MATURATIONAL CHANGES IN CYP2D16 EXPRESSION AND XENOBIOTIC METABOLISM IN ADRENAL GLANDS FROM MALE AND FEMALE GUINEA PIGS

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ABSTRACT:

CYP2D16 is expressed at high levels in the zona reticularis (ZR) of guinea pig adrenal glands and contributes to adrenal metabolism of xenobiotics. Studies were done to evaluate the effects of age and gender on adrenal CYP2D16 expression and xenobiotic metabolism. In both male and female guinea pigs at 1, 7, 14, or 30 weeks of age, in situ hybridization and immunohistochemistry confirmed that CYP2D16 was highly localized to the ZR of the adrenal gland. The steroidogenic P450 isozyme, CYP17, by contrast, was expressed in both the zona fasciculata and ZR. The intensity of CYP2D16 staining was not age- or gender-dependent. However, the proportion of each adrenal gland comprised by ZR and thus expressing CYP2D16 increased with aging in both sexes and was greater in males than in females. The rates of metabolism of bufuralol, a CYP2D-selective substrate, by adrenal microsomal preparations generally correlated with the amount of ZR (and CYP2D16) in the gland. Thus, adrenal xenobiotic-metabolizing activities were greater in males than in females at all ages and increased with aging in males. However, the rates of bufuralol metabolism declined in sexually mature females (14 weeks) from the levels found in prepubertal females (7 weeks) and then increased markedly in retired breeders (30 weeks), suggesting an inhibitory effect of estrogens on enzyme activity. The results indicate that the age and gender differences in adrenal CYP2D16 content are largely determined by differences in the size of the ZR rather than the concentrations of CYP2D16 within cells of the ZR. However, adrenal xenobiotic-metabolizing activities in females seem to be further modulated by an inhibitory effect of estrogens.

The mammalian adrenal cortex consists of three anatomical zones, the outermost zona glomerulosa, the intermediate zona fasciculata (ZF), and the innermost zona reticularis (ZR). Each of these zones has a unique role in the production of steroid hormones by the gland (Colby, 1987; Hanukoglu, 1992). The zona glomerulosa is the sole site of aldosterone synthesis, and adrenal androgens are secreted principally by the ZR. Glucocorticoids such as cortisol are produced by both the ZF and ZR, with the former dominating. The functional differences among the zones are attributable principally to the selectivity of steroidogenic enzyme expression in each zone (Hanukoglu, 1992; Keeney and Waterman, 1993).

In addition to synthesizing steroid hormones, the adrenal cortex can metabolize a variety of foreign compounds (Colby and Rumbaugh, 1980; Hallberg, 1990). Adrenal metabolism of xenobiotics may serve a protective function by detoxification of exogenous substances or, conversely, may convert relatively innocuous chemicals to reactive metabolites, causing adrenal toxicity (Hallberg, 1990; Colby and Longhurst, 1992; Colby et al., 1996). The capacity for adrenal metabolism of xenobiotics appears to be species-dependent. Very high levels of activity have been demonstrated in human fetal and guinea pig adrenal glands, with adrenal enzyme activities sometimes exceeding those in liver (Kupfer and Orrenius, 1970; Juchau and Pedersen, 1976; Eacho and Colby, 1983). In other species studied, varying amounts of adrenal xenobiotic metabolism are demonstrable (Colby and Rumbaugh, 1980; Hallberg, 1990; Colby and Longhurst, 1992; Colby et al., 1996).

The adrenal enzymes that catalyze foreign compound metabolism have not been studied as extensively as those found in the liver. We identified and cloned a P450 isozyme, CYP2D16, that is expressed at high levels in guinea pig adrenal microsomes and which contributes to xenobiotic metabolism in the gland. CYP2D16 is highly localized to the ZR of the guinea pig adrenal cortex as is xenobiotic-metabolizing activity (Jiang et al., 1995, 1996a; Yuan et al., 1997). In addition, both adrenal enzyme activities and CYP2D16 concentrations are similarly regulated by several physiological variables, including adrenocorticotropic, the major hormonal modulator of the adrenal cortex (Jiang et al., 1996b; Yuan et al., 1998). Metabolism of bufuralol, a CYP2D-selective substrate, by guinea pig adrenal microsomes further implicates CYP2D16 in adrenal xenobiotic metabolism (Jiang et al., 1996a,b).

