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J Clin Invest. 1989;83(4):1211-1216. <https://doi.org/10.1172/JCI114003>.

Research Article

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Downregulation of the Male-specific Hepatic Microsomal Steroid 16 α -Hydroxylase, Cytochrome P-450_{UT-A}, in Rats with Portal Bypass

Relevance to Estradiol Accumulation and Impaired Drug Metabolism in Hepatic Cirrhosis

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Abstract

Elevated serum estradiol concentrations and specific changes in the biliary excretion of some androstenedione metabolites have been reported in male rats with portal bypass produced by portal vein ligation (PVL). In this study, the hypothesis that male-specific forms of cytochrome P-450 are altered after PVL was tested by measuring microsomal steroid hydroxylase activities. Consistent with earlier findings in the intact animal, androstenedione 16 α -hydroxylase activity was reduced after PVL to 44% of control ($P < 0.05$). Other pathways of androstenedione hydroxylation, and total estrogen formation (after androstenedione aromatization) were unchanged. Although total estrogen formation was not different, a sevenfold greater proportion of estradiol was produced in PVL rat microsomes. Additional experiments revealed that PVL selectively reduced the rate of microsomal estradiol 16 α -hydroxylation (to 56% of control, $P < 0.02$). Levels of cytochrome P-450_{UT-A}, the microsomal steroid 16 α -hydroxylase, were lower after PVL (56% of control, $P < 0.05$), so that the present observations are consistent with the earlier suggestion that portal bypass is associated with the selective downregulation of this enzyme. Since downregulation of cytochrome P-450_{UT-A} also occurs in experimental hepatic cirrhosis, portal hypertension may well contribute significantly to altered drug metabolism in liver disease. Impaired hepatic elimination of androstenedione by hydroxylation may indirectly enhance extrahepatic aromatization of the androgen. The decreased activity of hepatic estradiol 16 α -hydroxylation after PVL would enhance the accumulation of estradiol, the biologically more potent estrogen.

Introduction

Diversion of portal blood away from the liver (portal bypass) occurs in portal hypertension due to cirrhotic liver disease or portal vein occlusion, as well as in surgical portacaval shunts. Portal bypass results in hepatic atrophy and altered drug metabolism (1-3). In the portal vein ligated (PVL)¹ male rat, a

simple model of portal bypass, a number of observations have suggested that the hypothalamic-pituitary-gonadal (HPG) axis is impaired. For example, PVL in male rats produces testicular atrophy and is associated with low serum testosterone concentrations, reduced serum levels of luteinizing hormone, increased serum estrone and estradiol concentrations, and enhanced production of urinary total estrogens (4). These experimental findings are of interest since humans with cirrhotic liver disease commonly exhibit clinical features of sexual disorder such as impotence, abnormal hair distribution, gynecomastia, and amenorrhea (3).

While the pathophysiology underlying these features is undoubtedly complex, involving gonadal damage, hypothalamic-pituitary defects, altered plasma protein binding of sex steroids, and changes in end organ responsiveness, it is possible that changes in the regulation of hepatic steroid metabolism also have an important role.

