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EXOCYCLIC-KETO REDUCTASE ACTIVITIES FOR PROGESTERONE AND S-WARFARIN IN HEPATIC MICROSOMES FROM ADULT MALE RATS[1]

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SUMMARY: Hepatic microsomes from adult male rats representing six inbred strains catalyzed quantitatively significant, NADPH dependent reductions of progesterone to the 20β (20R) alcohol and S-warfarin to its 11S-OH product. Microsomes from mature females and immature rats of both sexes were essentially devoid of these activities. Two strains of rat evidenced about 21% of these activities compared with the other strains and both activities were 25-81% repressed by treatment of rats with phenobarbital (PB). An excellent linear correlation was demonstrated for the two activities considering sex, age, NADPH>>NADH preference, PB-repression and strain differences. However, detergent latency (71%) and resistance to trypsinolysis were only observed for the keto-reductase activity with S-warfarin. Microsomes also catalyzed the reduction of progesterone to its 20α-OH derivative but this activity preferred NADH>NADPH, was induced 2.7-fold by PB and was essentially independent of age, sex and animal strain. Furthermore, unlike the 20β-OH activity, this reduction was resistant to proteolytic inactivation.

The metabolism of xenobiotics and steroid hormones prior to their secretion from mammals has been well studied with regard to their oxidation catalyzed by the cytochrome P450 (CYP) monooxygenase system but less attention has been afforded to studies of their reductive metabolism. Nevertheless, a variety of cytoplasmic enzymes catalyzing the reduction of carbonyl groups in aldehydes and/or ketones to corresponding alcohols have already been characterized in the rat (1,2). In general, these oxidoreductases are monomeric (M_r = 30-40 x 10^3) and use NADPH (or to a lesser extent NADH) as a reductant. Several purified forms have been shown to catalyze *in vitro* reactions involving

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Abbreviations used: CYP, cytochrome P450; PB, phenobarbital.

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a variety of xenobiotic and endogenous (e.g., steroid) substrates which has precluded the development of a logical nomenclature based on unique substrate specificities. Studies on microsomal versions of these reductases in rat liver have not been as extensive. Nevertheless, it is possible that some carbonyl reductase activities in microsomes might be affiliated with steroid dehydrogenases/reductases which have already been characterized in this subfraction (3,4).

Work in our laboratory concerning the genetic diversity and linkage of hepatic CYPs have employed various inbred strains of rat for electrophoretic studies of microsomal proteins (5). Recent efforts to provide functional correlations with the different electrophoretic phenotypes of CYPs affiliated with the different rat-strains have involved *in vitro* metabolic studies using hepatic microsomes with warfarin (6) and progesterone as substrates. During the course of these studies it was observed that significant reductive metabolism occurred with both substrates. In this report we demonstrate that the stereo-specific reduction of the exocyclic keto-groups of both substrates are subject to a variety of influences including animal age, sex, strain and PB-treatment.

MATERIALS AND METHODS

Racemic warfarin was obtained from Calbiochem and 20α-OH and 20β-OH derivatives of progesterone were obtained from Steraloids, Inc. All other chemicals were from Sigma. Warfarin enantiomers were resolved and purified as previously described (7). Immature (25 day-old) and sexually mature (10-11 week-old) male and female rats were used which represented the following inbred strains: ACI/NHsd, F344/NHsd, SHR/N, SHRsp/N, WF/Hsd and WKY/Crl (5). PB-treatment consisted of four consecutive 10:00 AM injections using a dose of 75mg/kg. Total hepatic microsomes were prepared by calcium precipitation (8). Protein concentrations were determined by the Lowry method using bovine serum albumin as a standard.

