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CYTOCHROME *P*450s IN THE GUINEA PIG ADRENAL THAT ARE IMMUNOLOGICALLY SIMILAR TO LIVER FORMS: ESTROGEN SUPPRESSION EXPLAINS MALE–FEMALE DIFFERENCES

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Summary—Several cytochrome *P*450s have been identified in guinea pig adrenal microsomes which are distinct from the known steroidogenic *P*450s, c17 and c21, and are immunochemically related to cytochrome *P*450s found in liver. One, a 52 K protein related to *P*450 I (CYP1), occurs almost exclusively in males, is localized to the inner zone, and is suppressed by ACTH. Its levels correlate with microsomal capacity for xenobiotic metabolism. The others, related to *P*450s II and III (CYP2 and 3), are more predominant in males, but not exclusive to them, are found in both the inner and outer zones, and are not suppressed by ACTH. Their functions remain to be elucidated. The male predominance of the CYP1-related protein has recently been shown to be due to suppression of the protein in females by estrogen. To determine if estrogen is also involved in the regulation of the CYP2-related proteins, ovariectomized and sham-operated animals were treated with a long-acting estrogen, estradiol valerate, or with the vehicle alone. These *P*450s reached male levels in ovariectomized females treated only with the vehicle. Their enhanced levels were suppressed by treatment with estrogen. Estrogen treatment also suppressed the levels of the *P*450s seen in sham-operated females. Endogenous estrogen produced similar effects. In hemi-ovariectomized females the contralateral ovary hypertrophied, a state in which estrogen levels would be maintained or increased. In these females no increase occurred in the immunodetectable *P*450s. In normal females, estrogen levels are low in prepubertal animals, rise at the time of puberty and drop again after ovarian cycling is completed. The CYP2-related proteins were present in adrenal microsomes of prepubertal females, but were suppressed after puberty. On the other hand, post-estrous females, in whom estrogen levels would be low, acquired male levels of these proteins in their adrenal microsomes. *P*450c17 and *P*450c21, as well as 3β-hydroxysteroid dehydrogenase, were not affected by surgery or estrogen. Taken together, these experiments indicate that suppression by estrogen in females can account, in large part, for the predominance of several immunochemical homologs of liver *P*450s in adult male guinea pig adrenals.

INTRODUCTION

Adrenocortical microsomes conduct cytochrome *P*450-mediated reactions involved not only in sterol and steroid synthesis, but also in the metabolism of steroids and foreign compounds [1–11]. Detailed analyses from many laboratories have contributed to the understanding of adrenal steroidogenic *P*450s [for reviews see 1–3], but relatively little is known about the *P*450s involved in the other functions [4–6]. In the rat adrenal low levels of immunodetectable *P*450 b and e (CYP2B1 and 2) and *P*450 c (CYP1A1) are present [7]. However, the majority of the metabolism of polycyclic aromatic hydrocarbons such as 7,12-dimethylbenz(*a*)anthracene (DMBA) by rat adrenocortical microsomes appears to be due to a unique 57 K cytochrome *P*450 [8]. Sex differentiation of DMBA metabolism in the rat adrenal has been reported [5], but has not been confirmed (Jefcoate, personal communication). In guinea pig adrenal microsomes we have identified a number of cytochrome *P*450s, distinct from the microsomal steroidogenic enzymes *P*450c17 and *P*450c21, that are immunochemically related to the CYP1, 2, and 3 families [9]. Although there is variability in their relative ratios, all are more prominent

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The nomenclature for cytochrome *P*450s used in this paper is as follows: $P450_{17\alpha}$ = *P*450c17; CYP17; $P450_{21}$ = *P*450c21, CYP21A1; $P450_{c,d}$ = *P*450c,d, *P*450 1A1,2, CYP1A1,2; $P450_{b,e}$ = *P*450b,e, *P*450 2b1,2, CYP2B1,2; $P450_{PB\text{-}1}$ = *P*450PB1, *P*450 2C6, CYP2C6; $P450_{2C}$ = *P*4502c, *P*450 2C11, CYP2C11; $P450_{PCN\text{-}E}$ = *P*450pcn1, *P*450 3A1, CYP3A1.

