[Chem. Pharm. Bull. 36(3)1104—1109(1988)]

Antioxidative Effect of Protoporphyrin on Lipid Peroxidation in Rat Liver Subcellular Fractions

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(Received June 5, 1987)

The effect of protoporphyrin IX (PP) on lipid peroxidation was investigated in rat liver homogenates, in hepatic subcellular organelles such as mitochondria and microsomes, and in a mixture of unsaturated fatty acids. PP inhibited both the Fe^{2+} and ascorbic acid (AsA)-stimulated lipid peroxidation and the concomitant increase of oxygen consumption in the homogenates. PP failed to inhibit auto-oxidation in the mixture of unsaturated fatty acids. PP inhibited the lipid peroxidation both in mitochondria stimulated by AsA and in microsomes stimulated by AsA or a reduced nicotinamide adenine dinucleotide phosphate-generating system. Its antioxidative effects were less pronounced in the heat-denatured subcellular fractions. Soluble fraction (SF) enhanced the antioxidative action of PP on the lipid peroxidation in boiled microsomes, but boiled SF lessened the action of PP.

These findings indicate that PP has an antioxidative effect on lipid peroxidation in liver, and that the antioxidative effect of PP is mediated by factors present in liver, some of which are heat-labile and some heat-stable.

Keywords——protoporphyrin IX; lipid peroxidation; antioxidant; rat liver; homogenate; subcellular fraction; unsaturated fatty acid; TBA value

Protoporphyrin IX (PP), an intermediate in heme biosynthesis, has been used in the treatment of liver diseases such as hepatitis.[1)]

In our previous studies, we have shown that sodium copper chlorophyllin (Cu-Chl-Na) has an antioxidative action on lipid peroxidation[2)] and that Cu-Chl-Na is a mixture of copper complexes of chlorophyll derivatives closely related to PP in their chemical structure.[3)]

Ogiso *et al.*[4)] have reported that PP, when given to rats, has a protective effect against lipid peroxidation in hepatic microsomes caused by CCl_4 intoxication, and they also found that PP suppresses, though to a small extent, lipid peroxidation induced by Fe^{2+} in rat liver microsomes *in vitro*.

It was, therefore, of interest to examine further the effect of PP on lipid peroxidation. In the present studies, we have demonstrated that PP has a potent antioxidative effect *in vitro* on lipid peroxidation caused by either ascorbic acid (AsA), Fe^{2+}-AsA or a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-generating system in homogenates and subcellular fractions of rat liver, and that PP has no antioxidative effect on auto-oxidation of unsaturated fatty acids.

Experimental

Materials——Protoporphyrin IX (disodium salt) was donated by Tokyo Tanabe Co., Ltd., Tokyo, Japan. 2-Thiobarbituric acid (TBA) was purchased from Wako Pure Chemical Ind., Ltd., Tokyo, Japan. Nicotinamide adenine dinucleotide phosphate (oxidized form, $NADP^+$) was obtained from Oriental Yeast Co., Ltd., Tokyo, Japan.

Glucose 6-phosphate (G6P, disodium salt) and G6P dehydrogenase (G6PDH, from yeast, 350 U/mg) were purchased from Boehringer Mannheim Yamanouchi Co., Ltd., Tokyo, Japan. Bovine serum albumin (BSA, crystallized and lyophilized) was purchased from Sigma Chemical Co., Ltd., St. Louis, MO., U.S.A. All other chemicals were of reagent grade.

Animals——Male Wistar rats, weighing approximately 200 g, were used. They were fed on a commercial chow and tap water *ad lib*, and were fasted for about 18 h before sacrifice, but were allowed free access to water.

