Toxic dark effects of protoporphyrin on the cytochrome *P*-450 system in rat liver microsomes

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In erythropoietic protoporphyria, accumulation of protoporphyrin has been found in various tissues and liver cirrhosis occurs frequently in this disease, probably due to toxic dark effects of protoporphyrin. We have studied the effect of porphyrins on various enzymic functions in rat liver microsomes. Incubation of microsomes with protoporphyrin resulted in a concentration-dependent inhibition of the oxidation of 7-ethoxycoumarin and aminopyrine by the cytochrome *P*-450 system. Kinetic analysis showed a decrease in $V_{\max}$, whereas the K_m was not affected (non-competitive inhibition). Furthermore, reduction of cytochrome *c* by the NADPH-cytochrome *P*-450 reductase and by the NADH-cytochrome b_5 reductase was inhibited. However, the activity of the reductases was only affected when the microsomes were pre-incubated with protoporphyrin, and it was found that the inhibition was dependent on the duration of the pre-incubation. Kinetic analysis again revealed non-competitive inhibition. When these experiments were repeated with uroporphyrin, no inhibition could be observed. With Stern–Volmer plots it was demonstrated that this was most likely caused by the localization of the porphyrins: protoporphyrin is localized in the membrane, whereas uroporphyrin remains in solution. From these results it is concluded that accumulation of protoporphyrin in the liver may markedly affect the cytochrome *P*-450 system and thus its detoxification function.

INTRODUCTION

The porphyrias are metabolic disorders characterized by an inborn deficiency of haem synthesis, resulting in excessive production, accumulation and excretion of haem precursors [1]. In protoporphyria (also known as erythropoietic protoporphyria or erythrohepatic protoporphyria) the defect is a deficiency of the enzyme ferrochelatase, which catalyses the insertion of iron into protoporphyrin to form haem. The enzyme defect appears to be present in all haem-forming tissues, including the liver and bone marrow [2].

The major clinical manifestation of protoporphyria is a pronounced hypersensitivity of the skin to visible light, caused by the sensitizing properties of protoporphyrin accumulated in the skin [3]. Furthermore, it has been found that liver disease occurs more frequently than normal, and protoporphyrin concentrations as high as 0.5–3.2 mg/g wet weight of liver have been measured in the livers of protoporphyria patients [4,5]. Investigation of livers of patients with hepatic failure showed that these livers were black, due to massive deposits of dark-brown pigment in hepatocytes, Kupffer cells, portal macrophages and small biliary structures [6]. When the liver biopsy specimens were examined by electron microscopy crystals could be observed, which upon isolation and characterization proved to be crystals of protoporphyrin [7].

In previous studies it has been shown that porphyrins can interfere with enzyme-catalysed redox reactions [8–11]. In the liver such reactions are carried out by the cytochrome *P*-450 system. Due to the broad reaction specificity of the *P*-450 system, many lipophilic drugs and xenobiotics are hydroxylated to more polar products, so that they can be more readily excreted. Most of these reactions begin with the transfer of electrons from NADPH (and NADH) through NADPH-cytochrome *P*-450 reductase (and NADH-cytochrome b_5 reductase) to cytochrome *P*-450. This leads to the reductive activation of molecular oxygen, followed by the insertion of one oxygen atom into the substrate [12,13].

As little is known about the cause of hepatic damage in protoporphyria, we decided to investigate the effect of protoporphyrin on the activity of the cytochrome *P*-450 system, using rat liver microsomes as a model system. If drug metabolism by the cytochrome *P*-450 system is affected by the accumulation of protoporphyrin, liver failure as observed in some protoporphyria patients could be the result.

MATERIALS AND METHODS

Protoporphyrin IX, uroporphyrin III and mesoporphyrin IX were purchased from Porphyrin Products (Logan, UT, U.S.A.). NADPH, NADH, superoxide dismutase and cytochrome *c* were obtained from Boehringer. Umbelliferone, aminopyrine and 7-ethoxycoumarin came from Sigma. All other reagents were of analytical grade and were used without further purification. All solutions were prepared in water that had been passed through a Millipore Milli-Q ultrapurification system and were made up freshly before use. Stock solutions of the porphyrins (1 mM) were made in 4 mM-NaOH. To avoid photoexcitation of the porphyrins the incubation mixtures were prepared and handled under dim light.

