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PRETRANSLATIONAL DOWN-REGULATION OF MALE SPECIFIC HEPATIC P450s AFTER PORTAL BYPASS

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Abstract—Diversion of portal blood away from the liver in the portal vein ligated (PVL) male rat results in dysfunction of the hypothalamo-pituitary-gonadal axis, as reflected by an increase in circulating oestradiol, a decrease in serum testosterone and decreased expression of the male-specific cytochrome P450 2C11 in hepatic microsomes. The present study assessed whether there was a decline in the hepatic concentrations of mRNA species corresponding to male-specific P450s and whether female-specific hepatic enzymes may be upregulated after PVL. In microsomes from PVL male rat liver the activities of P450s 2C11 and 3A2 (androstenedione 16α- and 6β-hydroxylation, respectively) were decreased to 45% ($P < 0.001$) and 43% ($P < 0.002$) of control. Slot blotting revealed a decline in the mRNAs for P450 2C11 and 3A2 to 46 ± 7 and 27 ± 4% of respective control (relative to β-actin mRNA). In contrast, mRNAs for the female specific P450 2C12 and Δ^4-ketosteroid-5α-oxidoreductase were unchanged from control (100 ± 13% and 122 ± 28%, respectively). P450 2C12 protein was not detected in either control or PVL male rat liver but a slight increase in the rate of 5α-dihydrotestosterone formation was noted after PVL (2.95 ± 0.40 vs 1.00 ± 0.22 nmol/min/mg protein, $P < 0.05$; corresponding, respectively, to 30 and 10% of the activity in female liver microsomes). These studies indicate that the male-specific P450 2C11 and 3A2 are down-regulated at a pretranslational level whereas upregulation of the female-specific enzymes P450 2C12 and Δ^4-ketosteroid-5α-oxidoreductase does not occur after PVL. Thus, the endocrine effects of portal bypass result in biochemical demasculinization, but not feminization, of hepatic enzymes involved in steroid biotransformation. Because male-specific P450s are effective steroid hydroxylating enzymes, their down regulation after PVL would significantly decrease hepatic androgen extraction.

Key words: cytochrome P450; portal bypass; microsomal steroid hydroxylases; androgen-dependent enzymes; RNA, messenger

Portal blood contains numerous agents of endogenous and exogenous origin that require deactivation by hepatic enzymes prior to elimination. Diversion of portal blood away from the liver (portal bypass) occurs in cirrhosis and other forms of liver disease [1, 2] and diminishes the hepatic extraction of chemicals, including steroid hormones [3, 4], present in portal blood.

Hyperoestrogenaemia has been reported in individuals with severe liver disease [5, 6] and in animals with experimental liver disease [7], portal bypass [8] or during liver regeneration [9]. A number of observations are consistent with the assertion that the normal function of the hypothalamo-pituitary-gonadal axis is impaired in liver disease. Thus, abnormal concentrations of gonadotropins and other neuroendocrine factors have been observed in humans with cirrhosis [6, 10, 11]. Previous studies from this laboratory have shown that, in male rats with portal bypass produced by ligation of the portal vein, serum leuteinizing hormone concentrations are suppressed even though concentrations of follicle stimulating hormone appear normal [8].

P450† or CYP is the multigene family that catalyses the oxidative biotransformation of many lipophilic substances, including steroid hormones. It has been demonstrated that the hepatic content of the male-specific cytochrome P450 2C11 enzyme is decreased in portal bypass [12]. This enzyme is regulated by several endogenous factors and is down regulated when serum oestrogen is elevated and when serum testosterone is low [13, 14]. Accordingly, the connection has been made between impairment of endocrine function and altered capacity of drug metabolizing enzymes in portal bypass and liver disease. The present study was undertaken to investigate whether female-specific enzymes may be regulated differently in male rat liver after portal bypass. Thus, the effect of PVL on the expression of male and female-specific hepatic enzymes involved in steroid biotransformation, as well as the corresponding mRNA species, was investigated.