Preparation of Subcellular Fractions——Liver homogenates were prepared in 150 mM KCl–10 mM phosphate buffer (pH 7.4) by the method of Sato *et al.*[2a] Hepatic mitochondrial and microsomal fractions and soluble fraction (SF) were prepared as described previously.[5]

Assay of Lipid Peroxidation——AsA, Fe^{2+}-AsA and an NADPH-generating system were used as stimulators of lipid peroxidation. Typical reaction mixtures were as follows. (1) Experiments with homogenates: 0.6 ml of 5% homogenates, 150 mM KCl, 20 μM $FeSO_4$, 0.5 mM AsA and 10 mM phosphate buffer (pH 7.4) in a final volume of 2.0 ml. (2) Experiments with subcellular fractions: appropriate amount of subcellular fractions, 90 mM KCl and 50 mM Tris–HCl buffer (pH 7.4) in a final volume of 1.0 or 2.0 ml. AsA at 0.5 mM or an NADPH-generating system (0.5 mM $NADP^+$, 5 mM G6P, 0.3 U/ml G6PDH and 5 mM $MgCl_2$) was added when necessary.

Incubation was carried out aerobically at 37°C for 60 min under room lights. The degree of lipid peroxidation was assayed by the TBA method of Ottolenghi[6] as modified by Sato *et al.*[7] The formation of lipid peroxides was expressed in terms of TBA value (absorbance at 532 nm due to malondialdehyde formation/ml of reaction mixture or mg of protein).

Measurement of Oxygen Uptake in Homogenates——Oxygen uptake was determined in a Warburg's manometer[8] using the reaction mixture as described by Sato *et al.*[2a]

Preparation of Boiled Samples——Mitochondria, microsomes and SF were heated at 100 °C for 5 min, cooled on ice for 5 min, and then homogenized.

Protein Determination——The protein in subcellular fractions was determined by the method of Lowry *et al.*[9] BSA was used as a standard.

Results and Discussion

The effect of PP on lipid peroxidation in liver homogenates was investigated in the presence of 20 μM $FeSO_4$ and 0.5 mM AsA. As shown in Table I, PP in the concentration range from 10 μM to 100 μM markedly suppressed the elevation of TBA values. The lipid peroxidation was almost completely inhibited by 50 μM PP. The effect of PP was equipotent to or

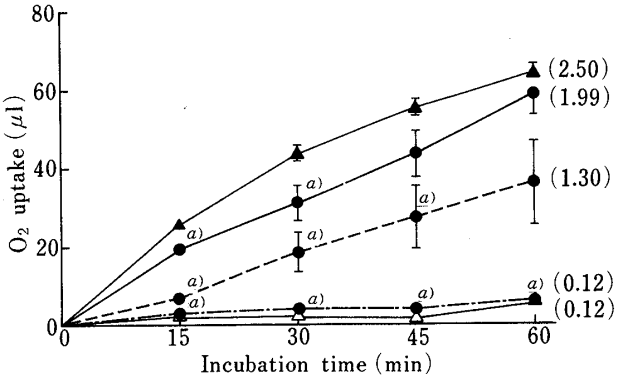

Fig. 1. Effect of PP on the Oxygen Consumption by Rat Liver Homogenates in the Presence of Fe^{2+} and AsA

The reaction mixture, consisting of 0.5 ml of 7.5% homogenates, 150 mM KCl, 20 μM $FeSO_4$, 0.5 mM AsA and 10 mM phosphate buffer (pH 7.0) in a total volume of 2.5 ml, was incubated at 37.5 °C. Each point represents the mean ± S.E. (vertical bars) for 3—4 rats. The values in parentheses represent the means of TBA values (A_{532}/ml of reaction mixture) at 60 min.

—△—, in the absence of Fe^{2+} and AsA; —▲—, 0 μM PP; —●—, 10 μM PP; ---●---, 20 μM PP; ---●---, 50 μM PP. *a*) $p<0.05$.

