Alteration in Membrane Fluidity of Rat Liver Microsomes and of Liposomes by Protoporphyrin and Its Anti-lipidperoxidative Effect

Kimie Imai, a, Tachio Aimoto, a Toshihide Shim a, b Toshiaki Nakashima, b Masaki Sato, 1) and Ryoei Kimura a

Faculty of Pharmaceutical Sciences, Setsunan University, 1 45–1 Nagato-cho, Hirakata, Osaka 573–0101, Japan, Third Department of Internal Medicine, Kyoto Prefectural University of Medicine, 4 465 Kajii-cho, Higashi-ku, Kawanishi, Nara 631–8506, Japan, and School of Pharmaceutical Sciences, University of Shizuoka, 5 52–1 Yada, Shizuoka 422–8526, Japan. Received September 10, 1999; accepted December 29, 1999

The effect of protoporphyrin (PP) on membrane fluidity was investigated by electron paramagnetic resonance spectroscopy using doxyl stearate spin probes in relation to the anti-lipidperoxidative effect of PP. PP decreased the membrane fluidity in rat liver microsomes at concentrations above 1 mM and also in phosphatidylcholine (PC)–cholesterol (Cho) (100 : 8, a molar basis) liposomes. The lipid peroxidation stimulated by Fe3+ and L-ascorbic acid in those membrane preparations was attenuated along with the decrease in membrane fluidity by PP. Similar results were also found in Cho-rich PC (100 : 30 to 100) liposomes having less fluidity. These results suggest that the decrease in the membrane fluidity caused by PP may be involved in the antioxidative action of PP.

Key words protoporphyrin; anti-lipidperoxidation; membrane fluidity; rat liver microsome; liposome; EPR spectroscopy

Protoporphyrin (PP) is an intermediate in heme biosynthesis and has been used to treat liver diseases such as hepatitis.1) We demonstrated that PP has an antioxidative effect on lipid peroxidation in rat liver subcellular fractions in vitro and that this effect is exerted via a factor(s) present in the liver.2) We also reported that the partition coefficient of PP between n-octanol and aqueous phase was very high, being about 150 at physiological pH, and that PP or a PP-derived porphyrin(s) was distributed at high concentrations in membrane-rich fractions such as mitochondria and microsomes in livers, inhibiting lipid peroxidation in these fractions when PP was given to rats.3)

Lipid peroxidation in biological membranes alters their biochemical functions such as enzyme inactivation4–9) and causes a decrease in membrane fluidity.10–12) Galeotti et al. described that a correlation existed between the lower rate of lipid peroxidation and the decrease in fluidity in tumor plasma membranes.13) There are some reports that tamoxifen, an antiestrogen drug, its derivatives, and known antioxidants diethylstilbestrol, hexestrol, and 17α-ethynylestradiol decrease membrane fluidity in ox-brain phospholipid liposomes or in rat liver microsomes and that these effects on membrane fluidity and their antioxidative action correlate to each other.14–16) Therefore, we studied whether the alteration of the membrane fluidity was involved in the mechanisms of the antioxidative action of PP.

We used EPR spectroscopy to examine the effect of PP on membrane fluidity in the liver microsomes of rats and in liposomes prepared from phosphatidylcholine (PC) with or without cholesterol (Cho). The results showed that PP decreased the membrane fluidity and that the decrease seemed to correlate with an increase of antioxidative effect of PP.

MATERIALS AND METHODS

Materials Protoporphyrin (disodium salt) was generously donated by Tokyo Tanabe Co., Ltd. (Tokyo, Japan). 5-And 16-doxyl stearic acids (5- and 16-DSA, respectively) were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, U.S.A.). Phosphatidylcholine (PC, from egg) and cholesterol (Cho) were obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). All other chemicals were of reagent grade.

Preparation of Microsomes Microsomes were prepared from the livers of male Wistar rats (weighing about 250 g) which had been fasted overnight. The liver was perfused in situ with ice-cold 1.15% KCl, then excised, minced and homogenized in 3 volumes of ice-cold 150 mM KCl–10 mM Tris–HCl buffer (pH 7.4). The homogenates were centrifuged at 9000 × g for 20 min and the supernatant was recentrifuged at 105,000 × g for 60 min at 4 °C. The pellet was weighed and suspended in the KCl–Tris buffer.

