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Uroporphyria caused by acetone and 5-aminolevulinic acid in iron-loaded mice

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A number of polyhalogenated aromatic compounds, such as hexachlorobenzene (HCBZ*), cause hepatic uroporphyria in experimental animals that is accompanied by decreased activity of hepatic uroporphyrinogen decarboxylase (URO-D) [for review see Refs 1 and 2]. A similar human condition occurred in Turkey in the late 1950s when HCBZ-treated wheat seed was ingested [1]. More recently, it has been found that uroporphyria can be produced in iron-loaded mice by polycyclic aromatic hydrocarbons that do not contain halogen atoms: Francis and Smith reported that continuous feeding of 3-methylcholanthrene (MC) and other polycyclic aromatic hydrocarbons for 10 weeks produces uroporphyria in iron-loaded mice [3], whereas Urquhart *et al.* reported that a single injection of MC causes uroporphyria in iron-loaded mice given 5-aminolevulinic acid (ALA) in their drinking water for 9 days [4]. Both the halogenated compounds and polycyclic aromatic compounds are inducers of cytochrome P450s of the IA subfamily. Cytochrome P450IA2, which is inducible in the rat by MC and HCBZ, catalyzes the oxidation of uroporphyrinogen, an activity that we have suggested is involved in the development of the uroporphyria [5]. In contrast, phenobarbital, an inducer of the IIB cytochrome P450, was inactive [4] or weakly active [3] in causing porphyria in the iron-loaded mice. In this paper, we report that acetone caused a massive uroporphyria and decreased hepatic URO-D activity in iron-loaded mice given 5-aminolevulinic acid in their drinking water. Although acetone is a highly effective inducer of cytochrome P450 of the IIE and IIB subfamilies in the rat [6], we found no evidence that the uroporphyria in acetone-treated mice was due to uroporphyrinogen oxidation catalyzed by these forms of cytochrome P450.

Materials and methods

Animals. Male C57BL/6 mice (22–25 g) were purchased from Charles River Breeding Laboratories (Wilmington, MA), and were fed RMH 3000 rodent chow (Purina) *ad lib.* Iron (12.5 mg/mouse) was administered i.p. as Imferon (iron-dextran, Carter-Golgau, Phoenix, AZ). Both acetone [ACS, Fisher (Fairlawn, NJ); 1% (v/v)] and ALA [Sigma Chemical Co., St Louis, MO; 2 mg/ml] were given continuously in the drinking water. (The details of the experiments are described in the table legends.) All mice drank approximately 2 ml liquid/day. MC (Sigma) was given i.p. in approximately 0.3 ml corn oil at 130 mg/kg, 48 hr before killing the animals, and sodium phenobarbital was given i.p. in approximately 0.3 ml saline at 100 mg/kg, once each day for 3 days before killing. Animals were kept in temperature-controlled rooms with 12-hr dark/light cycles. The animals were killed by cervical dislocation, and the livers were removed and homogenized in 4 vol. of 0.25 M sucrose. The homogenates were centrifuged at 10,000 *g* for 10 min, and then a portion of the supernatant fraction was centrifuged at 100,000 *g* for 1 hr for preparation of microsomes. Microsomes were washed with 0.15 M KCl, and the pellet was stored at −60° for up to 4 weeks before assay. These storage conditions did not affect enzyme activities.

Enzyme assays. Uroporphyrinogen decarboxylase activity was assayed in 10,000 *g* supernatant fractions as described [7] using 5 mM pentacarboxyporphyrinogen III as substrate. Aniline hydroxylase was assayed as described [8] using 0.15 mM aniline as substrate. The plot of the rate of the reaction was biphasic as a function of substrate concentration as has been found for hamster [9] and chicken hepatocytes [8]. Uroporphyrinogen oxidation was measured as described [5]. Cytochrome P450 was measured by the method of Omura and Sato [10]. Microsomal ethoxy- and pentoxyresorufin dealkylase activities were assayed as described [11]. Porphyrins were determined spectrofluorometrically [12] or by HPLC as described [13].

Results and discussion

Table 1 shows that high levels of URO accumulated in the livers of mice when treated with the combination of iron-dextran, acetone and ALA. Livers from three of the five mice had URO concentrations greater than 290 nmol/g wet wt. The urinary excretion of URO in animals given all three compounds was also elevated, being detectable

* Abbreviations: ALA, 5-aminolevulinic acid; HCBZ, hexachlorobenzene; MC, 3-methylcholanthrene; URO, uroporphyrin; and URO-D, uroporphyrinogen decarboxylase.

