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Absolute requirement for iron in the development of chemically induced uroporphyria in mice treated with 3-methylcholanthrene and 5-aminolevulinate

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Abstract Accumulating evidence, including experiments using cytochrome P450 1a2 (Cyp1a2) gene knock-out mice (Cyp1a2(−/−)), indicates that the development of chemically induced porphyria requires the expression of CYP1A2. It has also been demonstrated that iron enhances and expedites the development of experimental uroporphyria, but that iron alone without CYP1A2 expression, as in Cyp1a2(−/−) mice, does not cause uroporphyria. The role of iron in the development of porphyria has not been elucidated. We examined the in vivo effect of iron deficiency on hepatic URO accumulation in experimental porphyria. Mice were fed diets containing low (iron-deficient diet (IDD), 8.5 mg iron/kg) or normal (normal diet (ND), 213.7 mg iron/kg) levels of iron. They were treated with 3-methylcholanthrene (MC), an archetypal inducer of CYP1A, and 5-aminolevulinate (ALA), precursors of porphyrin and heme. We found that uroporphyrin (URO) levels and uroporphyrinogen oxidation (UROX) activity were markedly increased in ND mice treated with MC and ALA, while the levels were not raised in IDD mice with the same treatments. CYP1A2 levels and methoxyresorufin *O*-demethylase (MROD) activities, the CYP1A2-mediated reaction, were markedly induced in the livers of both ND and IDD mice treated with MC and ALA. UROX activity, supposedly a CYP1A2-dependent activity, was not enhanced in iron-deficient mice in spite of the fact of induction of CYP1A2. We showed that a sufficient level of iron is essential for the development of porphyria and UROX activity.

Keywords Porphyria · Uroporphyrin · CYP1A2 · Uroporphyrinogen oxidation · Iron

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Introduction

Porphyria cutanea tarda (PCT) is a human and animal disease characterized by massive hepatic accumulation and urinary excretion of uroporphyrin (URO) (Phillips et al. 2001). Inherited and non-inherited forms of PCT occur. Both of these forms are precipitated by the same etiologic agents, such as steroid contraceptives and alcoholic beverages (Becker 1965; Enriquez de Salamanca et al. 1982; Schoenfeld et al. 1996). An epidemic of hepatic uroporphyria was precipitated in humans in an accidental hexachlorobenzene poisoning in Turkey in the 1950s (Jarrell et al. 2002). Treatment of experimental animals with arylhydrocarbon receptor (AhR) ligands such as polycyclic aromatic compounds and polyhalogenated

aromatic compounds results in hepatic accumulation and urinary excretion of large amounts of uroporphyrin due to the marked decrease in uroporphyrinogen decarboxylase (UROD) activity and the shift in uroporphyrin metabolism to oxidation in the liver (Jones and Sweeney 1977; Smith and Francis 1983; Smith et al. 1998). CYP1A2 has been shown to be responsible for most of the oxidation of uroporphyrinogen to uroporphyrin (UROX) (Lambrecht et al. 1992; Sinclair et al. 1998; Smith et al. 2001).

It is also well known that uroporphyrin accumulation depends on the presence of iron. Intraperitonial injection of iron promotes uroporphyrin accumulation in the liver of rodents and decreases the time required for its development (Smith and Francis 1983; Smith et al. 1998). In some mouse strains, even a single dose of iron-dextran eventually caused uroporphyrin accumulation in the absence of cytochrome P-450 inducers (Deam and Elder 1991; Smith et al. 1989; Smith and Francis 1993; Gorman et al. 1999). It is also reported that accumulation of uroporphyrin was repressed in the liver of mice fed an iron-deficient diet (IDD), in spite of the fact that these mice received an injection of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a strong inducer of CYP1A (Sweeney et al. 1979). Unfortunately, the relationship between the induction of uroporphyria and iron and CYP1A was not resolved, probably because the presence of two species of CYP1A, namely, CYP1A1 and 1A2, was not known then. Interestingly, phlebotomy, which is presumed to remove excess iron stores (Epstein and Redeker 1965; Nonaka et al. 1986), is a successful treatment of human PCT. Thus, iron appears to be a very important factor in this disease. However, the mechanistic role of iron in PCT remains elusive.

