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INTERACTIONS OF HEPATIC CYTOCHROMES P-450 WITH STEROID HORMONES

REGIOSELECTIVITY AND STEREOSPECIFICITY OF STEROID METABOLISM AND HORMONAL REGULATION OF RAT P-450 ENZYME EXPRESSION*

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Endoplasmic reticulum-bound P-450 cytochromes play a central role in the oxidative metabolism of lipophilic compounds in eukaryotic cells. In mammalian systems, the microsomal P-450s are particularly prominent in hepatic tissue, where they catalyze an NADPH-dependent monooxygenation of their structurally diverse lipophilic substrates to yield polar (e.g. hydroxylated) derivatives. Many endogenous steroids and fatty acids are hydroxylated by these cytochromes, as are a large number of foreign compounds, including drugs and environmental chemicals [1–4]. In the case of steroid hormones, hydroxylation can lead to deactivation and elimination, or alternatively, may result in production of derivatives that have altered hormonal properties. In the case of drugs and other chemicals, hydroxylation often results in deactivation, but in some instances leads to formation of reactive derivatives that are responsible for the chemotherapeutic, mutagenic or carcinogenic properties of the parent compound (Scheme I) [5, 6]. There are multiple pathways for P-450-catalyzed steroid and xenobiotic biotransformation in the liver, and these pathways are modulated and regulated by a large number of environmental and hormonal factors.

The broad range of lipophilic compounds oxygenated by hepatic cytochrome P-450 in part reflects the presence of multiple forms (isozymes) of the P-450 hemeprotein. At least twenty distinct P-450 forms have been isolated from rat liver and characterized biochemically (see Table 1 for a summary of rat P-450 form nomenclatures currently in use). Corresponding P-450 forms expressed in rabbit and human liver tissue have also been identified and characterized [7–9]. Within any given species, the individual P-450 forms exhibit structural, immunochemical and spectral properties that are similar, although distinguishable. Many of these P-450s metabolize the commonly studied prototypic foreign compounds (e.g. 7-ethoxycoumarin, ethylmorphine, benzo(*a*)pyrene, etc.) with similar efficiencies and overlapping site specificities, making it difficult to uniquely characterize individual P-450 forms solely using these compounds as substrates. In contrast, as will be described below, steroid hormones are frequently oxygenated by P-450 enzymes with a high degree of specificity to yield unique metabolite profiles. This high specificity in turn suggests that these and other steroids may serve as physiological substrates for hepatic P-450s. Steroid hormones also play an important role in regulating the expression of several constitutive hepatic P-450s, by mechanisms that are only partially understood at the present time.

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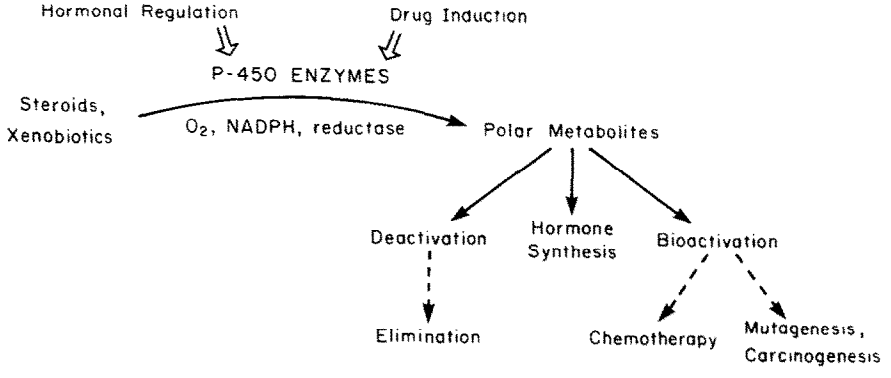
REGIOSELECTIVITY AND STEREOSPECIFICITY OF STEROID HORMONE HYDROXYLATION

A large number of distinct P-450 enzymes (~20) have been isolated from rat liver and characterized by investigators in several laboratories (Table 1).



Scheme 1. Pathways of P-450-dependent steroid and xenobiotic metabolism.

Table 1. Nomenclature for rat hepatic P-450 enzymes*

Waxman	Levin	Guengerich	Schenkman	Wolf	Gene designation†	Characteristic Properties
PB-1	k	PB-C	RLM5a	PB1b	IIC6	Phenobarbital-inducible warfarin 7-hydroxylase, hormone independent
2a	—	—	—	—	IIIA2	Male-specific testosterone 6β-hydroxylase‡
PB-2a	p	PCN-E	—	—	IIIA1	Synthetic steroid-inducible testosterone 6β-hydroxylase§
2c	h	UT-A	RLM5	PB2a	IIC11	Male-specific testosterone 2α/16α-hydroxylase‖
2d	i	UT-I	fRLM4	—	—	Female-specific steroid disulfate 15β-hydroxylase‖
3	a	UT-F	—	UT1	IIA1	Steroid hormone 7α-hydroxylase
PB-4	b	PB-B	PBRLM5	PB3a	IIB1	Major phenobarbital-inducible form, testosterone 16β-hydroxylase¶
PB-5	e	PB-D	PBRLM6	PB3b	IIB2	97% homologous to PB-4, low activity form
6	—	—	—	—	—	Constitutive form, crossreactive with PB-4
BNF-B	c	BNF-B	—	MC1b	IA1	Major 3-methylcholanthrene-inducible form¶
ISF-G	d	ISF-G	—	MC1a	IA2	Major isosafrole-inducible form¶
—	f	—	—	—	IIC7	Constitutive form, crossreactive with PB-4
—	g	—	RLM3	—	—	Male specific, strain-dependent
—	j	—	RLM6	—	IIE1	Ethanol-inducible nitrosamine demethylase

* Listed are the designations and some properties of the major well studied rat hepatic P-450s characterized in several laboratories. For further details see our earlier summary of rat P-450 nomenclature [64], as well as studies from the laboratories of Levin and Guzelian [16, 40, 65], Guengerich [66], Schenkman [38, 67], Wolf [68, 69] and references therein. Other distinct rat P-450s not listed include forms designated db1, db2, and UT-H (debrisoquine hydroxylases, gene subfamily IID) [70, 71], LAω (clofibrate-inducible lauric acid ω-hydroxylase gene IVA1) [72], Ch7α (cholesterol 7α-hydroxylase) [73, 74], and RLM2 (male-specific testosterone 7β/15α-hydroxylase) [47].