Assays of warfarin reduction by NADPH in the presence of microsomes used either 9R- or 9S-warfarin. Incubation conditions and the HPLC procedure for product analysis were as previously described (9) with the following exceptions. The reactions were terminated by centrifugation in 1.5 ml Eppendorf tubes at 14,000 x g for two minutes followed by filtration to facilitate the latter process. Also a Waters 600E system HPLC equipped with a 994 Programmable Photodiode Array Detector, a WISP 712 automatic injector, and a Baseline 810 Chromatography Workstation (Version 3.30, Dynamic Solutions) were employed. The column used was μBondapak C_{18}, 10 μm, 3.9 mm x 30.0 cm (Waters). Baseline separations of warfarin alcohols (i.e., enantiomeric pairs 9S,11S/9R,11R & 9S,11R/9R,11S) were accomplished.

Assays for microsomal catalysis of the C20-reduction of progesterone by NADPH to its 20β(R)-OH and 20α(S)-OH products used a published HPLC procedure (10) except that a Waters apparatus was used (see above). In this application a 25 cm APEX octadecyl (5μ) column (Jones Chromatography) was used. Spectra for the peak representing 20α-OH-progesterone (verified using an authentic standard) after microsomal metabolism of progesterone revealed the presence of Δ^6-progesterone which could be distinguished from the 20β-OH derivative by its ϵ_{max} at 285 nm (11).

All assays and digestions were performed at 37 ° C and studies on the trypsinolysis of microsomes employed 25 μg trypsin/mg microsomal protein under essentially the same conditions as those used in the activity assays. Tryptic digestions were terminated by the addition of two moles of bovine pancreas trypsin Inhibitor per mole of trypsin. Reductase activities for microsomes in the presence of detergents employed preincubations (5.00 min for warfarin and 3.25 min for progesterone) before initiation of the reaction by the addition of substrates (NADPH for the warfarin assay and progesterone for the progesterone assay).

RESULTS AND DISCUSSION

This study of hepatic microsomal reductase activities employed immature rats of both sexes representing four inbred strains and sexually mature male and female rats representing an additional two inbred strains. Catalysis of the NADPH reduction of the 11-keto group of 9R- and 9S-warfarin was assayed in all cases and significant activity was only observed for the conversion of the S-enantiomer exclusively to its 11-S-OH derivative by microsomes from adult males. (All other rats evidenced less than 3% of this activity.) Reduction of R-warfarin (only to its 11-R-OH-derivative) was barely detected having activities that were less than 3% of those for S-warfarin (except for adult-male SHR rats which showed 8% of this activity). The stereoselective and stereospecific reduction of warfarin to 11S-OH-S-warfarin by hepatic microsomes from adult male rats was reported previously (12). The present results which elucidated the age and sex dependence of this microsomal activity might explain discrepancies in the literature where this activity was not detected (12,13). Substantial progesterone reductase activity yielding 20β-OH-progesterone with NADPH as the reductant was observed only with microsomes from adult males except for trace amounts for immature F344 and ACI males (*i.e.*, 4% of corresponding activities in adult males) which probably reflected the onset of puberty in these strains. On the other hand, hepatic microsomes from all animals had essentially the same level of reductase activity for the production of 20α-OH-progesterone in the presence of NADPH (average = 0.12 ±0.05 nmoles/min/mg protein; n = 24)[3] with the possible exception of ACI rats which had higher activities compared with the other strains in all cases (average = 0.20 ±0.04 nmoles/min/mg protein; n = 4).

NADH was substituted for NADPH in metabolic assays using S-warfarin and progesterone as substrates with microsomes from adult ACI, F344 and SHR rats of both sexes. In all cases, reductase activity yielding 20β-OH-progesterone was not observed and that producing the 11S alcohol from S-warfarin using microsomes from adult-males was only about 15 ±1 % of that for NADPH. In contrast, the reduction of progesterone to its 20α-alcohol was significantly greater for NADH *vis à vis* NADPH in all cases (average fold-

[3] Mean and standard deviation; n = number of independent experiments.