In this study, we attempted to elucidate the mechanism by which iron deficiency inhibits uroporphyrin accumulation. We found that mice fed an IDD did not develop uroporphyrin accumulation even after treatment with 3-methylcholanthrene (MC) and 5-aminolevulinic acid (ALA), precursors of porphyrin and heme, respectively. This corresponded well with the low levels of UROX activity in the liver S9 fraction of these mice, indicating that the reduced uroporphyrin synthesis from uroporphyrinogen by UROX activity, which is mediated by CYP1A2, was the cause of absence of uroporphyria in these mice. However, CYP1A2 levels as well as the CYP1A2-dependent methoxyresorufin *O*-demethylase (MROD) activity were very high in the same group of mice, indicating that uroporphyrinogen may not be a direct substrate of CYP1A2.

Materials and methods

Chemicals

Uroporphyrin III was purchased from Frontier Scientific Co. 3-methylcholanthrene (MC), iron-dextran solution, methoxyresorufin, and resorufin were from Sigma Chemical Co. NADPH, glucose-6-phosphate (G-6-P), and glucose-6-phosphate dehydrogenase (G-6-PDH) were obtained from Oriental Yeast Co. Polyclonal anti-mouse CYP1A2 antibody and horseradish peroxidase-labeled anti-goat IgG were purchased from Daiichi Pure Chemicals. 5-Aminolevulinate (ALA), sodium amalgam, and other chemicals were obtained from Wako Pure Chemical Industries, Ltd. The iron-deficient (IDD; AIN93 additional without iron, 8.5 mg iron/kg) and normal (ND; AIN93 with ferric citrate as the iron source, 213.7 mg iron/kg) diets were from Nosan Co. Uroporphyrinogen III was freshly prepared from 0.75 mM uroporphyrin III (in 0.05 M Hepes buffer and 10 mM dithiothreitol) using 5% sodium amalgam (Smith and Francis 1987).

Animals and treatments

All experiments using animals were performed under the supervision and with the permission of the University's Institutional Animal Care and Use Committee. Six-week-old C57BL/6 male mice (Japan SLC, Inc.) were housed in a temperature- and humidity-controlled animal facility in 12-h light/dark cycles, and had free access to drinking water containing ALA (2 mg/ml) and either ND or IDD until sacrifice. During 7 days under a controlled tap water and diet (ND or IDD), MC in corn oil was injected intraperitoneally (ip) with a single daily dose of 50 mg/kg body wt for 3 days. Then animals were killed with carbon dioxide immediately after the last MC injection or 2 days, 1, 2, 3, or 4 weeks after the last injection. The liver S9 fraction and microsomes were obtained by differential centrifugation at 9,000 and 105,000 g, respectively, as described (Omura and Sato 1964), and stored at −86°C with 1.15% KCl

solution until use. The protein concentration was determined by the method of Lowry et al. (1951) with BSA as a standard. Heme in the blood was quantified by a Cobas Ready kit (Roche Diagnostics).

Porphyrin in liver

The liver S9 fraction was diluted with 0.05 M Hepes buffer (pH 7.5), 50 µl of which was injected into a 5 µm Inertsil ODS-2 HPLC column (25 cm × 4.6 mm, GL Sciences Inc.). A gradient elution was carried out using a Shimadzu LC-9A pump with a mobile phase of 0.02 M lithium citrate (pH 3.4) and methanol. The methanol concentration was increased linearly from 65 to 95% over 20 min, and then it was kept at 95% for 5 min. The porphyrin fluorescence was detected spectrophotometrically (Jasco FP-2020 Plus) at 397 nm (λex) and 617 nm (λem), respectively (Bonkovsky et al. 1986; Francis and Smith 1984). The concentration was estimated using a porphyrin standard.

