.

added liposome. Time course of S-thiolation was similar to that of S-thiolation by XOD in Fig. 2. However, the rate of oxidation was a lot higher than that of S-thiolation and reached maximum oxidation in 20 min. Addition of liposome did not affect S-thiolation of phosphorylase. But oxidation of phosphorylase by AAPH was increased rather than decreased as we expected when liposome was added. This increased oxidation might be caused by certain reactive species, possibly free radicals, produced as a result of peroxidative breakdown of liposome. AAPH also caused denaturation and degradation of phophorylase when GSH was not present, and addition of liposome did not give any significant protection from denaturation.

Inhibition of S-thiolation and oxidation of phosphorylase by several free radical scavengers was examined (Table 1). XOD-initiated S-thiolation and oxidation were inhibited by catalase, and the inhibition was potentiated by addition of SOD with catalase whereas SOD alone did not have any effect. Ascorbate and α-tocopherol also inhibited XOD-initiated S-thiolation and oxidation. With all of the scavengers tried, the degree of inhibition was same in S-thiolation and oxidation. The results of Fig. 2 and Table 1 suggest that the species causing S-thiolation and oxidation of phosphorylase in XOD

Table 1. Inhibition of XOD-, and AAPH-initiated S-thiolation and oxidation by radical scavengers. Phosphorylase b (3 μg/10 μl) was incubated for 30 min at 37℃ with 33 mU/ml xanthine oxidase and 1.5 mM xanthine or 20 mM AAPH in the presence of 0.2 mM GSH (for S-thiolation) or absence of GSH (for oxidation)

Additions	% Inhibition			
	XOD-initiated		AAPH-initiated	
	S-thiolation	oxidation	S-thiolation	oxidation
SOD[a] (100 U/ml)	0	0	0	0
Cat[b] (100 U/ml)	42	41	0	0
SOD+Cat[c]	83	90	0	0
Ascorbate (100 μM)	32	34	39	82
α-Tocopherol (50 μM)	58	61	0[d]	0[d]

[a] SOD : superoxide dismutase.
[b] Cat : catalase.
[c] 100 U/ml SOD+100 U/ml Cat.
[d] Inhibition was not observed up to 0.5 mM α-tocopherol.

reaction are reactive oxygen species including oxygen centered free radicals and that the reaction pathways for S-thiolation and oxidation by them are similar, in which protein sulfhydryls react with reactive oxygen species generated by XOD and the activated protein sulfhydryls react subsequently with glutathione in S-thiolation and with oxygen in oxidation. The results also support the idea that the reaction of protein sulfhydryls with reactive oxygen species seems to be rate limiting. And reactivities of the activated form of protein sulfhydryls with GSH and molecular oxygen seem to be almost same. The active species initiating S-thiolation and oxidation in AAPH reaction might be either water-soluble organic radicals themselves produced by thermal degradation of AAPH or oxygen free radicals generated by the reaction of dissolved molecular oxygen with AAPH-originated organic free radicals. AAPH-initiated protein S-thiolation and oxidation were not inhibited by SOD and catalase, but inhibited by ascorbate alone. This indicates that the species reacting with protein sulfhydryls are not oxygen free radicals. And a possible alternative mechanism for S-thiolation and oxidation in which certain activated species of GSH or molecullar oxygen which are generated by reaction with free radicals attack protein sulfhydryls to produce S-thiolated or oxidized proteins coluld be eliminated also by this result. The activated protein sulfhydryl species by XOD and that by AAPH seem to be significantly different in chemical reactivity. The former has the same reactivity with GSH and oxygen, but the latter has higher reactivity with molecular oxygen than GSH. AMVN-initiated S-thiolation and oxidation of the protein was not affected by any of the scavengers tried in Table 1.

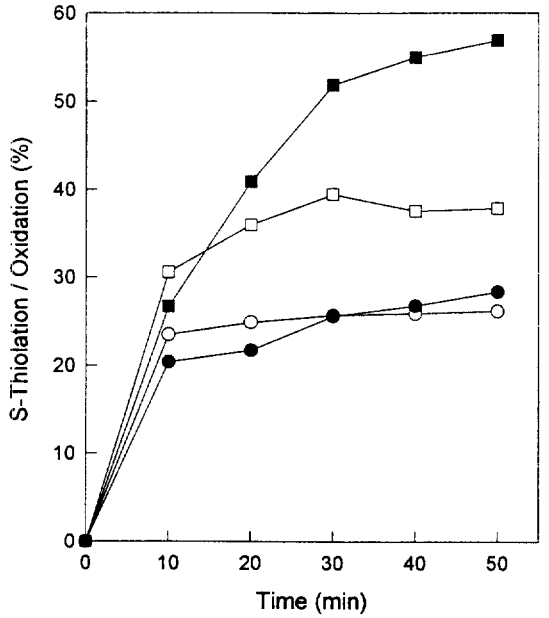

Fig. 4. S-thiolation and oxidation of phosphorylase b by AMVN and the effect of liposome. Phosphorylase b (3 μg/10 μl) was incubated at 37℃ with 20 mM AMVN under the identical conditions as in Fig. 3. □: S-thiolation; ○: oxidation; ■: S-thiolation with liposome added; ●: oxidation with liposome added.