Lipid Peroxidation of Microsomes In the L-ascorbic acid (AsA)-stimulated lipid peroxidation, the reaction mixture, containing about 10 mg of microsomal protein, 90 mM KCl, 0.5 mM AsA, 50 mM Tris–HCl buffer (pH 7.4) in a total volume of 10 ml, was incubated at 37 °C for 60 min, and the reaction was terminated by the addition of 0.1 ml of 10 mM EDTA. One half milliliter of the mixture was used for the assay of lipid peroxidation by the 2-thiobarbituric acid (TBA) method reported previously17) and the results were expressed in terms of TBA reacting substances(TBARS).

Preparation of Liposomes Known amounts of PC and Cho were dissolved in chloroform in a round-bottomed flask and the mixture was subjected to a rotary evaporation under reduced pressure to form a thin film of lipid on the wall inside the flask. Residual solvent was removed under vacuum at room temperature overnight. The lipids were dispersed by shaking with the KCl–Tris buffer and dispersed using a sonicator (Otake Model 5022 PZT, Tokyo) at 30 W for 10 min in ice water.

Lipid Peroxidation of Liposomes In the experiment with PP, a liposomal suspension containing 30 μmol PC, 135 mM KCl, 9 mM Tris–HCl buffer (pH 7.4) and various concentrations of PP in a total volume of 2.0 ml was incubated at 37 °C for 30 min and aliquots of the mixture were reacted as follows. The peroxidation reaction mixture consisting of 20 μmol PC, 150 mM KCl, 20 μM FeSO4, 0.5 mM AsA and 10

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mm Tris–HCl buffer (pH 7.4) in a total volume of 5.0 ml was incubated at 37°C. Five hundred microliters of the mixture was removed at various times during incubation and placed in tubes containing 0.25 ml of 2 mm α-tocopherol in ethyl alcohol, 2 ml of 12.5% trichloroacetic acid and 0.25 ml of water, and mixed. Two milliliters of 0.7% TBA was added and the mixture was boiled for 15 min. After being cooled, water was added to a final volume of 5.0 ml, then the mixture was shaken vigorously with 5 ml of chloroform to remove dispersed lipid, and centrifuged. The absorbance of the clear pink supernatant fraction was determined at 532 nm and the results were expressed in terms of TBARS.

Preparation of the Spin-Labeled Microsomes or Liposomes The spin probe, 5-DSA or 16-DSA, was dissolved in ethanol and stored at −20°C until use. Aliquots of the solution were transferred to test tubes and the solvent was evaporated to dryness to form a thin film of probes under a stream of nitrogen. Microsomal (0.3–0.4 g wet weight/ml) or liposomal (15 μmol lecithin/ml) suspensions were incubated with or without PP at 37°C for 30 min, 0.1 ml of the incubation mixture was transferred to tubes coated with 5-DSA or 16-DSA, and incubated at 37°C for 15 min. The ratio of total lipids to the spin label was about 200:1.

Electron Paramagnetic Resonance Spectroscopy EPR spectra were recorded with an EPR spectrometer (model JES- FE2XG, JEOL Ltd., Tokyo) at 24°C. The usual instrument settings were: radio frequency, 9.45 GHz; microwave power, 4.0 mW; modulation width, 0.32×10 gauss; response, 0.03 s; sweep width, ±50 gauss. The representative spectra of DSA spin labels embedded in the liver microsomal membrane are shown in Fig. 1. Similar spectra were obtained in the experiments in which liposomes were used (not shown). 5-DSA gave the upper spectrum where we could clearly define the outermost peak positions and the fluidity could be estimated by the order parameter (S), calculated as described by Gaffney. 16-DSA embedded in the deepest region of the membrane gave the lower spectrum in Fig. 1, where the outermost peaks collapsed into inner ones corresponding to its pronounced fluidity. Therefore, we could no longer calculate the order parameter (S), which compelled us to estimate the membrane fluidity by the rotational correlation time (τ), calculated as described by Henry and Keith. When the value of the order parameter or the rotational correlation time increases, it is interpreted as an indication that the freedom of anisotropic motion or the rate of rotational motion of the spin probe in the membranes, i.e., the membrane fluidity, decreases. 16-DSA is thought to reflect the condition of a more hydrophobic region (i.e. deeper sites) of the membrane as
RESULTS

Effect of PP on Membrane Fluidity and Lipid Peroxidation in Rat Liver Microsomes  We examined the variation in the membrane fluidity accompanied by AsA-stimulated lipid peroxidation in liver microsomes of rats (Fig. 2). By incubating microsomes in the presence of AsA, TBARS markedly increased and both the order parameter in 5-DSA and the rotational correlation time in 16-DSA also increased. The increases in the value in these parameters were inhibited by addition of 100 μM PP to the reaction mixture.

