Biochemical Pharmacology, Vol. 39, No. 11, pp. 1828–1830, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

Relationship between lucigenin-enhanced chemiluminescence and uroporphyrinogen oxidation in mouse and chick embryo liver microsomes

(*Received* 25 *May* 1989; *accepted* 1 *February* 1990)

Hexachlorobenzene (HCBZ*) and other polyhalogenated aromatic hydrocarbons (PHAs) cause an accumulation of uroporphyrin in the liver of rodents [1]. This increase has been attributed to decreased activity of the enzyme, uroporphyrinogen decarboxylase, which catalyses decarboxylation of uroporphyrinogen, with subsequent oxidation of uroporphyrinogen to uroporphyrin [2, 3]. However, in birds treated with PHAs, accumulation of uroporphyrin can occur without a decrease in the activity of the enzyme [4]. Recent *in vitro* studies suggest that a major initial effect of PHAs is to induce a cytochrome P450 which oxidizes uroporphyrinogen to uroporphyrin [5, 6]. It has been proposed that a product of this oxidation, possibly a polycarboxylate oligopyrrole, may act as an inhibitor of uroporphyrinogen decarboxylase [7–9].

Uroporphyrinogen oxidation (UROX) is catalysed by hepatic microsomes from rodents and chicken embryos treated with MC or PHAs [5, 6]. In rat microsomes, UROX is catalysed by the MC-inducible cytochrome P450IA2 isoenzyme [10]. Although the mechanism of the oxidation has not been elucidated, hydrogen peroxide, superoxide or hydroxyl radicals do not seem to be involved since catalase, superoxide dismutase and mannitol have no effect on the reaction [5, 6, 11]. Recently, several workers have implicated cytochrome P450-mediated generation of reactive oxygen species (ROS) in the pathogenesis of PHA-induced uroporphyria ([9, 12–14]; see review Ref. 15). Three approaches have been used to detect ROS production in such cytochrome P450-mediated reactions: lipid peroxidation [16], chemiluminescence (CL) [14] and UROX [5, 6]. We have now used liver microsomes from chick embryos and mice treated with known inducers of cytochrome P450 and compared the relative rates of CL and UROX. From the data obtained, we conclude that these two reactions do not always measure the same phenomenon.

Materials and Methods

Chemicals. Sources for most of the chemicals used were described previously [10]. Lucigenin (10,10′-dimethyl-9,9′-biacridinium dinitrate) was from the Sigma Chemical Co., Poole, U.K.

Animals, treatments and preparations of microsomes. C57BL/6, DBA/2 and AKR male mice (20–25 g) were purchased from Charles River Breeding Laboratories (Wilmington, MA). All mice were fed and watered *ad lib.* and were housed with 12 hr light/dark cycles. Four mice were treated in each group. MC (130 mg/kg) and OAT (75 mg/kg) were given as single i.p. doses in corn oil, and the animals were killed either 48 hr (MC) or 72 hr (OAT) later. Sodium PB (125 mg/kg) was given i.p. in saline for 3 consecutive days and the animals were killed 24 hr after the third treatment. Fifteen to sixteen-day-old White Leghorn chick embryos (15 per group) were injected with either 0.5 mg MC, 5 mg glutethimide or 1 mg OAT in 0.1 mL dimethyl sulfoxide (DMSO) 48 hr before being killed. Livers were homogenized (1/4; w/v) in 0.25 M sucrose–0.05 M HEPES–1 mM EDTA buffer (pH 7.6), and microsomes were prepared by differential centrifugation as previously described [17]. Microsomes were stored at −60° as suspensions in 20% glycerol–0.1 M sodium phosphate–0.1 mM EDTA, pH 7.4 for up to 8 weeks.

Assays. UROX and EROD were assayed essentially as described previously [5, 10]. With chick microsomes, UROX was initiated by addition of 10 μL TCB (0.2 μg/mL DMSO) to the 1 mL reaction mixture.

CL was assayed as described previously [14] with some modifications. The microsomal suspension was diluted 100-fold with glycerol–phosphate–EDTA buffer. The diluted suspension (50 μL) was then mixed with 0.45 mL 0.066 M Tris/HCl – 15 mM sodium azide (pH 7.4), and pre-incubated at 37° for 10 min. Azide was present to inhibit mitochondrial respiration and to quench singlet oxygen [18]. Lucigenin (10 μL of 5 mM solution in water) was added and the reaction initiated with 0.1 mL of NADPH (2.5 mg/mL in the Tris buffer). Luminescence was measured for 10 min at 37° in a Berthold Biolumat LB95000T luminometer and data are presented as total counts for the first 10 min. Cytochrome P450 concentrations were determined by the method of Omura and Sato [19]. Protein concentrations were determined by the method of Lowry *et al.* [20] using bovine serum albumin as standard.

