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EFFECTS OF LONG-TERM CHOLINE DEFICIENCY ON HEPATIC MICROSOMAL CYTOCHROME P-450-MEDIATED STEROID AND XENOBIOTIC HYDROXYLASES IN THE FEMALE RAT

MICHAEL MURRAY, ELIZABETH CANTRILL, LINDA FROST, ISHITA MEHTA and GEOFFREY C. FARRELL

Liver Research Group, Department of Medicine, University of Sydney, Westmead Hospital, Westmead, N.S.W., 2145 Australia

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Abstract—Total cytochrome P-450 levels decreased to about 80% of control in hepatic microsomes from female rats maintained for 30 weeks on a choline-deficient diet. Livers from these rats were fibrotic and had extensive fatty infiltration but, unlike livers of male rats on the same regimen, were not cirrhotic. Steroid hydroxylase activities were assessed in microsomes of female rats that received the choline-deficient diet and it was noted that the activity of the cytochrome P-450 *UT-F*-mediated steroid 7α-hydroxylase was decreased to about 50% of the activity present in choline-supplemented control rat microsomes. Similar decreases were observed for microsomal androstenedione 6β-hydroxylase and aniline 4-hydroxylase activities. In female rat hepatic microsomes these two activities are probably mediated by the isozyme cytochrome P-450 *ISF-G*. In contrast to these findings, the activities of four other xenobiotic metabolising enzymes, as well as rates of microsomal steroid 16α- and 16β-hydroxylation, were unchanged from control. Thus, in hepatic microsomes from choline-deficient female rats, it appears likely that levels of the non-sexually differentiated cytochromes P-450 *UT-F* and *ISF-G* are decreased. Unlike the situation in male rats, long term choline deficiency does not appear to influence levels of sexually-differentiated P-450 enzymes in the female rat.

The cytochrome P-450 (P-450)-mediated mixed function oxidase (MFO) system catalyses the oxidative biotransformation of lipophilic endogenous and exogenous substances to polar metabolites prior to elimination from the organism. A number of distinct P-450s have been purified to homogeneity [1–4]. It is the multiplicity of the P-450 system that gives rise to its apparent low substrate specificity since highly purified P-450 isozymes usually metabolise a narrower range of substrates than total microsomal P-450.

P-450 levels are low in humans with severe hepatocellular disease [5]. The capacity of such patients to catalyze drug oxidation is severely impaired. From *in vivo* studies of drug clearance by the liver [6] and estimation of microsomal MFO activities *in vitro* [5] it has been proposed that certain P-450s may be more susceptible to the effects of severe liver disease. To date no studies of P-450 composition in human liver disease have been reported.

Recently, several studies of the effects of experimental liver disease have been undertaken in the rat. Two distinct models of hepatic cirrhosis have been employed, namely, chronic intake of a choline-deficient diet [7] and repeated inhalation of carbon tetrachloride (CCl_4) [8]. Thus, it has been demonstrated that hepatic microsomes from cirrhotic male rats have a decreased capacity to bind [9] and to catalyze the oxidative metabolism of drug substrates [7, 8]. Further, it now appears that levels of two male-specific P-450s, and their associated drug and steroid hydroxylase activities are decreased in both models of hepatic cirrhosis [10, 11]. However, no information is yet available concerning hepatic drug metabolism in microsomes from female rats following prolonged intake of a choline-deficient diet.

The position-specific hydroxylation of C_{19}-steroids, including androst-4-ene-3,17-dione (AD) is catalyzed by individual forms of P-450. Thus, the P-450 isoform *UT-A* is the C_{19}-steroid 16α-hydroxylase that is present in male, but not female, rat liver microsomes [12]. P-450 *PCN-E*, another male-specific constitutive enzyme, is the steroid 6β-hydroxylase [12], whereas AD 7α- and 16β-hydroxylation are catalysed respectively by P-450 *UT-F* and P-450 *PB-B*, in microsomes from control rats [12]. (The P-450s *UT-A*, *PCN-E*, *UT-F* and *PB-B* have been designated P-450s IIC11, IIIA1/2, IIA1 and IIB1, respectively, in a recent compilation intended to unify the P-450 nomenclature in relation to gene families [13].) The present study was undertaken to examine the effects of hepatocellular injury produced by long-term intake of a choline-deficient diet on P-450 isozyme-specific steroid hydroxylation in microsomes from female rats. A series of xenobiotic MFO substrates was selected to provide additional information regarding the identity of the P-450s that are susceptible to the effects of severe liver disease.

