Antioxidant Effect of Protoporphyrin and Increase of Glutathione in Protoporphyrin-Administered Rat Liver

Kimie Imai, Tachio Aimoto, Masaki Sato, and Ryohi Kimura

Faculty of Pharmaceutical Sciences, Setsunan University, 45-1, Nagato-cho, Hirakata, Osaka 573-01, Japan and School of Pharmaceutical Science, University of Shizuoka, 52-1, Yada, Shizuoka 422, Japan. Received January 5, 1993

The effect of protoporphyrin (PP) administration on the activities of enzymes related to and/or involved in lipid peroxidation and on the content of reduced glutathione (GSH) was investigated in rat liver. PP, at an intravenous dose of 20 mg/kg, increased GSH content, caused a weak suppression of NADPH-cytochrome c reductase activity and a slight increase of γ-glutamyl transpeptidase activity 24 h after dosing, but had no effect on the activities of other enzymes such as xanthine oxidase, superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione S-transferase, γ-glutamylcysteine synthetase or glutathione synthetase. Treatment of rats with diethy maleate following PP injection resulted in the disappearance of antioxidant action of PP. Furthermore, sinusoidal, but not canalicular, efflux of hepatic GSH was decreased by the PP treatment. The increase of liver GSH content by PP treatment due to the decrease of sinusoidal efflux of GSH from the liver, thus would be involved in the exertion of antioxidant action of PP.

Keywords: protoporphyrin; glutathione; lipid peroxidation; rat liver

We previously found that protoporphyrin (PP) inhibited the lipid peroxidation stimulated by Fe2+ and/or ascorbic acid (AsA) or an NADPH-generating system in rat liver homogenates, mitochondria and microsomes in vitro. We also reported that PP inhibited the lipid peroxidation stimulated by Fe2+ and/or AsA in liver homogenates and subcellular fractions from rats given PP intravenously, and that the most potent antioxidant action of PP in the various tissues was found in liver and was noted at 24 h following administration. We further showed that both heat-labile and heat-stable factors present in liver might be involved in the exertion of the antioxidant action of PP, but that radical scavenging was not involved in this. The relation between lipid peroxidation and liver diseases such as alcoholic fatty liver and hepatitis has long been of interest. Since PP has been used in the treatment of hepatitis, understanding of the mechanism by which PP exerts its antioxidant effect was sought.

We therefore examined the effect of PP on the enzyme activities related to the lipid peroxidation and on the content of glutathione (reduced form; GSH, oxidized form; GSSG) in rat liver and found that PP increased the content of hepatic GSH. We extended our investigations to elucidate the mechanism of the increment of hepatic GSH content caused by PP treatment.

Materials and Methods

Chemicals

PP (disodium salt) was generously donated by Tokyo Tanabe Co., Ltd. NADPH and ATP (disodium salt) were purchased from Oriental Yeast Co., Ltd. GSH was from Boehringer Mannheim GmbH. Cytochrome c (from horse heart, Type III; cat c) was obtained from Sigma Chemical Co., Ltd. 2-Thiobarbituric acid (TBA) and diethy maleate (DEM) were purchased from Wako Pure Chemical Ind., Ltd. All other chemicals were of reagent grade.

Animal Treatments

Male Wistar rats, weighing about 200 g, were obtained from Japan SLC, Inc., and housed in an air-conditioned room (temperature 22–23 °C, humidity ca. 50%) with free access to a commercial chow (MF, Oriental Yeast Co., Ltd.) and tap water. PP solution containing 0.9% NaCl (5 ml/kg body weight) was administered intravenously to rats at a dose of 20 mg/kg. DEM (50% corn oil solution) was injected intraperitoneally at a dose of 1 ml/kg. The control animals received an equivalent volume of 0.9% saline or corn oil.

Rats were used in the following experiments 24 h after PP administration.

All animals were fasted for about 18 h but allowed free access to tap water prior to sacrifice.

GSH Determination

GSH contents in liver, bile and liver perfusate were measured by the method of Hissin and Hilf.

Assay of Lipid Peroxidation

Liver homogenates were prepared in 150 mM KCl–10 mM Tris–HCl buffer (pH 7.4). The reaction mixture, containing 1 ml of 10% (w/v) homogenate, 90 mM KCl, 2.5 mM FeSO4, 0.5 mM AsA and 50 mM Tris–HCl buffer (pH 7.4) in a final volume of 2.0 ml, was incubated at 37°C for 30 min, and the degree of lipid peroxidation was assayed by the TBA method.

Canalicular GSH Efflux

The animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). The bile duct was cannulated with a polyethylene tube (SP10, Natsume Seisakusho Co., Ltd.), placed before the hepatic hilus to avoid contamination with pancreatic juice. The animals were kept at about 30°C, and bile was collected for 15 min for the measurement of bile flow and GSH concentration. At the end of the experiment, the liver was perfused with 50 ml of 150 mM KCl–10 mM Tris–HCl buffer (pH 7.4), weighed, and used for the determination of GSH content.

