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IMMUNOREACTIVE UROPORPHYRINOGEN DECARBOXYLASE IS UNCHANGED IN PORPHYRIA CAUSED BY TCDD[1] AND HEXACHLOROBENZENE

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SUMMARY A method has been developed for the immuno-titration of rodent liver uroporphyrinogen decarboxylase (porphyrinogen carboxy-lyase, EC 4.1.1.37) and used to show that two porphyrogenic polyhalogenated aromatic hydrocarbons, 2,3,7,8-tetrachlorodibenzo-p-dioxin and hexachlorobenzene, cause porphyria in rodents by decreasing the catalytic activity of uroporphyrinogen decarboxylase without altering the amount of immunoreactive enzyme protein. Investigation of the nature of the inactive form of uroporphyrinogen decarboxylase produced by these compounds should provide new information about the mechanism of their toxicity.

TCDD[1], a contaminant of the herbicide 2,4,5-trichlorophenoxyacetic acid, is one of the most potent toxic, carcinogenic and teratogenic small molecules known (1). Other less potent, but widely used, PHAHs[1], for example some polyhalogenated biphenyls and hexachlorobenzene, share many of its actions. All cause hepatic porphyria and liver damage but these effects, unlike some others, are confined to certain species, including humans (1,2). In spite of extensive investigation, the mechanism of the toxicity of PHAHs is unknown (1). Porphyria is the only toxic effect that can be attributed to altered function of a single molecule, namely decreased activity of uroporphyrinogen decarboxylase (EC 4.1.1.37) (3). We now report that TCDD and hexachlorobenzene, one of the least toxic PHAHs, both inactivate uroporphyrinogen decarboxylase by abolishing catalytic activity without changing the amount of immunoreactive enzyme protein.

[1]abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; PHAH, polyhalogenated aromatic hydrocarbon.

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MATERIALS AND METHODS

Treatment of animals Male C57/BL6 mice (22 - 28 g body weight) were given a single intraperitoneal injection of either TCDD (125 µg/kg body weight) in arachis oil (0.2 ml) or arachis oil (controls) and were killed 30 days later. Female Wistar rats (100 - 150 g body weight) were fed powdered MRC diet 41B containing 0.3% (w/w) hexachlorobenzene (analytical reagent grade, BDH Limited, Poole, Dorset, U.K.) or powdered diet alone (controls) and were killed after 40 or 107 days. All animals were allowed free access to food and water.

Immunochemical reagents An antiserum to uroporphyrinogen decarboxylase was prepared by injecting rabbits with uroporphyrinogen decarboxylase partially purified (730-fold) from normal human erythrocytes (4). The antiserum precipitated uroporphyrinogen decarboxylase activity from human haemolysates but was shown to be polyspecific by crossed immunoelectrophoresis. A solid-phase antibody reagent (1.05 mg immunoglobulin protein/ml suspension) was prepared by reacting an IgG fraction of the antiserum with a diazonium salt of reprecipitated cellulose (5). Uroporphyrinogen decarboxylase adsorbed onto this reagent retained activity. The antiserum cross-reacted with enzyme from human, rat and mouse liver, although cross-reactivity with the rodent liver enzymes was poor as judged by uptake onto solid-phase antibody (8 - 10% of that from human liver homogenates of equivalent activity). The rodent liver enzyme could not be visualized by immunoelectrophoresis.

Titration of uroporphyrinogen decarboxylase with solid-phase antibody Liver homogenates (10 - 20%, w/v, in 0.1 M-KH_2PO_4/K_2HPO_4 buffer, pH 6.8, containing 0.1 mM-EDTA) prepared from animals treated as described above were centrifuged at 10,000 g_{av} for 2 min. Supernatants from control animals and from rats treated with hexachlorobenzene for 40 days were diluted with buffer to decrease enzyme activity to that of the supernatants from the corresponding treated (TCDD or hexachlorobenzene for 107 days) animals. Aliquots (0.1 ml) were mixed with solid-phase antibody from 0.1 - 0.5 ml of suspension, which had been washed four times with homogenization buffer, and kept, with occasional mixing, for one hour at room temperature followed by 16 - 20 h at 4°C. Tubes were wrapped in aluminium foil to protect the mixtures from light. Solid-phase antibody-enzyme conjugates were recovered by centrifugation, washed 3 - 5 times with buffer and resuspended in assay buffer (80 µl) containing 10 mM-dithiothreitol for measurement of enzyme activity.