Table 1. Strain differences and the effect of phenobarbital pre-treatment on the reduction of progesterone (P) to its 20α and 20β alcohols and S-warfarin (SW) to its 11S alcohol catalyzed by hepatic microsomes from adult male rats[a]

Strain	Pre-treatment	Activity (nmole/min/mg protein)		
		20α-OH-P	20β-OH-P	11S-OH-SW
ACI	None	0.230	1.332	0.194
F344	None	0.134(0.199)	0.996(1.337)	0.170(0.121)
	PB	0.453	0.748	0.078
SHR	None	0.124(0.159)	1.032(0.975)	0.168(0.185)
	PB	0.393	0.326	0.071
SHRsp	None	0.169	1.220	0.271
	PB	0.398	0.227	0.035
WF	None	0.124	0.253	0.044
	PB	0.260	0.115	0
WKY	None	0.089	0.233	0.041

[a] Assays were performed at least twice and these differed by less than 20% in all cases. Values in parentheses are for independent preparations of microsomes.

increase = 3.0 ±1.6; n = 6). Reactions using NADPH with microsomes from adult-males were also performed in the presence of carbon monoxide to test for the involvement of CYP and no effect of this addition on reductions of progesterone and S-warfarin were observed under conditions where at least 70% of the CYP-catalyzed oxidations were inhibited.

Reductase activities had characteristic strain differences and responses to treatment with PB as shown by the results in Table 1. It was noted that the formation of 20β-OH-progesterone and the 11S-OH derivative of S-warfarin were both significantly lower for WF and WKY rats compared with the other strains. Furthermore, PB-treatment significantly repressed the latter two activities (average = 64 ±25 % repression) whereas, the microsomal activity yielding the 20α-alcohol of progesterone was induced by this drug in all cases tested (average fold-increase = 2.7 ±0.5). A similar repression of microsomal S-warfarin (11S-OH) reductase activity by phenobarbital was previously reported but was not discussed (14).

The results discussed thus far suggest that the microsomal reductase activities yielding 20β-OH-progesterone and 11S-OH-9S-warfarin result from the action of the same enzyme because they share the same age, sex and strain dependence as well as NADPH preference and phenobarbital repression. This hypothesis is supported by the excellent linear correlation for the two activities as shown in Figure 1 which only considered data where at least one activity had a non-zero value (this excluded all experiments using microsomes from

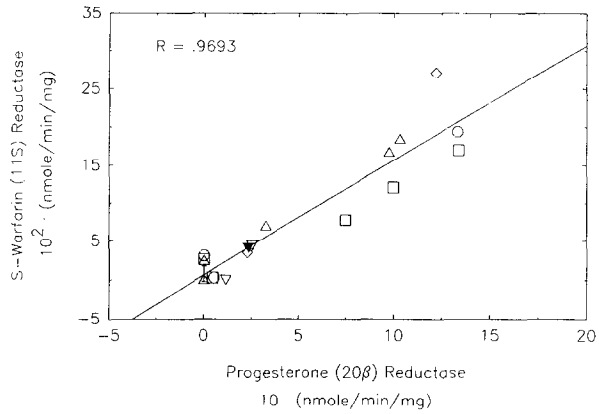

Figure 1. Plot of the reductase activity for Progesterone (20β-OH) *versus* that for S-Warfarin (11S-OH) for hepatic microsomes from untreated and PB-treated male rats representing different strains using either NADPH or NADH as a reductant. Circles, ACI; Squares, F344; Triangles, SHR; Diamond, SHRsp; Open Inverted Triangles, WF; Filled Inverted Triangle, WKY. The results for ACI, F344 and SHR rats clustered near zero represent the results of experiments using NADH and those employing microsomes from immature animals; the remaining data are from Table 1. The error bar is shown for the Y-intercept of the linear regression line and R is the correlation coefficient.

female rats). Additional support for this proposal is the fact that progesterone substantially inhibited the reductase activity producing 11S-OH-9S-warfarin and the reciprocal inhibition (i.e., S-warfarin inhibiting the production of 20β-OH-progesterone) was observed but appeared to be marginal under the conditions tested (see Table 2). In any event, if the same active site is employed for these two activities then Prelog's rule would be violated since NADPH-hydride attack would have to occur on opposite (Si or Re) faces of the substrate's exocyclic carbonyl group (15).