† See [7].

‡ Regulation of this P-450 is consistent with that deduced using an oligonucleotide probe for clone PCN2 [18].

§ Although the properties of these form(s) are consistent with their correspondence with clone PCN1 [18], recent studies suggest the existence of multiple inducible P-450s in this gene family [19].

‖ Other designations for P-450 2c include 16α [42], male [75], and M-1 [76], and for P-450 2d, 15β [43], female [75], and F-1 [76].

¶ Many other designations exist for these phenobarbital- or polycyclic-inducible P-450s (see [64]).

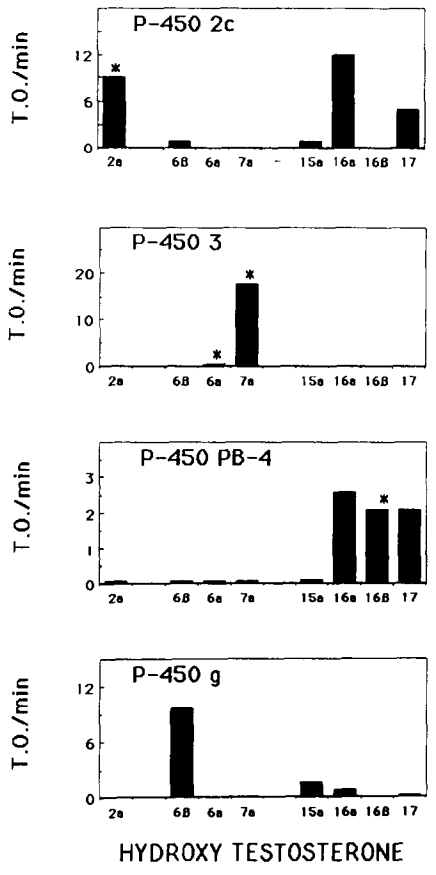


Fig. 1. Regioselectivity and stereoselectivity of testosterone hydroxylation catalyzed by purified P-450 enzymes after reconstitution with dialauroylphosphatidyl choline and purified NADPH P-450 reductase. Activities are expressed as turnover numbers (nmol product/nmol P-450/min), with individual P-450s isolated and product analyses carried out using methods detailed in [11]. Products are grouped into steroid A-ring metabolites (2αOH-T), B-ring metabolites (6βOH-T, 6αOH-T, 7αOH-T) and D-ring metabolites (15αOH-T, 16αOH-T, 16βOH-T and androstenedione, product of 17-oxidation) (T = testosterone). Metabolites characteristic of a single P-450 form are marked by asterisks. Purified P-450g [31] was kindly provided by Dr. J. Goldstein, NIEHS.

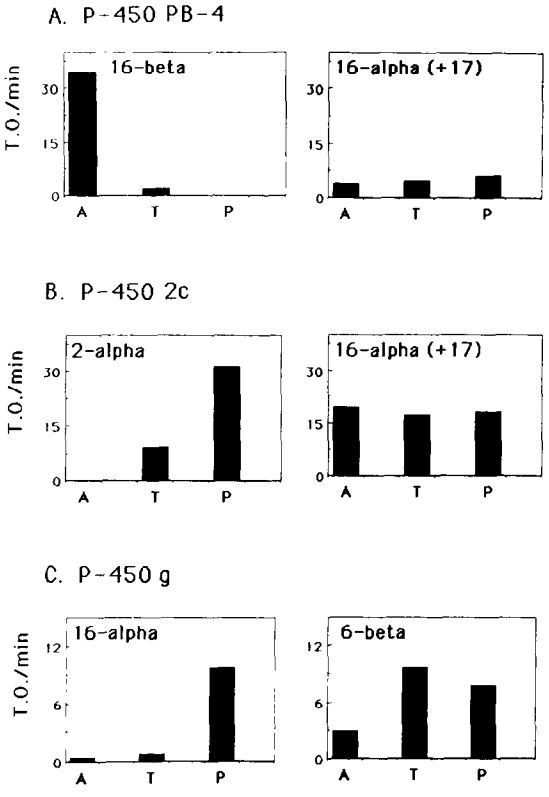


Fig. 2. Influence of steroid D-ring modifications on regioselectivity of hydroxylation catalyzed by purified, reconstituted P-450s. Turnover numbers are compared for hydroxylation of androstenedione (A), testosterone (T) and progesterone (P) at the indicated sites. 16-alpha (+17) refers to the rates of 16α-hydroxylation in the case of androstenedione and progesterone as substrates, and to the sum of the rates of 16α-hydroxylation + 17-oxidation (androstenedione formation) in the case of testosterone as substrate. For structures of the three steroid substrates, see Fig. 3.

Steroid hormones have proven most useful as substrates for distinguishing these multiple P-450 forms owing to the unique and characteristic metabolite profiles obtained. For example, experiments carried out using electrophoretically homogeneous rat hepatic P-450 form 2c (Table 1) establish that this enzyme can hydroxylate testosterone from the alpha face on either the A-ring (yielding 2αOH-testosterone) or the D-ring (yielding 16αOH-testosterone and androstenedione, the latter metabolite derived from oxidation at C17) (Fig. 1). P-450 form 3 also hydroxylates testosterone from the alpha face, but with this isozyme virtually all of the metabolism reflects B-ring hydroxylation at either the 7α or 6α position (7α/6α product ratio = 15:1). P-450 form g also catalyzes an efficient B-ring hydroxylation of testosterone, but from the beta face and at the C6-position. Finally, P-450 PB-4 yields both alpha and beta-derived metabolites, all at the D-ring (see Fig. 1). These and other observations establish that several of these hydroxy steroids correspond to P-450 enzyme-characteristic metabolites (Fig. 1, asterisks).