Of particular interest in this laboratory is the finding that reduced activity of the microsomal mixed-function oxidases (MFO) occurs in the PVL male rat (3). MFO activity is catalyzed by cytochrome P-450 (P-450), a family of isoenzymic hemoproteins that play a central role in the elimination of lipophilic compounds of endogenous and exogenous origin (5, 6). Although portal bypass leads to a decrease in total hepatic P-450 content, of greater interest is the recent demonstration that the PVL procedure results in a nonuniform decrease in some MFO activities (4). Whereas ethylmorphine *N*-demethylase activity was reduced in PVL rat liver to 35% of control, aniline 4-hydroxylase activity was unchanged. This observation has particular significance as ethylmorphine *N*-demethylase is an MFO activity that shows a striking sex-dependence; severalfold higher activity is present in male rat hepatic microsomes (7). It is now apparent that the HPG axis, via the pattern of pituitary growth hormone secretion, is responsible for the maintenance of sex-specific P-450s (8-10). An earlier report from this laboratory documented that, after androstenedione infusion in PVL male rats, biliary secretion of 16 α -hydroxyandrostenedione was decreased but secretion of estradiol was increased (11). Since steroid 16 α -hydroxylation is considered to reflect the activity of the male-specific P-450_{UT-A} (12, 13), it appeared likely that the regulation of this protein is altered after portal bypass. The present study was designed to address this possibility directly. Microsomal steroid hydroxylase activities, which are specific catalytic indicators of several P-450s (12-15), were measured in control and PVL rat liver. In addition, microsomal P-450_{UT-A} content was estimated by an immunoblot assay using highly purified P-450_{UT-A} from male rat hepatic microsomes and a monospecific rabbit anti-rat P-450_{UT-A} IgG preparation.

Methods

Portal bypass in the rat. Male Wistar rats were bred and housed in the small animal facility of Westmead Hospital. They were housed under

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Received for publication 8 July 1988 and in revised form 11 October 1988.

1. *Abbreviations used in this paper:* HPG axis, hypothalamic pituitary gonadal axis; MFO, mixed function oxidase; P-450, cytochrome P-450; PVL, portal vein ligation; TBS, Tris-buffered saline.

J. Clin. Invest.

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0021-9738/89/04/1211/06 \$2.00

Volume 83, April 1989, 1211-1216

conditions of constant temperature, humidity, and lighting (12 h light/dark cycle). Animals were allowed free access to food (commercial rat cubes) and water.

The portal vein ligation procedure has been described elsewhere (2). Briefly, the first stage involves subcutaneous transposition of the spleen to facilitate the development of perisplenic portasystemic anastomoses. The second stage, performed 4 wk after the first stage, involves ligation of the portal vein above the confluence of the splenic and superior mesenteric veins. Control animals were subjected to the first stage and to sham laparotomy at the time of the second stage. Animals were killed 6 wk after the completion of stage 2 of the operation.

Preparation of microsomal fractions. Livers from control and PVL male rats were removed and perfused with ice-cold saline. After initial homogenization (Potter-Elvehjem), hepatic microsomes were prepared by differential ultracentrifugation and then stored as suspensions at -70°C (in 50 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol) until required for use. Microsomal protein was assayed by the Lowry method with bovine serum albumin (BSA) as standard (16).

Steroids and biochemicals. 4- ^{14}C Androstenedione and 4- ^{14}C -estradiol (sp act 59 and 56 mCi/mmol, respectively) were purchased from Amersham Australia, Sydney, NSW. Steroid standards for use in TLC systems were obtained from either Sigma Chemical Co. (St. Louis, MO), Steraloids, Inc. (Wilton, NH), or the MRC Steroid Reference Collection (Queen Mary's College, London, UK). Biochemicals were purchased from Sigma and solvents and miscellaneous chemicals (at least analytical reagent grade) were from Ajax Chemicals, Sydney.

Androstenedione hydroxylase activity. Hepatic microsomal androstenedione hydroxylase activity was assayed essentially as described by Murray et al. (17). Incubations (4.0 ml) contained microsomal protein (3 mg); 4- ^{14}C androstenedione (175 μM ; 4×10^5 dpm), catalase (2,000 U), EDTA (1 mM), and an NADPH-generating system (4 mM glucose-6-phosphate, 2 U glucose-6-phosphate dehydrogenase, and 1 mM NADP) in 100 mM potassium phosphate buffer (pH 7.4). Reactions were terminated at several time points between 0 and 60 min by the addition of 10 ml ethyl acetate. After thorough mixing the organic phase was removed, evaporated to dryness under N_2 , and the residue reconstituted in a small volume of chloroform for application to TLC plates (silica gel 60 F₂₅₄ activated 15 min at 100°C before use; E. Merck, Darmstadt, FRG). Plates were developed twice in one of the two solvent systems-chloroform/ethyl acetate, 4:1 (14) or cyclohexane/ethyl acetate/ethanol, 10:9:1 (18). Metabolites were localized by UV light (3-oxo- Δ^4 -steroids), 10% phosphomolybdic acid in ethanol (estrogens) or 0.04% 2,4-dinitrophenyl hydrazine in 2 M HCl (5 α -androstane-3,17-dione). The former TLC system was superior for the estimation of 5 α -androstane-3,17-dione, 16 β -hydroxyandrostenedione, estradiol and 4-hydroxyestradiol whereas the latter system was preferable for 2-hydroxyestradiol and estriol production. Metabolites were scraped into vials for scintillation spectrometry (Aquasol scintillant; New England Nuclear, Boston, MA).