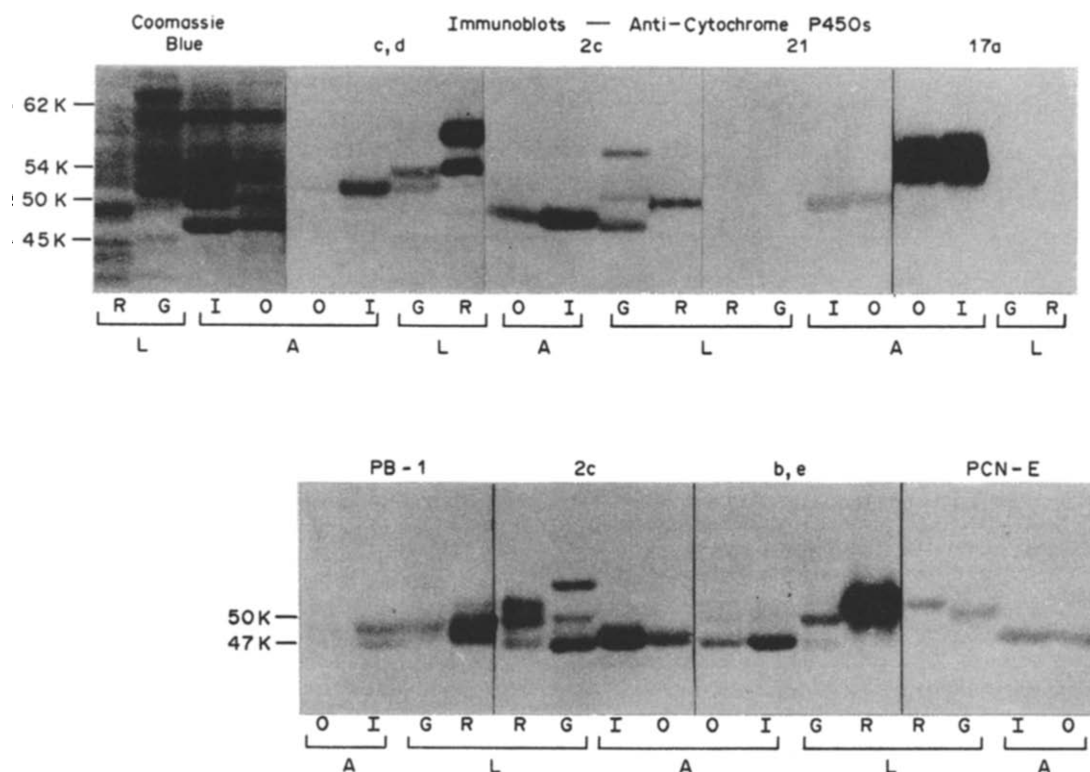

Fig. 1. Western blots of microsomal proteins using anti-cytochrome *P*450s and corun lanes stained with Coomassie blue. Microsomes from rat (R) and guinea pig (G) liver (L) are compared with those from male guinea pig adrenal inner (I) and outer (O) zones. Rat liver microsomes in the Coomassie blue and anti-*P*450c,d panels were from methylcholanthrene-treated animals. In the other panels the rat liver microsomes were from animals treated with phenobarbital. All guinea pig microsomes were from untreated animals.

in males than in females and are present in higher levels in the inner than in the outer zone [9, 10]. One, a 52 K protein related to the CYP1 family is found almost exclusively in adult males and in the inner zone [10, 11]. Its suppression, rather than stimulation, following ACTH treatment links it with the reported activity for xenobiotic metabolism in the guinea pig adrenal [9, 10]. The cytochrome *P*450s immunochemically related to the CYP2 and 3 families are not suppressed by ACTH [9]. Their functions remain to be determined. We have recently presented evidence that the CYP1-related protein is suppressed by estrogen in female guinea pig adrenals [12]. In this study we focus on the effects of estrogen on the levels of the CYP2 homologs. In the liver, several cytochrome *P*450s capable of metabolizing foreign compounds also metabolize steroids to less active compounds [13, 14]. That their immunochemical relatives in the guinea pig adrenal have steroid metabolizing capacities is a hypothesis which remains to be tested.