Structure–activity analysis carried out using three steroid hormones differing only in their C17 substituents (testosterone, R = 17β-hydroxyl; androstenedione, R = 17-keto; progesterone, R = 17β-acetyl; for structures, see Fig. 3) indicates that this site is an important determinant for the regioselectivity of hydroxylation catalyzed by P-450s PB-4, 2c and g (Fig. 2) but not P-450 3 (Fig. 3). Thus, when comparisons are made to androstenedione, increases in polarity (testosterone) or steric bulk at the 17β position (progesterone) markedly suppress P-450 PB-4-catalyzed hydroxylation at the adjacent 16β carbon. There is, however, little effect on the rates of alpha face hydroxylation at C16 and C17 (Fig. 2A). Although these structural changes at the 17β position also have little influence on the alpha face D-ring hydroxylase activities of P-450 2c, they significantly facilitate hydroxylation by this enzyme at the more distant 2α position (Fig. 2B). P-450 2c

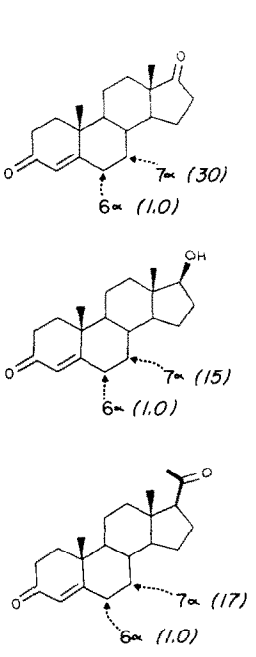


Fig. 3. Regio- and stereoselectivity of P-450 3-dependent steroid B-ring hydroxylation.

can therefore metabolize these steroid hormones with either the A-ring or the D-ring bound to the enzyme in close proximity to the active site Fe^{IV}-oxo species. Interestingly, in the case of P-450g, 16α-hydroxylation is markedly *enhanced* by introduction of an acetyl group at C-17β (Fig. 2C). As might be expected, these steroid D-ring modifications have much smaller effects on the regioselectivity of the B-ring hydroxylase P-450 3 (Fig. 3).

Although these studies clearly establish that several hepatic P-450s can exhibit high specificities when using steroid hormones as substrates, a number of important questions remain:

(1) What factors contribute to the high stereoselectivity exhibited by some P-450s but not others for hydroxylation of the same steroid substrate (e.g. 16αOH/16βOH testosterone product ratio of >30 for P-450 2c versus product ratio of ~1 for P-450 PB-4)?

(2) What structural features on the substrate and on the enzyme determine whether a given P-450 serves as a steroid B-ring hydroxylase (P-450 3), a D-ring hydroxylase (P-450 PB-4) or an A and/or D-ring hydroxylase, depending on the D-ring substituents (P-450 2c)?

(3) Which amino acid segments comprise the steroid binding sites on these enzymes?

(4) Do the monohydroxy steroids produced by these P-450s correspond to deactivated hormone metabolites, formed to facilitate steroid elimination, or do they (or perhaps their secondary metabolites [10]) serve physiological functions that are presently undefined? As will be described later in this study, the pathways for formation of these hydroxylated steroids are subject to multihormonal regulation and complex developmental control, making it tempting to speculate that unique hormonal properties are associated with at least some of these compounds.

P-450 ENZYME-SPECIFIC CONTRIBUTIONS TO MICROSOMAL STEROID METABOLISM

An important question is whether the high regio- and stereoselectivities exhibited by the purified hepatic P-450s in reconstituted enzyme systems provide an accurate monitor of their substrate specificities when bound to the endoplasmic reticulum. We have approached this question using isozyme-specific anti-P-450 antibodies to probe for the contributions of individual P-450 forms to microsomal steroid metabolism. Using antibodies to P-450 2c, adult male rat microsomal metabolism of testosterone at the 2α position can be inhibited in a selective and near-quantitative (up to 90%) fashion [11]. Microsomal steroid hormone 7α-hydroxylation is selectively inhibited by anti-P-450 3 antibodies, and 16β-hydroxylation by anti-P-450 PB-4 antibodies [12–15]. These findings are in good agreement with the catalytic specificities exhibited by the corresponding P-450 enzymes when purified. Several of the purified P-450s can catalyze 16α-hydroxylation of testosterone and androstenedione (e.g. Fig. 1), and accordingly, antibody inhibition experiments suggest that multiple P-450s can contribute to microsomal 16α-hydroxylation of these substrates. Thus, although P-450 2c catalyzes at least 90% of microsomal androstenedione 16α-hydroxylase activity in uninduced or β-naphthoflavone-induced adult male rat liver [11], P-450 PB-4 catalyzes about 60–70% and P-450 2c the remaining 30–40% of this activity in phenobarbital-induced adult male microsomes (Fig. 4). These findings are consistent with the virtual absence of P-450 PB-4 in the uninduced liver microsomes and its presence in the phenobarbital microsomes at a level several-fold higher than P-450 2c [12]. Similarly, in phenobarbital-induced immature (4 week-old) males or adult females, which both have high levels of P-450 PB-4 but undetectable P-450 2c, at least 90% of microsomal 16α-hydroxylation is P-450 PB-4-dependent. Androstenedione 16βOH/16αOH product ratios calculated for microsomal P-450 PB-4 on the basis of these experiments are in good agreement with product ratios determined directly using purified P-450 PB-4 (Table 2), indicating that the stereoselectivity of steroid hormone hydroxylation catalyzed by this microsomal P-450 is not markedly altered by membrane solubilization and enzyme purification.

It should be emphasized that results based on antibody inhibition experiments such as those just described are only as reliable as the specificities of the anti-P-450 antibodies employed. Potential crossreactivities with uncharacterized P-450 forms, or with closely related isozymes need to be considered. For example, recent studies [13] provide evidence for a P-450 form closely related to P-450 3 whose potential reactivity with the antibodies used in the above studies cannot yet be established. Moreover, in the case of P-450 PB-4, antibodies (both polyclonal and monoclonal) are highly crossreactive with P-450 PB-5 [14–17]. Therefore, the catalytic