Microsomes were prepared from untreated female Sprague–Dawley rats. In some experiments microsomes from rats treated for 8 consecutive days with phenobarbital (1%) in their drinking water were used. Livers were homogenized (1:2, w/v) in ice-cold 50 mM-potassium phosphate buffer, pH 7.4, containing 0.9% NaCl. After centrifugation at 12000 *g* (20 min, 4 °C), the supernatant was centrifuged at 100000 *g* for 60 min (4 °C). The microsomal pellet was resuspended and again centrifuged at 100000 *g* for 60 min (4 °C). The pellet was resuspended in 100 mM-potassium phosphate, pH 7.4, containing 0.1 mM-EDTA and 20% (w/v) glycerol. Microsomes (25 mg/ml) were stored at –70 °C. Protein was determined according to Lowry *et al.* [14] using BSA as a standard. All incubations were performed in 50 mM-Tris/HCl/150 mM-KCl, pH 7.4.

To determine the metabolism of 7-ethoxycoumarin, rat liver

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Fig. 1. Stern-Volmer plots

Stern-Volmer plots were prepared as described in the Materials and methods section. ○, Solutions contained porphyrins only (3 nM); ●, solutions contained porphyrins (3 nM) plus rat liver microsomes (1 µg of protein/ml). (a) Protoporphyrin, (b) uroporphyrin.

microsomes (0.125 mg of protein/ml) were incubated with NADPH (1 mM) and 7-ethoxycoumarin (0–1.2 mM) for 45 min at 37 °C. At all substrate concentrations used the reactions were linear with time. Reactions were terminated by the addition of 25 µl of 2 M-HCl to 1 ml samples. Umbelliferone, the metabolic product of 7-ethoxycoumarin, was extracted with 2 ml of chloroform. To 1 ml of the chloroform layer was then added 2.5 ml of 30 mM-sodium borate pH 9.2. After centrifugation the fluorescence of the water layer was determined (excitation 360 nm; emission 458 nm) [15]. Where necessary, values were corrected for the presence of porphyrins using standard curves with and without porphyrins present. The corrections were very small, because these porphyrins were hardly extracted into the chloroform layer. With aminopyrine, rat liver microsomes (0.125 mg of protein/ml, obtained from rats treated with phenobarbital) were incubated with NADPH (1 mM) and aminopyrine (0–7.5 mM) for 45 min at 37 °C. Again all reactions were linear with time. Reactions were terminated by addition of 1 ml of 10 % trichloroacetic acid to 1 ml samples. Formaldehyde formation was determined by the Nash procedure [16].

In the case of cytochrome *c* reduction by NADPH–cytochrome *P*-450 reductase, rat liver microsomes (0.125 mg of protein/ml) were incubated with cytochrome *c* (75 µM) and NADPH (0.25 mM) at 37 °C. For cytochrome *c* reduction by NADH–cytochrome *b*₅ reductase, rat liver microsomes (0.016 mg of protein/ml) were incubated with cytochrome *c* (75 µM) and NADH (0.2 mM) at room temperature. The reduction of cytochrome *c* was monitored spectrophotometrically at 550 nm (molar absorption coefficient: 21 000 M⁻¹·cm⁻¹) [17].

Stern-Volmer plots were constructed as described by Ricchelli & Jori [18,19]. Briefly, the fluorescence of protoporphyrin or uroporphyrin (3 nM) in 50 mM-sodium phosphate buffer, pH 7.4, was determined in the presence and absence of rat liver microsomes (1 µg of protein/ml) (excitation 405 nm; emission 635 nm). I⁻ was used as a fluorescence quencher (0–0.6 M). The ionic strength of the system was kept constant by addition of suitable amounts of KCl. The fluorescence quenching data were analysed by the Stern-Volmer equation:

$$F_0/F = 1 + K_q[X]$$

where F_0 and F represent the fluorescence intensities in the absence and presence respectively of the quencher, $[X]$ is the molar concentration of the quencher and K_q is the quenching constant.

Absorption measurements were carried out using a Beckman

DU 64 spectrophotometer. Fluorescence data were obtained using an Aminco SPF 500 spectrophotofluorometer. All experiments were carried out in triplicate and variability did not exceed 10%. Where indicated, results refer to means ± S.E.M. ($n = 3$).

