Volume 250, number 2, 349–352 FEB 07293 July 1989

Effects of iron-EDTA on uroporphyrinogen oxidation by liver microsomes

Judith M. Jacobs[+], Peter R. Sinclair*°, Richard W. Lambrecht* and Jacqueline F. Sinclair*°

*Veterans Administration, White River Junction, VT 05001, and Departments of [+]Pharmacology and °Biochemistry, Dartmouth Medical School, Hanover, NH 03756, USA

Received 18 April 1989

Uroporphyrinogen oxidation by hepatic microsomes from chick embryos or mice pretreated with methylcholanthrene was increased by addition of iron-EDTA. This increase was partially prevented by catalase, mannitol, ketoconazole and piperonyl butoxide, whereas only ketoconazole and piperonyl butoxide inhibited the oxidation in the presence and absence of iron-EDTA. These data suggest that the oxidations of uroporphyrinogen in the presence and absence of added iron occur by different mechanisms.

Uroporphyrinogen oxidation; Cytochrome P-450; Iron; (Liver)

1. INTRODUCTION

The uroporphyria caused by polyhalogenated hydrocarbons is similar to the human disease, porphyria cutanea tarda (PCT) in its clinical and biochemical manifestations [1,2]. Both conditions are characterized by massive hepatic accumulation of 7 and 8 carboxyl porphyrins [1,2]. Uroporphyrinogen is rapidly oxidized to uroporphyrin by hepatic microsomes from chick embryos, rats and mice pretreated with methylcholanthrene (MC) [3,4,6]. This observation has led to the hypothesis that uroporphyrinogen oxidation is mediated by a specific cytochrome P-450 isozyme, P450IA$_2$, which directly or indirectly has a major role in chemically induced uroporphyria [3–6].

Iron has been implicated in both experimental uroporphyria and clinical PCT, but its role is unknown [2]. Several investigators have described chemical systems in which iron plus hydrogen peroxide cause uroporphyrinogen oxidation, probably due to hydroxyl radical formation [5,7,8]. De Matteis found that iron-EDTA accelerates uroporphyrinogen oxidation by microsomes from β-naphthoflavone-induced chick embryo liver in the presence of 3,4,3′,4′-tetrachlorobiphenyl (TCB) and NADPH [7,8]. This observation suggests that in microsomes, reactive oxygen derived from uncoupled cytochrome P-450 electron transport interacts with an endogenous iron pool to cause porphyrinogen oxidation [7,8]. The purpose of the present study was to determine if uroporphyrinogen oxidation by cytochrome P-450 and the iron-mediated increase in the rate of this oxidation occur by the same mechanism.

Correspondence address: P.R. Sinclair, Research (151), VA Medical Center, White River Junction, VT 05001, USA

Abbreviations: DMSO, dimethyl sulfoxide; DFX, desferrioxamine mesylate; MC, 3-methylcholanthrene; Mes, 4-morpholineethanesulfonic acid; PCT, porphyria cutanea tarda; PIP, piperonyl butoxide; TCB, 3,4,3′,4′-tetrachlorobiphenyl

2. EXPERIMENTAL

Catalase (thymol-free), superoxide dismutase, ketoconazole, MC and mannitol were purchased from Sigma (St. Louis, MO); uroporphyrin I from Porphyrin Products (Logan, UT); desferrioxamine (DFX) from Ciba-Geigy (Summit, NJ); piperonyl butoxide (PIP) and TCB from Ultra-Science (Hope, RI); dimethyl sulfoxide (DMSO) from Fluka (Hauppauge, NY) and Imferon (iron dextran) from Merrill-Dow (Cincinnati, OH).

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

Animals, treatments and preparation of microsomal fractions are described in [6], except that, where indicated, Imferon was given to mice at a dose of 12.5 mg iron/mouse, 72 h prior to treatment with MC. The oxidation of uroporphyrinogen to uroporphyrin was followed fluorometrically as in [6]. Iron-EDTA was prepared by mixing 0.1 M EDTA with an equal volume of 0.05 M ferric chloride. PIP and TCB were added as solutions in DMSO, and the concentration of DMSO in the assay never exceeded 250 mM. Ketoconazole was dissolved in 50% acetone.