Enzyme measurement Uroporphyrinogen decarboxylase was measured using pentacarboxyporphyrinogen III as substrate (6).

RESULTS AND DISCUSSION

Severe hepatic porphyria and uroporphyrinogen decarboxylase deficiency was produced in male C57/BL6 mice with TCDD and in female Wistar rats with hexachlorobenzene (Table 1).

Figure 1 shows that the uptake by solid-phase antibody of uroporphyrinogen decarboxylase activity from liver homogenates from control animals increased progressively with the concentration of solid-phase antibody and could be clearly distinguished from non-specific uptake (compare control

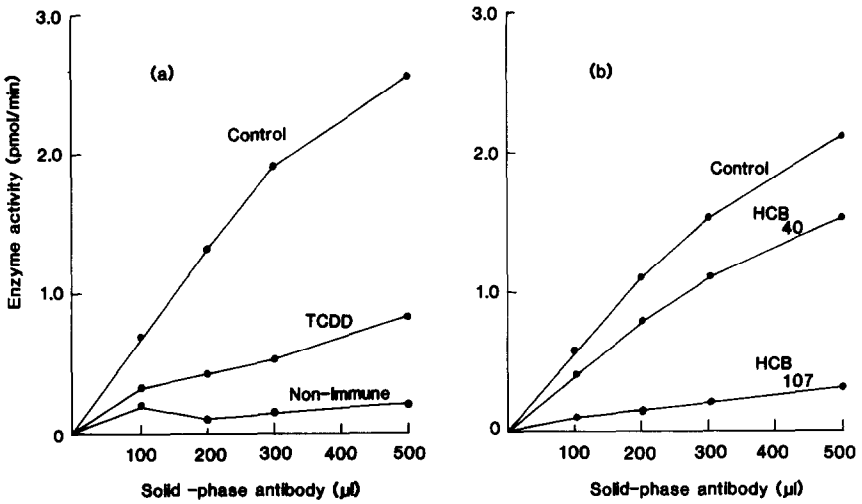

Figure 1 Uptake of uroporphyrinogen decarboxylase activity from mouse and rat liver preparations on to solid-phase antibody. a, Uptake of enzyme activity from mouse liver supernatant by solid-phase antibody (control and TCDD-treated mice). Uptake of enzyme activity from control mouse liver supernatant by equivalent amounts of a cellulose-rabbit IgG conjugate (non-immune) is also shown. b, Uptake of enzyme activity from rat liver supernatant (control and rats treated with hexachlorobenzene for 40 and 107 days). Uptake of enzyme activity by solid-phase non-immune rabbit IgG was identical to that shown for mouse liver.

and non-immune curves, Fig. 1). In this type of experiment, the slope of the initial, linear part of the curve depends on the amount of enzyme bound by the antibody and the specific activity (activity/mg enzyme protein) of the enzyme. Provided there is no change in the immunoreactivity of the enzyme, or introduction into the assay system of compounds that effect the antigen-antibody reaction, this technique can therefore be used to assess enzyme specific activity.

When liver homogenates from mice treated with TCDD and rats treated with hexachlorobenzene were reacted with solid-phase antibody under identical conditions, and at equivalent enzyme activities, there was a marked decrease in the uptake of enzyme activity (Fig. 1) which was dose-dependent (Fig. 1b). The antigen-antibody reaction was not affected by the large amounts of porphyrin present in the homogenates from treated animals because neither addition of equivalent amounts of uroporphyrin III to control homogenates nor removal of porphyrin from treated homogenates with an ion-exchange resin (Dowex 1-X2) (7) altered uptake. The low uptake of enzyme

from treated animals could not be explained by decreased enzyme stability because loss of activity during the enzyme solid-phase antibody reaction was similar (20 - 30%) for all homogenates.

