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Effect of Protoporphyrin on Peroxidative Damage of Rat Liver Lysosomes

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The effects of protoporphyrin IX (PP) on lipid peroxidation and lysosomal membrane stability were investigated using the lysosome-containing ($3500 \times g$) fraction from rat liver. In a concentration-dependent manner, PP inhibited the release of lysosomal marker enzymes, aryl sulfatase and acid phosphatase, which followed lipid peroxidation. The inhibition pattern of enzyme release by PP was similar to that of lipid peroxidation. These findings indicate that PP protects lysosomes from peroxidative damage. In hypo-osmotic medium, PP inhibited the release of two marker enzymes from lysosomes at low concentration, but at relatively high concentration it increased the release of the enzymes, suggesting that PP has biphasic effects on the lysosomal membrane.

Keywords——protoporphyrin IX; lipid peroxidation; antioxidative effect; rat liver lysosome; lysosomal enzyme release; membrane stability

We have reported that protoporphyrin IX (PP) has an antioxidative effect on lipid peroxidation in subcellular organelles such as mitochondria and microsomes stimulated by Fe^{2+}, ascorbic acid (AsA) or a reduced nicotinamide adenine dinucleotide phosphate-generating system. We have also reported that PP has no function as a radical scavenger and requires biological factors to exert its antioxidative effect.[1)]

It is known that the peroxidation of biomembrane lipids causes perturbation of the biomembrane, followed by changes such as altered activities of membrane-bound enzymes[2)] and facilitated permeability of the membrane, *e.g.*, the release of enzymes from lysosomes.[3)] Mak *et al.*[4)] reported that the lysosomal membrane is susceptible to free radical-induced lipid peroxidation, and this process may be the immediate cause of disintegration of the lysosome. There are some reports that antioxidative agents such as α-tocopherol[3b,4)] and ethylenediaminetetraacetic acid[3c)] inhibit the enzyme release from lysosomes induced by lipid peroxidation. It has been also reported that the alterations of lysosomal membranes and the subsequent release of hydrolytic enzymes as a consequence of peroxidative injury can contribute to liver damage.[5)]

It was of interest to investigate whether PP is effective in the inhibition of the lysosomal membrane disintegration induced by lipid peroxidation, since PP has been used in the treatment of liver diseases such as hepatitis.[6)]

In the present studies, we have demonstrated that PP inhibits the enzyme release from lysosomes which is stimulated by lipid peroxidation and induced by a hypo-osmotic condition.

Experimental

Chemicals——Protoporphyrin IX (disodium salt) was kindly donated by Tokyo Tanabe Co., Ltd., Tokyo, Japan.

AsA, sodium *p*-nitrophenyl phosphate and dipotassium *p*-nitrocatechol sulfate were obtained from Nakarai Chemicals, Ltd., Kyoto, Japan. 2-Thiobarbituric acid (TBA) was purchased from Wako Pure Chemical Ind., Ltd., Tokyo, Japan. All other chemicals were of reagent grade.

Animals——Male Wistar-KY rats weighing 180—220 g were used. The animals were fasted for about 18 h prior to sacrifice, but were given tap water *ad libitum*.

Assay of Lysosomal Membrane Stability——The lysosome-containing ($3500 \times g$) fraction was prepared according to the method of Tanaka and Iizuka[7] with a slight modification by Sato *et al.*[3c] The protein concentration was measured by the method of Lowry *et al.*[8] The labilization and stabilization of lysosomes were assayed by determining the release of aryl sulfatase (ASase) and acid phosphatase (APase) as lysosomal marker enzymes, as described by Sato *et al.*[3c] Experimental details on the reaction mixture are given in the legends to figures. After incubation of the reaction mixture at 37 °C for 30 min, 0.5 and 1.5 ml aliquots were removed for the assays of lipid peroxidation and of marker enzymes released from lysosomes, respectively. The total activity of each enzyme was assayed in a suspension of $3500 \times g$ fraction treated with 0.2% Triton X-100 at 37 °C for 30 min. The enzyme release was expressed as percent of the total enzyme activity.