Preliminary experiments using chick embryo microsomes tested whether the buffer (0.05 M Hepes, pH 7.6) or the chelating agent (1 mM EDTA) used routinely in these studies affected uroporphyrinogen oxidation. Since Hepes participates in free radical reactions [9], liver microsomes from MC-induced chick embryos were also prepared using Mes (0.05 M, pH 7.6) as buffer. Neither Hepes nor Mes affected the oxidation rates. Iron-EDTA promotes hydroxyl radical formation, but desferrioxamine iron chelates do not [10]. Hepatic microsomes from chick embryos induced with MC were isolated in the presence of 1 mM DFX or 1 mM EDTA and were assayed for uroporphyrinogen oxidation as described in table 1 except that the buffer contained DFX, EDTA, or no chelator. The rate of uroporphyrinogen oxidation was not affected by the chelating agent used for preparation of the microsomes or present in the assay.

3. RESULTS

3.1. *Chick embryo liver microsomes*

In the presence of TCB, iron-EDTA markedly stimulated uroporphyrinogen oxidation by hepatic microsomes isolated from MC-treated chick embryos (table 1) as previously shown for hepatic microsomes from β-naphthoflavone-treated embryos [7]. In the absence of TCB, the oxidation was much slower, but it was also stimulated by iron (table 1).

To ascertain if reactive oxygen species were involved in the iron stimulation, the effects of catalase and mannitol on uroporphyrinogen oxidation were examined. Catalase addition to the assay inhibited the stimulatory effect of iron-EDTA, but had no effect on uroporphyrinogen oxidation in the absence of added iron (table 1). Mannitol, a hydroxyl radical scavenger, also markedly inhibited iron stimulation, but had no effect on uroporphyrinogen oxidation in the absence of added iron (fig.1).

We also examined the effect of cytochrome P-450 inhibitors on the iron-EDTA stimulation of uroporphyrinogen oxidation, since previously we demonstrated that uroporphyrinogen oxidation, in the absence of added iron, is catalyzed by a specific, MC-induced isozyme of cytochrome P-450 [3,6]. The cytochrome P-450 inhibitors, ketoconazole [12] and PIP [13], almost completely prevented uroporphyrinogen oxidation by MC-induced chick embryo liver microsomes, both with and without added iron-EDTA (fig.2A,B). These results suggest that uroporphyrinogen oxidation is cytochrome P-450 dependent, in the presence as well as the absence of added iron.

Table 1

Effect of iron-EDTA on uroporphyrinogen oxidation by hepatic microsomes from chick embryos treated with MC

Addition to assay	Uroporphyrinogen oxidation (pmol/min per mg protein)	
	No Fe-EDTA	Fe-EDTA (50 μM)
None	6.6 ± 0.0	31.9 ± 1.8
Catalase	6.6 ± 0.0	17.7 ± 1.9
TCB	40.9 ± 4.0	74.0 ± 10.0
TCB + catalase	40.8 ± 4.7	51.3 ± 4.0

Oxidation of uroporphyrinogen to uroporphyrin was followed at room temperature spectrofluorometrically. The assay mixture contained 1 ml assay buffer (0.25 M sucrose, 0.05 M Hepes, 1 mM EDTA; pH 7.6), 0.01 ml microsomal suspension containing 0.15–0.2 mg protein, 0.02 ml NADPH-generating system [6]. Uroporphyrinogen I was reduced with sodium amalgam [6] and used at a final concentration of 2 μM. The reaction was started by the addition of 0.01 ml TCB (0.2 mg/ml solution in DMSO). Iron-EDTA was used at a final concentration of 50 μM Fe. Catalase was used at 0.03 mg/ml (greater quantities of catalase did not increase the inhibitory effect). Data from a representative experiment are shown (mean ± SD of triplicate assays). In 6 separate experiments, catalase inhibited the iron-EDTA stimulation of microsomal uroporphyrinogen oxidation from 40 to 90%

3.2. *Mouse liver microsomes*

Table 2 shows that iron-EDTA increased uroporphyrinogen oxidation by hepatic microsomes from mice treated with MC or corn oil. This increase was prevented by catalase and superoxide dismutase. In an experiment analogous to the one with chick embryo liver microsomes, mannitol did not affect uroporphyrinogen oxidation by microsomes from an MC-induced mouse, but almost completely prevented the stimulation by iron-EDTA (not shown). At 10 μM, PIP inhibited uroporphyrinogen oxidation by microsomes from an MC-induced mouse by 67%; in the presence of iron-EDTA, the inhibition was 63%.

