# Low rate of NADPH/ADP-iron dependent lipid peroxidation in hepatic microsomes of DBA/2 mice

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Hepatic microsomal lipid peroxidation has been studied in 4 inbred strains of mice: C57BL/6, BALB/c, AKR and DBA/2. The rates of lipid peroxidation stimulated in vitro by carbon tetrachloride, ascorbate-iron and cumene hydroperoxide were similar in all 4 strains. Lipid peroxidation induced by NADPH/ADP-iron, however, proceeded at a substantially lower rate in the hepatic microsomes of DBA/2 mice. It is suggested that this low rate of enzymic iron-induced lipid peroxidation is a factor that may be involved in the resistance of this strain of mice to experimental hepatic porphyria induced by polyhalogenated aromatic hydrocarbons.

*Lipid peroxidation* *Hepatic microsome* *DBA/2 mouse* *Ah phenotype* *Hepatic porphyria* *Polyhalogenated aromatic hydrocarbon*

## 1. INTRODUCTION

In certain inbred mice strains (e.g. C57BL/6) the administration of 3-methylcholanthrene induces cytochrome $P_1$-450 and aryl hydrocarbon hydroxylase (AHH) activity; such strains are termed Ah responsive [1]. The most potent inducer of AHH activity is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [2]. Certain other strains show little or no response to 3-methylcholanthrene and are termed Ah non-responsive; the typical non-responsive strain is DBA/2 [1].

TCDD and related polyhalogenated aromatic hydrocarbons such as hexachlorobenzene (HCB) are highly toxic in many species and their toxicity has been associated with their ability to induce AHH activity. The relative toxicity of a range of chlorinated dibenzodioxin isomers correlates well with their relative potency as AHH inducers [3] and, in mice, sensitivity to TCDD-induced thymic atrophy, skin lesions and hepatic porphyria are all associated with the Ah phenotype [2]. However, the susceptibility of mice strains to porphyria induced by TCDD and hexachlorobenzene does not completely correlate with Ah responsiveness [4,5].

It has been suggested that polyhalogenated aromatic hydrocarbons such as TCDD and HCB induce porphyria by inhibiting the activity of hepatic uroporphyrinogen decarboxylase [6,7] but the exact mechanism of this effect is not known. It has been proposed that lipid peroxidation of cellular membranes may play a role in the induction of this type of chemical porphyria principally because of the aggravating effects of iron, an important catalyst of lipid peroxidation: iron-deficient mice are protected against TCDD-induced porphyria and liver damage [7,8] while on the other hand, iron-overloaded rats are more susceptible to HCB-induced porphyria [9]. Iron and HCB act synergistically to inhibit uroporphyrinogen decarboxylase and so induce hepatic porphyria in mice [4].

The proposed association between iron-dependent lipid peroxidation and chemical porphyria, together with the reported lack of complete correlation between TCDD- and HCB-induced hepatotoxicity and the Ah phenotype in mice have led us to investigate the susceptibility to lipid

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peroxidation of hepatic microsomal fractions from various inbred strains of mice. We have used two strains classified as Ah responsive (BALB/c and C57BL/6) and two strains classified as Ah non-responsive (DBA/2 and AKR).

Four methods of stimulating microsomal lipid peroxidation in vitro have been used. The ascorbate-iron-stimulated system is non-enzymic and utilises ascorbate to maintain iron in the reduced form. Cumene hydroperoxide-dependent lipid peroxidation utilises the microsomal hemoprotein cytochrome P-450 to catalyse the production of pro-oxidant radical species from the organic hydroperoxide; microsomal electron transport activity is not required. $CCl_4$-stimulated lipid peroxidation requires the NADPH-dependent activation of $CCl_4$ to an electrophilic radical species and the NADPH/ADP-iron-dependent system utilises NADPH : cytochrome *c* reductase to maintain the pro-oxidant ADP-iron complex in the reduced state.