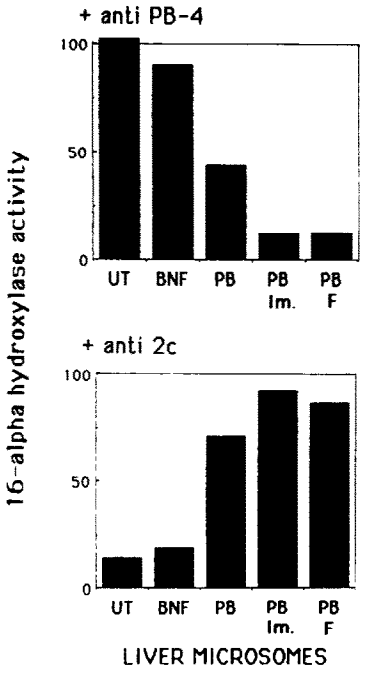


Fig. 4. Antibody inhibition of liver microsomal androstenedione 16α-hydroxylation. Shown are the effects of saturating amounts of monoclonal antibodies directed against P-450 PB-4 or P-450 2c on 16α-hydroxylation catalyzed by untreated or β-naphthoflavone-induced adult male microsomes (UT, BNF) or by phenobarbital-induced adult male (PB), immature male (PB Im) or adult female (PB F) microsomes. Based on data in [15].

contributions of these two closely related P-450 forms (97% identical in primary structure) cannot be adequately resolved using available methodologies. Although one might presume that the contribution by P-450 PB-5, for instance, to microsomal androstenedione 16β-hydroxylation is minimal on the basis of the much lower activity exhibited by purified PB-5 as compared to purified PB-4 in reconstituted enzyme systems [16, 17], at least one rat P-450 form appears unstable to purification (P-450 PB-2a; see below), indicating that such a presumption might not be warranted.

STEROID HORMONE 6β-HYDROXYLASE P-450s

Antibody inhibition experiments have indicated that the majority (≥85%) of microsomal steroid hormone 6β-hydroxylase activity is catalyzed by immunoreactive P-450 PB-2a [12]. This P-450 is part of a gene family which apparently contains three and perhaps more members [18, 19]. At least two of these P-450s, namely the constitutive P-450 2a and the xenobiotic inducible P-450 PB-2a, probably catalyze 6β-hydroxylation of testosterone [12, 18]. Unfortunately, these cytochromes deactivate during the course of microsome solubilization and enzyme purification [12, 19, 20], precluding direct studies of their substrate specificities. However, induction and other indirect studies suggest that these cytochromes (or perhaps closely related P-450 forms) also contribute significantly to formation of Δ^6-testosterone, product of a steroid C5–C6 desaturation reaction [21], in addition to three other minor microsomal testosterone metabolites: 2βOH, 15βOH and 18-OH testosterone [22]. We have recently shown [23] that two of these same metabolites (2βOH-testosterone and 15βOH-testosterone) are formed by an immunochemically related isozyme expressed in human liver microsomes, designated P-450$_{NF}$ [24] or P-450 HLp [25]. Efforts aimed at the preparation of specific, inhibitory monoclonal antibodies to rat P-450 PB-2a [26] (or to its rabbit homolog, P-450 form 3c [E. Johnson, personal communication]) that might be

Table 2. Androstenedione 16βOH/16αOH product ratio: microsomal versus purified P-450 PB-4

Metabolite	Total microsomal activity*	×	Fractional P-450 PB-4 Contribution†	=	Calculated P-450 PB-4-dependent activity*	16βOH-A/16αOH-A Product ratio‡
A. PB microsomes, adult male						
16βOH-A	10.0		90–100%		9.0–10.0	8.2–10.0
16αOH-A	1.6		60–70%		1.0–1.1	
B. PB microsomes, adult female						
16βOH-A	4.7		90–100%		4.2–4.7	7.0–9.4
16αOH-A	0.6		85–95%		0.5–0.6	
C. PB microsomes, immature male						
16βOH-A	7.3		95–100%		6.9–7.3	6.3–7.3
16αOH-A	1.1		90–100%		1.0–1.1	
D. Purified, reconstituted P-450 PB-4						
16βOH-A	—[a]		—		34.2[b]	9.0
16αOH-A	—		—		3.8[b]	

* nmol metabolite/min/mg microsomal protein

† Values based on the maximal inhibition observed in the presence of saturating levels of inhibitory antibody (e.g. Fig. 4).

‡ Ratio of P-450 PB-4-dependent microsomal androstenedione 16β-hydroxylation to 16α-hydroxylation.

[a] Not applicable.

[b] Experimental values.

Table 3. Androstenedione hydroxylase turnover numbers determined for microsomal versus purified P-450 enzymes*

	β-naphthoflavone microsomes		Untreated microsomes	Phenobarbital microsomes		
	P-450 BNF-B	P-450 2c	P-450 2c	P-450 3	P-450 PB-4	P-450 PB-2a
Androstenedione metabolite	6βOH-A	16αOH-A	16αOH-A	7αOH-A	16βOH-A	6βOH-A
Total microsomal hydroxylase activity (nmol metabolite/mg/min)	1.6	1.0	2.4	0.45	8.4	6.3
P-450 enzyme specific content (nmol/mg)	1.2	0.19	0.30	0.22	1.0	0.66
Turnover numbers (nmol metabolite/min/nmol P-450 enzyme)						
Microsomal P-450 (calculated)†	*0.07*	*4.7*	*7.2*	*1.8*	*7.6*	*8.6*
Purified P-450 (experimental)	1.5	19.3	19.3	8.9	34.2	(~0.5)§
Relative activity‡	5%	24%	37%	20%	22%	(1700%)

* Microsomal activities and isozyme specific contents are based on data presented in [12].

† Values are calculated by multiplying the observed microsomal activity by the fraction attributed to the indicated P-450 form as determined in antibody inhibition experiments. This fraction is equal to 5% of the total activity in the case of P-450 BNF-B and ~90% of the total activity for the other P-450 forms. The resultant value is divided by the P-450 enzyme specific content to yield the indicated turnover numbers. Thus, P-450 2c-dependent androstenedione 16α-hydroxylation is catalyzed with a calculated microsomal turnover of $1.0/0.19 = 5.3 \times 90\% = 4.7$ nmol product/min/nmol P-450 2c in the β-naphthaflavone-induced liver microsomes.

‡ Microsomal P-450 turnover number/purified P-450 turnover number × 100%.