MATERIALS AND METHODS

Rat model of portal bypass and hormone treatments. Male wistar rats were obtained from the Westmead Hospital animal facility and were subjected to the

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† Abbreviations: androstenedione, androst-4-ene-3,17-dione; P450, cytochrome P450; 5α-oxidoreductase, Δ^4-ketosteroid-5α-oxidoreductase; PVL, portal vein ligation.

two-stage PVL procedure described previously [15]. Briefly, stage one consisted of subcutaneous transposition of the spleen and was performed when rats were ~90 g in weight (approximately 3 weeks of age). Stage two was performed 4 weeks later and involved ligation of the portal vein above the confluence of the superior mesenteric and splenic veins. Control rats were sham-operated littermates. All animal studies were performed in accordance with the guidelines established by the Australian National Health and Medical Research Council and the Westmead Hospital Animal Care and Ethics Committee.

Animals were killed and liver and testes were removed and weighed. Liver for RNA analysis was snap frozen in liquid nitrogen and was stored at −70°. Microsomal fractions were isolated from homogenates of rat livers or testes by differential ultracentrifugation [16] and were stored at −70° until required for experiments; testicular microsomes were only stored for up to 1 week prior to use.

Chemicals. [4-^{14}C]Androstenedione (sp. act. 59 mCi/mmol), [4-^{14}C] testosterone (sp. act. 56 mCi/mmol) and [γ-^{32}P]ATP (sp. act. 5,000 Ci/mmol) were purchased from Amersham Australia (Sydney, NSW, Australia). Authentic steroid metabolites for use as standards in TLC were supplied by Sigma Chemical Co. (St Louis, MO, U.S.A.), Steraloids Inc. (Wilton, NH, U.S.A.) or the MRC Steroid Reference Collection (Queen Mary's College, London, U.K.). Biochemicals were purchased from Sigma or Boehringer–Mannheim Australia (Castle Hill, NSW, Australia). Analytical grade reagents and miscellaneous chemicals were from Ajax Chemicals (Sydney, NSW, Australia).

Oligonucleotide and cDNA probes. Oligonucleotides were prepared using a DNA synthesiser and were obtained from Rachel Forster Hospital, Sydney or Bresatec Ltd, Adelaide. Sequences (complementary) were: *CYP2C11*, 5′-ATC-CAC-GTG-TTT-CAG-CAG-CAG-CAG-GAG-TCC-3′ [17] corresponding to nucleotides 925-954 of the coding sequence of the reported cDNA [18], *CYP3A2*, 5′-ACT-GCC-TTT-GTG-AAG-ATC-CCA-ATA-AAA-TTC-3′ [17] corresponding to nucleotides 1594-1623 of the reported cDNA [19], *CYP2C12*, 5′-AAT-AGC-AGC-AAA-ATG-TTT-TGA-ATG-TGT-CTT-3′ [20] complementary to nucleotides 691-720 [21] and 5α-*oxidoreductase*, 5′-GAC-TCA-GCT-CAT-GGG-AGG-CAA-C-3′ [20] corresponding to nucleotides 793–814 of the reported cDNA [22]. The β-*actin* probe was a cDNA isolated from the plasmid clone pHFβA-1 [23].

Assays of androstenedione hydroxylation and testosterone reduction in rat hepatic microsomes. The assay of androstenedione hydroxylation has been outlined elsewhere [24]. Incubations (0.4 mL) were run for 2.5 min and contained substrate ([4-^{14}C]-androstenedione, 0.18 μCi, 20 nmol), microsomal protein (0.15 mg) and the standard NADPH-generating system in 0.1 M potassium phosphate buffer, pH 7.4. Reactions were terminated by the addition of chloroform and removal to ice. After extraction, products were applied to silica gel TLC plates (Type 60 containing F_{254} indicator and activated at 100° for 15 min). Plates were developed twice in the solvent system of Waxman *et al.* [25] (chloroform:ethyl acetate, 4:1) and metabolites were located by autoradiography (Hyperfilm-MP, Amersham). Metabolite formation was quantitated by scintillation counting (Aquasol, New England Nuclear, Sydney, Australia) and was linear under the conditions described.