## 2. MATERIALS AND METHODS

Adult male mice of 4 inbred strains were used: DBA/2 and C57BL/6 mice were obtained from Avonvale Ltd (Ashley Heath, Hants), AKR mice from the National Institute for Medical Research (Mill Hill, London) and BALB/c mice were bred in Brunel University. All were maintained on the same diet (Expanded Breeder Diet no.3, Special Diet Services, Witham, Essex); they were deprived of food overnight before killing. After removing the gall bladders, livers from 3–4 mice were pooled, microsomes were prepared as described in [10] and washed once by resuspension and recentrifugation in 0.15 M KCl. Cytochrome P-450 and NADPH : cytochrome *c* reductase were assayed as described [10] except at 25°C. Microsomal lipid peroxidation was assayed as malonaldehyde (MDA) production except where indicated. Carbon tetrachloride-induced lipid peroxidation was determined as in [11,12]. Cumene hydroperoxide-stimulated lipid peroxidation was estimated by incubating microsomes (1 mg protein/ml) with 0.1 mM cumene hydroperoxide in 23 mM Tris-HCl, pH 7.4, and 73 mM KCl at 37°C for 15 min [13]. Ascorbate-iron-induced lipid peroxidation utilised the same system but substituted the cumene hydroperoxide with ascorbate (0.5 mM) and ferrous sulphate (5 $\mu$M); the incubation time was 5 min. NADPH/ADP-iron-dependent lipid peroxidation was measured as the rate of oxygen uptake at 25°C as described [14] and as MDA production at 37°C using reactant concentrations as for the oxygen uptake experiments but using an NADPH-generating stock [12] and an incubation time of 10 min. Throughout, MDA was measured in the protein-free supernatant by the thiobarbituric acid assay [12] and the level of MDA in the absence of pro-oxidant (i.e. $CCl_4$, cumene hydroperoxide, ascorbate-iron or ADP-iron) was subtracted in each case. Microsomal fatty acids were determined as described [15]; microsomal protein was measured by the method of Lowry et al. [16].

## 3. RESULTS AND DISCUSSION

In all 4 strains of mice studied here, the rates of lipid peroxidation were broadly similar in the ascorbate-iron-dependent system, the cumene hydroperoxide-dependent system and in that stimulated by $CCl_4$. However, in the case of NADPH/ADP-iron-stimulated lipid peroxidation it was found that the microsomal suspensions from DBA/2 mice exhibited markedly decreased rates in comparison with preparations from all 3 of the other strains being approx. 50 and 20%, in terms of MDA production and of oxygen consumption respectively, of the rates found with the other microsomal preparations (table 1). The apparent discrepancy between the two methods of measuring lipid peroxidation in this system was investigated in one experiment by measuring the NADPH/ADP-iron-dependent oxygen uptake and MDA production at the same temperature. Microsomes were incubated in the oxygen electrode apparatus at 25°C [14] for 2 min, and then MDA was assayed. DBA/2-derived microsomes produced 68 pmol MDA per nmol oxygen consumed; AKR-derived microsomes produced 36 pmol MDA per nmol oxygen consumed. It is evident that this is a real difference in stoichiometry and it may be of interest to investigate further the stoichiometry of this reaction by measuring the utilisation of polyunsaturated fatty acids and the production of carbonyls other than MDA.

The reason for the lower rate of NADPH/ADP-iron-stimulated lipid peroxidation in DBA/2-

Table 1

Lipid peroxidation, microsomal enzymes and fatty acid composition in hepatic microsomes from 4 inbred mouse strains