EXPERIMENTAL PROCEDURES

English short-haired (Hartley) guinea pigs were obtained from Camm Research Labs (Camden, NJ) and fed standard lab chow *ad libitum* in a controlled lighting environment (light on, 6 a.m., light off, 7 p.m.). Sexually mature male and female guinea pigs weighing 600–800 g were used. They were compared with ovariectomized and sham-operated females, as well as with females of increasing age, spanning prepubertal to retired breeder (250–1300 g). Ovariectomy was performed via bilateral dorsal incisions. The ovary, oviduct and companion vessels were drawn out through the incision, the oviduct and vessels tied off with surgical thread, and the ovary excised. The duct and vessels were then returned to the peritoneal cavity and the incision closed with surgical clips. In sham-operated animals an incision was made, but the gonads were not disturbed so as to not compromise their vascular supply. In all cases blood loss was minimal and healing rapid. Some of the gonadectomized and sham-operated

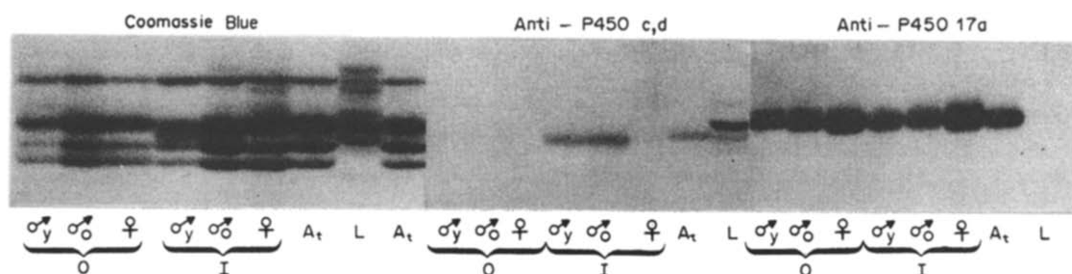

Fig. 2. Coomassie blue-stained gel and Western blots of corun lanes comparing the distribution of microsomal proteins detectable with anti-$P450$c17 and anti-$P450$c,d in outer (O) and inner (I) adrenocortices of female (♀) and both younger ($♂_y$, 450 g) and older ($♂_o$, 800 g) male guinea pigs. Microsomal proteins obtained from the whole adrenal (A_t) and liver (L) are shown for comparison.

females were left untreated. Others were injected with the long-acting compound, estradiol valerate (β-estradiol 17-valerate, Sigma Chem Co., St Louis, MO) (0.5 mg/100 g body wt in sesame oil, s.c.), or with the vehicle alone, the day after surgery. At least three animals were included in each group. After 2 weeks, subcellular fractions were prepared and analyzed for protein, by SDS–PAGE and by immunoblotting [11]. The 8% gels were loaded with 12 μg of microsomal protein per lane in all cases. Antibodies made against rat liver cytochrome $P450$s were received from Drs M. Negishi, M. Adesnik and T. Morimoto ($P450$ c and d), Dr M. Adesnik ($P450$ b and e), and Dr David Waxman ($P4502$c and $P450$PB1) [10]. Antibodies to $P450$c17, and $P450$c21 were received from Dr Peter Hall [9–11]. Antibodies to some of these proteins were also purchased from Oxygene (Dallas, TX). Antibody to 3β-hydroxysteroid dehydrogenase (3βHSD) was obtained from Dr John Stavley [15]. All were polyclonal antibodies. Control blots were incubated with nonimmune sera. Representative immunoblots are shown for each experiment.

RESULTS

Guinea pig adrenals contain several proteins which are detectable with antibodies made against rat liver cytochrome $P450$s (Fig. 1) [9]. One, 52 K, reacts with antibody recognizing the two members of the CYP1 family, $P450$ c and d. Several, 47–50 K, react with antibody recognizing members of the CYP2 family: $P450$s b and e, PB1, and 2c. Another, 48.5 K, reacts with antibody recognizing a member of the CYP3 family, PCN-E (pcn1). All of these immunodetectable proteins are more prominent in microsomes of the inner zona reticularis than in those of the combined outer zones, glomerulosa and fasciculata. This inner zone predominance is most distinct for the protein related to CYP1 and least so for that related to CYP3. With the exception of the latter, anti-$P450$ pcn1 detectable protein, all comigrate with protein(s) detectable in guinea pig liver microsomes (Fig. 1). While we cannot be certain of their precise relationship to their immunochemical relatives in the liver, the identification of these proteins as cytochrome $P450$s has been established by their suppression following treatment with spironolactone [9, 11], a compound whose metabolite(s) destroys cytochrome $P450$s in the guinea pig adrenal [16]. The sole exception was the larger of the two proteins detectable with anti-$P450$PB1 [9]. All of the immunodetectable $P450$s are more prominent in microsomes from males than in those from females, with the CYP1-related protein being the