Hepatic microsomal 5α-dihydrotestosterone formation was performed in a similar fashion except that the substrate was [4-^{14}C]testosterone (0.18 μCi, 20 nmol) and NADH (1 mM) was used to initiate the reaction. Reactions were terminated by the addition of chloroform and, after extraction, products were applied to TLC plates. Plates were developed sequentially in the solvent systems of Waxman *et al.* [25] (first:—dichloromethane:acetone 4:1, second:—chloroform:ethyl acetate:ethanol, 4:1:0.7) and metabolite formation was quantitated by scintillation counting. Reactions were linear under these conditions.

Assay of androstenedione reduction to testosterone in rat testicular microsomes. Incubations (0.4 mL) contained substrate (0.18 μCi [4-^{14}C]androstenedione, 20 nmol) and microsomal protein (0.5 mg) in 0.1 M potassium phosphate buffer, pH 7.4. Reactions were initiated with NADPH (1 mM) and terminated by the addition of chloroform and removal to ice. After extraction, radioactive products were resolved by TLC, detected by autoradiography and quantitated by scintillation counting essentially as described above. Reactions were linear under the incubation conditions described.

Immunochemical detection of P450 enzymes in hepatic microsomes from sham-control and PVL male rats. The isolation of cytochrome P450 2C11 from adult male rat liver and the preparation of monospecific anti-P450 2C11 IgG from the serum of rabbits inoculated with the purified P450 have been described previously [12]. The IgG preparation detected an antigen that was present in male, but not female, rat liver and preferentially inhibited microsomal androstenedione 16α-hydroxylation in male rat liver; these findings confirmed that the antibody was anti-P450 2C11. A monoclonal antibody to the female-specific P450 2C12 was generously provided by Drs A. Mode and J.-Å. Gustafsson, Department of Medical Nutrition, Huddinge Hospital, Sweden. The characteristics of this antibody have been outlined elsewhere [26].

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis of hepatic microsomes and electrophoretic transfer of proteins to nitrocellulose were performed as reported previously, except that 2 and 4 μg of microsomal protein was loaded onto gel lanes for the detection of P450 2C11 and P450 2C12, respectively [12]. Immunodetection was performed using primary antibody concentrations of 3.7 μg/mL (2C11) or 1 μg/mL (2C12) and enhanced chemiluminescence (Amersham) followed by autoradiography (Hyperfilm-MP). Laser densitometry was used for P450 quantitation (LKB Ultroscan-XL, Bromma, Sweden).

RNA extraction from liver and analysis of P450 mRNA species. Total hepatic RNA was extracted by the acid guanidinium isothiocyanate–phenol–chloroform procedure of Chomczynski and Sacchi

Table 1. Androstenedione metabolism in hepatic microsomes from PVL and control male rats

Treatment	Androstenedione metabolite (nmol/min/mg protein)				
	Testosterone	6β-Hydroxy	7α-Hydroxy	16α-Hydroxy	16β-Hydroxy
Sham control	1.78 ± 0.27	2.57 ± 0.30	0.28 ± 0.02	2.87 ± 0.20	0.56 ± 0.07
PVL	1.36 ± 0.27	1.09 ± 0.23	0.33 ± 0.04	1.28 ± 0.13	0.52 ± 0.04
Percentage of control	77	43	119	45	93
P	NS	<0.002	NS	<0.001	NS

Data are means ± SE of estimates obtained from eight separate microsomal suspensions per group. Differences between groups were analysed for significance by the Student's *t*-test.