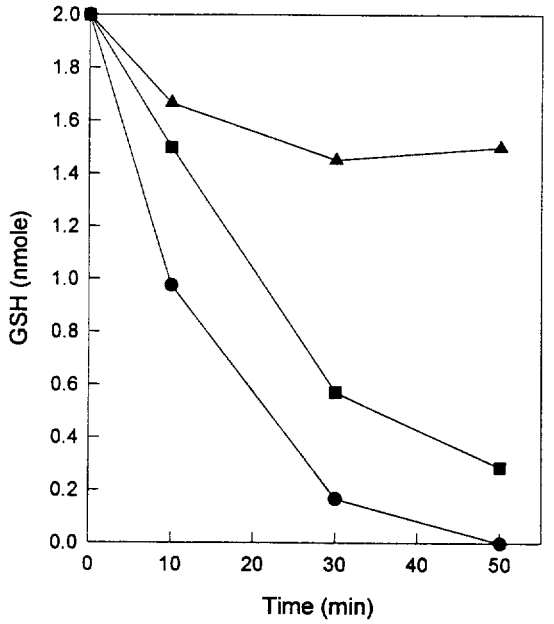

Fig. 5. GSH in XOD-, AAPH-, and AMVN-initiated reaction. 0.2 mM of GSH was reacted with XOD (33 mU/ml) and xanthine (1.5 mM) (●), 20 mM AAPH (■), or 20 mM AMVN (▲). At the indicated time, 100 μl aliquot of reaction mixture was analyzed by HPLC as described in Materials and Methods.

Fig. 4 shows the same experiment as Fig. 3 but with AMVN as a free radical generator. Several differences between the results of Fig. 3 and Fig. 4 were observed. Overall rates of S-thiolation and oxidation were lower in Fig. 4 which might be due to lower effective concentration of free radicals in solution because AMVN is lipid-soluble. The rate of AMVN-initiated S-thiolation was higher than that of oxidation, while the rate of oxidation was higher in AAPH-initiated reaction. The effect of liposome was also different. Addition of liposome resulted remarkably increased S-thiolation, so that the rate of S-thiolation was almost same as in XOD-initiated S-thiolation, whereas oxidation was not affected. This increased S-thiolation seemed to be caused by certain water-soluble reactive species of peroxidative breakdown products of liposome. The results suggest that activated intermediate of protein sulfhydryls generated by AMVN reaction is quite different from that by AAPH reaction, and that the former has higher reactivity with GSH than molecular oxygen.

The reaction rates of GSH in XOD, AAPH, and AMVN systems were determined by measuring residual GSH in reaction mixture (Fig. 5). XOD-generated reactive oxygen species and AAPH-genetated free radicals showed very high reactivity with GSH, while AMVN-generated radicals had very limited reactivity with GSH.

Peroxidation of phosphatidyl choline liposome was examined with XOD, AAPH, and AMVN as free radical generators. No measurable peroxidation of liposome

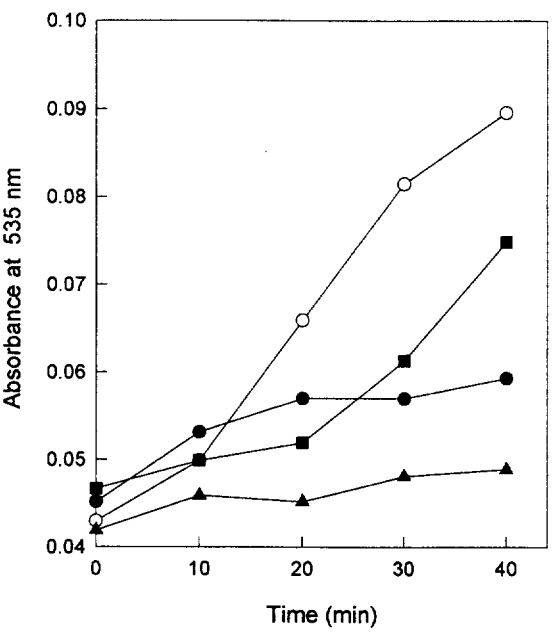

Fig. 6. Peroxidation of liposome by AAPH and the effect of GSH and phosphorylase b. 5 mM liposome was incubated at 37℃ with 20 mM AAPH at pH 7.0 (○). And the effect of addition of 0.2 mM GSH (●), 0.5 mg/ml phosphorylase b (■), or both GSH and phosphorylase (▲) on lipid peroxidation was determined.