MROD or UROX activity

MROD activity due to CYP1A2 was assayed as published previously with a small modification (Clark et al. 1995; Burke et al. 1985). The reaction solution (1 ml) contained 0.5 mg of liver S9 fraction, 0.22 ml of an NADPH-regenerating system (0.1 ml 100 mM $MgCl_2 \cdot 6H_2O$, 0.1 ml 100 mM G-6-P, 0.01 ml 50 mM NADPH, and 0.01 ml 200 U/ml G-6-PDH), and 0.05 ml 0.4 mM methoxyresorufin in 0.05 M Hepes buffer (pH 7.5). After pre-incubating the S9 fraction for 5 min in a shaking water bath at 40°C, the reaction was initiated by the addition of G-6-PDH and NADPH to the reaction mixture, incubated for 10 min in the dark, and stopped by adding 4.0 ml cold methanol. After the sample was centrifuged at 1,000 g for 5 min, the reaction product resorufin was measured fluorometrically using a fluorescence spectrophotometer (FP-777, JASCO Co.) at 530 nm (λex) and 590 nm (λem). UROX activity was assayed with the method published previously (Lambrecht et al. 1990; Smith et al. 1990) with a small modification. In 25 ml test tubes were placed 2.52 ml 0.25 M sucrose/0.05 M Hepes/0.5 mM EDTA, pH 6.8 (sucrose/Hepes/EDTA buffer), 0.4 mg S9 fraction (final protein concern, 133 mg/ml), 0.42 ml NADPH-generating system [(0.01 ml 50 mM NADPH, 0.01 ml 200 U/ml G-6-PDH, 0.1 ml 100 mM G-6-P, and 0.3 ml 100 mM $MgCl_2 \cdot 6H_2O$) dissolved in 0.05 M Hepes buffer, pH 6.8]. The reaction was initiated by the addition of 0.02 ml uroporphyrinogen III solution, and the tubes were incubated at 40°C for 30 min in the dark. The uroporphyrinogen oxidation was traced fluorometrically at 399 nm (λex) and 616 nm (λem), respectively.

Western blotting determination of expression of cyp1A2

The CYP1A2 expression level was determined by immunoblotting using an anti-mouse CYP1A2 polyclonal antibody according to Towbin et al. (1979). S9 protein was separated on a 10% polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane. Polyclonal antibody to mouse CYP1A2 (goat serum) was used as the primary antibody, and horseradish peroxidase-labeled anti-goat IgG was used as the secondary antibody. The staining was performed with an ECL plus Western Blotting Detection system (Amersham Biosciences) as the substrate. After scanning the membrane with a computer, a digital image of the spectral configurations was analyzed by the computer-assisted image analysis program, NIH Image ver. 1.61 (National Institutes of Health, Bethesda, MD, USA). The mean intensity from the reference control mouse was defined as 1.

Statistical analyses

Results are presented as means ± SEM. Significance was determined by ANOVA, in which $P < 0.05$ was considered to be significant.

Results

In the group of IDD mice that received ALA, the blood hemoglobin concentration tended to decrease, but not to a fatal level, over the entire experimental period (Table 1). Thus, iron deficiency cannot be regarded as an obstacle to our experiment.

Figure 1 shows the accumulation of URO in the livers of mice that were fed ND or IDD and tap water containing ALA and injected with MC. The ND mice began to accumulate URO 1 week later, and after 2 weeks the concentration had increased from

Table 1 Heme concentration in blood

Day	0	2	7	14	21	28
Iron-deficient (g/dl)	19.63 ± 0.67	19.23 ± 0.38	17.88 ± 0.21	16.05 ± 0.33	15.38 ± 0.65	15.23 ± 0.70
Normal (g/dl)	16.55 ± 0.86	ND	ND	ND	ND	ND

For the treatment regimen, see the "Materials and Methods". Day 0: mice who had been fed ALA-supplemented water and iron-deficient or normal diet for 1 week were killed without receiving MC injection. The blood was extracted from hearts when the mice were killed. Heme was quantified using a Cobas Ready kit (Roche Diagnostics). The number of mice in each group was three or four. ND means "not done". Values are means ± SEM

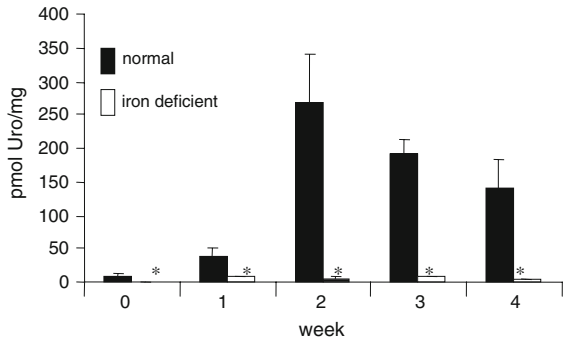

Fig. 1 Accumulation of uroporphyrins in the livers of mice fed normal and iron-deficient diets after a single i.p. injection of MC. *Closed bar*, normal diet; *open bar*, iron-deficient diet. Results are means and ranges for three or four animals per time point. There ware no increase in uroporphyrin levels in mice fed the iron-deficient diet. *All sections significantly different from mice fed normal diet ($P < 0.05$)