TABLE I. Effect of PP on Lipid Peroxidation in Rat Liver Homogenates in the Presence of Fe^{2+} and AsA

Concn. of PP (μM)	TBA value[a]
0	1.596 ± 0.118
10	0.352 ± 0.057[b]
20	0.137 ± 0.030[b]
50	0.017 ± 0.002[b]
100	0.007 ± 0.003[b]

The incubation mixture, consisting of 0.6 ml of 5% homogenates, 150 mM KCl, 20 μM $FeSO_4$, 0.5 mM AsA and 10 mM phosphate buffer (pH 7.4) in a total volume of 2.0 ml, was incubated at 37 °C for 60 min. Each value is the mean ± S.E. for 4—5 rats. The TBA value in the absence of Fe^{2+} and AsA was 0.073. *a*) A_{532}/ml of reaction mixture. *b*) $p<0.05$.

more potent than that of Cu-Chl-Na.[2a)]

In another experiment with tetraethoxypropane, PP at the concentrations used in the above experiments did not interfere with the reaction between TBA and malondialdehyde which is easily formed by the decomposition of tetraethoxypropane (data not shown).

We examined the effect of PP on oxygen consumption by liver homogenates. As shown in Fig. 1, oxygen uptake by the homogenates reached about 12 times the basal level when Fe^{2+} and AsA were added to the homogenates. It is known that oxygen uptake by homogenates in the presence of Fe^{2+} and AsA is regarded as an index of lipid peroxidation.[2a)] PP at the concentrations of 10 to 50 μM inhibited the increase in oxygen uptake in a concentration-related manner. A maximum effect was observed at 50 μM PP, at which the inhibition by PP was almost complete. TBA values were also determined after 60 min of incubation. PP at 10, 20 and 50 μM decreased the values by 20, 48 and 95%, respectively. These results indicate that PP has an antioxidative effect on lipid peroxidation in liver homogenates.

We also examined whether PP had an antioxidative action on the auto-oxidation of unsaturated fatty acids such as linolenic acid and linoleic acid, when the acids were incubated aerobically for 90 min. Oxygen consumption by the oxidation of the acids was, unexpectedly, enhanced to 33, 37 and 58% above the control value by the addition of PP at 20, 50 and 100 μM, respectively (data not shown).There was no difference among the TBA values. These results indicate that PP has no protective property against the auto-oxidation of unsaturated fatty acids. It is thought that PP may require a biological system to exert its antioxidative action on lipid peroxidation.

In the following experiments, we determined the effect of PP on AsA-stimulated lipid peroxidation in intact and heat-denatured (boiled) hepatic subcellular organelles such as mitochondria and microsomes. As shown in Table II, in intact mitochondria, the TBA values increased when the subcellular particles were incubated with AsA. PP completely inhibited the lipid peroxidation at a concentration of 20 μM. In boiled mitochondria, the TBA values were also elevated by AsA and the elevation was inhibited to 72% by the addition of 20 μM PP. AsA stimulated lipid peroxidation more markedly in boiled than in intact mitochondria. The inhibitory effect of PP at 20 μM was less pronounced in boiled than in intact mitochondria.

In the experiments with microsomes, AsA stimulated lipid peroxidation to the same extent in both intact and heat-denatured microsomes (Table II) and oxygen consumption also increased as the TBA values did in both specimens (data not shown). PP inhibited the increases of lipid peroxidation in both specimens, and in intact microsomes PP began to inhibit lipid peroxidation at a concentration as low as 2 μM (Fig. 2). As in mitochondria, the inhibition by PP was much stronger in intact than in boiled microsomes. These results suggest

TABLE II. Effect of PP on AsA-Stimulated Lipid Peroxidation in Mitochondria and Microsomes in Rat Liver

Concn. of PP (μM)	Mitochondria				Microsomes			
	Intact		Boiled		Intact		Boiled	
	TBA value[a)]	%	TBA value[a)]	%	TBA value[a)]	%	TBA value[a)]	%
Nil[b)]	0.021±0.002		0.017±0.001		0.035±0.003		0.067±0.011	
0	0.476±0.107	100	0.893±0.024	100	1.321±0.042	100	1.263±0.088	100
20	0.008±0.001[c)]	2	0.646±0.062[c)]	72	0.309±0.104[c)]	23	0.970±0.082	77
50	0.007±0.001[c)]	1	0.046±0.021[c)]	5	0.078±0.020[c)]	6	0.857±0.106[c)]	68