Experiments with iron administered to mice in

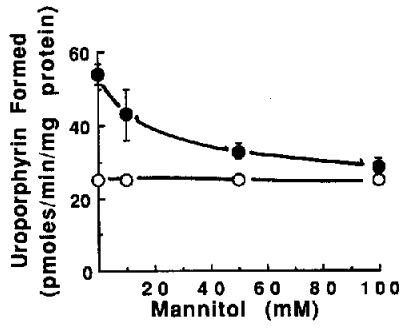

Fig.1. Effect of mannitol on iron-EDTA stimulation of uroporphyrinogen oxidation by liver microsomes from MC-induced chick embryos. Assay conditions are described in table 1. (○) Activity in the presence of TCB; (●) activity in the presence of 50 μM iron-EDTA and TCB. Data are presented as means and ranges of 2 assays for each data point.

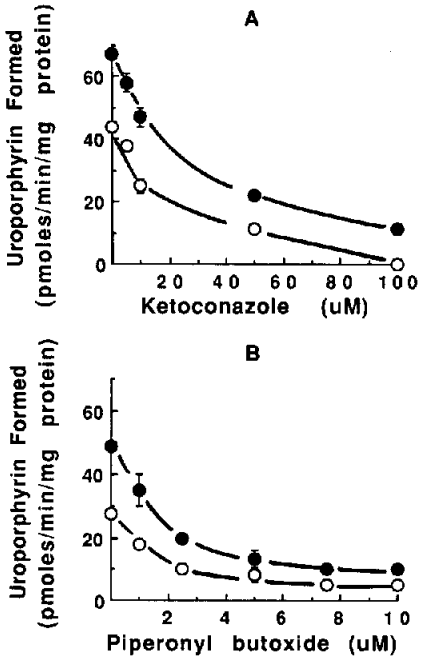

Fig.2. Effect of ketoconazole (A) and piperonyl butoxide (B) on iron-EDTA stimulation of uroporphyrinogen oxidation by liver microsmes from MC-induced chick embryos. Chick embryo treatment and microsome preparation are described in [6]. The assay is described in table 1. (○) Activity in the presence of TCB; (●) activity in the presence of 50 μM iron-EDTA and TCB. Data are presented as means and ranges of 2 assays for each data point. The assay mixture was incubated with PIP at room temperature for 10 min before TCB and uroporphyrinogen were added to allow formation of the P-450–PIP complex.

Table 2

Effect of catalase and superoxide dismutase on iron-EDTA stimulation of uroporphyrinogen oxidation by mouse liver microsomes

Treatment	Addition to assay	Uroporphyrinogen oxidation (pmol/min per mg protein)	
		No Fe-EDTA	Fe-EDTA (50 μM)
Corn oil	none	7.5 (7.0–8.0)	29.0 (21.0–37.0)
	catalase	6.6 (6.6–6.6)	13.3 (13.3–13.3)
	SOD	6.2 (6.2–6.2)	12.3 (12.3–12.3)
MC	none	35.0 (33.3–36.4)	53.0 (51.5–54.5)
	catalase	33.3 (33.3–33.3)	37.5 (36.4–42.0)
	SOD	32.0 (30.3–33.3)	35.7 (35.7–35.7)
	catalase + SOD	30.6 (27.8–33.3)	35.7 (35.7–35.7)

Treatments and assay conditions are described in [6] and table 1, except that in rodents, TCB is not needed to start the reaction [6]. Catalase was used at 0.03 mg/ml (greater quantitites of catalase did not increase the inhibitory effect). Superoxide dismutase was used at 114 U/ml. Results are expressed as means and ranges for 2 or more assays for each addition

vivo were conducted to see if excess iron present in the liver would increase uroporphyrinogen oxidation in vitro. Prior to treatment with MC, the mice were treated with Imferon to increase liver stores of iron [14]. The iron status of the animal had no effect on the rate of uroporphyrinogen oxidation either by hepatic microsomes or 10000 × *g* supernatants from corn oil or MC-treated animals (not shown). These results indicate that the excess iron present in the isolated microsomes is not equivalent to iron-EDTA added to the assay.

4. DISCUSSION

De Matteis proposed that cytochrome P-450-catalysed uroporphyrinogen oxidation requires reactive oxygen species and non-heme iron [7,8]. This raises the question of whether uroporphyrinogen oxidation, in the presence or absence of added iron, occurs by the same mechanism. However, our results suggest that uroporphyrinogen is oxidized by different mechanisms in the presence or absence of added iron. The iron-EDTA stimulation of microsomal uroporphyrinogen oxidation involved reactive oxygen species, since it was inhibited by catalase and man-

nitol. In the absence of added iron, microsomal uroporphyrinogen oxidation was not inhibited by catalase, mannitol, or superoxide dismutase, which indicates that reactive oxygen species were probably not responsible for this reaction. If such species were involved, the reaction may have occurred within the hydrophobic membrane where it would be inaccessible to catalase and mannitol, as has been suggested for metal-dependent lipid peroxidation [15].