[27]. Oligonucleotides were labelled with [γ-^{32}P]ATP using polynucleotide kinase. For northern blotting RNA (20 μg) was subjected to electrophoresis on 1.2% agarose under denaturing conditions (2.2 M formaldehyde) and transferred to nylon membranes (Hybond-N$^+$, 0.45 μm, Amersham) [28]. For slot blotting RNA was applied directly to the nylon filters using a BioDot SF slot blot apparatus (BioRad, Richmond, CA, U.S.A.). Hybridization and washing temperatures were 42 and 50° for oligonucleotide and cDNA probes, respectively. Filters were exposed for 15–72 hr to Hyperfilm-MP with Cronex Lightning Plus intensifying screens (NEN-DuPont). After development, the membranes were stripped and rehybridized to β-actin. After autoradiography, the resultant films were scanned by laser densitometry and the levels of the P450 or the 5α-oxidoreductase mRNA in individual livers relative to those of β-actin were determined.

Determination of serum sex hormone concentrations. Serum oestradiol and testosterone concentrations were estimated by commercial RIA kits (CIS, Gif Sur Yvette, France). Cross-reactivities of the oestradiol kit with oestrone, oestriol, testosterone, androstenedione and progesterone were less than 1%. Less than 1% cross-reactivities with androstenedione, dehydroepiandrosterone, oestrogens and progesterone occurred with the testosterone RIA. However, the kit was 7.2% cross-reactive with dihydrotestosterone.

Statistics. Data are expressed as means ± SEM throughout. Data from multiple treatment groups were subjected to single factor analysis of variance and Student–Newman–Keuls testing. Comparisons between groups were made using the Student's *t*-test.

RESULTS

Androstenedione hydroxylation and testosterone reduction in hepatic microsomes from PVL and sham-operated control male rats

Consistent with previous reports [12], the data in Table 1 demonstrate that androstenedione hydroxylations catalysed by male-specific P450s were decreased in PVL rat liver. 16α-Hydroxylation catalysed primarily by P450 2C11 [26], was decreased to 45% of the control rate (1.28 ± 0.13 vs 2.87 ± 0.20 nmol/min/mg protein; P < 001) whereas

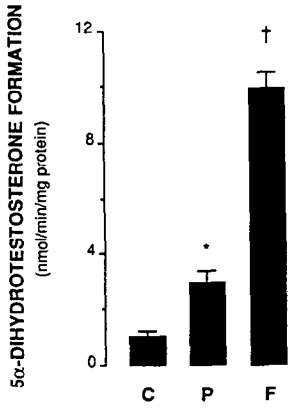

Fig. 1. NADH-mediated formation of 5α-dihydrotestosterone from testosterone in hepatic microsomes from sham-operated control male (C), PVL male (P) and control female (F) rats. Significant difference from control male: *P < 0.05, †P < 0.01.

6β-hydroxylation, mediated principally by P450 3A2, was decreased to 43% of sham-operated control (1.09 ± 0.23 vs 2.57 ± 0.30 nmol/min/mg protein; P < 0.002). In contrast, 7α-hydroxylation of the steroid, mediated by the P450s 2A1 and 2A2 [29], and 16β-hydroxylation, involving as yet unidentified P450s, were unaffected by portal bypass. Similarly, the activity of the 17-ketosteroid-17β-oxidoreductase enzyme that supports testosterone formation from androstenedione [30], was not impaired in hepatic microsomes from PVL male rats.

The formation of 5α-dihydrotestosterone from testosterone was estimated in hepatic microsomes from untreated and PVL male rats because the 5α-oxidoreductase enzyme is known to be expressed at a higher level in female rat liver. As shown in Fig. 1, microsomal dihydrotestosterone formation was 1.00 ± 0.22 nmol/min/mg protein in untreated rat liver and was increased after PVL (2.95 ± 0.40 nmol/min/mg protein, P < 0.05). However, in microsomes from female rat liver, the activity was considerably higher (9.91 ± 0.59 nmol/min/mg protein) than in either control or PVL male rat liver.