The incubation mixture, consisting of about 2 mg protein of mitochondria or microsomes, 90 mM KCl, 0.5 mM AsA and 50 mM Tris–HCl buffer (pH 7.4) in a total volume of 2.0 ml, was incubated at 37 °C for 60 min. Each value represents the mean ± S.E. for 3—4 rats. *a*) A_{532}/mg protein. *b*) Without AsA. *c*) $p<0.05$.

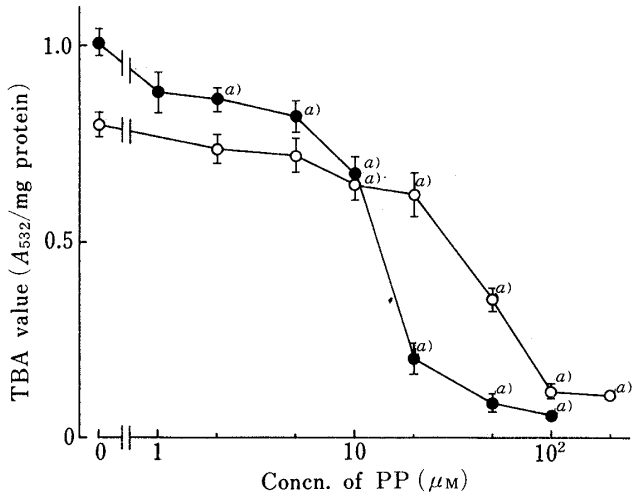

Fig. 2. Effect of PP on Lipid Peroxidation in Rat Liver Microsomes

The reaction mixture, consisting of 1 mg protein of microsomes, 90 mM KCl, 0.5 mM AsA or an NADPH-generating system, and 50 mM Tris-HCl buffer (pH 7.4) in a final volume of 1.0 ml, was incubated at 37 °C for 60 min. Each point represents the mean ± S.E. (vertical bars) for 4—8 rats.
—●—, AsA-stimulated; —○—, NADPH-stimulated. *a*) $p<0.05$.

TABLE III. Effect of PP on AsA-Stimulated Lipid Peroxidation in Boiled Microsomes of Rat Liver in the Presence of Soluble Fraction

Concn. of PP (μM)			+ Intact SF		+ Boiled SF	
	TBA value[a)]	%	TBA value[a)]	%	TBA value[a)]	%
0	1.467 ± 0.144	100	1.287 ± 0.176	100	1.431 ± 0.196	100
20	1.021 ± 0.229	70	0.577 ± 0.228[b)]	45	1.262 ± 0.269	88
50	0.547 ± 0.093[b)]	35	0.293 ± 0.125[b)]	23	0.678 ± 0.066	47

The incubation mixture, consisting of about 0.6 mg of boiled microsomal protein, 90 mM KCl, 0.5 mM AsA, 50 mM Tris-HCl buffer (pH 7.4), and with or without soluble fraction (SF, about 2 mg protein) in a final volume of 1.0 ml, was incubated at 37 °C for 60 min. Each value represents the mean ± S.E. for 3—4 rats. *a*) A_{532}/mg of microsomal protein. *b*) $p<0.05$.

that both heat-labile and heat-stable factors, which mediate the antioxidative action of PP, exist in liver mitochondria and microsomes.

We also examined whether PP showed an inhibitory effect on lipid peroxidation induced by other stimulators such as an NADPH-generating system in microsomes. The NADPH-generating system caused an elevation of TBA values, though the extent of elevation was small, as compared to that by AsA (Fig. 2). PP significantly inhibited the elevation at concentrations of 10 μM or above. The degree of antioxidative effect of PP was smaller in NADPH-stimulated than AsA-stimulated microsomes.