Liver Perfusion and Sinusoidal GSH Efflux

Livers obtained from rats under pentobarbital sodium (50 mg/kg, i.p.) anesthesia were perfused via the portal vein with a single-pass of Krebs-Henseleit bicarbonate buffer supplemented with 10 mM glucose, equilibrated with an O2/CO2 (95/5, v/v) mixture to give pH 7.4. Perfusions were carried out at 37°C and at constant flow rates (3.97 ± 0.18 ml/g liver/min in control rats, 3.91 ± 0.10 ml/g liver/min in PP injected rats). The perfusate was collected from a cannula inserted into the inferior vena cava. Following a 30-min stabilization period, five equally spaced perfusate samples, 1-min collection each, were taken in a 1-h perfusion for the determination of GSH. The mean of the five determinations and the respective perfusion rate were employed to calculate the rate of GSH influx. Concomitantly, lactate dehydrogenase (LDH) activity and protein content in the perfusate were measured as a marker of liver damage by the method of Wrblewski and LaDue and Lowry et al. respectively. At the end of the perfusion, liver samples were taken to determine GSH content.

Statistical Analysis

The statistical significance of differences between control and PP-injected rats was determined by Student's t test and p values of less than 0.05 were considered to be significant.

Results

The activities of several enzymes related to lipid peroxidation were examined in rat liver. PP decreased the activity of NADPH-cytochrome c reductase, which catalyzed the generation of superoxide anion in liver microsomes (control 116.7 ± 3.2 vs. PP-treated 96.2 ± 5.0 mU cyt c reduced/mg protein/min, p < 0.05). PP did not affect the activities of the other hepatic enzymes examined: xanthine
oxidase, superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase or glutathione S-transferase (data not shown).

PP caused a 36% increase in liver GSH content (Fig. 1). No difference was observed in GSSG content in livers of rats between control and PP treatment (control 0.64 ± 0.05 vs. PP-treated 0.83 ± 0.07 nmol/mg protein). DEM, a depletor of liver GSH, markedly reduced the content of hepatic GSH when given to rats, and the lipid peroxidation was strongly enhanced (Fig. 2). Although PP increased the hepatic GSH content with resultant prevention of lipid peroxidation, it failed to prevent the rise of lipid peroxidation in GSH-depleted livers by DEM (Fig. 2). When added to the intact rat liver homogenate, GSH decreased the level of lipid peroxidation by about 50% at a concentration equivalent to that increased by PP administration (data not shown).

PP did not alter the activity of hepatic γ-glutamylcysteine synthetase or glutathione synthetase which was involved in GSH synthesis (data not shown). The activity of hepatic γ-glutamyl transpeptidase (γ-GTP), which constituted the initial step enzyme in the degradation of GSH, was increased by PP injection (control 0.686 ± 0.050 vs. PP-treated 0.893 ± 0.038 nmol p-nitroaniline formed/mg protein/min, p < 0.05).

The influence of PP on the canalicular efflux rate of GSH was studied in anesthetized rats. As shown in Table I, the rate of GSH in control livers was 2.01 nmol/g liver/min accounting for 17% of the total efflux from the organ (13.8 nmol/g liver/min, Tables I and II). PP showed no effect on the biliary concentration or canalicular efflux rate of GSH or on the bile flow, though hepatic GSH content was increased by PP treatment. We also observed the canalicular efflux of GSSG in agreement with reports, but there was no difference in the rate of efflux of GSSG between control and PP-administered rats (data not shown).

The sinusoidal efflux of GSH, in contrast, was inhibited by PP, resulting in a higher GSH content (Table II). Leakage of LDH and protein into the perfusate was found, but no significant difference was noted in the values between control and PP-treated groups.

### Table I. Liver Canalicular Efflux of GSH in PP-Administered Rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>PP</th>
</tr>
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<tbody>
<tr>
<td>Biliary GSH concentration (^a)</td>
<td>0.96 ± 0.09</td>
<td>1.12 ± 0.08</td>
</tr>
<tr>
<td>Bile flow (^d)</td>
<td>2.12 ± 0.16</td>
<td>2.08 ± 0.14</td>
</tr>
<tr>
<td>Canalicular GSH efflux (^b)</td>
<td>2.01 ± 0.18</td>
<td>2.35 ± 0.28</td>
</tr>
<tr>
<td>Liver GSH content (^c)</td>
<td>2.43 ± 0.20</td>
<td>3.02 ± 0.09</td>
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</table>

Treatment of rats was carried out as described in the legend to Fig. 1, except that animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg) before the bile duct cannulation. Data are expressed as the mean ± S.E. for 5–8 rats. \(^a\) Significantly different from the corresponding control, p < 0.05. \(^b\) μmol/ml bile. \(^c\) μg liver/min. \(^d\) nmol/g liver/min.