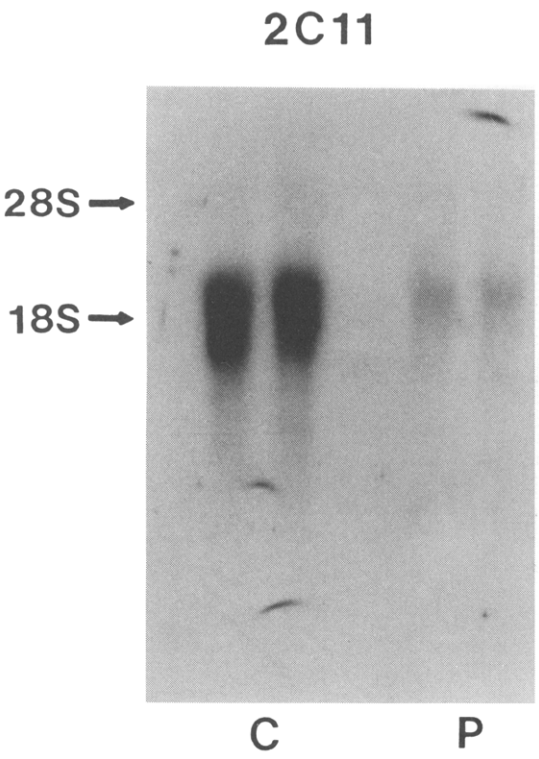

Fig. 2. Northern blot analysis of P450 2C11 mRNA in sham-operated control (C) and PVL (P) male rat liver. Locations of the 28S and 18S RNAs are indicated.

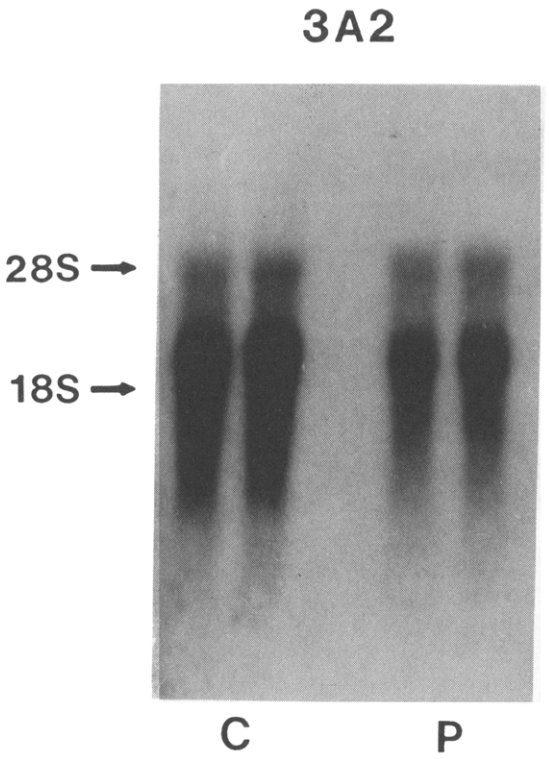

Fig. 3. Northern blot analysis of P450 3A2 mRNA in sham-operated control (C) and PVL (P) male rat liver. Locations of the 28S and 18S RNAs are indicated.