The inhibitory effects of ketoconazole and PIP imply that uroporphyrinogen oxidation, both in the presence and absence of added iron, is dependent on cytochrome P-450. The mechanism by which cytochrome P-450 oxidizes uroporphyrinogen is unknown, but we have no evidence that endogenous iron is required for the reaction catalysed by the specific, MC-induced cytochrome P-450, since the chelators, DFX and EDTA, had no effect on the reaction. Furthermore, uroporphyrinogen oxidation was not increased in microsomes from iron-loaded mice. Cytochrome P-450 may produce some reactive oxygen species required for stimulation of uroporphyrinogen oxidation by added iron [7,8]. The reactive oxygen species involved in iron-stimulated uroporphyrinogen oxidation may be hydroxyl radical, which may also be generated by the interaction of iron-EDTA with NADPH-cytochrome P-450 reductase [16,17].

Our data do not explain the role of iron in uroporphyria. One possible role is to increase δ-aminolevulinic acid synthetase [18], which would increase the liver concentration of uroporphyrinogen. Products of uroporphyrinogen oxidation may inhibit uroporphyrinogen decarboxylase [4–6,19]. Thus the role of iron in vivo may be to increase the concentration of uroporphyrinogen and hence, the inhibitory products. Furthermore, the results presented here suggest that the addition of iron-EDTA to microsomes may not be an appropriate model for the events that lead to uroporphyria.

Acknowledgements: The authors wish to thank Drs G. Elder, N. Jacobs, E.L. Smith, S. Aust and A. Cederbaum for advice and helpful comments. This work was supported by funds from the US Veterans Administration and grants from the National Institutes of Health (CA 25012 and AA 07146). J.M.J. was supported in part by NIEHS Training Grant ES07104 and the Ryan Foundation.

REFERENCES

[1] Marks, G.S. (1985) Crit. Rev. Toxicol. 15, 151–179.
[2] Sweeney, G.D. (1986) Clin. Biochem. 19, 3–15.
[3] Sinclair, P.R., Lambrecht, R. and Sinclair, J. (1987) Biochem. Biophys. Res. Commun. 146, 1324–1329.
[4] De Matteis, F., Harvey, C., Reed, C. and Hempenius, R. (1988) Biochem. J. 250, 161–169.
[5] Francis, J.E. and Smith, A. (1988) FEBS Lett. 233, 311–314.
[6] Jacobs, J.M., Sinclair, P., Bement, W., Lambrecht, R., Sinclair, J. and Goldstein, J. (1989) Biochem. J. 258, 247–253.
[7] De Matteis, F. (1988) Mol. Pharmacol. 33, 463–469.
[8] De Matteis, F. (1988) Sem. Hematol. 25, 321–325.
[9] Woods, J.S. (1988) Biochem. Biophys. Res. Commun. 152, 1428–1434.
[10] Grady, J.K., Chasteen, N. and Harris, D. (1988) Anal. Biochem. 173, 111–115.
[11] Gutteridge, J.M.C., Richmond, R. and Halliwell, B. (1979) Biochem. J. 184, 469–472.
[12] Higashi, Y., Omura, M., Suzuki, K., Inano, H. and Oshima, H. (1987) Endocrinol. Jap. 1, 105–115.
[13] Anders, M.W. (1968) Biochem. Pharmacol. 17, 2367–2370.
[14] Smith, A. and Francis, J.E. (1985) Biochem. J. 214, 909–913.
[15] Borg, D. and Schaich, K. (1988) in: Oxyradicals in Molecular Biology and Pathology (Cerutti, P. et al. eds) pp.427–441, A.R. Liss, New York.
[16] Morehouse, L.A., Thomas, C. and Aust, S. (1984) Arch. Biochem. Biophys. 232, 366–377.
[17] Winston, G.W., Feierman, D. and Cederbaum, A. (1984) Arch. Biochem. Biophys. 232, 378–390.
[18] Bonkowsky, H., Healey, J., Sinclair, P., Sinclair, J. and Pomeroy, J. (1981) Biochem. J. 196, 57–64.
[19] Urquhart, A., Elder, G., Roberts, A., Lambrecht, R., Sinclair, P., Bement, W., Gorman, N. and Sinclair, J. (1988) Biochem. J. 252, 357–362.