### Table II. Sinusoidal Efflux of GSH, LDH and Protein in Perfused Livers from PP-Administered Rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>PP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sinusoidal GSH efflux (^b)</td>
<td>11.8 ± 0.4</td>
<td>10.4 ± 0.3</td>
</tr>
<tr>
<td>Liver GSH content (^c)</td>
<td>2.60 ± 0.10</td>
<td>3.00 ± 0.11</td>
</tr>
<tr>
<td>Sinusoidal LDH efflux (^b)</td>
<td>0.060 ± 0.016</td>
<td>0.072 ± 0.019</td>
</tr>
<tr>
<td>Sinusoidal protein efflux (^b)</td>
<td>59.9 ± 5.6</td>
<td>60.5 ± 7.6</td>
</tr>
</tbody>
</table>

Treatment of rats was carried out as described in the legend to Fig. 1. Preparation of perfused livers was described in the text. Data are expressed as the mean ± S.E. for 6–8 rats. \(^a\) Significantly different from the corresponding control, p < 0.05. \(^b\) nmol/g liver/min. \(^c\) μmol/g liver. \(^d\) 1 μg liver/min. \(^e\) μg/g liver/min.

![Fig. 1. Effect of PP Administration on GSH Content in Rat Liver](image1)

Rats were given PP (20 mg/kg) intravenously and were killed 24 h after the administration. Data are expressed as the mean with S.E. (vertical bars) for 5 rats. \(^a\) p < 0.05.

![Fig. 2. Effect of DEM Injection on GSH Content and Lipid Peroxidation in PP-Administered Rat Liver](image2)

Rats were given PP (20 mg/kg) intravenously 22 h before intraperitoneal treatment with DEM (1 ml/kg), and were killed 2 h after DEM administration. Data are expressed as the mean with S.E. (horizontal bars) for 5–8 rats. \(^a\) Significantly different from each other, p < 0.05.
Discussion

The study was undertaken to clarify the mechanism(s) by which PP given to rats exerted its anti-lipid peroxidative action in the liver.

The effects of PP on the activities of hepatic enzymes involved in production and degradation of active oxygen species were evaluated. While PP suppressed by about 20% of the activity of microsomal NADPH-cytochrome reductase which catalyzed generation of $O_2^-$, it showed no effect on the activities of the other enzymes examined. Thus, the slight suppression of the reductase may contribute, at least in part, to the exertion of the antioxidative action of PP.

PP administration caused an increase in hepatic GSH content. GSH, a heat-labile tripeptide, is present in high concentration in liver cytosol and can scavenge by itself radicals such as $\cdot$OH or $O_2^-$. We also confirmed that GSH, when added to the intact liver homogenate, decreased lipid peroxidation. Therefore, the antioxidative effect of PP is also thought to be expressed through a mediation of elevated GSH. This is supported by the present findings that lipid peroxidation was strongly stimulated in homogenates of livers deprived of GSH by DEM treatment, while the oxidation in those of livers from rats treated with PP alone was markedly inhibited.

There is a report that GSH and hemin can associate under physiological conditions and that the two are linked through the iron of the latter. PP would not be associated with GSH, however, since PP had no chelated metal and the heme content in the liver from PP-administered rats, as determined by the pyridine–hemochromogen method, was not increased (data not shown).

PP did not affect the activity of either $\gamma$-glutamylcysteine synthetase or glutathione synthetase which catalyzed the biosynthesis of GSH. On the other hand, $\gamma$-GTP, the enzyme involved in the initial biodegradation of GSH, was slightly increased by the administration of PP. The enzyme activity of rat is reported to be very low in liver as compared with that in kidneys (about 0.2% of the renal activity). Therefore, the increase in hepatic activity caused by PP seems to have no significant role in the increase of hepatic GSH content.

Turnover of hepatic GSH in the basal state is accounted for entirely by efflux of GSH from the liver. PP treatment did not modify the canalicular rate of this efflux, whereas the sinusoidal efflux rate of GSH was reduced by PP. The increase in hepatic GSH in PP-treated rats was again observed, and can be ascribed mainly to the decrease of GSH efflux from the liver into the sinusoidal space. No variation in the plasma membrane permeability of the hepatocyte by PP treatment would occur, since the leakage of LDH and protein from livers into perfusate did not significantly differ between control and PP-treated. There was a slight efflux of GSSG into the perfusate from livers of both control and PP-treated rats with no significant difference between them, although no sinusoidal efflux of GSSG was reported (less than 0.5 nmol/min/g liver).

The difference between our findings and those earlier reports could arise from different methods employed, since the sensitivity to GSSG of the present method was much higher than that employed earlier.

There are several reports about the mechanisms concerning the sinusoidal efflux of GSH. Some of the reports have shown evidence of carrier-mediated transport systems for GSH and its derivatives in rat liver sinusoidal membrane vesicles, perfused livers and isolated hepatocytes. Others stated that GSH transport was related to the movement of K+ or was stimulated by hormones such as vasopressin, phenylephrine and adrenaline and by cAMP. Ooikhtens et al. reported that the organic anions, sulfobromophthalein, rose bengal, indocyanine green and bilirubin, might competitively inhibit the carrier for GSH efflux from inside the hepatocyte. A similar mechanism is thought to be involved in the suppression of GSH efflux from rat livers by PP, since it is anionic in the physiological pH; details of the mechanism, however, remain to be clarified.

References and Notes