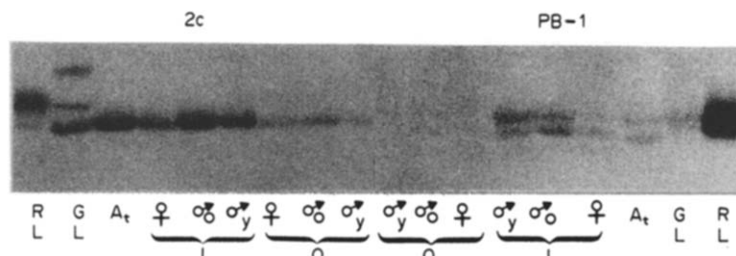

Fig. 3. Western blots of adrenal microsomes illustrating differences in distribution of anti-$P4502$c and anti-$P450$PB1 detectable proteins between the inner (I) and outer (O) zones of adult female (♀) and both young ($♂_y$, 450 g) and adult ($♂_o$, 800 g) male guinea pigs. Microsomes obtained from whole adrenals (A_t) and from rat (R) and guinea pig (G) liver (L) are shown for comparison.

Anti – P450s
b, e 2c PB-1

O S S O O S S O O S S O O S S O O S

Fig. 4. Western blots comparing the levels of immunodetectable cytochrome P450s in adrenal microsomes from three ovariectomized (O) and three sham-operated (S) females.

Anti – P450s 17α 2c PB-1 c, d a 3β HSD

S S O O O O S S S S O O O O S S S S O O
EV: + – + – – + – + + – + – – + – + + – + –

Fig. 5. Western blots comparing the levels of immunodetectable cytochrome P450s in adrenal microsomes from ovariectomized (O) and sham-operated (S) females treated with estradiol valerate (EV+) or with the vehicle alone (EV–).

Anti – P450s : PB – 1 2c

S S O O O 10 8 4 3 2.5 S S O O O 10 8 4 3 2.5
+ – + – hemi x 10^2 g + – + – hemi x 10^2 g

Fig. 6. Western blots comparing immunodetectable P450s in adrenal microsomes from females of increasing age, prepubertal (250–400 g), sexually mature (800 g), and retired breeder (1000 g) (2.5–4, 8, 10 × 10^2 g, respectively), with those in adrenals of hemi-ovariectomized (hemi-O) females and ovariectomized (O) vs sham-operated (S) animals treated with estradiol valerate (+) or with the vehicle alone (–).

most distinctly sex-differentiated (Figs 2 and 3) [9, 10]. Cytochromes P450c21 (not shown) and P450c17 do not show such a marked difference in distribution between the zones or between males and females (Fig. 2).

When female guinea pigs were ovariectomized, the levels of all of the cytochrome CYP2-related P450s, as did the CYP1-related proteins [12], increased over those seen in corresponding sham-operated animals. They approached the levels found in male adrenal microsomes (Fig. 4). Treatment of females with the long-acting estrogen, estradiol valerate, suppressed the levels of the CYP1- and 2-related proteins in both ovariectomized and sham-operated females (Figs 5 and 6). The only protein which did not change with ovariectomy and hormone replacement was the larger protein reacting with anti-P450PB1, which as noted above was not suppressible by spironolactone. Neither immunodetectable P450c17 or P450c21, nor protein detectable with antibody to 3βHSD, another microsomal enzyme involved in steroidogenesis, showed any significant change following surgery or estrogen treatment. The broad band reacting with anti-P450c17 can be resolved into two closely

migrating proteins, as shown in Fig. 2, and is typical of guinea pig adrenocortical microsomes [9–11].