As it is well known that hepatic SF inhibits the microsomal lipid peroxidation,[7)] it was of interest to observe whether SF affects the antiperoxidative action of PP. The experiments were carried out with boiled microsomes as a substrate for the peroxidation reaction induced by AsA, for the purpose of excluding the participation of heat-labile mediator(s) in the antioxidative action of PP. The results are shown in Table III. Intact SF showed a tendency to decrease the lipid peroxidation in boiled microsomes, when added to the incubation mixture. Moreover, intact SF enhanced the effect of PP, indicating a synergism between the actions of the SF and PP. On the other hand, the addition of boiled SF to the reaction mixture in the presence of PP lessened, rather than strengthened, the antioxidative action of PP. Boiled SF had no effect on boiled-microsomal lipid peroxidation.

All the results mentioned above indicate again that the antioxidative action of PP on lipid peroxidation in hepatic subcellular organelles is mediated by factors present in mitochondria and microsomes, some of which are heat-labile and some heat-stable. A heat-labile factor(s) is

also present in SF.

In the next experiment, we tested whether PP reduced 1,1-diphenyl-2-picrylhydrazyl (DPPH), which is used as a model of free radicals. α-Tocopherol, a typical radical scavenger, reduced DPPH. However, PP even at a high concentration of 50 μM did not reduce it (data not shown). The result indicates that the antioxidative action of PP is not related to radical-scavenging.

In the present study, we have found that PP shows an inhibitory effect on the peroxidation of lipids in liver homogenates, mitochondria and microsomes, whereas it shows no antioxidative effect on the auto-oxidation of unsaturated fatty acids, and it does not reduce DPPH. Therefore, it is considered that the mechanism of the antioxidative action of PP is different from that of Cu-Chl-Na, possibly a radical scavenger,[2b] and PP appears to exert its action through the mediation of some factor(s) present in liver.

There are several reports that show the existence of heat-labile mediators of glutathione-dependent anti-lipid peroxidation in hepatic subcellular fractions such as mitochondria,[10] microsomes[11] and SF.[10,12] Burk has found that rat liver microsomal mediator is a protein which scavenges free radicals.[11a] Ursini *et al.*[13] have shown that mediator in SF of pig liver is a protein which exhibits glutathione peroxidase activity, while McCay *et al.*[10] have demonstrated that a factor in rat liver SF has no glutathione peroxidase activity and seems to be a glutathione transferase. Our present results, however, do not provide any evidence concerning the nature of the factors which mediate the protective effect of PP on lipid peroxidation in rat liver subcellular fractions.

We confirmed the results and speculation by Ogiso *et al.*[4] that PP suppressed microsomal lipid peroxidation and its action might be indirect rather than a direct one on lipid peroxidation. We extended their findings to mitochondria and found that PP also inhibited mitochondrial lipid peroxidation. Furthermore, we showed that PP required heat-labile or heat-stable mediators for exerting its antioxidative action in these two fractions.

Porphyrins including PP are photosensitizers and singlet oxygen, which is known to be an initiator of lipid peroxidation, is formed by reaction of the sensitizer triplet with ground-state oxygen. The excited states of porphyrins and singlet oxygen cause the photodynamic inactivation of biological systems. Thus, light absorption by porphyrins or their metal complexes, energy transfer to oxygen, and subsequent attack of singlet oxygen on unsaturated groups elsewhere in the systems would cause the light-mediated damage in the systems.[14] This would be likely in the present experiments because the experiments were carried out under room lights. Thus, it is considered that PP would cause lipid peroxidation.

In contrast to this consideration, however, PP showed an antioxidative action on lipid peroxidation in biological systems.

The mechanisms by which PP exerts its antioxidative effect remain to be elucidated and are under investigation in our laboratories.

Acknowledgement We are grateful to Tomoka Kotani, Akiko Iwasaki and Mayumi Okamoto for their excellent technical assistance.

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