Effect of PP on the Fluidity of Microsomal Membrane  The effects of PP at various concentrations on the order parameter and rotational correlation time in rat liver microsomes with 5- or 16-DSA spin labels are shown in Fig. 3. The order parameter obtained with 5-DSA in the membrane incubated with PP was higher than that in the control at concentrations above 1 mM. The rotational correlation time obtained with 16-DSA was also increased by PP at concentrations above 1 mM. These results indicate that PP decreases the fluidity of the hepatic microsomal membrane.

Effects of PP on the Fluidity and Lipid Peroxidation of Liposomal Membrane  We performed similar experiments using liposomes with a PC–Cho ratio similar to that of rat liver microsomes. The results are shown in Table 1. PP increased significantly the order parameter obtained with 5-DSA at concentrations above 0.5 mM, whereas the rotational correlation time obtained with 16-DSA increased only at 5 mM PP. These results show that PP decreases the fluidity of the liposomal membrane.

Lipid peroxidation in those liposomes stimulated by Fe^{2+} and AsA was investigated. In the liposomes with fluidity decreased by 0.5 mM PP, the extent of lipid peroxidation was lower than that in the control (without PP) and the prior exposure of the liposomes to 5 mM PP completely suppressed the induction of peroxidation throughout the incubation period. The liposomes with fluidity decreased by PP were less susceptible to lipid peroxidation (Fig. 4).

The Fluidity of Liposomal Membrane and Lipid Peroxidation  To clarify whether the alteration in membrane fluidity affects lipid peroxidation, we studied peroxidation in liposomal membranes with various degrees of fluidity. Table 2 shows the order parameter and the rotational correlation time of the spin labels in these liposomes. Those with a high Cho content had a larger order parameter and a larger rotational correlation time than those of the PC liposomes. We also investigated lipid peroxidation in those liposomes and the results are shown in Fig. 5.

Table 1. Effect of PP on the Order Parameter of 5-DSA and Rotational Correlation Time of 16-DSA in Liposomes

<table>
<thead>
<tr>
<th>PP concn. (mM)</th>
<th>Order parameter</th>
<th>Rotational correlation time (×10^{-10} s)</th>
</tr>
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<tbody>
<tr>
<td>0.5</td>
<td>0.63 ± 0.003</td>
<td>12.63 ± 0.14</td>
</tr>
<tr>
<td>0.5</td>
<td>0.63 ± 0.001*</td>
<td>12.83 ± 0.24</td>
</tr>
<tr>
<td>5.0</td>
<td>0.659 ± 0.001*</td>
<td>14.20 ± 0.06*</td>
</tr>
</tbody>
</table>

Liposomes were prepared from PC and Cho (100:8, molar ratio). A liposomal suspension containing 30 μmol PC, 135 μmol KCl, 9 mM Tris-HCl buffer (pH 7.4) and various concentrations of PP in a total volume of 2.0 ml, was incubated at 37°C for 30 min. Each value represents the mean ± S.E. of 4–5 separate experiments. *p < 0.05.
Liposomes were prepared from PC and Cho (100:8, molar ratio). A liposomal suspension containing 30 μmol PC, 155 mM KCl, 9 mM Tris-HCl buffer (pH 7.4) and various concentrations of PP in a total volume of 2.0 ml, was incubated at 37°C for 30 min. Aliquots of the mixture were then incubated with Fe³⁺ and AsA as described in Materials and Methods. Data are expressed as the value minus the corresponding value at 0 time. Each point represents the mean with S.E. (vertical bars) of 3—4 separate experiments. The PP concentration in the first incubation: 0 μmol (□), 0.5 μmol (○), 5 μmol (▲).

Table 2. Order Parameters of 5-DSA and Rotational Correlation Time of 16-DSA in Liposomes of Various Lipid Compositions

<table>
<thead>
<tr>
<th>Molar ratio of PC and Cho</th>
<th>Order parameter</th>
<th>Rotational correlation time (×10⁻¹⁰(s))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0</td>
<td>0.605±0.005</td>
<td>11.06±0.08</td>
</tr>
<tr>
<td>1:0.3</td>
<td>0.667±0.003</td>
<td>14.56±0.75</td>
</tr>
<tr>
<td>1:1</td>
<td>0.702±0.001</td>
<td>19.07±0.23</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.E. for 3 separate experiments.