Prior studies demonstrated that adrenal xenobiotic metabolism in guinea pigs varied with age and gender. Enzyme activities are greater in males than in females and tend to increase with aging in both sexes (Martin and Black, 1983; Black et al., 1989). The studies presented here were done to pursue the mechanisms involved by determining if CYP2D16 contributed to the age and gender dependence of adrenal xenobiotic metabolism in guinea pigs.
**Experimental Procedures**

**Materials.** 3-Aminopropyltriethoxysilane, proteinase-K, 3,3'-diaminobenzidine tetrahydrochloride, DNase, and RNase were purchased from Sigma Chemical Co. (St. Louis, MO). Normal rabbit serum and the HistoMark kit including the blocking solution, biotin-labeled goat anti-rabbit antibody, and horseradish peroxidase-labeled streptavidin were obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Normal rabbit IgG was from Vector Laboratories (Burlingame, CA). Anti-rat P4502D1 antiserum and anti-guinea pig P450c17 IgG were kindly provided by Dr. Y. Funae (Osaka City University Medical School, Osaka, Japan) and Dr. S. Takemori (University of Hiroshima, Hiroshima, Japan), respectively (Ishibashi et al., 1988; Ohishi et al., 1993). The in situ hybridization kit including fluorescein-dUTP terminal transferase, caccodylate buffer, control unlabeled probe, hybridization buffer, blocking agent, anti-fluorescein alkaline phosphatase conjugate, nitroblue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) was purchased from Amersham Pharmacia Biotech (Arlington Heights, IL). Triethanolamine was obtained from Roche Molecular Biochemicals (Indianapolis, IN).

**Animals.** Male and female English Short-Hair guinea pigs were obtained from Camm Research Institute (Wayne, NJ). Animals were maintained under standardized conditions of light (6:00 AM–6:00 PM) and temperature (22°C) on a diet of Purina Mills (Richmond, IN) Guinea Pig Diet and water ad libitum. Guinea pigs were killed in the morning by CO2 inhalation, and the adrenal glands were quickly removed. One adrenal from each animal was placed in 1.15% KCl-0.05 M Tris-HCl (pH 7.4) on ice for use in enzyme assays and Western blotting. A 2-mm section from the middle part of the other adrenal was fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline at 4°C and processed for histological analyses as described below. Adrenals from four guinea pigs were included in each experimental group.

**Enzyme Assays.** Whole adrenals were homogenized in KCl-Tris buffer, and microsomal fractions were obtained by differential centrifugation. Bufuralol 1'-hydroxylase activity was assayed by high-performance liquid chromatography as the rate of conversion of bufuralol to 1'-hydroxybufuralol (Kronbach et al., 1987). The metabolite was monitored by UV absorbance at 247 nm. Ethoxycoumarin O-deethylase activity was determined by the rate of formation of the fluorescent metabolite, 7-hydroxyethoxycoumarin; fluorescence was measured at excitation and emission wavelengths of 368 and 456 nm, respectively (Greenlee and Poland, 1978). Steroid 17α-hydroxylase activity was determined as the rate of conversion of progesterone to 17α-hydroxyprogesterone plus 11-deoxycorticisol by adrenal microsomes. Steroid metabolites were separated by high-performance liquid chromatography and quantified by UV absorbance at 254 nm as described previously (Colby et al., 1993). The conditions of the enzyme assays were established to ensure linearity of product formation with respect to protein concentrations and incubation times.

**SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analyses.** Electrophoresis of adrenal microsomal proteins was done as described previously (Jiang et al., 1995, 1996). Briefly, 10 µg of microsomal protein were separated on an 8% polyacrylamide gel made with an acrylamide-bisacrylamide ratio of 19:1. Electrophoresis was done under constant voltage (125 V for stacking gel and 250 V for separating gel) in half-strength Laemmli tank buffer (12.5 mM Tris, 125 mM glycine, and 0.1% SDS). Visualization of protein bands was achieved by Coomassie blue staining. Western blotting analyses were done using goat anti-rabbit IgG coupled to alkaline phosphatase with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium as substrate for detection. To check protein transfer and to match Coomassie-stained protein bands with immunoreactive proteins, the nitrocellulose membranes were stained with Poncieux S for 10 min and then rinsed with nanopure water before incubation with primary antibodies. In microsomes from both male and female guinea pigs, P4502D1 antiserum was immunoreactive only with the protein band previously identified as CYP2D16 (Jiang et al., 1996). Quantitation of immunoreactive bands as well as analyses of immunohistochemical and in situ hybridization staining intensities were done with a Multiscan-R video densitometry system (Interactive Technologies International, St. Petersburg, FL), using one-dimensional video densitometry software.

**Light Microscopic Immunohistochemistry.** Immunohistochemical analyses were done as described previously (Yuan et al., 1997). The anti-P4502D1 antiserum and anti-P450c17 IgG were previously characterized for their specificities (Ishibashi et al., 1988; Ohishi et al., 1993). To establish the specificity of the staining, control sections were incubated with dilutions of normal rabbit serum or normal rabbit IgG equivalent to those of the P4502D1 antiserum and P450c17 IgG, respectively.