§ Purified P-450 PB-2a is inactivated during the course of enzyme isolation. Testosterone 6β-hydroxylase activities as high as 3.1 nmol metabolite/min/nmol P-450 enzyme have recently been reported using a partially purified preparation of P-450 PB-2a [22].

used to confirm the catalytic specificities thus attributed to members of this P-450 gene family have proven unsuccessful. The possible contribution of these P-450s to 6β-hydroxylation of other physiologically important steroids such as cortisol [27] and various bile acids [28, 29] remains to be established.

Although P-450s 2a and PB-2a are probably the major microsomal catalysts of steroid hormone 6β-hydroxylation in uninduced and drug-induced rat liver, respectively, several other P-450s (e.g. forms BNF-B and g) exhibit significant 6β-hydroxylase activity when purified (e.g. Fig. 2). This raises the question whether they might contribute to 6β-hydroxylation in liver microsomes enriched with one or both of these P-450 forms. Surprisingly, though, one cannot detect significant catalytic contributions of P-450 BNF-B or P-450g to 6β-hydroxylation catalyzed by β-naphthoflavone-induced microsomes or microsomes expressing high levels of P-450g, respectively [15, 30, 31]. In the case of β-naphthoflavone-induced microsomes, steroid 6β-hydroxylase turnover numbers estimated for microsomal P-450 BNF-B are at most 5% of those observed in reconstituted systems. This contrasts with microsomal turnover numbers ranging from 20 to 40% of the purified enzyme activities in the case of P-450s 2c, 3 and PB-4 (Table 3). That these latter microsomal activities are also significantly lower than the corresponding reconstituted activities probably reflects their dependence on NADPH P-450 reductase, which is rate limiting [32, 33] in these microsomal incubations: addition of exogenous, purified P-450 reductase stimulates each of their respective associated microsomal steroid hydroxylase activities up to 2–3-fold (data not shown). Interestingly, preliminary reports indicate that P-450g-dependent microsomal activities are, in fact, susceptible to inhibition by specific antibody once excess P-450 reductase has been added to microsomes of the high P-450g phenotype [34]. This suggests that P-450g in these microsomes (and perhaps also P-450 BNF-B in β-naphthoflavone-induced microsomes) may compete for endogenous P-450 reductase much less effectively than the other P-450s when the monooxygenase substrate is a steroid that is metabolized efficiently by multiple P-450 forms.

PERTURBATION OF ENDOGENOUS STEROID METABOLISM BY XENOBIOTICS

It is well established that the broad capacity of hepatic tissue for P-450-dependent xenobiotic metabolism can be modulated by exposure of individuals to various foreign compounds, some of which can induce the levels of specific P-450 enzymes by up to 50- or 100-fold. Xenobiotic suppression of constitutively expressed P-450s has also been observed and can result in a 90% loss of select P-450 forms [11, 35–37]. Possible mechanisms for this suppression of constitutive P-450 enzyme expression might include: (a) direct transcriptional suppression by xenobiotic–receptor complexes (e.g. by the *Ah* receptor–polycyclic ligand complex); (b) competition between constitutive and inducible enzymes for limited cellular heme pools, leading to accumulation and ultimately degradation of constitutive P-450 apoproteins; or perhaps (c) perturbation of the hormonal regulation of P-450 enzyme expression (see below).

Since several of the P-450 forms thus subject to modulation by xenobiotics are active in steroid hormone hydroxylation, xenobiotics can perturb endogenous steroid hormone metabolism in any of several ways (Fig. 5). These include: (a) induction of new hydroxylation pathways [e.g. 50-fold elevation of P-450 PB-4-dependent androstenedione 16β-hydroxylation upon exposure to phenobarbital]; (b) suppression of hormonally regulated pathways [e.g. up to 90% decrease in P-450 2c-dependent steroid hormone 16α and 2α hydroxylations upon administration of anti-cancer drugs or certain polyhalogenated biphenyls); and (c) induction of pathways otherwise subject to strict endocrine control [e.g.

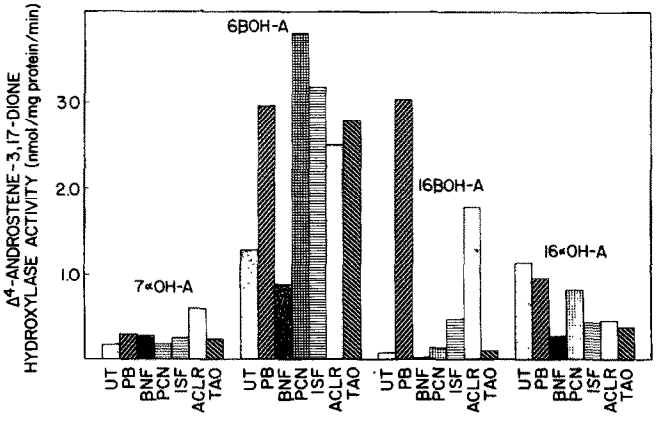


Fig. 5. Modulation of microsomal androstenedione hydroxylation by xenobiotics. Androstenedione metabolism was assayed in incubations containing liver microsomes isolated from untreated adult male rats (UT) or from adult male rats induced with phenobarbital (PB), β-naphthoflavone (BNF), 16α-cyanopregnenolone (PCN), isosafrole (ISF), Aroclor 1254 (ACLR) or troleandomycin (TAO). Shown are the rates for androstenedione 7α-hydroxylation (P-450 3-dependent), 6β-hydroxylation (P-450 2a + PB-2a-dependent), 16β-hydroxylation (P-450 PB-4(+PB-5)-dependent) and 16α-hydroxylation (P-450 2c(+PB-4)-dependent).

SEX-DEPENDENCE OF RAT P-450s

	P-450 2a	P-450 2c	P-450 2d
Characteristic activities	6β-OH T, A, P	2αOH-T 16αOH-A	15βOH-steroid sulfate
Sex-specificity (adult rats)	Male-specific	Male-specific	Female-specific
Developmental profile:			
males	maintenance	induction	suppression
females	suppression	(absent)	induction

Hormonal Regulation:

- P-450s 2a and 2c are neonatally imprinted by androgen.
- P-450 2d is expressed at a hormone-independent basal level that can be stimulated by estrogen and suppressed by androgen.
- Pituitary hormone(s) appear to play a key role in regulating the expression of these sex-dependent P-450 enzymes.

Scheme 2. Sex-dependence of rat hepatic P-450 enzymes.