MATERIALS AND METHODS

Chemicals. Ethylmorphine was from McFarlan Smith Ltd. (Edinburgh), 4-chloro-*N*-methylaniline from Calbiochem (San Diego, CA), and 7-ethoxy-

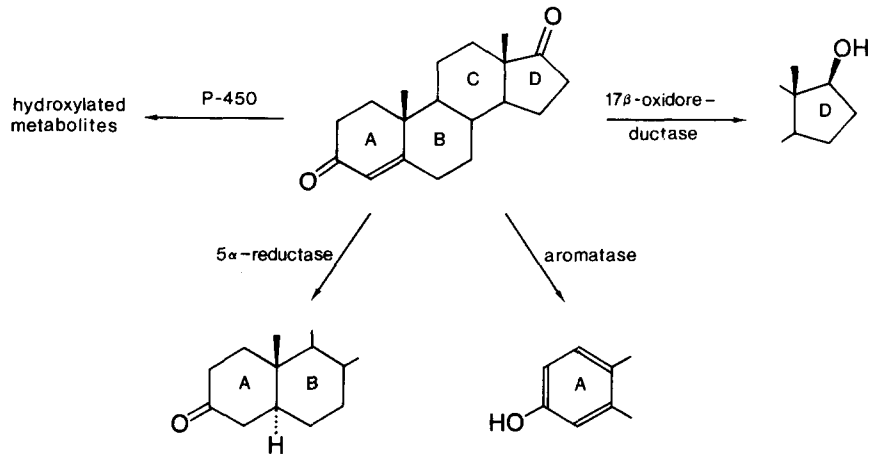

Fig. 1. General scheme for the microsomal metabolism of androst-4-ene-3,17-dione by cytochromes P-450 and other enzymes.

coumarin, umbelliferone and resorufin were from Aldrich Chemical Co. (Milwaukee, WI). 7-Ethoxyresorufin was purchased from Pierce Chemicals (Rockford, IL) and aniline from Ajax Chemicals (Sydney, N.S.W.); aniline was redistilled from zinc dust prior to use.

[4-^{14}C] Androst-4-ene-3,17-dione (AD; sp. act. 59 mCi/mmol; 98% purity by TLC) was purchased from Amersham Australia, Sydney, N.S.W. Unlabelled AD, 6β-hydroxy- and 16α-hydroxyAD, as well as all biochemicals, were obtained from Sigma Chemical Co. 7α-HydroxyAD was obtained from Professor D. N. Kirk of the MRC Steroid Reference Collection, Queen Mary's College, London, U.K. 16β-HydroxyAD was prepared enzymatically [14] by the action of 3β-hydroxysteroid dehydrogenase (Sigma) on 3β, 16β-dihydroxyandrost-5-ene-17-one (MRC Collection). Solvents and miscellaneous chemicals were from Ajax Chemicals, Sydney, and were at least analytical reagent grade.

Animals. Female Wistar rats (initial weight 90–130 g) were obtained from the animal facility of the Institute of Clinical Pathology and Medical Research at Westmead Hospital.

Choline-deficient diet. Components of the choline-deficient diet (containing no choline) were obtained from the suppliers previously indicated [7]. Choline-supplemented diet was prepared by the addition of 0.4% choline chloride to the choline-deficient synthetic diet. (Commercial rat diet, supplied by Allied Feeds, Sydney, Australia, contains 0.13% choline chloride).

Animal treatment. Animals received water and diet *ad libitum* over a 30-week period, until the time of sacrifice. After an overnight fast, microsomal fractions were prepared from individual rat livers as before [7]. The final microsomal fractions were stored at −70° as suspensions in 50 mM potassium phosphate (pH 7.4) containing 20% glycerol, until required for experiments.