Estradiol hydroxylase activity. Microsomal estradiol metabolism was estimated under similar incubation conditions to those described for androstenedione hydroxylase, except that estradiol (175 μM ; 4×10^5 dpm) was the substrate. Reactions were initiated by the addition of NADP and terminated by the addition of 10 ml ethyl acetate. Incubation extracts were applied to silica gel 60 TLC plates that did not contain the F₂₅₄ indicator and were run twice in the solvent system cyclohexane/ethyl acetate/ethanol, 10:9:1 (18). Estrogens were located on TLC plates that had been sprayed with the 10% phosphomolybdic acid reagent in ethanol after heating to 100°C for 10 min. Product formation was quantified by liquid scintillation spectrometry.

Purification of the male-specific P-450_{UT-A}. The procedure of Kamataki et al. (19) for the isolation of the principal male-specific P-450 was employed in this study, with some modifications. First, cholate-solubilized male rat liver microsomes were subjected to hydrophobic affinity chromatography at 4°C on *n*-octylamino Sepharose 4B. The

fraction eluted by 10 mM potassium phosphate buffer (pH 7.25) containing 0.06% Lubrol PX and 0.35% sodium cholate was applied to DEAE-Sephacel (rather than Whatman DE-52), and eluted at room temperature. The unbound fraction was then applied to hydroxylapatite (Bio-Gel HT; Bio-Rad Laboratories, Sydney, Australia) at 4°C , and a 20–100 mM potassium phosphate gradient (pH 7.25) was applied. An additional purification step was performed at room temperature on CM-Trisacryl M (LKB, Bromma, Sweden) at pH 6.5 before detergent removal on hydroxylapatite. The specific content of the preparation was 13.7 nmol P-450/mg protein.

Preparation of antiserum to P-450_{UT-A}. Female New Zealand rabbits were injected at four dermal sites with purified P-450_{UT-A} mixed (1:1 vol/vol) with either Freund's complete (first injection only) or incomplete (second and third injections) adjuvant. Injections were spaced 3 wk apart and serum was collected from an ear vein 10 d after the final inoculation. IgG was prepared by ammonium sulfate fractionation and was rendered monospecific by recycling for 15 h through a column of cholate/Lubrol PX-solubilized isofaerole-induced female rat liver microsomes coupled to CNBr-activated Sepharose 4B. Recovery of IgG was 90% and Western blot analysis revealed that the monospecific anti-UT-A IgG recognized an antigen present in cholate-solubilized microsomes from male, but not female, rat liver (Fig. 1). The IgG preparation also preferentially inhibited androstenedione 16 α -hydroxylase activity in control rat hepatic microsomes as described elsewhere (20).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Hepatic microsomes (33 μg protein per lane) were prepared for electrophoresis by incubation with 2% SDS and 5% 2-mercaptoethanol at 100°C for 5 min. Electrophoresis was conducted according to the method of Laemmli (21), modified by doubling the concentrations of Tris and glycine in all buffers and gels. This has been found to effect superior resolution of individual proteins in the P-450 molecular weight region (22). Lanes containing standard amounts of pure P-450_{UT-A} (0.1–2.0 μg protein) were also electrophoresed.