A variety of topologies are possible for enzymes associated with microsomes and these include peripheral proteins associated with either the outer or inner surfaces, integral proteins mainly exposed on either the outer or inner surfaces, transmembrane proteins exposed on both surfaces and soluble proteins trapped in the microsomal interior. It is also possible that peripheral proteins on the outer surface were scavenged during the course of preparation. Additional complications regarding microsomal enzymes with active sites in the interior is their accessibility to NAD(P)H coenzymes which are not readily permeable to the microsomal membrane (16). Lastly, it is possible that hydrophobic substrates such as warfarin and progesterone might bind with low molecular weight Fatty Acid Binding Proteins (FABP) that occur in high concentrations in liver cells (17) and include a form in rat liver that binds tightly to racemic warfarin (18). In an effort to elucidate the topology of active sites involved in the three reductase activities studied, the latency of these activities

Table 2. Reciprocal inhibition studies of S-warfarin and progesterone and the effect of non-ionic detergents on reductase activities in hepatic microsomes from adult male rats

Addition[a]	Activity (nmole/min/mg protein)	
	11S-OH-9S-warfarin[b]	20β-OH-progesterone[b]
None	0.166 ±0.012	-
Carrier	0.113 ±0.008	-
Progesterone	0.022 ±0.012	-
None	0.136 ±0.029	-
Emulgen 913	0.460 ±0.064	-
Triton X-100	0.495 ±0.016	-
None	-	1.15 ±0.02
S-Warfarin	-	1.03 ±0.03
None	-	1.39 ±0.04
Emulgen 913	-	0.98 ±0.20
Triton X-100	-	1.18 ±0.11

[a] Progesterone was added in 50 μl of acetonitrile to give a final concentration of 0.8 mM; carrier was 50 μl of acetonitrile; S-warfarin was added to give a final concentration of 0.5 mM. Detergent concentrations were 0.2 %. Standard deviations are for experiments that were performed at least in triplicate. All other conditions were as described under Materials and Methods.

[b] Microsomes from ACI and F344 rats were used for assays employing S-warfarin and progesterone as substrates, respectively.

to detergents was investigated (%-latency = $((SA_d - SA)/SA_d)\cdot 100$; where SA_d and SA are the specific activities in the presence and absence of detergent, respectively). It was found that S-warfarin (11S-OH) reductase was markedly latent (71 %) whereas, progesterone (20β-OH) reductase was slightly inhibited by both detergents tested as shown in Table 2. A similar, minor inhibition by these detergents was also observed for progesterone (20α-OH) reductase activity (not shown).

An additional test to distinguish the topology for enzymes catalyzing the different reductase activities involved measurements of their proteolytic susceptibility as a function of digestion time for microsomes in the presence of trypsin (see Figure 2). None of the three activities evidenced the same behavior. A monophasic decay for the progesterone (20β-OH) reductase occurred which had a half-life that was only 3-5 times longer than those observed for CYP activities (not shown). A rapid, initial activation phase was observed for the S-warfarin (11S-OH) reductase activity which was followed by a relatively slow decay phase which had an apparent half-life about five times longer than that for the progesterone (20β-OH) activity. The maximal observed activation of the S-warfarin reductase activity with tryptic digestion was about 50% higher than the 0-time control. A rapid decay phase