Microsomal metabolism of androstenedione. The microsomal metabolism of AD (including hydroxylation, 17β-reduction and 5α-reduction; Fig. 1) was assayed as described previously [15]. Briefly, incubations containing hepatic microsomes (3 mg protein/4 ml), AD (0.175 mM, 4×10^5 dpm, added in 100 μl of acetone) and an NADPH-generating system were terminated after 10 min with 5.5% zinc sulphate. After centrifugation, the supernatant was extracted with chloroform and the chloroform phase evaporated to dryness under N_2. The extracts were applied, in a small quantity of chloroform, to TLC plates (silica gel 60 F_{254} type, 20 cm × 20 cm × 0.25 mm thickness; activated 15 min at 100° before use; E. Merck, Darmstadt, F.R.G.). Plates were developed twice in the solvent system chloroform:ethyl acetate (1:2) [16]. Zones corresponding to hydroxylated AD standards were visualised under UV light whereas 5α-androstane-3,17-dione was identified after spraying the plate with 2,4-dinitrophenylhydrazine (0.4% in 2 M HCl). Formation of metabolites was quantitated by liquid scintillation spectrometry (Aquasol scintillant, New England Nuclear Corp., Boston, MA).

Mixed-function oxidase assays. Ethylmorphine *N*-demethylase, 4-chloro-*N*-methylaniline *N*-demethylase [17] and aniline 4-hydroxylase [18] were assayed by established procedures. 7-Ethoxycoumarin and 7-ethoxyresorufin *O*-deethylase activities were measured in an Aminco SPF-125 spectrofluorometer by the time-dependent formation of umbelliferone (7-hydroxycoumarin) and resorufin, respectively [19].

Other assays. Microsomal protein was estimated by the method of Lowry *et al.* [20]. Cytochrome P-450 was assayed by difference spectrometry [21].

Miscellaneous serum biochemistry. Serum biochemistry analyses were performed in the Clinical Chemistry Laboratory of the Institute of Clinical Pathology and Medical Research at Westmead Hospital using automated procedures.

Analysis of data. Mean values of data from choline-deficient and choline-supplemented animals were compared using the Student's *t*-test (two tailed).

Table 1. Choline-deficient and choline-supplemented female rats: serum biochemistry

	Bilirubin (μmoles/l)	Albumin (mg/ml)	Alkaline phosphatase (units/l)	Alanine aminotransferase (units/l)	γ-Glutamyl transpeptidase (units/l)
Choline-supplemented	3.2 ± 0.5 (5)	41 ± 1 (5)	120 ± 40 (5)	29 ± 12 (6)	3.6 ± 1.7 (5)
Choline-deficient	9.5 ± 2.6 (8)	41 ± 3 (8)	320 ± 130 (8)	64 ± 40 (10)	2.0 ± 1.2 (10)
	P < 0.001	NS	P < 0.01	NS	NS

Data are mean ± SD. Values in parentheses indicate the number of individual rats assayed.

RESULTS

Effects of long-term choline-deficiency on female rats

Female rats that received the synthetic choline-deficient diet for 30 weeks had significantly lower body weights than choline-supplemented control rats (260 ± 40 g vs 340 ± 50 g, $P < 0.005$). Livers from choline-deficient female rats were significantly larger ($P < 0.02$) than those from choline-supplemented control rats (not shown). Fatty infiltration and moderate to severe fibrosis were apparent from histological evaluation of livers from choline-deficient female rats (not shown). Splenic weight was greater in choline-deficient animals when expressed as a percentage of body weight (0.28 ± 0.04% vs 0.21 ± 0.03%; $P < 0.005$).

Compared with control animals, serum concentrations of bilirubin and alkaline phosphatase were significantly elevated in choline-deficient rats (Table 1). However, serum concentrations of albumin, alanine aminotransferase and γ-glutamyl transpeptidase in the choline-deficient female animals were not altered from control levels. Thus there is evidence of mild hepatic dysfunction following long-term choline-deficiency.

Effects of long-term choline deficiency on hepatic microsomal cytochrome P-450 and related enzyme activities

Long-term intake of the synthetic choline-deficient diet was studied for its effects on cytochrome P-450 and P-450-dependent steroid hydroxylation in female hepatic microsomes. Levels of P-450 were found to be reduced in these microsomal fractions to 78% of control ($P < 0.001$; Table 2).

In choline-deficiency, formation of 6β-hydroxy- and 7α-hydroxyAD was decreased to 52% and 49% of control, respectively ($P < 0.02$, Table 2). In contrast, formation of the 16α- and 16β-hydroxyAD metabolites was unchanged from control. The activities of two important cytochrome P-450-independent steroid metabolizing enzymes, 17-ketosteroid 17β-oxidoreductase and Δ^4-steroid 5α-reductase, were also estimated in hepatic microsomal fractions. Testosterone formation (by the action of the microsomal 17β-oxidoreductase on AD) in choline-deficient female rat liver microsomes was 1.11 ± 0.33 nmole/min/mg protein and was not significantly different from control microsomes (0.83 ± 0.18 nmole/min/mg protein; Table 2). However, the activity of the 5α-reductase enzyme, that converts AD to 5α-androstanedione, was reduced in microsomes from choline-deficient female rat liver to 5% of control levels ($P < 0.02$, Table 2).