RESULTS

Stern-Volmer plots

Information about the localization of porphyrins can be obtained by determining the degree of accessibility of these porphyrins to a hydrophilic fluorescence quencher. Addition of the quencher iodide to porphyrin solutions resulted in a concentration-dependent decrease in porphyrin fluorescence. These data were analysed in Stern-Volmer plots, which relate the decrease in fluorescence intensity (F_0/F) to the quencher concentration (Figs. 1a and 1b). From these plots the quencher constant K_q could be calculated according to the Stern-Volmer equation. In the case of protoporphyrin, K_q amounted to 12.3 M⁻¹ and for uroporphyrin a K_q of 4.3 M⁻¹ was found. In the presence of microsomes the K_q for protoporphyrin decreased to 1.8 M⁻¹, whereas in the case of uroporphyrin the K_q did not change significantly (Figs. 1a and 1b).

Effect of porphyrins on the oxidation of 7-ethoxycoumarin and aminopyrine by rat liver microsomes

The results in Fig. 2 illustrate the effect of protoporphyrin on the oxidation of 7-ethoxycoumarin to umbelliferone by the cytochrome *P*-450 system. Addition of protoporphyrin resulted in a concentration-dependent inhibition of 7-ethoxycoumarin metabolism (Fig. 2). When another dicarboxylic porphyrin, namely mesoporphyrin, was used, 7-ethoxycoumarin metabolism was only slightly inhibited: at a mesoporphyrin concentration of 150 µM, 7-ethoxycoumarin metabolism was inhibited by 20 % (results not shown). Incubation of the microsomes with protoporphyrin for 30 min prior to the addition of 7-ethoxycoumarin and NADPH slightly increased the degree of inhibition (Fig. 2). Similar results were found when the microsomes were pre-incubated with protoporphyrin and NADPH. When protoporphyrin was replaced by uroporphyrin in the incubation mixtures, no inhibition of 7-ethoxycoumarin metabolism could be observed in all cases (Fig. 2).

In further experiments the effects of protoporphyrin on the kinetics of 7-ethoxycoumarin metabolism were determined. Michaelis constants (K_m) and maximum velocity (V_{max}) were

Fig. 2. Effect of porphyrins on the oxidation of 7-ethoxycoumarin

Product formation was measured as described in the Materials and methods section. Results are expressed as percentages of the total amount of product formed without porphyrin present, which amounted to 141 pmol of umbelliferone/min per mg of protein. Pre-incubation of the microsomes at 37 °C for 30 min lowered this to 98 pmol of umbelliferone/min per mg of protein. ●, ■, In the presence of protoporphyrin; ○, □, in the presence of uroporphyrin; ●, ○, no pre-incubation; ■, □, pre-incubation for 30 min with a porphyrin.

Fig. 3. Hanes plot of the effect of protoporphyrin on the oxidation of 7-ethoxycoumarin

Reaction conditions were as described in Fig. 2. ●, Without protoporphyrin; ■, 150 μM-protoporphyrin. The unit of $[S]/v$ is mg of protein · min⁻¹ · μl⁻¹.

determined using Hanes plots, where the intercept with the x-axis represents $-K_m$ and the slope is $1/V_{max}$. (Fig. 3) [20]. It was found that the inhibition was a result of a decrease in V_{max} , from 171 ± 15 to 46 ± 4 pmol/min per mg of protein, whereas the K_m did not change significantly and remained at 0.14 ± 0.05 mM (Fig. 3). Similar results were obtained with aminopyrine as the substrate for the cytochrome *P*-450 system. In these experiments microsomes from phenobarbital-treated rats were used to enhance the metabolism of aminopyrine. With aminopyrine as substrate the K_m remained at 5.5 ± 0.78 mM and the V_{max} decreased from 384 ± 34 to 241 ± 27 pmol/min per mg of protein in the presence of 150 μM-protoporphyrin.

Fig. 4. Effect of pre-incubation time on the inhibition by protoporphyrin of cytochrome *c* reduction

Cytochrome *c* reduction was measured as described in the Materials and methods section. Protoporphyrin (75 μM) was added 0–75 min prior to the addition of cytochrome *c* and NAD(P)H. Results are expressed as percentages of the amount of cytochrome *c* reduced without protoporphyrin present. In the case of NADPH (■) this amounted to 42.4 nmol/min per mg of protein, and with NADH (●) it was 1837 nmol/min per mg of protein.

Fig. 5. Effect of porphyrins on the reduction of cytochrome *c*

Reaction conditions were as described for Fig. 4. Rat liver microsomes were incubated with protoporphyrin (■, ●) or uroporphyrin (□, ○) (0–125 μM) for 30 min prior to the addition of cytochrome *c* and NADPH (■, □) or NADH (●, ○).