20-fold induction of P-450 PB-2a-catalyzed steroid hormone 6β-hydroxylation in adult female rats administered 16α-cyanopregnenolone].

The consequences of these perturbations in steroid hydroxylation pathways are largely unknown, as are possible physiological functions of the individual hydroxylated metabolites. The overall rate of oxidative deactivation is, however, likely to be altered in response to xenobiotics. This may be of particular importance in cancers such as endometrial and breast, which appear to be dependent on circulating hormone levels. In the case of estradiol, for example, 16α-hydroxylation has been linked to the incidence of mammary tumors [41]. Alterations in the levels of the particular P-450s catalyzing the competing pathways of estrogen 2 and 16α hydroxylation [38–40] may therefore be of consequence to hormonal carcinogenesis.

SEX-DEPENDENCE AND DEVELOPMENTAL REGULATION OF HEPATIC P-450 ENZYMES

Modulation of P-450 protein and activity levels also occurs in response to hormonal and developmental factors. Studies on the hormonal regulation of P-450 expression have been facilitated by recent studies demonstrating the identity of several rat hepatic P-450s with specific microsomal steroid hydroxylase activities that are sex-dependent and developmentally regulated (Scheme 2). P-450 2c catalyzes the steroid hormone 2α and 16α hydroxylase activities induced at puberty in male rat liver [11, 42] while P-450 2d catalyzes the female-specific 15β-hydroxylation of steroid disulfates [43, 44]. Induction of the male-specific P-450 2c protein parallels induction of microsomal 16α-hydroxylase activity in the maturing males (Fig. 6A). Correspondingly, the female-specific P-450 2d is developmentally induced in maturing females, although it (or a closely related isozyme) is also expressed in immature (4-week-old) rats of both sexes [12, 45]. Immunoreactive P-450 2a catalyzes the major steroid hormone 6β-hydroxylase activity expressed in mature male rats and in immature rats of both sexes [12]. This P-450, which is male-specific in adult rats, is developmentally suppressed in maturing females

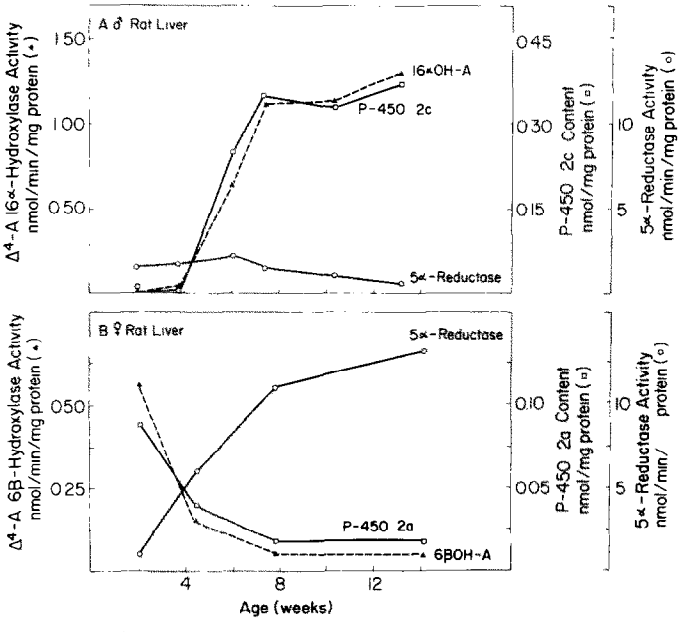


Fig. 6. Developmental regulation of P-450s 2c and 2a and their associated microsomal androstenedione hydroxylase activities. Panel A- levels of P-450 2c polypetide (determined by Western blotting) and its associated microsomal steroid 16α-hydroxylase activity determined in developing male rats. Panel B- levels of P-450 2a polypeptide and its associated microsomal steroid 6β-hydroxylase activity determined in developing female rats. Age dependence of the adult female-specific 5α-reductase is shown for comparison. Based on data in [12].

in parallel to the loss of microsomal 6β-hydroxylase activity (Fig. 6B). Other sex-dependent P-450 enzymes subject to developmental regulation include the male-specific P-450 forms g [31, 46] and RLM2 [47]. Not all constitutively expressed P-450s are sex regulated, however, since, for example, the constitutive P-450 PB-1 is expressed at comparable levels in both sexes and is not significantly affected by hormonal factors [Fig. 7D; refs 12, 48, 49]. Although P-450s 2a and 2c are both male-specific in adult rats, they exhibit distinct developmental profiles, with P-450 2c markedly induced at puberty and P-450 2a maintained at the levels found in prepubertal animals (Scheme 2). This maintenance of P-450 2a in males versus its suppression in developing females has recently been confirmed by Parkinson and coworkers in studies on the developmental regulation of microsomal testosterone 6β-hydroxylation [22] as well as by Gonzalez and associates in hybridization studies using a specific oligonucleotide probe [18]. Although Cresteil *et al.* [50] could not detect immunoreactive P-450 2a in immature rats of either sex in the absence of phenobarbital induction, those studies were carried out using polyclonal antibodies raised to the inducible P-450 PCN-E (P-450 PB-2a), which may have exhibited an affinity or cross-reactivity with P-450 2a that was too low to permit its detection in the uninduced animals using Western blotting techinques.

STEROID HORMONAL REGULATION OF HEPATIC P-450 EXPRESSION

The hormonal basis for these developmental profiles has been examined by our laboratory to provide a more detailed understanding of the role of gonadal hormones [51–53] in programming for expression of these P-450s and other hepatic proteins [12, 54]. Ovariectomy does not lead to expression of the male-specific P-450s 2a and 2c in female rats, indicating that these P-450s are not negatively regulated by estrogen. In contrast, castration of male rats at birth both abolishes expression of P-450 2a and P-450 2c in adult male rats and blocks the supression of P-450 2d that normally occurs in the males at puberty (*BC*, Fig. 7, lane 3). These effects are not observed in rats castrated at five weeks of age (i.e. subsequent to neonatal androgen exposure; *LC*, lane 2) and are substantially reversed by two single testosterone injections during the first three days of life (*BC/T*, lane 4). Thus neonatal androgen "imprints" on the male rat a program for the developmental regulation of these three sex-dependent P-450s. This program entails induction of P-450 2c, suppression of P-450 2d and maintenance of P-450 2a as the male rat goes through puberty. These findings also establish that changes in the corresponding microsomal steroid hydroxylase activities with development and with hormonal status are primarily due to alterations in P-450 protein levels rather than to modulation of their catalytic activities [12].