Enzyme Assay——ASase (EC 3.1.6.1) activity was determined as described by Dodgson and Spencer[9] using dipotassium *p*-nitrocatechol sulfate as a substrate. APase (EC 3.1.3.2) activity was assayed by the method of Nakanishi *et al.*[10] using *p*-nitrophenyl phosphate as a substrate.

Assay of Lipid Peroxidation——Lipid peroxidation was measured by determining malondialdehyde formed in the reaction mixture according to the method of Fong *et al.*[11] with a slight modification. The method was able to overcome interference with the TBA color reaction by sucrose; To an aliquot (0.5 ml) of the reaction mixture, 0.5 ml of 20% trichloroacetic acid, 1 ml of 8×10^{-3}% sodium copper chlorophyllin (Cu-Chl-Na), 1 ml of distilled water and 1 ml of 0.7% TBA were added, and the mixture was heated at 60 °C for 90 min. In some studies, antioxidant was added to the TBA reaction mixture in order to prevent auto-oxidation of lipids during heating with TBA.[12] In this experiment, because PP caused a slight elevation of the value during heating at 60 °C, Cu-Chl-Na, a water-soluble antioxidant,[13] was added to the mixture at a final concentration of 2×10^{-3}%; this level of Cu-Chl-Na completely prevented elevation of the TBA value during the heating period. After the mixture had been cooled, 1 ml of 60% trichloroacetic acid was added and the whole was mixed gently. Chloroform (3 ml) was then added and the mixture was shaken vigorously to remove dispersed lipid. The absorbance of the clear pink supernatant fraction was determined at 532 nm, and expressed in terms of TBA values.

Statistical Analysis——The data were analyzed statistically by using Student's *t*-test.

Results and Discussion

We examined the antioxidative effect of PP at various concentrations in the present experimental system. The results are shown in Fig. 1A. In the $3500 \times g$ fraction of rat liver, the TBA value was elevated by the addition of AsA to the incubation mixture, as observed with mitochondria and microsomes.[1] PP significantly suppressed the elevation of TBA value at a concentration as low as 5 μM, and inhibited it almost completely at 20 μM or above.

Release of lysosomal marker enzymes, ASase and APase, from lysosomes in the $3500 \times g$ fraction during incubation was quite small in the absence of AsA, but was increased by the addition of AsA (Fig. 1B). PP inhibited the increase of the enzyme release at the concentration of 5 μM or above, in agreement with the results on its inhibitory effect on lipid peroxidation. The results with Fe^{2+} were similar to those with AsA (data not shown). Most of the PP added to the incubation mixture was adsorbed or bound to the subcellular organelles during incubation and co-sedimented with the organelles during centrifugation, and PP remaining in the supernatant, which was used as the enzyme source, did not interfere with the assay of ASase and APase activities (data not shown). The pattern of the decrease in the release of the two marker enzymes is similar to that in TBA value. The results show that PP decreases the peroxidation of lysosomal membrane lipids and protects the lysosomal membrane from peroxidative damage, namely, PP stabilize the lysosomal membrane through its antioxidative effect. In relation to the results with PP, we confirmed that α-tocopherol, a typical antioxidant, inhibited both the lipid peroxidation and the release of lysosomal enzymes in our experimental system, in agreement with the reported results,[3b,4] but its inhibitory effect was less than that of PP (data not shown).

To examine whether PP has a direct effect on lysosomal membranes, the effect of PP at