Lipid peroxidation was markedly stimulated by Fe³⁺ and AsA in the liposomes prepared from PC alone. In those containing Cho, however, the TBARS were also elevated with a longer incubation, but the values were smaller than those in the PC liposomes. Increasing the molar ratio of Cho to PC attenuated the extent of lipid peroxidation in the liposomes.

**DISCUSSION**

The fluidity of the membrane reportedly decreases follow-

ing lipid peroxidation in the membranes of rat liver micro-

somes and liposomes. We confirmed that the membrane fluidity was decreased by AsA-stimulated lipid peroxida-

tion in the rat liver microsomal membrane and that PP inhibited this decrease by inhibiting the peroxidation (Fig. 2).

PP has a lipid-water partition coefficient and is dis-

tributed throughout membrane-rich fractions such as liver microsomes and mitochondria. We showed that PP decreased membrane fluidity in rat liver microsomes, which indicates that PP interacts with or binds to the microsomal membrane. In the present study, the concentration of PP in the reaction mixture (1 mM) corresponds to about 28 nmol/mg of microsomal protein. We also reported that PP completely inhibited the AsA-stimulated lipid peroxidation in rat liver microsomes at a concentration of 100 nmol/mg of microsomal protein. Thus, PP decreased the membrane fluidity at the concentration at which it suppressed lipid peroxidation in microsomes. PP also decreased the membrane fluidity in liposomes having a similar PC–Cho ratio to that of the microsomal membrane. Therefore, the decrease in the membrane fluidity by PP is thought to be due to the interaction of PP with the membrane lipids.

Ricchelli et al. reported that PP molecules are deeply embedded in the very rigid, hydrophobic core of the lipid bilayer using three types of liposomes consisting of dipalmi-

toylphosphatidylcholine (DPPC) alone and DPPC mixed with cardiolipin or cholesterol. Such disposition of PP might contribute to the decrease in membrane fluidity, although the detailed mechanism is not clear.

The lipid peroxidation stimulated by Fe³⁺ and AsA was inhibited in the liposomes in which the fluidity was reduced by PP. PP would not chelate a ferrous ion, because PP did not decrease the peroxidation of linolenic acid stimulated by Fe³⁺ and the fluorescence intensity of PP was not altered by its incubation with Fe³⁺, though the Fe-chelated-PP does not emit fluorescence (data not shown). The lipid peroxidation stimulated by Fe³⁺ and AsA was also suppressed in Cho-

rich liposomes with low fluidity. The inhibition rate of the lipid peroxidation in the liposomes consisting of PC and Cho in molar ratio 1:1 was about 70% at 30 min incubation, consistent with the data of Wiseman et al. Nagatsuka and Nakazawa reported that in PC liposomes the lipid peroxidation caused by the protracted irradiation of ⁶⁰Co γ-ray was suppressed by adding cepharanthin, which caused a decrease in the membrane fluidity. Wiseman et al. also suggested that the ability of tamoxifen to decrease membrane fluidity may be the mechanism of its antioxidant action using ox-

brain phospholipid liposomes. Lipid peroxidation in the membrane with reduced fluidity could also hardly be induced in our present experiments. Thus, the decrease of membrane fluidity caused by PP seems to be involved in the antioxidative action of PP.

Shima et al. have described that the rate of nitroxide radical decay in the membrane of isolated hepatocytes from rats increases with an increase in the membrane fluidity, and vice versa. They considered that variation in this fluidity changed the permeability of the antioxidants into the membrane and influenced radical decay. The penetration of the factor(s) which initiates the peroxidation reaction into the membrane might be hampered in the membrane with low fluidity. As PP decreased the membrane fluidity in this study,
the suppressed penetration of peroxidation initiators into the membrane with reduced fluidity would be partly responsible for inhibiting the lipid peroxidation by PP.

The inhibition of lipid peroxidation was more potent in the membranes with decreased fluidity by PP than in those by Cho. There is a report that the rate of lipid peroxidation is rapid in neutral egg PC liposomes but the lipid peroxidation is decreased in egg PC liposomes charged with dicetylphosphate or stearyl amine in Fe^{2+}/AsA-system. We have no data on how PP affects the state of charge of liposomes and this remains to be clarified.

REFERENCES AND NOTES

2) "Drugs in Japan (Ethical Drugs)," ed. by Japan Pharmaceutical Information Center, Yakugyo Jiho Co., Tokyo, 1998, p.1539.