Table 1. Effects of acetone, ALA and iron on hepatic porphyrins and uroporphyrinogen decarboxylase activity in mice

Treatment	N	Porphyrins (nmol/g liver) URO	COPRO	PROTO	Uroporphyrinogen decarboxylase (pmol/min/mg protein)
Iron-dextran	3	0.10 ± 0.08	0.02	0.22 ± 0.09	86.5 ± 8.9
ALA	3	ND*	ND	40.3 ± 11.0†	92.0 ± 8.3
Iron-dextran + acetone	3	0.12 ± 0.03	0.12	0.24 ± 0.02	78.3 ± 5.0
Acetone + ALA	5	2.73 ± 4.39	ND	54.4 ± 10.7†	74.2 ± 10.4
Iron-dextran + ALA	3	7.44 ± 4.9†	2.02	7.4 ± 8.1	65.4 ± 16.3
Acetone + iron-dextran +ALA	5	208.8 ± 126.8†‡	0.68	17.6 ± 16.2†	26.0 ± 6.4‡

Mice were treated as indicated with a single i.p. injection of iron dextran, followed 3 days later with 1% (v/v) acetone and 11.7 mM ALA, as indicated in the drinking water, continuously for 35 days. Hepatic porphyrin was measured spectrofluorometrically, and UROD activity was measured as described in Materials and Methods.

Values are means ± SD except for COPRO where an average is given.

* Not detectable.

† Significantly different from iron-dextran treatment alone at 95% confidence by analysis of variance.

‡ Significantly different from all other treatments at 95% confidence by analysis of variance.

Table 2. Induction of cytochrome P-450 and mixed-function oxidase activities in iron-treated mice subsequently treated with methylcholanthrene, phenobarbital or acetone

Treatment	Cytochrome P-450 (nmol/mg protein)	EROD (pmol/min/mg protein)	PROD (pmol/min/mg protein)	Aniline hydroxylase (nmol/min/mg protein)
Control	0.82	46	12	0.95
MC	1.48	7245	30	1.28
PB	1.34	131	156	1.18
Acetone	1.13 ± 0.13	66 ± 12	26 ± 5	2.27 ± 0.14

Microsomes were prepared from iron-dextran-treated mice killed after 10 days of treatment with 1% (v/v) acetone in the drinking water or with MC or PB as described in Materials and methods. Values from control (untreated), MC- or PB-treated mice are from pools of 3, 8 and 3 mice respectively. Values for acetone-treated mice are means ± SD for 5 individuals.

after 23 days of treatment (data not shown). The accumulated hepatic porphyrins were mainly 8- and 7-carboxyl porphyrins, as determined by reverse phase HPLC. This pattern was very similar to that previously observed in mice treated with either HCBZ or MC [3–4]. The finding that mice treated with iron-dextran, acetone and ALA had massively increased hepatic URO concentrations was observed in two separate experiments, using four mice in the first, and five mice in the second experiment. The livers of animals treated with the combination of all three compounds also had significantly decreased URO-D activities with an average decrease in URO-D activities of 70% compared to animals treated with iron-dextran alone. This decrease in URO-D activity was similar to that caused by polyhalogenated aromatic compounds [4, 14]. Table 1 also shows that treatment with only two of the compounds did not produce significant decreases in the activity of the URO-D.

As determined spectrofluorometrically, even in the absence of acetone, hepatic URO accumulation was increased following iron-dextran and ALA treatment compared to treatment with ALA alone (Table 1). (This result was confirmed by reverse phase HPLC by which an 8- to 80-fold increase in URO was detected (data not shown).) However, the amount of URO accumulated was much less than when acetone was also included in the treatment. In a longer study, others have shown that treatment of mice with iron-dextran and ALA without acetone for 7 weeks causes a severe uroporphyria associated with decreased UROD activity [15].

Protoporphyrin also accumulated in the livers of animals treated with ALA but the accumulation was less in the iron-dextran-loaded mice. The decreased accumulation was possibly due to increased conversion of protoporphyrin to heme. These data suggest that iron may be limiting for the ferrochelatase reaction, as indicated previously for homogenates of rat and human liver [16].