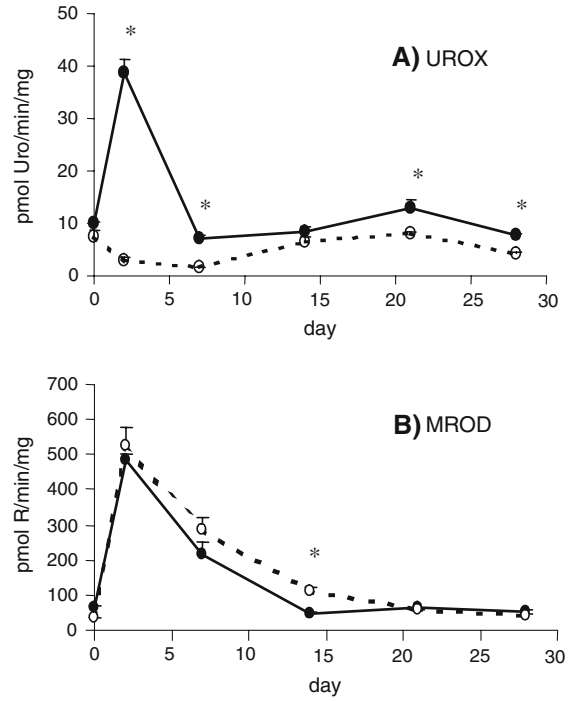

Fig. 2 UROX and MROD activities of liver S9 fraction of mice fed normal and iron-deficient diets. Experimental conditions are described in "Materials and Methods". **a** UROX, **b** MROD. *Closed circle*, normal diet; *open circle*, iron-deficient diet. Results are means and ranges for three or four animals per time point. *Values of mice fed the iron-deficient diet were significantly different from normal ($P < 0.05$)

9.62 ± 2.39 to 267.79 ± 71.72 pmol/mg, i.e., about 28 times the value before injection of MC. This level remained high and declined slowly as reported previously (Smith and Francis 1987). IDD mice, on the other hand, showed a different pattern: the accumulation of URO remained low, around 8 pmol/mg, which was less than the value of ND mice before MC injection at 9.62 ± 2.39. Thus, it was confirmed that iron deficiency represses URO accumulation in the liver of mice in a porphyrogenic regimen, in agreement with the previous paper (Sweeney et al. 1979).

Figure 2 shows the time courses of the respective UROX (a) and MROD (b) activities in the S9 fraction of IDD and ND mice. IDD mice were found to have low UROX activity for the entire experimental period, in spite of high MROD activity 2 days after the last MC injection. For ND mice, on the other hand, a good parallelism was observed between the activities of UROX and MROD (a metabolic marker of CYP1A2).

The ratios of UROX/MROD activities of IDD and ND mice at 2 days after the last MC injection indicate that UROX activity was repressed in IDD mice (IDD: 0.006 ± 0.003, ND: 0.08 ± 0.009). Regardless of the amount of iron intake, the MROD activities were significantly higher at 2 days after the last MC injection, and then decreased to normal levels.

Figure 3 depicts the results according to Western immunoblot detection of CYP1A2 in the S9 fractions

of both ND and IDD mice killed 2 days after the last MC injection. Similar levels of CYP1A2 were detected regardless of the quantity of iron intake.

Discussion

Several lines of experimental evidence indicate that CYP1A2 is essential to uroporphyria and UROX activity in rodents. URO accumulation and UROX activity were repressed in the liver of cyp1A2(−/−) null mutant mice in spite of MC, PCB, or TCDD treatment (Sinclair et al. 1998; Smith et al. 2001; Gorman et al. 2002). The present work showed a parallel between UROX and MROD activities only in ND mice. In IDD mice, on the other hand, URO accumulation was prevented and UROX activity unchanged regardless of the induced levels of CYP1A2 expression.

Sweeney et al. (1979) and Smith and Francis (1987) have reported that UROD activity increased in the liver of mice fed an IDD and decreased in the livers of mice overloaded with iron, respectively.