Regulation of male-specific and female-specific hepatic enzymes in portal bypass

Further studies were undertaken to correlate the apparent effects of PVL on steroid biotransformation pathways with the hepatic content of the corresponding enzymes or their mRNAs. As reported previously [12], P450 2C11 protein was decreased after PVL from 28 ± 1 to 14 ± 2 μg/mg protein ($P < 0.001$). From the northern analysis of P450 2C11 mRNA shown in Fig. 2, it now appears clear that the effect of PVL on 2C11 protein is pretranslational. A similar effect was noted in the case of the mRNA for P450 3A2 (Fig. 3). Thus, northern analysis revealed a marked decrease in the level of the 3A2 signal after PVL. In contrast, the expression of neither P450 2C12 nor the 5α-oxidoreductase was increased after PVL (Fig. 4). In agreement with the mRNA studies, western blot analysis suggested that PVL did not increase the microsomal content of P450 2C12 apoprotein (not shown). Slot blotting was undertaken to provide quantitative estimates of the alterations of the hepatic mRNAs after PVL. Thus, the appearance of Fig. 5 (mRNAs for 2C11 and 3A2) is in close agreement with the findings from northern analysis. Similarly, the appearance of the slot blots of the mRNAs for 2C12 and the 5α-oxidoreductase in control and PVL male rat liver did not appear different (not shown). After laser densitometry it was found that PVL decreased the hepatic content of the mRNA species for P450 2C11 and P450 3A2 to $47 \pm 7\%$ and $27 \pm 4\%$ of respective control levels, relative to β-actin (Fig. 6). PVL did not alter the relative hepatic content of the mRNAs corresponding to P450 2C12 ($100 \pm 13\%$ of sham-operated control) or that of the 5α-oxidoreductase ($122 \pm 28\%$ of control male; Fig. 6).

Serum sex hormone concentrations in PVL male rats

Previous studies have indicated that dysfunction of the hypothalamo-pituitary-gonadal axis occurs in male rats with portal bypass produced by the PVL procedure [8, 12]. In accord with these findings, serum oestradiol was increased in male rats after portal bypass (250 ± 20 pM vs 80 ± 10 pM in sham-controls, $N = 8$ per group; $P < 0.001$) and serum testosterone was decreased to 67% of sham-operated control (6.4 ± 2.5 vs 9.6 ± 1.8 nM, $N = 8$ per group; $P < 0.02$).

Testicular microsomal testosterone production

In view of the decline in serum testosterone concentrations in portal bypass, further studies were undertaken to establish a mechanism for this effect. As shown in Fig. 7, PVL was associated with a 56% reduction in the final step of the testicular biosynthesis of androgen. Thus, the conversion of androstenedione to testosterone, mediated by a testicular 17β-hydroxysteroid oxidoreductase, was 0.088 ± 0.018 nmol/min/mg protein in testicular

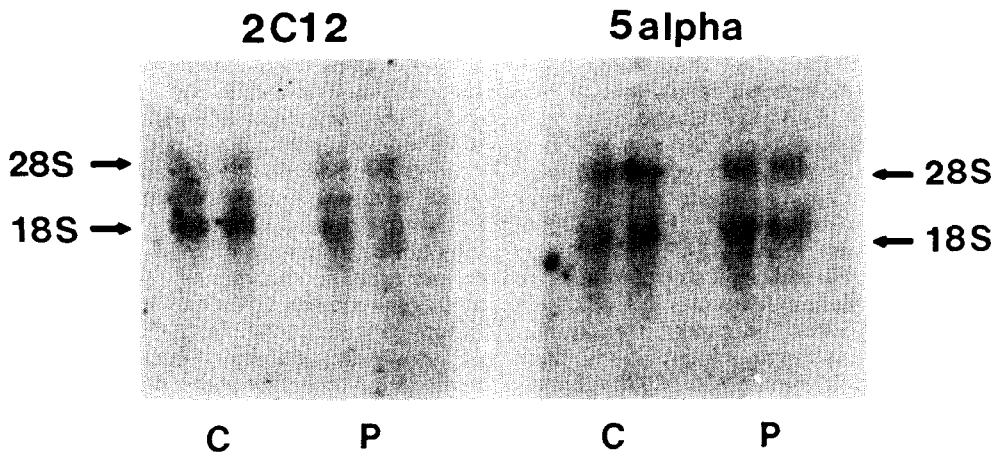

Fig. 4. Northern blot analysis of P450 2C12 and 5α-oxidoreductase mRNA in sham-operated control (C) and PVL (P) male rat liver. Locations of the 28S and 18S RNAs are indicated.