| | Strain | | | |
|---|---|---|---|---|
| | BALB/c | C57BL/6 | DBA/2 | AKR |
| Lipid peroxidation stimulated by: | | | | |
| Ascorbate-Fe[a] | 3.09 ± 0.32 | 3.24 ± 0.13 | 3.62 ± 0.77[e] | 3.66 ± 0.21 |
| Cumene hydroperoxide[a] | 0.62 ± 0.06 | 0.50 ± 0.03 | 0.59 ± 0.05[e] | 0.59 ± 0.01 |
| $CCl_4$[a] | 0.35 ± 0.03 | 0.29 ± 0.02 | 0.27 ± 0.02 | n.d. |
| NADPH/ADP-Fe[a] (MDA produced) | 1.67 ± 0.19 | 1.87 ± 0.20 | 0.94 ± 0.14[e] | 2.31 ± 0.06 |
| NADPH/ADP-Fe[b] ($O_2$ consumed) | 73.8 ± 10.2 | 78.2 ± 4.8 | 16.4 ± 2.8[e] | 83.2 ± 2.4 |
| Microsomal enzymes | | | | |
| Cytochrome P-450[c] | 0.87 ± 0.08 | 0.68 ± 0.06 | 0.65 ± 0.07 | 0.67 ± 0.04 |
| NADPH:cyt $c$[d] reductase | 62.6 ± 3.2 | 73.5 ± 2.8 | 63.9 ± 11.0[e] | 96.3 ± 14.4 |
| Fatty acid (% of total) | | | | |
| 16:0 | 24.4 ± 1.8 | 27.2 ± 0.8 | 26.5 ± 0.7 | 25.4 ± 1.3 |
| 18:0 | 14.6 ± 1.2 | 14.0 ± 0.4 | 14.2 ± 0.4 | 15.8 ± 0.5 |
| 18:1 | 10.3 ± 0.4 | 12.9 ± 0.2 | 14.5 ± 0.5 | 15.3 ± 1.8 |
| 18:2 | 18.7 ± 0.2 | 18.3 ± 0.4 | 18.0 ± 0.4 | 15.8 ± 1.0 |
| 20:4 | 15.8 ± 1.8 | 13.9 ± 0.6 | 12.8 ± 0.7 | 15.2 ± 0.3 |
| 22:6 | 15.6 ± 2.3 | 13.8 ± 0.2 | 14.3 ± 0.6 | 12.4 ± 2.6 |
| Total ($\mu$g/mg protein) | 248.6 ± 34.5 | 225.7 ± 9.2 | 230.0 ± 22.1 | 269.2 ± 13.3 |

Results are expressed as [a]nmol/mg protein per min, [b]nmol/mg protein per min, [c]nmol/mg protein and [d]nmol cytochrome $c$ reduced/mg protein per min or as otherwise indicated. Data are mean ± SD from 4 or [e] 8 groups each group containing pooled liver microsomes from 3–4 animals. n.d., not determined

derived liver microsomes is not readily apparent. An elevated antioxidant in these microsomes would have been reflected in a decreased extent of peroxidation in all systems and an extended induction period would have been observed; the $\alpha$-tocopherol content of these microsomes was not elevated (not shown). The activity of NADPH:cytochrome $c$ reductase, which catalyses NADPH/ADP-iron-stimulated lipid peroxidation, was normal in DBA/2 derived microsomes (table 1). The polyunsaturated fatty acid content of the microsomal preparations from all 4 strains was found to be broadly similar (table 1); this accords with the similar rates of non-enzymic lipid peroxidation.

The relative rates of enzymic ADP-iron-dependent lipid peroxidation in microsomes from the 4 strains of mice did not correlate with Ah responsiveness, since AKR-derived microsomes were fully active in relation to lipid peroxidation. However, there is some degree of correlation between this lipid peroxidation activity and the relative susceptibility of the 4 strains to chemical porphyria in which iron-dependent lipid peroxidation has been proposed to be involved. Greig et al. [5] found that iron-enhanced, TCDD-induced porphyria was as high in AKR mice as in BALB/c and C57BL/6 mice, whereas TCDD did not induce porphyria in DBA/2 mice, even with concomitant iron administration; Ah responsiveness alone is therefore not the sole requirement for the inhibition of uroporphyrinogen decarboxylation. One explanation proposed by Greig et al. was that an important factor in determining the induction of porphyria by iron and TCDD might be hepatic

lipid peroxidation and that this activity might be greater in AKR mice than in DBA/2 mice. The data presented here are consistent with that hypothesis.

However, enzymic iron-dependent lipid peroxidation is not completely absent in DBA/2 liver microsomes yet porphyria cannot be induced in DBA/2 mice by TCDD or HCB, even in siderotic animals [4,5]. The inhibition of uroporphyrinogen decarboxylation by polyhalogenated aromatic compounds and the synergistic effect of iron are probably processes involving many steps, not least the induction of cytochrome $P_1$-450. Subsequent metabolism of the toxic compounds, possibly to reactive intermediates, and iron-catalysed free radical-mediated processes, such as lipid peroxidation, may also be involved. Clearly, the interactions of these various factors require further investigation. Future studies in this area comparing DBA/2 mice with (for example) C57BL/6 mice must consider not only the difference in Ah phenotype but also the low rate of enzymic iron-dependent lipid peroxidation in the former strain.

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