Effect of porphyrins on the reduction of cytochrome *c* by NADPH-cytochrome *P*-450 and NADH-cytochrome *b*₅ reductase

In rat liver microsomes cytochrome *c* can be reduced either by NADPH-cytochrome *P*-450 reductase in the presence of NADPH, or by NADH-cytochrome *b*₅ reductase in the presence of NADH. Cytochrome *c* reduction was dependent on the presence of NAD(P)H and microsomes. The addition of superoxide dismutase (75 units/ml) did not affect cytochrome *c* reduction. This indicates that superoxide, which can be formed by the cytochrome *P*-450 system [21], was not involved in the reduction of cytochrome *c*.

Addition of protoporphyrin to the incubation mixtures had no

Table 1. Effect of protoporphyrin on cytochrome *c* reduction

Cytochrome *c* reduction was measured as described in the Materials and methods section. The cytochrome *c* concentration was varied from 12.5 to 75 μM . K_m is expressed in μM and V_{max} in nmol/min per mg of protein.

[Proto- porphyrin] (μM)	NADPH-cytochrome <i>P</i> -450 reductase		NADH-cytochrome <i>b</i> ₅ reductase	
	K_m	V_{max}	K_m	V_{max}
0	48.9 $\pm$ 6.5	73.5 $\pm$ 4.7	38.9 $\pm$ 7.6	2782 $\pm$ 231
75	46.9 $\pm$ 6.6	52.1 $\pm$ 3.5	32.5 $\pm$ 3.3	1277 $\pm$ 50
125	42.9 $\pm$ 8.7	30.3 $\pm$ 2.8	30.8 $\pm$ 7.0	810 $\pm$ 68

effect on the reduction of cytochrome *c*. However, when microsomes were incubated with protoporphyrin prior to the addition of cytochrome *c* and NAD(P)H, cytochrome *c* reduction was inhibited. Fig. 4 illustrates the effect of pre-incubation with protoporphyrin on the inhibition of cytochrome *c* reduction. Based on these results, and on the fact that the activity of the NADPH-cytochrome *P*-450 reductase decreased after prolonged incubation at 37 °C, a pre-incubation period of 30 min was used in further experiments. Under these conditions it was found that protoporphyrin induced a concentration-dependent inhibition of cytochrome *c* reduction (Fig. 5, closed symbols). Protoporphyrin concentrations higher than 125 μM could not be used because the absorbance at 550 nm became too high to determine cytochrome *c* reduction accurately. Similar results were obtained when the microsomes were washed after incubation with protoporphyrin for 30 min at 37 °C, indicating that protoporphyrin bound to the microsomes was responsible for inhibition of the reductases (results not shown).

Kinetic analysis revealed that the effect of protoporphyrin was caused by a concentration-dependent decrease in V_{max} , whereas the K_m was not affected (Table 1). Pre-incubation with uroporphyrin, on the other hand, did not affect the rate of cytochrome *c* reduction at all, in the presence of either NADPH or NADH (Fig. 5, open symbols).

DISCUSSION

The hepatic cytochrome *P*-450 system, which is localized in the membrane of the endoplasmic reticulum, plays a central role in the oxidative metabolism of a wide range of both endogenous and exogenous compounds. The results described in this paper demonstrate that protoporphyrin is an inhibitor of the activity of the cytochrome *P*-450 system. Uroporphyrin, on the other hand, did not affect the activity of the *P*-450 system at all (Figs. 2 and 4). A possible explanation for this difference could be that the inhibition is a property of dicarboxylic porphyrins: uroporphyrin contains eight carboxylic acid side groups and protoporphyrin only two. However, experiments with mesoporphyrin, also a dicarboxylic porphyrin, rule out such a possibility, as mesoporphyrin inhibited 7-ethoxycoumarin metabolism by only 20 %, whereas at the same concentration protoporphyrin caused an inhibition of 80 % (Fig. 2). Because of the number of carboxylic acid side groups, uroporphyrin is a highly polar compound, whereas protoporphyrin is highly hydrophobic. It can be expected that these porphyrins will be distributed in membrane structures such as microsomes according to their solubility properties. This was confirmed using Stern-Volmer plots (Figs. 1a and 1b). In the presence of microsomes the quencher constant K_q for protoporphyrin decreased dramatically, indicating that protoporphyrin became inaccessible to the quencher iodide. With

uroporphyrin, on the other hand, K_q did not change, indicating that protoporphyrin binds to the microsomes, whereas uroporphyrin remains in solution (Figs. 1a and 1b). Thus the fact that protoporphyrin did but uroporphyrin did not inhibit the activity of the *P*-450 system can be explained by this distinct difference in localization.