Transfer of proteins to nitrocellulose and immunochemical detection. After electrophoresis, proteins were transferred to nitrocellulose sheets by the method of Towbin et al. (23). The sheets were then

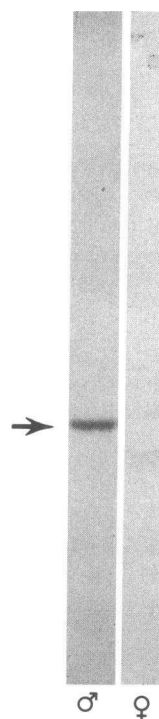


Figure 1. Western immunoblot incubated with an antibody against rat liver P-450_{UT-A}. The SDS-polyacrylamide gels were electrophoresed, the proteins transferred to nitrocellulose sheets, and the blots visualized as described in Methods. (Left) Immunoblot of P-450_{UT-A} in hepatic microsomes from untreated male rats and (right) immunoblot of P-450_{UT-A} in female rat microsomes.

shaken in Tris-buffered saline (TBS; 50 mM Tris, 200 mM NaCl, pH 7.4) for 30 min, followed by an overnight incubation in 3% BSA in TBS. The next step involved incubation of the nitrocellulose sheets with anti-UT-A IgG (1:1,000 dilution of 37.5 mg protein/ml) for 2 h, followed by five washes in TBS (5 min each wash), and then a 10-min wash in TBS containing 3% BSA. Sheets were incubated with a combination of ^{125}I -labeled and peroxidase-labeled donkey anti-rabbit IgG (Amersham, Bucks, UK; 2×10^5 cpm/ml; dilution as recommended by supplier) in TBS containing 3% BSA. Blots were visualized with 4-chloro-1-naphthol/hydrogen peroxide (24), excised and subjected to gamma counting (LKB multigamma 1260). Radioactivity was found to be proportional to the amount of antigen (0.3–6.0 μg) used in this study for the construction of standard curves.

Statistics. Differences between groups were evaluated by the Student's *t* test (unpaired, two-tailed). $P < 0.05$ was considered to indicate a significant difference between means.

Results

Time dependent formation of hydroxyandrostenedione metabolites in microsomal incubations in vitro. The formation of 16α -hydroxyandrostenedione in hepatic microsomes from PVL rats (1.45 ± 0.76 nmol/mg per min during a 10-min incubation) was significantly lower than that observed in control microsomes (3.30 ± 1.14 nmol/mg per min, $P < 0.05$, Table I). In contrast, the rates of androstenedione 6β -, 7α -, and 16β -hydroxylation were unchanged from control (Table I). As the direct consequence of reduced 16α -hydroxylase activity, total androstenedione hydroxylation after 60 min of incubation was less in PVL microsomes (98 ± 13 nmol products/mg protein) than in control microsomes (132 ± 15 nmol/mg, $P < 0.02$).

Effect of PVL on other pathways of androstenedione metabolism and estradiol metabolism. Androstenedione is also subject to metabolism by other microsomal enzymes. 17β -Hydroxysteroid oxidoreductase catalyzes the formation of testosterone and 3-oxo- Δ^4 -steroid 5α -oxidoreductase converts androstenedione to 5α -androstane-3,17-dione (25–28). Aromatase (also a P-450 enzyme) catalyzes A-ring conversion of androgens to estrogens (29–31). The PVL procedure did not significantly alter the microsomal formation of testosterone or 5α -androstane-3,17-dione (not shown) a finding that suggests that neither the 17β -oxidoreductase nor the 3-oxo- Δ^4 -steroid oxidoreductase was affected in this model.