Although neonatal androgen thus imprints (programs) the expression of P-450s 2a and 2c in adult rat liver, the imprinted levels of P-450 2c (defined as the level of enzyme expressed by rats castrated postpubertally) are typically only 50–60% of those found

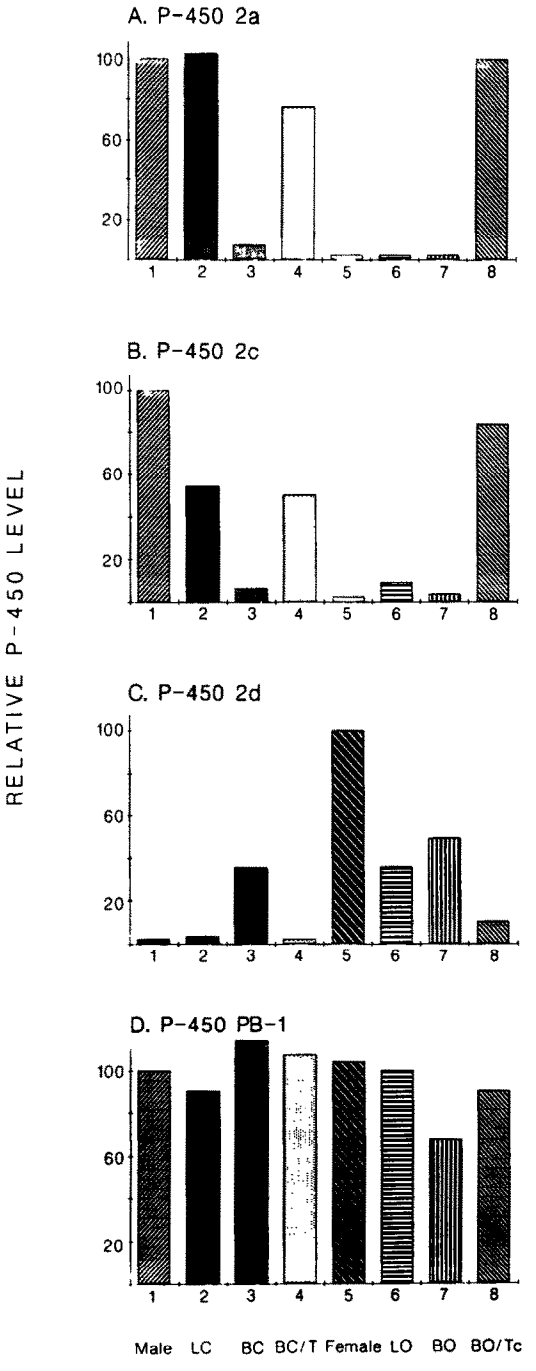


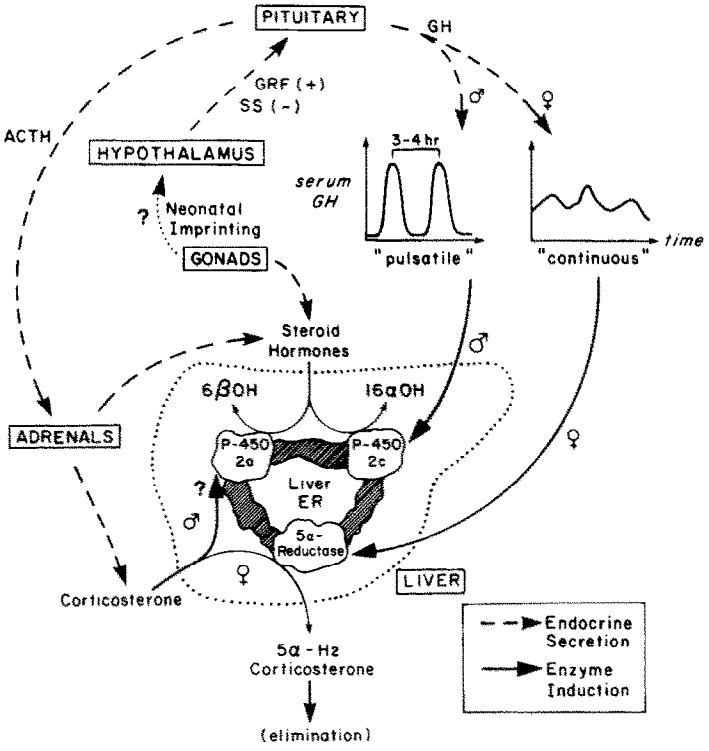
Fig. 7. Effects of gonadectomy and testosterone replacement on expression of rat hepatic P-450s. Shown are the relative levels of hepatic P-450 forms 2a, 2c 2d and PB-1 in adult (10 week old) male rats (lanes 1–4) or female rats (lanes 5–8). Rats were either (a) untreated (lanes 1, 5), (b) gonadectomized at 5 weeks of age (*LC*, late castration, lane 2; *LO*, late ovariectomy, lane 6) or (c) gonadectomised at birth (*BC*, birth castration, lane 3; *BO*, birth ovariectomy, lane 7). Lane 4—birth castrates exposed to androgen neonatally by testosterone injections on days 1 and 3 of life (*BC/T*). Lane 8—birth ovariectomized rats implanted subcutaneously with a testosterone-packed Silastic capsule at 5 weeks of age (*BO/Tc*). P-450 levels were determined by Western blotting of liver microsomes isolated from animals killed at 10 weeks of age. Based on data in [12, 54].

in untreated adult males (Fig. 7B). This suggests that adult androgen may be required for full expression of this P-450 form. Consistent with this idea, continuous exposure to androgen after the age of 5 weeks elevates the level of P-450 2c to that found in untreated adult males. However, adult androgen exposure can also stimulate the expression of P-450 2c and also P-450 2a in rats castrated neonatally, even in the absence of neonatal androgen imprinting [54]. In contrast to this marked dependence on androgen for expression of the male-specific P-450s 2a and 2c, expression of the female-specific enzymes P-450 2d and steroid hormone 5α-reductase appears to be only partially dependent on estradiol, since birth gonadectomy does not abolish, but rather reduces these latter two enzymes to the basal levels found in immature rats of either sex (Fig. 7C and [54]).