Previous work has indicated an association of uroporphyria with the forms of cytochrome P-450 induced by MC and HCBZ [4, 5, 12, 14]. To investigate whether the URO accumulation caused by acetone was associated with the same form of cytochrome P450 induced by MC, ethoxyresorufin deethylase activity was used as a marker for such induction. Table 2 shows that there was less than 1.5-fold increase in this activity in mice treated with acetone for 10 days compared to the 160-fold increase by MC. Pentoxyresorufin dealkylase, an activity increased most effectively by phenobarbital and which appears to be relatively specific for rat P450IIB [17], was increased 2-fold by acetone treatment. These results suggest that, if a specific form of cytochrome P450 is involved in the uroporphyria caused by acetone, then the form participating is not that inducible

by MC or phenobarbital. The low K_m form of aniline hydroxylase was measured to indicate the presence of the form of cytochrome P450 from the IIE subfamily that is induced in rats, rabbits and mice by acetone [18, 19]. Acetone treatment of the mice for 10 days caused increased activity of the low K_m form of aniline hydroxylase (Table 2). In an experiment with 35 days of acetone treatment, aniline hydroxylase was increased about 2-fold whether iron-dextran or ALA was present or absent (data not shown). However, in mice treated with both iron-dextran and ALA with acetone for 35 days, aniline hydroxylase activities were similar to untreated mice (data not shown). The cause of this apparent lack of induction is not known but may be due to some hepatotoxicity from the combination of all three treatments.

We previously reported that microsomes from MC-induced rats and mice catalyze oxidation of uroporphyrinogen [5]. We investigated whether microsomes from acetone-treated mice also catalyzed this oxidation. However, we found no increase in uroporphyrinogen oxidation activity by the microsomes from iron-dextran-loaded mice treated with acetone for 10 days compared to controls (data not shown). The oxidation rates were the same with or without acetone in the reaction mixture or whether or not mice had been pretreated with iron. Thus, we found no evidence that a specific form of cytochrome P450 induced by acetone, such as P450IIE, catalyzes uroporphyrinogen oxidation or is involved in the uroporphyria caused by acetone.

The human disease, porphyria cutanea tarda (PCT), is characterized by hepatic accumulation of URO and decreased URO-D activity. The disease is often associated with consumption of alcoholic beverages and some degree of hepatic siderosis (for review, see Ref. 20). Further work will be needed to show whether the uroporphyria caused by the combination of acetone, iron-dextran and ALA may be an experimental model for the role of ethanol-like inducing compounds in the pathogenesis of human PCT.

In summary, hepatic uroporphyria and decreased URO-D activity can be produced in 35 days in mice treated with the combination of iron-dextran, acetone and ALA, but not when mice are treated with combinations of only two of these compounds.

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Antiperoxidant effects of dihydropyridine calcium antagonists

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While sharing the ability to impede calcium (Ca^{2+}) entry into the heart-muscle cell (myocyte) via the voltage-dependent slow Ca^{2+} channel [1], Ca^{2+} antagonists may also possess "cytoprotective" properties independent of Ca^{2+}-channel blockade [2, 3]. Other than myocyte Ca^{2+} overload [4], superoxide (O_2^-)- and iron-dependent membrane phospholipid peroxidation, with xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2) (XOD) as a potential O_2^- source, may promote myocardial ischemic and reperfusion injury [5, 6]. A previous investigation from this laboratory [7] demonstrated that some Ca^{2+} antagonists reduce the susceptibility of cardiac-membrane phospholipid to free radical-induced peroxidation. In that study, the dihydropyridine class, represented by nifedipine and nitrendipine [8], was the only one of the four major chemical classes of Ca^{2+} antagonists which did not demonstrate at least some antiperoxidant efficacy. Since the prototype nifedipine is among the most potent Ca^{2+} antagonists known [1, 9], and a number of structurally-related dihydropyridines hold promise for the clinical management of cardiovascular [2] and cerebral [10] disorders, we have investigated the antiperoxidant potential of five other dihydropyridine Ca^{2+} antagonists: niludipine, nimodipine, nisoldipine, nicardipine, and felodipine. Our findings show that dihydropyridine Ca^{2+} antagonists have a wide range of antioxidant potencies and may indeed protect myocardial-membrane phospholipid from peroxidative injury. One dihydropyridine, nisoldipine, was found to inhibit an enzymatic O_2^- source, XOD.