Although total estrogen formation from androstenedione after PVL (11.8 ± 1.7 nmol/mg per h) was unchanged from control (14.5 ± 4.4 nmol/mg per h) it appeared that estradiol

accumulation occurred to a greater extent in PVL microsomes ($P < 0.05$, Table II). The data in Table II also suggested that a trend toward lower estradiol formation from androstenedione occurs after PVL but the difference did not reach statistical significance. The capacity of PVL male rat hepatic microsomes to convert estradiol to estrone was also assessed directly and, as shown in Table III, the microsomal fraction from rats subjected to total portal bypass catalyzed estrone formation at $\sim 55\%$ of the control microsomal rate. This finding is of particular significance since estrone is formed by the P-450_{UT-A}-catalyzed 16α -hydroxylation of estradiol (32) which, in the rat, is a major deactivation pathway for estradiol, the estrogen with the greatest biological potency. In the case of estradiol as the P-450 substrate, the reduction in microsomal hydroxylase activities was again nonuniform. Two other hydroxylations, at the 2-position of estradiol (probably catalyzed to a large degree by the P-450s PCN-E and UT-A; 33) and at the 17α -position to yield the $17\alpha,\beta$ -gem diol that spontaneously dehydrates to estrone, were not decreased in PVL rat hepatic microsomes (Table III).

Immunoquantitation of P-450_{UT-A}. Microsomal levels of the male-specific P-450_{UT-A} were decreased in PVL rat liver to about 56% of control (0.31 ± 0.09 vs. 0.55 ± 0.06 nmol/mg microsomal protein, $P < 0.05$). This finding is quite consistent with steroid 16α -hydroxylase data. Indeed, the decreases in 16α -hydroxylation in PVL rat hepatic microsomes to 44% and 56% of respective control levels are in good agreement with the UT-A quantitation data.

Discussion

This study provides definitive evidence in support of the assertion that microsomal levels and activity of the male-specific steroid 16α -hydroxylase, P-450_{UT-A}, are decreased in the PVL male rat. Evidence based on *in vivo* studies, for the proposal that sex-specific P-450 isoenzyme(s) may be downregulated in the PVL model of portal bypass had been presented in earlier reports from this laboratory (4, 11). Of the four microsomal androstenedione hydroxylase activities measured in this study three pathways of metabolism were unaffected in portal bypass. Thus, androstenedione 6β - and 7α -hydroxylation, which reflect the activities of the P-450s PCN-E and UT-F, respectively (12, 34), were unchanged after PVL. Although the identity of the constitutive steroid 16β -hydroxylase has not been assigned unequivocally (35, 36), this activity was also unchanged in microsomal fractions after PVL; P-450_{UT-A} was therefore selectively decreased in PVL male rat liver.

To a large extent, the present findings in the PVL male rat model of portal bypass are also in agreement with our earlier observations made in two distinct models of hepatic cirrhosis, produced by prolonged intake of a choline-devoid diet (37) or by repeated inhalation of carbon tetrachloride (38). In both models clear evidence was presented to demonstrate the preferential downregulation of P-450_{UT-A} and its associated steroid 16α -hydroxylase activities.

Some relevance of our findings to the human situation is now apparent. It has been reported that male patients with cirrhosis exhibit characteristics of demasculinization and feminization, including testicular atrophy and gynecomastia (3). In patients, evidence supporting the impaired function of the

Table I. Hydroxyandrostenedione Formation in Rat Hepatic Microsomal Incubations In Vitro

Microsomal type	Hydroxyandrostenedione			
	6β	7α	16α	16β
	nmol/mg/min			
Control	1.38 ± 0.36	0.36 ± 0.13	3.30 ± 1.14	0.23 ± 0.05
PVL	1.09 ± 0.53	0.47 ± 0.13	1.45 ± 0.76	0.32 ± 0.14
<i>P</i>	NS	NS	<0.05	NS

Values are given as means \pm SD of four individual microsomal suspensions in each group.