PITUITARY HORMONES AND HEPATIC P-450 SYNTHESIS

Although the studies described above clearly establish the importance of neonatal androgen for programming the expression of several hepatic P-450s, they do not address the question of whether androgen acts directly on the liver, or whether its effects are mediated indirectly, perhaps via another endocrine organ. Other studies, however, support the concept that the effects of androgen on hepatic enzyme expression are indirect, mediated perhaps by pituitary growth hormone secretions [e.g. 55]. Adult rats are sexually differentiated with respect to the temporal pattern of pituitary growth hormone secretion, with adult males exhibiting an episodic, or pulsatile pattern of growth hormone secretion, and adult females a more continuous secretory pattern [56]. These secretory patterns are regulated by hypothalamic secretion of the positive effector growth hormone releasing factor (GRF) and the negative regulator somatostatin (SS) (Scheme 3). This hypothalamic regulation is determined by neonatally secreted testicular androgen, which appears to imprint the high amplitude growth hormone pulses characteristic of adult male rats, as well as by testosterone secretions during adult life, which seem important for maintaining the low baseline growth hormone level found between pulses [57].

Experiments designed to assess the role(s) of these sexually differentiated growth hormone secretory



Scheme 3. Roles of hepatic P-450s in steroid metabolism and proposed mechanisms for endocrine regulation of hepatic enzyme expression. Steroid hormones produced by the gonads and by the adrenals are converted to their 6β-hydroxy and 16α-hydroxy derivatives by hepatic P-450s 2a and 2c, respectively. Although these derivatives are generally believed to be less active hormonally and more readily eliminated, they may serve important biological functions that are presently undefined. The pulsatile pattern of pituitary growth hormone (GH) secretion characteristic of adult male rats [56], neonatally imprinted via the hypothalamus and regulated by growth hormone releasing factor (GRF) and somatostatin (SS), is required for expression of the male-specific P-450 2c at puberty. Pituitary secretion of growth hormone in adult female rats is more continuous and leads to induction of the female-specific 5α-reductase and probably also the suppression of P-450 2a at puberty. Maintenance of P-450 2a in mature males may also be partially dependent on adrenal corticosteroids, which are known to induce immunoreactive P-450 2a in cultured hepatocytes [60].

patterns in hepatic P-450 expression suggest that P-450 2c expression is dependent on pulsatile growth hormone secretion, while P-450 2d expression requires more continuous serum growth hormone levels. Thus P-450 2c levels in adult male rats are greatly reduced by either hypophysectomy or continuous growth hormone infusion. Partial to complete restoration of this P-450 form can be achieved by subcutaneous injection of growth hormone twice daily, a schedule chosen to mimic the adult male pulsatile secretory pattern [42, 58]. By contrast, although expression of the female-specific P-450 2d is also abolished by hypophysectomy, continuous growth hormone infusion (but not twice daily injection) effectively restores enzyme expression [43, 58]. P-450 2a is apparently not dependent on the adult male pulsatile secretory pattern, since this P-450 form is also expressed at significant levels in two-week old rats of both sexes [12, 18], which secrete growth hormone at lower levels and in a more continuous fashion than do the adult males [56]. P-450 2a-dependent androgen 6β-hydroxylation is, however, suppressed by continuous infusion of growth hormone to adult male rats [55]. This suggests that the developmental suppression of P-450 2a in female rats may, in fact, reflect the onset of higher and more continuous growth hormone secretion characteristic of adult females. Precedent for such a possibility is provided by recent studies demonstrating that adult growth hormone secretions suppress basal (uninduced) levels of the phenobarbital-inducible P-450s PB-4 and PB-5 [59]. Alternatively, the decrease in P-450 2a in developing female rats may, in part, reflect the decrease in circulating corticosteroids [51] that results from the marked post-pubertal induction of hepatic steroid 5α-reductase activity (Scheme 3). Both synthetic and natural corticosteroids have been shown to induce immunoreactive P-450 2a in cultured rat hepatocytes [60], suggesting that these steroids might serve as endogenous inducers of this or related P-450 forms. More detailed studies are required to fully evaluate the roles of growth hormone and adrenal corticosteroids in expression of P-450 2a and other hepatic enzymes.

In summary, the studies described above demonstrate that steroids can serve as highly specific substrates for hepatic P-450 enzymes and as hormonal regulators of isozyme expression. Although much has been learned about the developmental control and hormonal regulation of these enzymes during the past few years, several important questions remain unanswered.

(1) Is pulsatile growth hormone secretion sufficient for full expression of P-450 2c, or are other endocrine factors required, as is the case for another adult male-specific liver gene product, alpha 2U globulin [61]?

(2) Are the immunoreactive forms of P-450s 2a and 2d that are detected in immature rats identical to those expressed in adult male and female rats, respectively?

(3) Does P-450 2a suppression in maturing female rats reflect the onset of continuous growth hormone secretion? Does hypophysectomy of adult females lead to masculinization (i.e. induction) of P-450 2a?

(4) Does growth hormone act directly to activate P-450 gene expression, or are its effects mediated indirectly, perhaps by somatomedins produced in the liver in response to growth hormone stimulation [62]?

(5) How does growth hormone, when secreted in a pulsatile fashion, effect a male pattern of hepatic P-450 metabolism, while the same total growth hormone levels, when secreted in a more continuous fashion, result in a female pattern? Does the more continuous occupancy of growth hormone receptors in the females lead to down regulation of IGF_1 receptors as proposed [63] in the case of the mouse major urinary protein (MUP)?

(6) How does neonatal androgen imprint hypothalamic regulation of pituitary growth hormone secretion?

(7) Do analogous endocrine pathways regulate human P-450 enzymes?

Although marked age and sex-differences in human hepatic P-450 metabolism have not been reported, it is conceivable that interindividual differences due to genetic and environmental factors may mask developmental patterns or hormonal influences that do have a significant effect on human P-450 enzyme expression.

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