Uptake of enzyme activity from livers from PHAH-treated animals was also decreased when the reaction with solid-phase antibody was carried out with homogenates at equivalent protein concentrations under conditions where antigen remained in excess with only 1.9 - 4% of total enzyme activity bound (Table 2). Under these conditions, there was close agreement between the extent to which PHAHs decreased uptake by solid-phase antibody and depressed enzyme activities in liver (compare columns showing activity ratios in Tables 1 and 2).

The results shown in Figure 1 and Table 2 suggest that the decreased uroporphyrinogen decarboxylase activity in PHAH-induced porphyria is not caused by a decreased concentration of normal enzyme. Had this been so, the uptake curves at equivalent activities (Fig. 1) would have superimposed. Furthermore, the low uptake shown in Table 2 cannot be explained as a

Table 1: Hepatic uroporphyrinogen decarboxylase activities and porphyrin concentrations

Animals	Treatment	Porphyrins (nmol/g)	Uroporphyrinogen decarboxylase (nmol/min/g)	Enzyme activity ratio (control/ treated)
Mice(4)	Control	0.28 (0.24-0.32)	7.13 (6.84-8.68)	-
Mice(4)	TCDD	67.5 (9.1-146.4)*	1.70 (1.27-3.14)*	4.2
Rats(5)	Control	0.62 (0.43-0.74)	5.32 (4.85-6.54)	-
Rats(5)	Hexachloro-benzene (40 days)	22.8 (4.8-46.7)**	2.11 (1.29-3.09)**	2.5
Rats(5)	Hexachloro-benzene (107 days)	808.7 (206.4-1441.4)**	0.48 (0.36-0.72)**	11.1

Values are means with ranges in parentheses.
*, ** significantly different from control group, Mann-Whitney test,
*$p < 0.03$, **$p < 0.01$.
Enzyme activities and porphyrin concentrations are expressed per g wet wt. liver.

Table 2: Uptake of uroporphyrinogen decarboxylase activity by solid-phase antibody

Animals	Treatment	Solid-phase enzyme activity (pmol coproporphyrinogen/min)	Solid-phase enzyme activity ratio (control/treated)
Mice(4)	Control	3.59 (3.29-3.87)	-
Mice(4)	TCDD	0.79 (0.64-1.01)*	4.5
Rats(5)	Control	1.58 (1.41-1.70)	-
Rats(5)	Hexachloro-benzene (40 days)	0.84 (0.51-1.33)**	1.9
Rats(5)	Hexachloro-benzene (107 days)	0.17 (0.13-0.24)**	9.3

Liver supernatant fractions were prepared as described under Methods but were not diluted before reaction of 0.1 ml aliquots with solid-phase antibody from 0.2 ml suspension. For all samples uptake of enzyme activity by solid-phase non-immune rabbit IgG was < 10% of the uptake by solid-phase antibody, except for those from rats treated with hexachlorobenzene for 107 days where it was approx. 50%. Values are means with ranges in parentheses.
*, ** significantly different from control group, Mann-Whitney test, *$p < 0.03$, **$p < 0.01$.

consequence of low antigen (enzyme) concentration because, although in these experiments uptake was dependent on antigen concentration (Fig. 2, curve a), the effect is clearly too small to be responsible for the large differences in uptake that were found (Fig. 1 and Table 2).

However, our experimental findings can be explained by postulating that PHAHs decrease the catalytic activity of uroporphyrinogen decarboxylase without altering the concentration of immunoreactive enzyme protein; that is, they decrease its specific activity. The decreased uptake of enzyme activity by solid-phase antibody (Fig. 1 and Table 2) would thus reflect the ratio of enzymatically-active to inactive molecules in the homogenates from PHAH-treated animals. This interpretation is supported by our observation that addition of TCDD-treated homogenate to control homogenate decreased uptake of uroporphyrinogen decarboxylase activity to a greater extent than addition of buffer (Fig. 2, compare curves a and b), indicating that treated homogenates contain material that competes with active uroporphyrinogen decarboxylase for uptake.