Table II. Formation of Estrogen Metabolites of Androstenedione in Microsomal Incubations In Vitro

Microsomal type	Estrogen metabolite				
	Estradiol	16 α -Hydroxyestradiol (estriol)	2-Hydroxyestradiol	4-Hydroxyestradiol	Total estrogen
	nmol/mg/h				
Control	0.28 \pm 0.33	7.04 \pm 5.68	3.94 \pm 1.92	0.58 \pm 0.30	14.52 \pm 4.35
PVL	1.90 \pm 1.16	3.04 \pm 2.00	4.98 \pm 0.98	1.27 \pm 0.66	11.78 \pm 1.66
P	<0.05	NS	NS	NS	NS

Values are mean \pm SD of four microsomal suspensions in each group.

HPG axis in hepatic cirrhosis includes altered circulating levels of growth hormone (39) and somatomedins (40, 41), low gonadotrophin levels in the presence of reduced serum testosterone concentrations (42), and abnormal response of growth hormone after thyrotropin-releasing hormone administration (43). In relation to the regulation of sex-dependent P-450s in rats it is now recognized that the pulsatility of plasma growth hormone concentrations is the primary regulator of UT-A expression in male rats (8–10). Sex differences in human steroid metabolism have also been documented (44). Accordingly, the logical extension of our studies in experimental animals is an evaluation of the effects of chronic liver disease on homologous P-450s in human liver.

The likely consequences of downregulation of P-450_{UT-A} in rats with portal bypass are several. Since we have demonstrated that the whole body clearance of androstenedione is unchanged in portal bypass (11), the decrease in microsomal steroid 16 α -hydroxylation requires a compensatory elevation of androstenedione elimination by alternate routes. The most likely event is extrahepatic metabolism of the steroid, perhaps involving the adrenal glands. This proposal is consistent with our earlier findings that the plasma concentrations of 11 β -hydroxyandrostenedione as well as 3 α , 17 β -dihydroxy-5 α -androstane are elevated after PVL (11). The well-documented increase in serum estrogens (4) is also extrahepatic since the present study noted that total hepatic microsomal aromatization of androstenedione was not different in control and PVL rat liver. An additional complication is that the downregulation of P-450_{UT-A} decreases the rate of deactivation of estradiol (to estriol). Elevated estradiol concentrations would be ex-

pected to maintain an abnormal release of growth hormone from the pituitary which, in turn, would continue to keep hepatic P-450_{UT-A} at a reduced level. It therefore seems likely that the shunting of portal blood away from the liver, which is a feature of the portal hypertension of chronic liver disease, may be an important factor leading to the downregulation of P-450_{UT-A} previously observed in experimental cirrhosis (37, 38).

Apart from the abnormal physiology of sex hormone production and disposition in portal bypass it is also clear that marked alterations in the capacity of the liver to handle drugs of therapeutic importance would occur. Rat P-450_{UT-A} is an efficient catalyst in the oxidative metabolism of a wide range of drugs and foreign compounds (13, 45). Impaired regulation of analogous human P-450s in portal bypass would be expected to significantly influence the metabolic handling of therapeutic agents and, because of altered pharmacokinetics, predispose to drug accumulation and toxicity. Knowledge of the hormonal regulation of the human proteins may ultimately lead to pharmacological attempts to reverse P-450 repression in chronic liver disease.

Acknowledgments

The authors are grateful to Drs. M. C. Berndt and W. J. Booth, Westmead Hospital, for valuable advice in the production of antisera and in the Western blot technique. The secretarial assistance of Ms. Diane West in the preparation of this manuscript is gratefully acknowledged.

This work was supported by the Australian National Health (NH) and Medical Research Council (MRC). M. Murray is an NH & MRC Research Fellow.

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Table III. Formation of Major Hydroxylated Metabolites of Estradiol in Hepatic Microsomal Incubations In Vitro

Microsomal type	Estradiol metabolite		
	2-Hydroxyestradiol	16 α -Hydroxyestradiol (estriol)	Estrone
	nmol/mg/min		
Control	0.11 \pm 0.03	0.18 \pm 0.03	0.41 \pm 0.06
PVL	0.15 \pm 0.05	0.10 \pm 0.04	0.51 \pm 0.15
P	NS	<0.02	NS

Values are mean \pm SD of four individual microsomal fractions in each group.

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