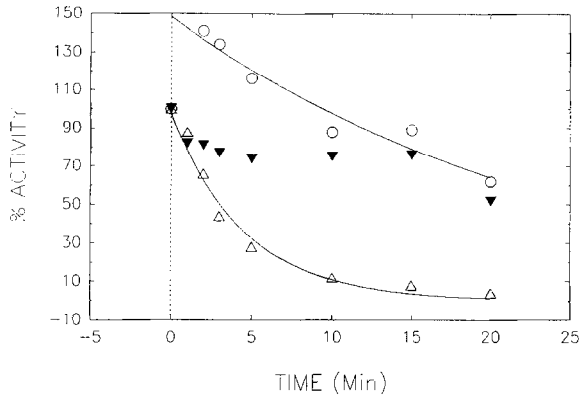

Figure 2. Time course of reductase activity changes for hepatic microsomes during trypsinolysis. Microsomes were from adult-male F344 rats. 100 % activity is the zero-time control (0.132 nmole/min/mg protein for S-warfarin (11S-OH) reductase (Circles); 1.71 nmole/min/mg protein for progesterone (20β-OH) reductase (Triangles); 0.226 nmole/min/mg protein for progesterone (20α-OH) reductase (filled inverted triangles)). Theoretical curves are for first order decays with half lives of 16.5 and 3.2 minutes characterized data for 11S-OH-9S-warfarin (excluding the 0-time value of 100%) and 20β-OH-progesterone data (excluding the 15 and 20 minute points), respectively. These curves also gave best-fit 0-time intercepts that were 148 ±9% and 97 ±8% for the formation of 11S-OH-9S-warfarin and 20β-OH-progesterone activities, respectively.

(to about 75% of the control activity) was observed for progesterone (20α-OH) reductase and this was followed by a much slower phase that appeared to mimic the slow decay phase for S-warfarin reductase activity.

Considering the excellent correlation between the male-specific reductase activities with progesterone and S-warfarin (Table 1 and Figure 1), the gross disparities in their behavior with detergents and trypsin (Table 2 and Figure 2) were not expected. Nevertheless, the latter findings do not disprove the hypothesis that the same enzyme catalyzes both of the male-specific reactions since other differentiating factors for the two activities might be involved; e.g., substrate delivery systems (FABPs ?) might be different for S-warfarin and progesterone regarding their functional stability in the presence of detergents or trypsin.

For ACI, F344, SHR and SHRsp rats, the adult male-specific microsomal reduction of S-warfarin by NADPH in the presence of O_2 represent 51 ±2% of the total observed *in vitro* metabolism (*i.e.*, the sum of reductase and CYP-generated products; data not shown) of this substrate. The significance of S-warfarin reduction to its 11S-alcohol is also suggested by the facts that warfarin alcohols are pharmacologically less active than the parent drug and that cytoplasmic reductases greatly prefer reducing the R-enantiomer (23). Therefore, it is reasonable to suggest that the reduction of S-warfarin by hepatic microsomes from adult male rats may be an important detoxification mechanism *in vivo*.

The reduction of progesterone to its 20β-OH product by microsomes from adult male rats (*i.e.*, not including WF and WKY strains) represents 17 ±3% of the total CYP plus reductase metabolites. A previous report on a cytosolic progesterone (20β-OH) reductase from neonatal pig testes indicated that the purified enzyme had a specific activity of 3 nmoles/min/mg protein (24) which is only about three times larger than the specific activity found in microsomes (see Table 1). Therefore, it is possible that the microsomal activity studied here also represents a significant reaction *in vivo*. On the other hand, the microsomal progesterone (20α-OH) reductase activity generates only 2.4 ±0.2% of the total metabolic CYP plus reductase activity and had a specific activity that was about 10^4-fold lower than that reported for a purified cytosolic enzyme from rat ovary which catalyzes the same reaction (25).

The reductase activities producing 20β-OH-progesterone and 11S-OH-9S-warfarin studied here might correspond with other adult male activities previously reported to characterize rat-hepatic microsomes which include "Sulphate Specific 20β-Reductase" (19), acetohexamide reduction by "Carbonyl Reductase" (20), 2-(2-Amino-5-bromobenzoyl)-pyridine reduction by "Carbonyl Reductase" (21) and "Glycyrrhetinate Dehydrogenase" (22). Since all of these activities prefer NADPH>NADH, it will be interesting to test whether they also correlate with the adult male activities in the present study respecting strain-dependence and the effect of PB. In this regard, it has already been shown that 3-keto-glycyrrhetinate (3β-OH) reductase activity was 66% repressed after treatment with PB (22).

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