Subsequent experiments assessed the activities of several microsomal xenobiotic-metabolizing enzymes in choline-deficient and choline-supplemented rat liver. From the data presented in Table 3 it is apparent that aniline 4-hydroxylase activity was significantly lower in microsomes from choline-deficient animals. Four other MFO activities (ethylmorphine *N*-demethylase, 4-chloro-*N*-methylaniline *N*-demethylase, 7-ethoxyresorufin *O*-deethylase) were unchanged from control levels.

DISCUSSION

From the results of the present study, it is apparent that decreases in certain P-450-mediated pathways of drug metabolism occur in the female rat after long-term intake of a choline-deficient diet. Earlier studies demonstrated that levels and activities of sex-specific forms of P-450 are decreased in microsomes from male rats with hepatic cirrhosis produced by the same dietary regimen [10]. Similar findings were made in hepatic cirrhosis induced by chronic CCl_4-inhalation [11]. It was therefore suggested that changes in male-specific P-450s were independent of the means by which the liver disease was produced. It is now clear that changes in female rat liver that result from dietary choline deficiency are not as striking as in the male. Livers were not severely cirrhotic, although extensive fibrosis and fatty infiltration of hepatocytes were observed, and serum biochemical markers indicated hepatocellular dysfunction of a minor nature. P-450 levels were decreased by approximately 20% in choline-deficient female rat hepatic microsomes compared with about a 50% reduction of levels in male fractions [7].

The steroid AD was used as a probe of relative changes in individual P-450s produced by choline-deficiency. Although microsomes from female rats have a lower capacity to catalyze steroid hydroxylation than microsomes from male rats, the steroid probes remain useful in the identification of specific P-450s that may be down regulated in female rats with experimental liver injury. The major finding of this study was that AD 7α-hydroxylation was decreased to 49% of control microsomal levels after the dietary regimen. This pathway of steroid oxidation is catalyzed by P-450 *UT-F* [12], an enzyme that is present at relatively high levels in female hepatic microsomes (0.17 nmole/mg protein) but is generally an ineffective catalyst in most MFO reactions [1]. AD 6β-hydroxylation was also reduced in microsomes from choline-deficient female rats to 52% of control levels. Whereas this activity is catalyzed by P-450 *PCN-E* in male rat liver microsomes

Table 2. Cytochrome P-450 and androstenedione metabolism in choline-deficient and choline-supplemented female rat liver microsomes

Microsomes	Cytochrome P-450 (nmol/mg protein)	Hydroxyandrostenedione 6β (pmol/min/mg protein)	Hydroxyandrostenedione 7α (pmol/min/mg protein)	Hydroxyandrostenedione 16α (pmol/min/mg protein)	Hydroxyandrostenedione 16β (pmol/min/mg protein)	Testosterone (pmol/min/mg protein)	5α-Androstanedione (pmol/min/mg protein)
Choline-supplemented	1.17 ± 0.07 (6)	98 ± 35	430 ± 130	37 ± 5	36 ± 16	830 ± 180	310 ± 240
Choline-deficient	0.91 ± 0.08 (10)*	51 ± 15†	210 ± 120†	31 ± 15	22 ± 10	1110 ± 330	16 ± 26†

Data are mean ± SD. Values in parentheses indicate the number of individual microsomal suspensions assayed. In the case of assays of androstenedione metabolism, N = 5 (choline-supplemented) and N = 6 (choline-deficient). Different from control: * $P < 0.001$, † $P < 0.02$.

Table 3. Mixed-function oxidase activities in choline-supplemented and choline-deficient female rat liver microsomes

	Ethylmorphine *N*-demethylase (nmol CH_2O/mg protein/min)	4-Chloro-*N*-methyl aniline *N*-demethylase (nmole CH_2O/mg protein/min)	7-Ethoxycoumarin *O*-deethylase (pmole umbelliferone/ mg protein/min)	7-Ethoxyresorufin *O*-deethylase (pmole resorufin/ mg protein/min)	Aniline 4-hydroxylase (nmole 4-aminophenol/mg protein/min)
Choline-supplemented	3.51 ± 0.51 (6)	1.82 ± 0.28 (6)	154 ± 28 (6)	81 ± 14 (6)	1.06 ± 0.07 (6)
Choline-deficient	3.75 ± 0.30 (6)	1.63 ± 0.21 (6)	155 ± 47 (10)	99 ± 32 (10)	0.69 ± 0.09 (6)
% Choline-supplemented controls	107	90	101	122	65
P value	NS	NS	NS	NS	<0.001

Data are mean ± SD. Values in parentheses indicate the number of individual microsomal suspensions assayed.