Data were analysed using analysis of variance.

Results and Discussion

Effect of different inducers of cytochrome P450 on UROX and CL catalysed by hepatic microsomes from several inbred strains of mice. In these experiments we used three mouse strains that differ in their inducibility by MC for the IA sub-family of cytochrome P450s [21]. The DBA strain is unresponsive, the AKR strain unresponsive or weakly responsive and the C57 strain fully responsive to induction

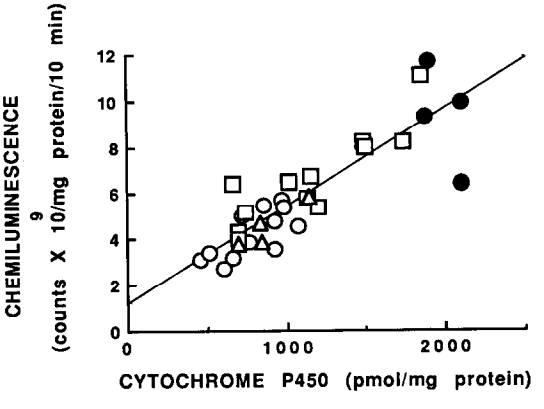

Fig. 1. Relationship of CL to mouse microsomal cytochrome P450. Each symbol represents an individual mouse as shown in Table 1. Symbols: (○) corn oil; (□) MC; (△) OAT; (●) PB.

* Abbreviations used: CL, NADPH-dependent lucigenin chemiluminescence; DMSO, dimethyl sulfoxide; EROD, 7-ethoxyresorufin deethylase, HCBZ, hexachlorobenzene; MC, 3-methylcholanthrene; OAT, *o*-aminoazotoluene; PB, phenobarbital; PHAs, polyhalogenated aromatic compounds; ROS, reactive oxygen species; TCB, 3,4,3′,4′-tetrachlorobiphenyl; UROX, uroporphyrinogen oxidation.

Table 1. Relationships between hepatic microsomal uroporphyrinogen oxidation, chemiluminescence, ethoxyresorufin deethylase and cytochrome P450 content in mice

Strain	Treatment	UROX*	CL†	EROD‡	Cyt P450§
DBA	Corn oil	14 ± 3	3.9 ± 0.6	0.19 ± 0.04	0.78 ± 0.19
	MC	21 ± 6	5.0 ± 1.0	0.30 ± 0.11	0.70 ± 0.03
AKR	Corn oil	16 ± 2	5.3 ± 0.50	0.22 ± 0.02	0.97 ± 0.09
	MC	28 ± 4‖	6.1 ± 0.61	0.37 ± 0.11	1.13 ± 0.08¶
C57BL/6	Corn oil	13 ± 3	3.5 ± 1.0	0.22 ± 0.13	0.62 ± 0.10
	MC	51 ± 11‖¶	8.9 ± 1.5‖¶	4.68 ± 1.19‖¶	1.64 ± 0.18‖¶
	PB	26 ± 7	9.5 ± 2.0‖	0.32 ± 0.02	1.99 ± 0.12‖
	OAT	45 ± 6‖	4.6 ± 1.0	0.44 ± 0.20	0.88 ± 0.19

For treatments see Materials and Methods. Four mice per group.
* pmole uroporphyrin/min/mg protein.
† Counts × 10^9/10 min/mg protein.
‡ nmole resorufin/min/mg protein.
§ nmole/mg protein.
‖ Significantly different from corn oil treatment of the same strain at the 95% confidence level.
¶ Significantly different from the other mouse strains given the same treatment, at the 95% confidence level.

Table 2. Relationship between microsomal uroporphyrinogen oxidation, chemiluminescence, ethoxyresorufin deethylase and cytochrome P450 in chick embryo

	UROX*		CL†			
Treatment	DMSO	TCB	DMSO	TCB	EROD‡	Cyt P450§
Untreated	2	3	0.2	0.3	0	0.32
MC	4	48	1.3	1.2	2.08	0.66
OAT	2	30	0.1	0.2	0.66	0.61
Glutethimide	4	4	1.1	1.3	0	1.84

For treatments see Materials and Methods. Values represent means of duplicate determinations of microsomes from pooled livers. Footnotes *–§, see Table 1.

by MC. As expected, Table 1 shows the difference between these strains for the inducibility by MC of total cytochrome P450 and EROD, an activity mainly catalysed by the mouse P1 isozyme (IA1) of cytochrome P450 [22]. MC increased both UROX and CL in C57 mice. However, in AKR mice, MC increased UROX by 1.8-fold with no significant increase in CL.