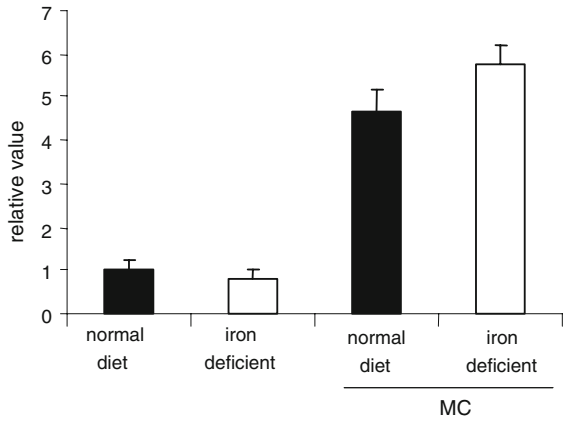

Fig. 3 Immunoblots of liver S9 fraction for detection of hepatic CYP1A2 expressed in mice fed iron-deficient and normal diets. Immunoblotting was performed as described in "Materials and Methods" using an antibody that detects both CYP1A1 and CYP1A2. Twenty microgram of liver S9 protein was applied to each well. *Closed bar*, normal diet; *open bar*, iron-deficient diet. Mice that had been fed normal diet and not been treated with MC were used as the standard of relative value. The mean staining intensity of the band of CYP from reference control mouse was defined as 1. The intensities from other samples were described by the proportion of the mean intensities from reference control. "MC" means the mice treated with MC and sacrificed 2 days later

Thus it appeared to us that iron plays the switching role at the point of bifurcation of the uroporphyrinogen metabolic pathway, one route directed to the UROD pathway and the other to the UROX pathway. When iron levels exceeded a certain level, the metabolic pathway shifted in the direction of UROX pathway, resulting in the accumulation of uroporphyrin, and when lower, toward the UROD pathway (Fig. 4). The formation of an inhibitor of UROD in livers of mice treated with iron and hexachlorobenzene has been demonstrated (Chaufan et al. 2001). Inhibition of the UROD pathway naturally increases the substrate available for the UROX pathway, and therefore, results in the accumulation of the oxidation product, URO. In addition, our results indicated the induction of UROX activity in ND mice treated with MC. Because CYP1A2 is induced in MC-treated mice and has been shown to be essential for UROX activity, it is natural to assume that CYP1A2 is responsible for the induction of UROX in ND mice treated with MC. However, this does not explain the absence of induced levels of UROX activity in IDD mice treated with MC. Ascorbic acid was reported to inhibit URO accumulation in primary cultures of chick embryo hepatocytes and hepatic microsomes from chickens and mice (Sinclair et al. 1995). Iron deficiency, however, rather decreases the ascorbic acid and α-tocopherol concentrations in the livers of mice. If iron bridges the reaction between CYP1A2

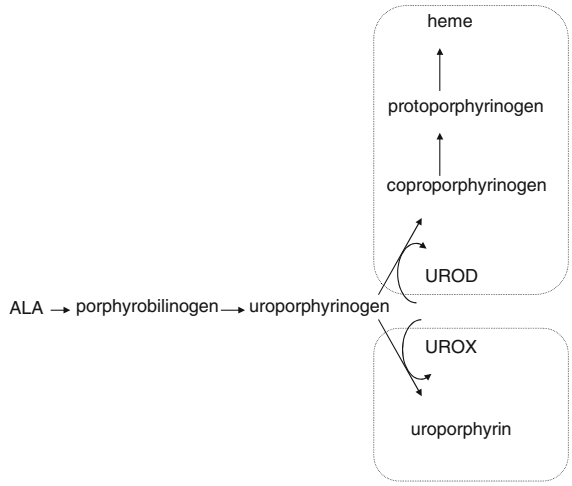

Fig. 4 Heme biosynthetic pathway. ALA, 5-aminolevulinic acid; UROX, uroporphyrinogen oxidase (CYP1A2); UROD, uroporphyrinogen decarboxylase

and uroporphyrinogen, this apparent discrepancy is reconciled. For example, iron, in whatever form, in the form of heme, for example, may receive an electron from CYP1A2 and transfer it to uroporphyrinogen to be oxidized. Or, alternatively, iron may be oxygenated by CYP1A2, and the oxygenated iron in turn oxidizes uroporphyrinogen. It is also suggested that reactive oxygen species generated during redox cycling of iron, which is reduced by CYP1A2, may be directly involved in the oxidation of uroporpyrinogen to uroporphyrin. In this manner, even if CYP1A2 is induced, the UROX activity does not take place without iron.

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