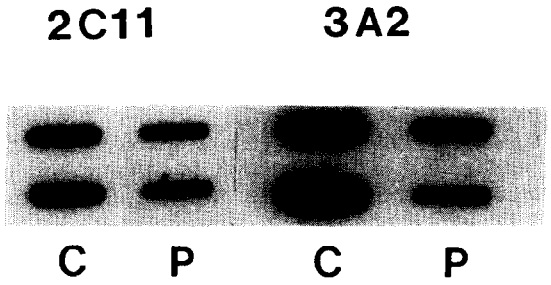

Fig. 5. Slot blot analysis of P450 2C11 and 3A2 mRNAs in sham-operated control (C) and PVL (P) in male rat liver.

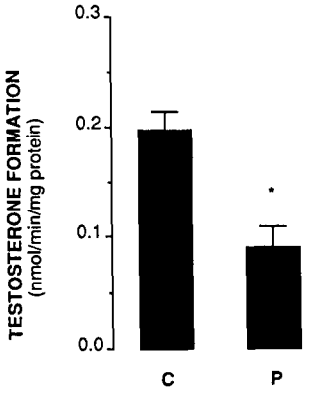

Fig. 7. Formation of testosterone from androstenedione in testicular microsomes from sham-operated control (C) and PVL (P) male rats. *P < 0.001.

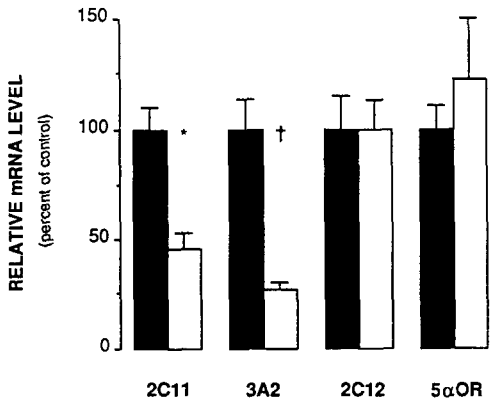

Fig. 6. Relative levels of specific mRNAs hybridising to P450s 2C11, 3A2 and 2C12 and 5α-oxidoreductase (5αOR) in sham operated control (solid bars) and PVL (open bars) male rat liver. Different from sham-operated control *P < 0.002, †P < 0.001.

microsomes from PVL rats vs 0.198 ± 0.016 in control fractions (N = 4 per group; P < 0.001). Considered together, therefore, the present data indicate that portal bypass is associated with a decrease in testicular androgen output.

DISCUSSION

It is clear from numerous reports that patients with chronic liver disease and portal bypass have increased serum oestrogen levels [5, 6]. It is held that this hormonal abnormality could be responsible, in part, for the hypogonadism, gynaecomastia and feminization (or demasculinization) observed in such patients [6, 11]. It is also possible that other biochemical defects observed in these patients are attributable to altered sex steroid production.

Experimental cirrhosis, portal bypass and liver regeneration in the male rat have also been associated with hyperoestrogenaemia [7–9, 31–33]. An impor-

tant biochemical marker of liver disease in these models has been found to be the microsomal content of the quantitatively important P450 2C11 [7, 12]. In each case, apparent down regulation of P450 2C11 and its associated enzyme activities have been described and a functional consequence of this effect would be decreased hepatic clearance of androgens (by 2α- and 16α-hydroxylation) and oestrogens by 2-, 4- and 16α-hydroxylation [34, 35]. In addition, the apparent decrease in P450 3A2 that has also been noted in experimental cirrhosis [36, 37] and the reduced 6β-hydroxylation of steroids in the present PVL model, would also be expected to further decrease steroid clearance. Apart from these biochemical effects, it is evident that portal bypass (in PVL or due to portal-systemic shunting in chronic liver disease) decreases the possible interaction between sex hormones present in portal blood and the hepatic enzymes involved in their deactivation, because of reduced delivery of steroids to the liver. It is therefore envisaged that two mechanisms contribute to the increase in serum oestradiol levels: down regulation of hepatic microsomal P450 enzymes that deactivate androgens (and oestrogens), and shunting of androgens to extrahepatic sites where aromatization occurs. Earlier studies in the PVL male rat have provided direct evidence for increased production of oestradiol from androstenedione [33] and testosterone [38].