We also used compounds that are known to preferentially induce different forms of cytochrome P450 to compare CL and UROX in the C57BL/6 strain of mice. Table 1 shows that MC and OAT significantly increased UROX whereas MC and PB significantly increased CL. As previously shown in rats, UROX correlates with increased cytochrome P450 1A2 which is preferentially induced by MC and OAT [10]. PB preferentially induces cytochromes P450 IIB and III [23]. Figure 1 shows that CL correlated with the total amount of cytochrome P450 ($r = 0.87$ for all mice and 0.89 for C57 mice only) with no apparent relationship to particular cytochrome P450 isozymes. These results with inducers of different isozymes of cytochrome P450 suggest that UROX and CL are not equivalent.

Comparison of UROX and CL catalysed by chicken embryo liver microsomes. We next compared UROX and CL in microsomes from chicken embryos treated with different inducers of cytochrome P450 (Table 2). The maximum rate of MC-induced UROX was similar to that in the mouse; however CL activities were lower in the chick embryo. The reason for the lower CL activity of chick liver microsomes is not known, but chick embryo microsomes are more stable than rodent microsomes when incubated with NADPH [24]. OAT increased UROX but not CL whereas glutethimide, a powerful phenobarbital-like inducer of cytochrome P450 in the chick embryo [25], increased CL but not UROX. Table 2 also shows another major difference between CL and UROX. TCB did not affect CL. In contrast, UROX was inactive without addition of TCB, as shown previously [5].

Conclusions. Our results show that UROX is increased following treatments that do not increase species of ROS that react with lucigenin at pH 7.4. Peroxidative mechanisms for UROX of the type suggested by others (e.g. Ref. 8) are unlikely to produce ROS species that are detected by lucigenin [26, 27]. However, the resistance of UROX to catalase and other inhibitors [5, 6, 11] suggests that the oxidation of uroporphyrinogen is not mediated by H_2O_2, superoxide anion or hydroxyl radical species that are freely available in solution. We suggest that cytochrome P450 may catalyse UROX either directly (without generation of ROS) or indirectly without releasing ROS to the aqueous environment. Finally, those compounds which induce UROX and cytochrome P450 IA2 such as MC and HCBZ, also cause uroporphyria in mice, whereas compounds such as PB that increase CL but not UROX, are either ineffective or weakly effective in causing uroporphyria in rodents [28].

Acknowledgements—We thank Mr W. J. Bement for technical assistance and Dr E. L. Smith for helpful comments.

This research was supported by funds from the VA, and grants from NCI (CA25012) and NIAAA(AA07146). J. M. J. was supported by NIEHS Training grant ES07104.

PETER R. SINCLAIR*†
JUDITH M. JACOBS*‡
SUSAN M. DEAM§
RICHARD W. LAMBRECHT*
JACQUELINE F. SINCLAIR*†
GEORGE H. ELDER§

*Veterans Administration
White River Junction
VT 05001; Departments of
†Biochemistry and
‡Pharmacology
Dartmouth Medical School,
Hanover, NH 03756
U.S.A.
§Department of Medical
Biochemistry
University of Wales College
of Medicine
Heath Park
Cardiff CF4 4XN, U.K.