was observed with XOD system. Fig. 6 shows the time course of peroxidation by AAPH and the effect of GSH and phosphorylase on it. Liposome was gradually peroxidized by incubation with AAPH. Addition of GSH

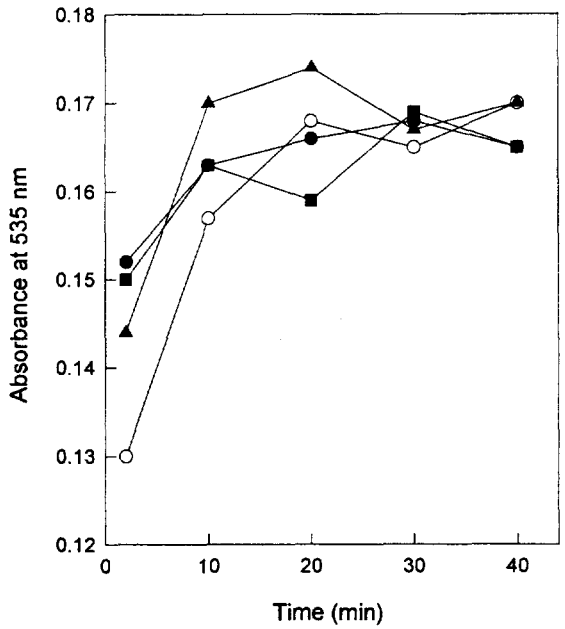

Fig. 7. Peroxidation of liposome by AMVN and the effect of GSH and phosphorylase b. 5 mM liposome was incubated at 37℃ with 20 mM AMVN at pH 7.0 (○). And the effect of addition of 0.2 mM GSH (●), 0.5 mg/ml phosphorylase b (■), or both GSH and phosphorylase (▲) on lipid peroxidation was determined.

or phosphorylase b at the concentration same as in Fig. 3 decreased peroxidation significantly and addition of both GSH and phosphorylase inhibited almost 90% of peroxidation. This result is consistent with that of Fig. 5 which shows relatively high reactivity of GSH with AAPH-generated free radicals.

Fig. 7 shows peroxidation of liposome by AMVN. The reaction proceeded extremely fast so that initial absorbance value was not able to be determined. And addition of GSH and phosphorylase did not have significant effect on the rate of lipid peroxidation.

The effect of radical scavengers on AAPH- and AMVN-initiated peroxidation of liposome was examined (Table 2). Ascorbate inhibited AAPH-initiated peroxidation at relatively high concentration whereas it had limiting effect on AMVN-initiated peroxidation. This indicates that water-soluble free radical scavengers have very poor effect when radicals are produced in lipid phase. On the other hand, α-tocopherol was very effective in inhibiting peroxidation by AAPH and AMVN. AMVN-initiated lipid peroxidation required higher concentration of α-tocopherol probably due to extremely high reactivity of AMVN-originated radicals with lipid. β-Carotene also inhibited AAPH-initiated peroxidation. The effect of α-tocopherol and β-carotene which can be incorporated into lipid layer seems to be as a result of radical chain-breaking effect rather than direct removal of AAPH- and AMVN-generated free radicals.

Table 2. Inhibition of AAPH- and AMVN-initiated peroxidation of liposome by radical scavengers. 5 mM liposome was incubated for 30 min at 37℃ with 20 mM AAPH or 20 mM AMVN

Additions		% Inhibition	
		AAPH-initiated	AMVN-initiated
Ascorbate	5 mM	0	0
	10 mM	31	0
	20 mM	42	8
α-Tocopherol	2 μM	22	N.D.[a]
	5 μM	53	N.D.
	20 μM	82	N.D.
	50 μM	N.D.	24
	0.5 mM	N.D.	82
	2.0 mM	N.D.	100
β-Carotene	10 μM	32	0
	50 μM	44	0

[a]N.D. indicates not determined.

This study was attempted in order to understand the reactions of two important cellular components, soluble proteins and cell membrane, in the situation of free radical generation either in cytosol or in membrane lipid phase by using mixture of phosphorylase b and phosphatidyl choline liposome as a model system. The results suggest that cytosolic soluble proteins seem to be the primary target of modification in the initial stage of water-soluble, either oxygen centered or organic, free radical generation. Water-soluble organic free radicals also react with membrane lipids. On the other hand, membrane lipids seem to be the preferential target when free radicals are generated in lipid layer. Cytosolic proteins, possibly membrane proteins too, can also be damaged by lipid soluble radical species. And proteins in cytosol and in membrane can be modified by certain reactive species which seem to be the degradation products or intermediates of membrane lipids during peroxidative free radical chain reaction. In most of the cases, proteins can be protected from degenerative effects of free radical species if the proteins have reactive sulfhydryl groups on surface by forming S-thiolated proteins with glutathione.

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