Materials and methods

Materials. All reagents and buffers were from commercial sources at the highest available grade, as specified [7, 11]. Ca^{2+} antagonists were obtained from their manufacturers: felodipine (H 154/82; A.B. Hässle, Mölndal, Sweden); nicardipine (YC-93; Yamanouchi Pharmaceutical Co. Ltd, Tokyo, Japan); and niludipine (Bay 7168), nimodipine (Bay 9736), and nisoldipine (Bay K5552) (Bayer AG, Wuppertal, F.R.G.). Concentrated stock solutions of Ca^{2+} antagonists were made in ethanol such that the final solvent concentration in the peroxidation reaction system did not affect lipid peroxidation. XOD (analytical preparation from bovine milk; 1 unit/mg) and superoxide dismutase (superoxide:superoxide oxidoreductase, EC 1.15.1.1; analytical preparation from bovine erythrocytes; 5000 units/mg) (SOD) were from Boehringer-Mannheim (Indianapolis, IN). Water was purified with a Milli-Q system (Millipore, Bedford, MA). All experiments were carried out under amber lighting.

Peroxidation reaction system. Liposomes were prepared from purified rat myocardial-membrane phospholipid, as detailed [11]. The phospholiposomes were subjected to O_2^-- and iron-dependent peroxidation at 37° in a reaction system containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–0.145 M KCl, pH 7.4; 0.1 mM Fe^{3+}–1.0 mM ADP complex; 1.0 mM hypoxanthine; 10 mUnits XOD/ml; and 125 μg cardiac phospholipid/ml. These conditions are optimal for initial linear rates of O_2^- production and O_2^--dependent peroxidation [11, 12]. After 60 min, peroxidation was terminated by acidifying each milliliter of peroxidation reaction with 0.15 ml of ice-cold 76% (w/v) trichloroacetic acid in 2.3 N HCl.

Quantification of lipid peroxidation. Lipid peroxide formation was measured as thiobarbituric acid (TBA)-reactive material [11]. Under these conditions, TBA-reactivity largely reflects malondialdehyde (MDA) produced from cardiac phospholipid peroxidation [11]. The Ca^{2+} antagonists studied did not influence the TBA-reactivity of MDA standard and were not themselves TBA-reactive. Concentration–response curves were generated with the assistance of RS/1 software (BBN Corp., Cambridge, MA).

Assessment of O_2^- trapping. The O_2^--scavenging potential of test substances (i.e. Ca^{2+} antagonists) was assayed as prevention of the SOD-inhibitable reduction of ferricytochrome *c* at 22° [13]. The assay contained: 0.25 mM potassium-phosphate buffer, pH 8.6; 10^{-4} M EDTA; 2 mM NaOH; air-saturated dimethyl sulfoxide (DMSO) containing 0.55 M water, and 76 μM ferricytochrome *c*. The linear rate of SOD-inhibitable cytochrome *c* reduction was monitored at 550 nm, and attentuation of this rate was considered evidence of the O_2^--trapping ability of a substance. The extinction coefficient 21 mM^{-1} cm^{-1} was used to calculate the amount of O_2^- produced.

XOD activity. XOD was assayed spectrophotometrically by monitoring the conversion of xanthine substrate to uric acid at 25° [14]. The standard assay mixture contained: 50 mM potassium-phosphate buffer, pH 7.8; 10 μM EDTA; 4.2×10^{-9} M catalytically flavin-active XOD; and 0.5 mM xanthine. Some incubations included a Ca^{2+} antagonist at concentrations specified in the text.

Results

Antiperoxidant effect of dihydropyridine Ca^{2+} antagonists. Five dihydropyridine Ca^{2+} antagonists were tested for their ability to inhibit O_2^-- and iron-dependent myocardial-membrane phospholipid peroxidation (Table 1). Niludipine could not protect the phospholipid from oxidative injury, even at a 500 μM final concentration. Nimodipine, at 500 μM, inhibited peroxidation by 15%; solubility limits prevented its being tested at higher concentrations. In contrast, nisoldipine, felodipine, and nicardipine effectively protected myocardial-membrane phospholipid from oxidative injury at low micromolar concentrations. The concentration–response curves of the Ca^{2+} antagonist-antiperoxidants allowed determination of their respective antiperoxidant IC_{50} values (i.e. the concentration of Ca^{2+} antagonist at which peroxidation was inhibited by 50%). Nisoldipine, the most potent dihydropyridine Ca^{2+} antagonist-antiperoxidant identified with an antiperoxidant IC_{50} of 80 μM, was able to prevent completely myocardial phospholipid peroxidation at a concentration of ~200 μM. Felodipine and nicardipine, with respective IC_{50} values of 110 and 150 μM, were somewhat less potent than nisoldipine and inhibited peroxidation by ~75% at 500 μM; higher concentrations were not attainable without the need