The inhibition of 7-ethoxycoumarin and aminopyrine metabolism by protoporphyrin was concentration-dependent (Fig. 2). Hanes' analysis showed that the V_{max} values decreased whereas the K_m remained unchanged, indicating non-competitive inhibition (Fig. 3). Non-competitive inhibition is consistent with a decrease in the quantity of active enzyme without a change in the affinity of the enzyme for its substrate. An interaction of protoporphyrin with the substrate-binding site of the cytochrome *P*-450 system seems therefore very unlikely. Binding of protoporphyrin elsewhere in the cytochrome *P*-450 system could cause conformational changes or interfere with the electron flow through the *P*-450 system. It is feasible, for example, that an interaction of protoporphyrin with the reductases would result in a decreased flow of electrons towards cytochrome *P*-450 and subsequently in a decrease in V_{max} . Such a decreased flow of electrons could be caused either by inhibition of the activity of the reductase(s) or by interference with the transport of electrons from the reductase(s) to the cytochrome.

The activities of the NADPH-cytochrome *P*-450 reductase and NADH-cytochrome *b*₅ reductase were monitored by measuring the reduction of cytochrome *c*. Similar results were obtained in the presence of either NADPH or NADH, although the NADH-cytochrome *b*₅ reductase seemed slightly more susceptible to inhibition by protoporphyrin (Figs. 4 and 5). In both cases the inhibition by protoporphyrin was characterized by a decrease in V_{max} , without a change in K_m , as was also found for 7-ethoxycoumarin metabolism (Table 1 and Fig. 3). However, the following observations argue against a relationship between the inhibition of the *P*-450 system and the inhibition of the reductase(s). First, the inhibition of cytochrome *c* reduction was dependent on pre-incubation of the microsomes with protoporphyrin (Fig. 4). These results differ from those obtained with 7-ethoxycoumarin, where pre-incubation of the microsomes with protoporphyrin was not necessary for inhibition to occur (Fig. 2). Secondly, the activity of the reductases greatly exceeded the overall activity of the cytochrome *P*-450-dependent mixed-function oxidase. In the case of 7-ethoxycoumarin, the V_{max} amounted to 0.171 nmol/min per mg of protein, whereas for cytochrome *c* reduction by the NADPH-cytochrome *P*-450 reductase a value of 73.5 nmol/min per mg of protein was obtained. Thus it seems unlikely that the decrease in *P*-450 activity is explained by a decrease in activity of one or both of the reductases *per se*. A decrease in activity of the reductases might, however, have other effects. NADPH-cytochrome *P*-450 reductase plays a role in haem catabolism, whereas NADH-cytochrome *b*₅ reductase and cytochrome *b*₅ are involved in fatty acid and sterol desaturase activity, as well as in fatty acid elongation [22–24]. Inhibition of these activities may, therefore, have important structural and functional consequences.

Although the decrease in cytochrome *P*-450 activity cannot be caused by the inhibition of cytochrome *P*-450 reductase, the results do suggest that protoporphyrin can interfere with the electron flow through the *P*-450 system, e.g. by interference with transport of the electrons from the reductase(s) to the cytochrome *P*-450 or somewhere on the cytochrome *P*-450. Mason and co-workers reported the one-electron reduction of uroporphyrin by hepatic microsomes in the presence of NADPH or NADH, resulting in the formation of a porphyrin anion free radical [10]. Their results indicated that the reductase was the primary donor of electrons, but cytochrome *P*-450 may have been a secondary

donor. It is feasible that, by binding to the cytochrome *P*-450 system, protoporphyrin is able to scavenge electrons from the *P*-450 system, subsequently inhibiting its activity. Damage due to the formation of a porphyrin radical could not be detected. Pre-incubation with either protoporphyrin alone or protoporphyrin plus NADPH gave similar results, indicating that under our conditions porphyrin radicals were not formed or did not inhibit 7-ethoxycoumarin metabolism (Fig. 2).

In conclusion, the results described in this paper demonstrate that protoporphyrin inhibits the microsomal cytochrome *P*-450 system. The accumulation of massive amounts of protoporphyrin in the livers of protoporphyria patients could thus result in inhibition of enzymes such as the cytochrome *P*-450 system *in vivo*. It seems possible that inhibition of these enzymes contributes to the liver damage observed in some of these patients.

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