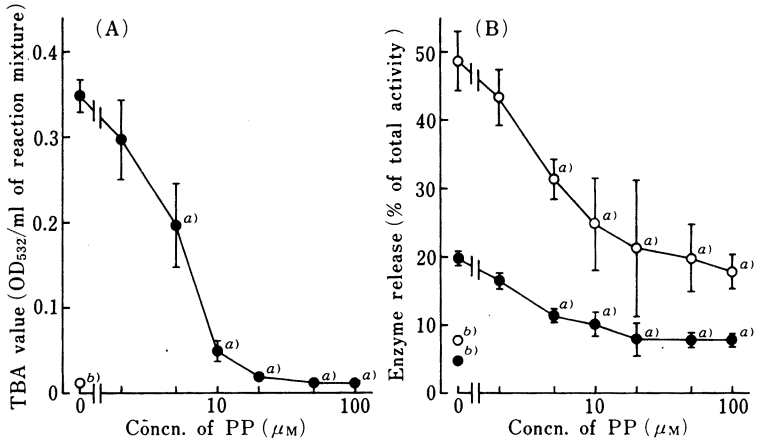

Fig. 1. Effects of PP on Lipid Peroxidation and Lysosomal Enzyme Release in 3500 × *g* Fraction of Rat Liver Stimulated by AsA

The reaction mixture, consisting of 6 mg of protein of 3500 × *g* fraction, 0.25 M sucrose, 0.5 mM AsA and 40 mM Tris–acetate buffer (pH 7.4) in a total volume of 3.0 ml, was incubated at 37 °C for 30 min. Each point represents the mean ± S.E. (vertical bars) for 4—6 separate experiments.

(A) Lipid peroxidation. (B) Enzyme release —○—, ASase; —●—, APase.

a) Significantly different from the control (0 μM) at a *p* value of 0.05 or less. *b*) In the absence of 0.5 mM AsA.

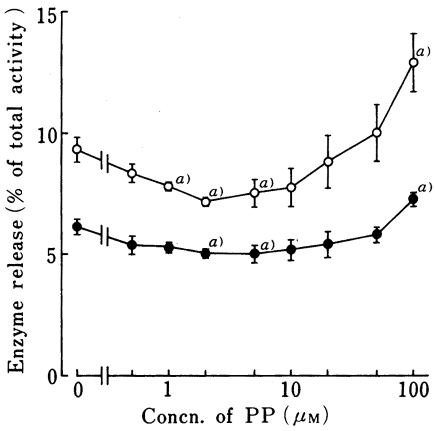

Fig. 2. Effect of PP on Lysosomal Enzyme Release from 3500 × *g* Fraction of Rat Liver Incubated with 0.18 M Sucrose

The reaction mixture, consisting of 6 mg of protein of 3500 × *g* fraction, 0.18 M sucrose and 40 mM Tris–acetate buffer (pH 7.4) in a total volume of 3.0 ml, was incubated at 37 °C for 30 min. Each point represents the mean ± S.E. (vertical bars) for 4—6 separate experiments.

—○—, ASase; —●—, APase.

a) Significantly different from the control (0 μM) at a *p* value of 0.05 or less.

various concentrations on the release of lysosomal enzymes during incubation of 3500 × *g* fraction in a hypo-osmotic medium, 0.18 M sucrose, was investigated. The results are shown in Fig. 2. The release of ASase and APase was accelerated by the hypo-osmotic condition, while no elevation of the TBA value was observed (data not shown). PP moderately reduced the release of ASase and APase at 1—5 μM and 2—5 μM, respectively. The maximum extent of the reduction was 23% for ASase and 18% for APase. However, PP at the relatively high concentration of 100 μM significantly accelerated the release of both enzymes, and showed 38% and 18% increases in release of ASase and APase, respectively. These results indicate that PP has biphasic effects on lysosomal membranes, a stabilizing effect at low concentrations and a labilizing effect at relatively high concentrations.

Although the lipid peroxidation stimulated by AsA was almost completely inhibited at 20 μM PP, enzyme releases stimulated by AsA were not decreased to the control level even at the concentration of 100 μM of PP. This might be due to a membrane-labilizing action of PP at higher concentration.

Sato *et al.*[14] have reported that PP acts as both a stabilizer and a labilizer on hypo-osmotic hemolysis of human erythrocytes, and they have speculated that the effects of PP are brought about by changes of membrane structure which are caused by insertion of PP into the lipid layers of the erythrocyte membrane. Their speculation may be applicable to the mechanism of lysosomal membrane alteration by PP, but the details remain to be clarified.

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