The changes produced by treatment with estradiol valerate reflect those produced by endogenous estrogen. For example, the CYP2-related proteins were detectable at male levels in prepubertal females (250–400 g), but were suppressed after puberty, which occurs in females of about 450 g (Fig. 6). In older females, in whom ovarian cycling was no longer occurring, male levels of these proteins were again achieved (Fig. 6). This post-estrous increase was also seen in the CYP1-related protein. Hemi-ovariectomy, which resulted in hypertrophy of the contralateral gonad, maintained the lower protein levels seen in sexually mature females (500–800 g), in ovariectomized females treated with estrogen, and in sham-operated females (Fig. 6).

DISCUSSION

Sex differentiation of a group of microsomal cytochrome $P450$s in the guinea pig adrenal which are distinct from $P450$c17 and $P450$c21 has been previously reported [9, 10]. This paper supports a role for estrogen in the creation of their male predominance. Ovariectomy resulted in increased levels of these hepatic cytochrome $P450$ homologs in the female guinea pig adrenal. The increases were prevented by estrogen administration. Changes in endogenous estrogen produced similar results. Levels of the $P450$s seen in prepubertal females were suppressed by increased estrogen levels at the time of puberty. In post-estrous females, in whom estrogen levels would be low, the proteins increased to approximate levels seen in normal males. On the other hand, in hemi-ovariectomized females, where hypertrophy of the contralateral ovary maintains estrogen production, the low levels of the proteins normally seen in sexually mature females were maintained. These results indicate that the sex differences (male > female) in levels of these $P450$s is a result of suppression of their expression by estrogen in females. The steroidogenic $P450$s, on the other hand, showed no such sex differentiation and were not influenced by ovariectomy or estrogen, consistent with observations in other species [17].

In the rat liver there are several cytochrome $P450$s whose levels of expression differs in males and females [13, 14]. In all cases, male predominance is produced by testosterone imprinting, not estrogen suppression [13, 14]. Ovariectomy of female rats does not alter the levels of these proteins [13]. This is quite distinct from the results reported here for male predominant cytochrome $P450$s in the guinea pig adrenal where ovariectomy results in increases that can be suppressed by estrogen replacement. Although some positive effect of testosterone cannot be ruled out, the effects of estrogen reported here seem sufficient to account for male predominance. In rat liver, the effects of testosterone are often mediated via growth hormone [13, 14]. Testosterone imprinting produces highly pulsatile secretion of growth hormone (GH) in males. GH secretion in females is more uniform. Whether the male predominance of the guinea pig adrenal cytochrome $P450$s involves direct effects of sex steroids or is mediated by pituitary hormones remains to be determined.

The liver homologs of the $P450$s found in the guinea pig adrenal have well-recognized capacities for metabolism of foreign compounds. At least one of the adrenal $P450$s, the CYP1-related protein, correlates with the capacity for xenobiotic metabolism [9]. If the others have similar functions, the higher levels of these proteins in the inner zone may explain the greater sensitivity of this region to toxic compounds [18]. On the other hand, their zonal distribution may be physiologically relevant. Several of the sex-differentiated cytochrome $P450$s in the rat liver are capable not only of metabolizing various foreign compounds, but also of hydroxylating particular sites on the steroid ring [13, 14]. For example, microsomal CYP3A1 ($P450$pcn1) and 3A2 are capable of 6β-hydroxylation of testosterone, CYP2B1 ($P450$b) is capable of 16β-hydroxylation of testosterone and CYP2C11 ($P4502$c) is capable of 2α- and 16α-hydroxylation of testosterone. In addition, purified CYP1A1 ($P450$c) and $P450$g are able to perform 6β-hydroxylation [13] and recombinant CYP1A2 expressed in Hep G2 cells can hydroxylate estradiol in the 2α and 4α positions [19]. In most cases, these hydroxylations are thought to create less active steroids, which are excreted from the body. However, the catecholestrogens formed by 2- and 4-hydroxylation of estrogen can be oxidized to quinones and have been linked with estrogen-induced cancer [20], as has a 16α hydroxylated derivative of estradiol, 16α-hydroxyestrone [21]. In the guinea pig adrenal, the outer zones are more active in steroidogenesis than the inner zona reticularis [22]. The predominance of immunochemical homologs of liver $P450$s in the guinea pig adrenal inner zone places

them in an ideal anatomical position to metabolize steroids produced by the outer zones before they leave the adrenal. If these cytochrome *P*450s do possess site specific steroid metabolic capacities they may serve as intraadrenal regulators of total adrenal output of metabolically active steroids. This hypothesis is currently being tested.

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