REFERENCES

1. Marks G, Exposure to toxic agents: the heme biosynthetic pathways and hemoproteins as indicator. *Crit Rev Toxicol* **15**: 151–179, 1985.
2. Elder G, Porphyria caused by hexachlorobenzene and other polyhalogenated aromatic hydrocarbons. In: *Heme and Hemoproteins* (Eds. De Matteis F and Aldridge W), pp. 157–200. Springer, Berlin, 1978.
3. Sweeney G, Porphyria cutanea tarda, or the uroporphyrinogen decarboxylase deficiency disease. *Clin Biochem* **19**: 3–15, 1986.
4. Lambrecht R, Sinclair P, Bement W, Sinclair J, Carpenter H, Buhler D, Urquhart A and Elder G, Hepatic uroporphyrinogen decarboxylase activity in cultured chick-embryo hepatocytes and in Japanese quail and mice treated with polyhalogenated aromatic compounds. *Biochem J* **253**: 131–138, 1988.
5. Sinclair PR, Lambrecht R and Sinclair J, Evidence for cytochrome P450-mediated oxidation of uroporphyrinogen by cell-free extracts from chick embryos treated with 3-methylcholanthrene. *Biochem Biophys Res Commun* **146**: 1324–1329, 1987.
6. De Matteis F, Harvey C, Reed C and Hempenius R, Increased oxidation of uroporphyrinogen by an inducible liver microsomal system. *Biochem J* **250**: 161–169, 1988.
7. Urquhart A, Elder G, Roberts A, Lambrecht R, Sinclair P, Bement W, Gorman N and Sinclair J, Uroporphyria produced in mice by 20-methylcholanthrene and 5-aminolevulinic acid. *Biochem J* **253**: 357–362, 1988.
8. De Matteis F, Role of iron in the hydrogen peroxide-dependent oxidation of hexahydroporphyrins(porphyrinogens): a possible mechanism for the exacerbation by iron of hepatic uroporphyria. *Mol Pharmacol* **33**: 463–469, 1988.
9. Francis J and Smith AG, Oxidation of uroporphyrinogens by hydroxyl radicals. Evidence of non-porphyrin products as potential inhibitors of uroporphyrinogen decarboxylase. *FEBS Lett* **233**: 311–314, 1988.
10. Jacobs J, Sinclair P, Bement W, Lambrecht R, Sinclair J and Goldstein J, Oxidation of uroporphyrinogen by methylcholanthrene-induced cytochrome P450: essential role of cytochrome P450 d. *Biochem J* **258**: 247–253, 1989.
11. Jacobs JM, Sinclair PR, Lambrecht RW and Sinclair JF, Effects of iron-EDTA on uroporphyrinogen oxidation by liver microsomes. *FEBS Lett* **250**: 349–352, 1989.
12. Ferioli A, Harvey C and De Matteis F, Drug-induced accumulation of uroporphyrin in chicken hepatocyte cultures. *Biochem J* **224**: 769–777, 1984.
13. Elder G, Roberts A and Urquhart A, Acquired uroporphyrinogen decarboxylase defects: molecular mechanisms. In: *Porphyrins and Porphyrias* 19–22 June, 1985 (Ed. Y Nordmann), pp. 147–152. Colloque INSERM, John Libbey, London, 1986.
14. Urquhart A and Elder G, Hexachlorobenzene-induced oxygen activation by mouse liver microsomes: comparison with phenobarbitone and 20-methylcholanthrene. *Biochem Pharmacol* **36**: 3795–3796, 1987.
15. De Matteis F, Toxicological aspects of liver heme biosynthesis. *Semin Hematol* **25**: 321–329, 1988.
16. Morehouse L and Aust S, Reconstituted microsomal lipid peroxidation: ADP-Fe-dependent peroxidation of phospholipid vesicles containing NADPH-cytochrome P450 reductase and cytochrome P450. *Free Radical Biol Med* **4**: 269–277, 1988.
17. Sinclair J, Sinclair P and Bonkowsky H, Hormonal requirements for the induction of cytochrome P450 in hepatocytes cultured in a serum-free medium. *Biochem Biophys Res Commun* **87**: 710–714, 1979.
18. Cadenas E and Sies H, Low level chemiluminescence as an indicator of singlet molecular oxygen in biological systems. *Methods Enzymol* **105**: 221–231, 1984.
19. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes. *J Biol Chem* **239**: 2370–2378, 1964.
20. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
21. Poland A and Knutson J, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. *Annu Rev Toxicol Pharmacol* **22**: 517–554, 1982.
22. Goldstein J, Linko P, Luster M and Sundheimer D, Purification and characterization of a second form of hepatic cytochrome P-448 from rats treated with a pure polychlorinated biphenyl isomer. *J Biol Chem* **257**: 2702–2707, 1982.
23. Wolf C, Seilman S, Oesch F, Mayer R and Burke M, Multiple forms of cytochrome P450 related to forms induced by phenobarbital. *Biochem J* **240**: 27–33, 1986.
24. McCluskey S, Racz W and Marks G, Properties of 17- to 19-day-old chick embryo liver microsomes. *J Pharmacol Methods* **16**: 111–123, 1986.
25. Sinclair P, Frezza J, Sinclair J, Bement W, Haugen S, Healey J and Bonkovsky H, Immunochemical detection of different isoenzymes of cytochrome P450 induced in chick hepatocyte cultures. *Biochem J* **258**: 237–245, 1989.
26. Weimann A, Hildebrandt A and Kahl R, Different efficiency of various synthetic antioxidants towards NADPH induced chemiluminescence in rat liver microsomes. *Biochem Biophys Res Commun* **125**: 1033–1038, 1984.
27. Allen RC, Phagocytic leukocyte oxygenation activities and chemiluminescence. *Methods Enzymol* **133B**: 449–493, 1986.
28. Francis J and Smith A, Polycyclic aromatic hydrocarbons cause hepatic porphyria in iron-loaded C57BL/10 mice: comparison of uroporphyrinogen decarboxylase inhibition with induction of alkoxyphenazone dealkylations. *Biochem Biophys Res Commun* **146**: 13–20, 1987.