The enzymes studied in the present report are subject to complex multihormonal control involving androgens and estrogens, but also thyroid and peptide hormones and glucocorticoids [17, 20, 39–41]. The PVL model of portal bypass is characterised by hyperoestrogenaemia and hypogonadism, giving rise to reduced serum testosterone concentrations. As may be anticipated from other studies, altered hormone synthesis and degradation in the PVL male rat results in impaired expression of hepatic enzymes that are regulated by androgen. It now appears that this impaired regulation of androgen-dependent P450 enzymes occurs at a pretranslational level. This may be a consequence of decreased *CYP* gene transcription or of altered rates of mRNA degradation but clearly results in a decreased level of expression of these P450s in rat liver. Evidence has been presented that sex-specific P450s such as P450 2C11, 2C12 and 3A2 are under transcriptional hormonal control in the rat [42–44]. Thus, it may be anticipated that decreases in hepatic mRNAs for male-specific P450 2C11 and 3A2 in the PVL rat reflect decreased rates of gene transcription.

Reciprocal upregulation of the female-specific hepatic enzymes P450 2C12 and 5α-oxidoreductase was not observed in the present study in PVL rat liver. Thus, 2C12 expression was not increased at either the protein or mRNA level. Similarly, no evidence was obtained for upregulation of the 5α-oxidoreductase at the mRNA level although an apparent increase in dihydrotestosterone formation from testosterone was apparent in microsomes from PVL male rat liver. This increase in enzyme activity was still, however, substantially lower (about 30%) than the activity normally found in control female rat liver. The reason for the discrepancy between the mRNA and enzyme activity measurements is not clear but it has been noted recently that there are at least two distinct 5α-oxidoreductases in human liver [45]. There may be more than a single isoenzyme that contributes to the 5α-oxidoreductase of rat liver. Taken together, these observations are consistent with the assertion that PVL produces demasculinization, but not feminization, of hepatic enzymes involved in steroid biotransformation. It is noteworthy that the hepatic 5α-oxidoreductase(s) may not contribute significantly to serum dihydrotestosterone concentrations. When required for androgen receptor activation, dihydrotestosterone is generally produced within target cells by the action of specialised 5α-oxidoreductases. Instead the hepatic microsomal enzyme probably has a more important function in the processing of 4-ene-steroids prior to elimination as conjugates.

The present study demonstrates for the first time that PVL decreases the testicular formation of testosterone from androstenedione by 17-keto reduction. Thus, decreased serum testosterone levels in these rats is not due solely to enhanced aromatization of androgens to oestrogens. It was shown previously that serum levels of leuteinizing hormone (but not follicle-stimulating hormone) were reduced in association with testicular atrophy, loss of Leydig cells and arrest of spermatogenesis in the PVL male rat [8]. Thus, it appears that the source of enhanced serum oestrogen concentrations in PVL male rats is, indirectly, testicular androgen. Rerouting of these androgens away from the liver, and therefore hepatic deactivation enzymes, results in enhanced aromatisation to form oestrogens. Although the underlying reason for the decrease in testosterone production was not addressed a plausible hypothesis is that oestradiol accumulation results in the suppression of steroidogenesis, perhaps via a decrease in the testicular 17β-hydroxysteroid oxidoreductase enzyme. Thus, the disordered pattern of sex steroid production is maintained.

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