**In Situ Hybridization.** In situ hybridization studies were done with a mixture of four antisense oligonucleotides corresponding to different regions of P4502D16 as described previously (Yuan et al., 1997). The specific binding of the probes was detected with anti-fluorescein alkaline phosphatase conjugate.
gate, and nitroblue tetrazolium/BCIP was used for color development. Negative controls used for in situ hybridization included 1) incubation in solution containing each sense oligo (complementary to the antisense sequences), 2) DNase or RNase pretreatment of the slides before hybridization, and 3) M13 forward-sequencing primer (nonspecific probe).

Image Analyses. The microscopic image of each adrenal gland was projected onto a video screen, and the borders of the stained areas and those of the total gland were traced onto a transparency sheet. The transparency was then photocopied, and the areas corresponding to the stained and unstained regions were cut out and weighed. The percentage of the whole adrenal cortex comprised by the stained area was calculated for each sample.

Data Analyses. All data are expressed as means ± S.E.M. Statistical analyses of differences between group means were done with the Newman-Keuls multiple-range test; p < 0.05 was considered significant.

Results

Adrenal bufuralol 1'-hydroxylase and ethoxycoumarin deethylase activities were low in 1-week-old male guinea pigs but increased progressively with age (Figs. 1 and 2). The greatest incremental change occurred between 1 and 7 weeks. In females, xenobiotic-metabolizing activities were similarly low at 1 week of age and increased approximately 10-fold by 7 weeks but declined in sexually mature (14 weeks) animals. In female retired breeders (~30 weeks), adrenal bufuralol 1'-hydroxylase and ethoxycoumarin deethylase activities were 5- to 6-fold greater than in 14-week-old animals (Figs. 1 and 2). At all ages after 1 week, enzyme activities were significantly greater in males than in females.

In contrast to the age-related changes in adrenal xenobiotic metabolism, steroid 17α-hydroxylase activity was relatively unaffected by age or gender (Fig. 3). Activity increased somewhat between 1 and 7 weeks of age in both males and females. 17α-Hydroxylation did not further change after 7 weeks of age, and activities were similar in males and females at all ages (Fig. 3).

Immunoblotting, immunohistochemical, and in situ hybridization analyses were done to investigate the relationship of CYP2D16 to the age and gender dependence of adrenal xenobiotic metabolism. Western blots of microsomal preparations from whole adrenal glands demonstrated increasing concentrations of CYP2D16 with age in males and females, and higher levels in males than in females at all

Fig. 3. Age and gender effects on adrenal 17α-hydroxylase activities in guinea pigs.

Values are means ± S.E.M. of four animals per group.

Fig. 4. Quantitative analyses of age and gender effects on microsomal CYP2D16 protein levels in the guinea pig adrenal cortex.

Protein concentrations were determined by densitometric analysis of Western blots as described under Experimental Procedures. Values are the means of four animals per group and are expressed as a percentage of the 30-week-old male value ± S.E.M.

Fig. 5. Localization of CYP2D16 and CYP17 in the guinea pig adrenal cortex.

Sections are from a 14-week-old male guinea pig. A and B, immunostaining with anti-CYP17 IgG (A) or normal rabbit IgG (B). C and D, immunostaining with anti-CYP2D1 antisera (C) or normal rabbit serum (D). E and F, in situ hybridization with CYP2D16 antisense oligonucleotide probe (E) or sense oligonucleotide probe (F). Bar, 450 μm. ZG, zona glomerulosa; M, medulla.
ages (Fig. 4). Thus, adrenal CYP2D16 content generally paralleled xenobiotic-metabolizing activities except for the absence of any decline in 14-week-old females. CYP17 protein concentrations did not vary significantly with age or gender (not shown).

As reported previously (Yuan et al., 1997), immunostaining for CYP2D16 protein as well as in situ hybridization demonstrated that CYP2D16 was highly localized to the ZR, whereas CYP17 was distributed throughout the ZF and ZR (Fig. 5). Negative controls for both techniques revealed a complete absence of staining (Fig. 5). Comparison of the immunohistochemical and in situ hybridization results with H&E-stained serial sections confirmed the anatomical localization of CYP2D16 and CYP17 in each sample (not shown).

In male and female guinea pigs, both immunohistochemical (Fig. 6) and in situ hybridization (Fig. 7) analyses revealed increasing areas of CYP2D16 staining with increasing age, corresponding with the greater size of the ZR in older animals (Ito, 1952; Black, 1972). The relative amount of adrenal cortex staining positively for CYP2D16 was somewhat greater in males than females at 1, 7, and 14 weeks of age; the gender differences increased in 30-week-old animals (Fig. 8). There were no consistent effects of either age or gender on the intensity of CYP2D16 staining resulting from immunohistochemistry or in situ hybridization (Figs. 6 and 7). In all samples, CYP17 protein was distributed throughout the ZF and ZR and the staining intensity was not age- or gender-dependent (not shown).