[12, 22], this enzyme is virtually absent from control female liver. Instead, the isosafrole-inducible *ISF-G*, also termed P-450 IA2 [13], is present at measurable levels in female microsomes [12] and is a possible candidate as the female 6β-hydroxylase. The observations that aniline 4-hydroxylase and AD 6β-hydroxylase activities were decreased to a similar extent in microsomes from choline-deficient female rats is consistent with the suggestion that P-450 *ISF-G* levels may be lowered in this model of liver injury.

The finding that AD 16α- and 16β-hydroxylase activities are unchanged by choline deficiency suggests that levels of the phenobarbital-inducible P-450 *PB-B* are not decreased [12]. Equally, there is no evidence of up-regulation of the male-specific 16α-hydroxylase, P-450 *UT-A*, such as occurs in ovariectomized female rats following testosterone administration [23, 24]. Similarly, the data in Table 3, that show no change in 7-ethoxyresorufin *O*-deethylase activity in choline deficiency strongly suggest that the polycyclic hydrocarbon-inducible P-450 *BNF-B* [1] (P-450 IA1 [13]) is present in control and diseased livers at similar levels. As ethylmorphine *N*-demethylase activity is also unchanged in choline deficiency it appears likely that levels of the relevant P-450s (the female-specific P-450 *UT-I* [25], and the major sexually undifferentiated enzyme present in female rat liver, P-450 *PB-C* [12], also termed P-450 IIC6 [3]) are similar to those in controls. Certainly, both enzymes are active in the microsomal *N*-demethylation of ethylmorphine [1], and are quantitatively important in female rat liver.

The activity of the enzyme NADPH: Δ^4-3-oxosteroid 5α-oxidoreductase, which is responsible for the reductive metabolism of A-ring unsaturated steroids, was decreased in choline-deficient female microsomes. This enzyme is not a P-450 but is sexually differentiated with approximately 20–80-fold higher levels in adult female microsomes [24, 26]. The regulation of this enzyme is not well understood although testosterone administration to gonadectomized females decreases 5α-reductase activity to male levels [24]. The situation in choline-deficient female rats does not appear to be straight forward since, although a similar decrease in 5α-reductase activity was observed, there was no concomitant increase in the male-specific P-450 *UT-A*. It has been shown that testosterone supplementation of ovariectomized female rats results in the expression of P-450 *UT-A* [23, 24]. It remains a possibility that the change in 5α-reductase activity is independent of changes in sex hormones and may instead be a consequence of direct liver cell injury produced by the choline-deficient diet.

Earlier studies from this laboratory documented the altered regulation of the male-specific P-450 *UT-A* and *PCN-E* in the male rat with hepatic cirrhosis produced by two different treatment regimen [10, 11]. Thus it was suggested that the pattern of serum growth levels, the regulator of sex-specific P-450 expression [27–29], could well be altered in male rats with cirrhosis. From the present study there is no evidence for a similar effect on the female-specific P-450 *UT-I*. Instead, the effects of choline deficiency in female rats appear restricted to P-450 *UT-F* and *ISF-G*, two of the sexually undifferentiated forms of the enzyme. It is noteworthy that a decrease in P-450 *UT-F*, and its associated 7α-hydroxylase activity, was observed in microsomes from male rats with choline deficiency cirrhosis [10]. It therefore appears that there may be two effects on P-450s elicited by the choline-deficient diet. First, a sex-dependent effect in male rats and, second, a sex-independent effect on P-450s such as P-450 *UT-F*, that appear to be regulated by non-gonadal factors. Other P-450s do not appear to be affected in either sex, e.g. P-450 *PB-C*.

The extent of correlation between the changes in P-450-mediated metabolism in this model of liver injury and analogous human disease situations remains to be assessed. Whereas most workers agree that feminization may occur in human males with severe liver disease [30], the situation in women is less well documented. The implications of the present data are that the effects of chronic liver disease in the female are less pronounced than in the male.

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