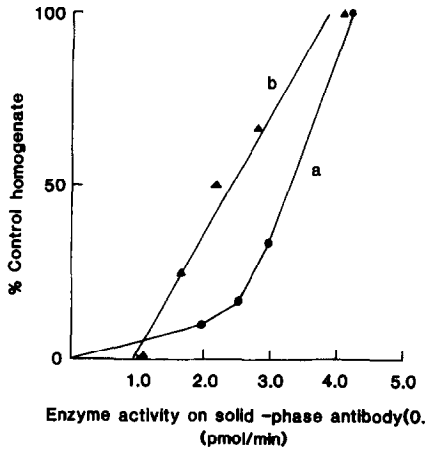

Figure 2 Effect of dilution on uptake of uroporphyrinogen decarboxylase activity by solid-phase antibody. Control homogenate was diluted with phosphate buffer (●, curve a) or TCDD-treated mouse liver homogenate (▲, curve b) to give mixtures of the percentage composition indicated. Supernatant fractions were prepared from the mixtures and reactions with solid-phase antibody carried out as described under Methods.

These experiments appear to exclude mechanisms for the production of porphyria by PHAHs which postulate decreased synthesis of structurally-normal uroporphyrinogen decarboxylase (7,8), either as part of the pleiotropic response initiated by binding of PHAHs to the cytosolic receptor for TCDD and its congeners (8) or through some other action (7). Increased catabolism leading to low concentrations of normal enzyme is similarly excluded.

The nature of the inactive form of uroporphyrinogen decarboxylase that appears to be produced by PHAHs remains to be identified. It seems likely that there is selective modification of the active centre but it is not clear how this might occur. Possible explanations include direct inhibition of the enzyme by PHAHs (9), inactivation by reactive PHAH metabolites (2,10), inactivation by oxygen radicals generated by an iron-dependent toxic response to PHAHs (11), or interference with the synthesis of uroporphyrinogen decarboxylase so that a closely related, but inactive, protein is produced. Of these mechanisms, reaction of a group, essential for catalytic activity, with a metabolite of PHAH to form an inactive, covalent bonded

enzyme-metabolite conjugate seems the most likely. Metabolism of hexachlorobenzene may be required for its porphyrogenic activity (2,10,12,13) and there is indirect evidence that reactive metabolites of hexachlorobenzene inactivate uroporphyrinogen decarboxylase by reacting with thiol groups that are essential for activity (2,13). Some problems arise when this hypothesis is extended to explain the action of TCDD. Although this compound is now known to be metabolized slowly to phenolic and other derivatives (14,15), covalent-binding in vivo is so low as to be barely detectable (16). Also, some selective affinity of the metabolites for the active centre of uroporphyrinogen decarboxylase would be required because the porphyrogenic dose of TCDD is so low (approx. 9 nmol in our experiments). However, neither the metabolism nor the covalent-binding of TCDD has yet been investigated in susceptible species receiving porphyrogenic doses and the possible formation of reactive metabolites cannot be excluded. Experiments with radio-labelled PHAHs, employing immunochemical isolation of inactive enzyme, should give a direct answer to this problem.

Finally, our results strengthen the view that hexachlorobenzene, TCDD and probably other PHAHs, while differing widely in their toxicity, cause porphyria, and possibly liver damage and other related toxic reactions, by the same mechanism (2,3). Determination of the structure of inactivated uroporphyrinogen decarboxylase should provide new information about this mechanism. Furthermore, they suggest that the molecular lesion produced by PHAHs may be sufficiently characteristic to provide a means of determining whether uroporphyrinogen decarboxylase defects of unknown pathogenesis, such as that in the 'sporadic' form of human porphyria cutanea tarda (17), are due to this cause.

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