**Discussion**

Prior investigations demonstrated that adrenal xenobiotic metabolism in guinea pigs is age- and gender-dependent (Colby et al., 1980; Martin and Black, 1983; Black et al., 1989; Black, 1994). Enzyme activities are greater in males than in females and increase with age in both sexes. In addition, virtually all xenobiotic-metabolizing activity is localized to the innermost zone of the guinea pig adrenal cortex, the ZR (Eacho and Colby, 1983; Martin and Black, 1983; Black et al., 1989; Jiang et al., 1995, 1996a). Expression of CYP2D16 in the gland is similarly limited to the ZR (Jiang et al., 1995, 1996a; Yuan et al., 1997), suggesting a major role for this P450 isozyme in adrenal metabolism of foreign compounds.

The results presented here confirm and extend prior observations concerning the effects of age and gender on adrenal metabolism of foreign compounds. In male guinea pigs, enzyme activities increased progressively with age, as did adrenal concentrations of CYP2D16 (Fig. 4). However, the increases seem largely attributable to the disproportionate increase in mass of the ZR with aging. At 1 week of age, the ZR makes up approximately 10% of the cortex, but it increases to more than 50% in 30-week-old males (Fig. 8). Thus, the zone responsible for xenobiotic metabolism comprises a greater fraction of the gland in older animals (Ito, 1952; Black, 1972). This change in the zonal composition of the adrenal cortex seems to account for most, if not all, of the age-related increases in adrenal xenobiotic-metabolizing activities and CYP2D16 content in male
guinea pigs. The mechanism(s) responsible for this disproportionate increase in size of the ZR with age have not been resolved.

In females, the relative size of the ZR also increased with age but reached a maximum of approximately 40% of the cortex, which is less than that in males. This difference may at least partly account for the gender differences in adrenal xenobiotic-metabolizing activities in 30-week-old animals. However, the age-dependent changes in enzyme activities in females do not uniformly follow the changes in the size of the ZR. The most notable inconsistency is the decline in the rates of xenobiotic metabolism that occurs between 7 and 14 weeks of age despite growth of the ZR and an increase in CYP2D16 concentrations during the same period. Since this period approximates the time of sexual maturation in female guinea pigs, increasing estrogen production may be a contributing factor to the apparent discrepancies. Black (1994) has demonstrated that estrogen administration to guinea pigs decreases adrenal ethylmorphine demethylase activity and, like our results, found enzyme activity to be lower in sexually mature females than in prepubertal animals or retired breeders (older than 6 months). Estrogen production declines in older females, which probably accounts for the high levels of adrenal enzyme activity in 30-week-old animals. Thus, the age-related changes in adrenal xenobiotic metabolism in females seem to reflect an inhibitory effect of estrogens superimposed upon an increasing mass of ZR that would otherwise increase metabolism. The decrease in adrenal bufuralol metabolism concomitant with an increase in CYP2D16 protein content in sexually mature females suggests the possibility that estrogens exert their effects at a post-translational level. It is also possible that another, as yet unidentified P450 isozyme contributes to adrenal xenobiotic metabolism in female guinea pigs. Further studies are now needed to determine the specific mechanism(s) involved.

The age-related changes in the zonal composition of the guinea pig adrenal cortex may affect steroid as well as xenobiotic metabolism. Because CYP17 is expressed in both the ZF and ZR, changes in the ratio of these two zones had little impact on overall adrenal CYP17 concentration or 17α-hydroxylase activity. However, activity of the rate-limiting reaction in cortisol synthesis, cholesterol side chain cleavage, is far greater in the ZF than the ZR of the guinea pig adrenal cortex (Colby and Eacho, 1984; Eacho and Colby, 1985). Consequently, the ZF is the major source of cortisol synthesis and secretion in guinea pigs. Thus, the decline in cortisol secretion (per unit adrenal mass) that occurs with aging in guinea pigs (Fajer and Vogt, 1963; Greiner et al., 1976) may be attributable to the decreasing fraction of the gland comprised by the ZF. It is also possible that the increasing size of the ZR (and expression of CYP2D16) results in greater intra-adrenal degradation of cortisol before secretion. Steroid metabolism (testosterone 6β-hydroxylation) by other CYP2D isozymes has
previously been demonstrated (Wong et al., 1989; Schenkan, 1992), and high cortisol 2α- and 6β-hydroxylase activities are found in adrenal microsomes from guinea pigs that express high levels of CYP2D16 (Burstein et al., 1967; Huang et al., 1997). Because the pattern of adrenal blood flow directs hormones produced in the ZF through the ZR before release into the general circulation (Vinson et al., 1985), CYP2D16 may have a role in the modulation of adrenal hormone secretion. Accordingly, down-regulation of CYP2D16 by adrenocorticotropicin (Jiang et al., 1996b) could serve as a mechanism to increase adrenal steroid secretion.

References