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Strain Differences in Adrenal Microsomal Steroid Metabolism in Guinea Pigs

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We recently reported that CYP2D16, a xenobiotic-metabolizing P450 isozyme, was expressed at higher levels in adrenal microsomes from inbred Strain 13 guinea pigs than in those from outbred English Short Hair (ESH) animals. Studies were done to determine if there also were strain differences in adrenal microsomal steroid metabolism. In both inner (zona reticularis) and outer (zona fasciculata plus zona glomerulosa) zone preparations of the adrenal cortex, 21-hydroxylase activities were greater in microsomes from ESH than from Strain 13 guinea pigs. By contrast, 17α-hydroxylase activities were similar in the two strains. In both strains, 21-hydroxylase activities were greater in inner than outer zone microsomes, but the opposite was found for 17α-hydroxylase activities (outer>inner). Northern and Western analyses revealed higher levels of CYP21 mRNA and protein in adrenals from ESH than Strain 13 guinea pigs, but there were no strain differences in CYP17 mRNA or protein concentrations. Despite the zonal differences in adrenal 17α-hydroxylase and 21-hydroxylase activities, CYP17 and CYP21 mRNA and protein levels were similar in the inner and outer zones within each strain of guinea pig. The results demonstrate strain differences in microsomal steroid metabolism that are explained by differences in CYP21 expression. By contrast, the zonal differences in steroid hydroxylase activities may be attributable to post-translational mechanisms. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

The major functions of the adrenal cortex are the synthesis and secretion of various steroid hormones [1–3]. Among the enzymes required for adrenal steroidogenesis are both mitochondrial and microsomal cytochrome P450 isozymes that catalyze site-selective hydroxylations on the steroid nucleus. Because of their importance in hormone production, the steroidogenic cytochromes P450 have been extensively investigated with respect to both function and regulation [1–3].

There are also enzymes expressed in the adrenal cortex that function in xenobiotic metabolism [4–9]. The capacity for foreign compound metabolism contributes to the adrenotoxic actions of some chemicals by effecting the formation of reactive products within the gland [4–8]. It appears that most adrenal xenobiotic metabolism is catalyzed by P450 isozymes that are distinct from those involved in steroidogenesis [4–11]. However, far less is known about adrenal xenobiotic- than steroid-metabolizing enzymes.

We recently cloned a P4502D subfamily member (CYP2D16) that is expressed at high levels in guinea pig adrenal microsomes [10, 11], and may account for much of the xenobiotic-metabolizing activity in the gland. In studies on the regulation of adrenal CYP2D16, we found that expression was significantly greater in highly inbred Strain 13 guinea pigs than in the more common outbred strains such as English Short Hair (ESH) guinea pigs [12]. Thus, CYP2D16 expression was correlated with strain differences in adrenal xenobiotic-metabolizing activities [13]. The studies presented in this report were done to determine if the strain differences were limited to the expression of CYP2D16 or were also applicable to adrenal steroidogenic enzymes. The results demonstrate strain differences in adrenal microsomal steroid metabolism that are attributable to differences in CYP21 expression.

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MATERIALS AND METHODS

Materials

A partial-length guinea pig CYP17cDNA corresponding to bp 1–1280 [14] was cloned in our laboratory. A partial-length human CYP21 cDNA [15] was generously provided by Dr. Walter L. Miller (University of California, San Francisco). Anti-guinea pig CYP17 and anti-bovine CYP21 antisera were elicited in rabbits as previously reported [16]. The random primer DNA labeling kits were obtained from Stratagene (La Jolla, CA). T4 polynucleotide kinase was purchased from New England Biolabs (Beverly, MA). Tri Reagent-RNA/DNA/Protein Isolation Reagent was purchased from Molecular Research Center (Cincinnati, OH). Nitrocellulose and Nytran membranes were from Schleicher and Schuell (Keene, NH). Bufuralol and 1′-hydroxybufuralol were provided by Dr. Bruce Mico (Hoffman-LaRoche, Nutley, NJ). Goat anti-rabbit IgG, protein assay kits, and chemicals for gel electrophoresis were obtained from BioRad (Hercules, CA). Protein and RNA molecular weight standards were purchased from Promega (Pittsburgh, PA). All other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Animals and tissue preparations

Adult male guinea pigs weighing approximately 850 g were used in all experiments. English Short-Hair guinea pigs were obtained from Camm Research Institute (Wayne, NJ) and Strain 13 guinea pigs were from Crest Caviary (Prunedale, CA). Animals were killed by CO_2 inhalation and the adrenal glands were quickly removed and trimmed free of adhering fat and connective tissue. Adrenals were then bisected longitudinally, and the dark-brown inner zone, consisting primarily of zona reticularis, was dissected from the tan outer zone which is comprised of zona fasciculata and zona glomerulosa [17]. Microsomes were obtained by differential centrifugation as described previously [18].

SDS-PAGE and Western blot analyses

Polyacrylamide gel electrophoresis of adrenal microsomal proteins was done as described previously [10]. Briefly, 10 μg of microsomal protein were separated on an 8% polyacrylamide gel made with an acrylamide to 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, 0.1% SDS). Visualization of protein bands was by Coomassie blue staining. Western blot analyses were done with anti-CYP17 and anti-CYP21 according to Towbin *et al.* [19] using goat anti-rabbit IgG coupled to alkaline phosphatase with BCIP/NBT 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, then rinsed with nanopure water before incubation with primary antibodies. Quantitation of immunopositive bands was done with a Multiscan-R Video Densitometry System (Interactive Technologies International, L.C., St. Petersburg, FL), using the 1D video densitometry software.

Northern blot analyses

Total RNA was isolated using RNeasy® total RNA kits from Qiagen (Chatsworth, CA). RNA preparations (10 μg) were denatured, electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde, and transferred onto a nylon membrane by alkaline transfer [20]. The same membrane was sequentially probed with CYP21 cDNA, CYP17 cDNA, and the loading standard. Hybridization and washing were carried out under conditions described by Ausubel *et al.* [21]. The final wash was 15 min in 0.2 × SSPE and 0.1% SDS, 60°C for the CYP17 probe and 50°C for the CYP21 probe. A rat 18 s ribosomal RNA oligodeoxyribonucleotide probe, end labeled with [γ-^{32}P]ATP, was used to assess the amount and integrity of RNA loaded [22]. Hybridization with the oligodeoxyribonucleotide probe was done according to Clements *et al.* [22] with a final wash of 15 min at 50°C in 0.2 × SSPE and 0.1% SDS. All Northern blots were visualized by autoradiography following

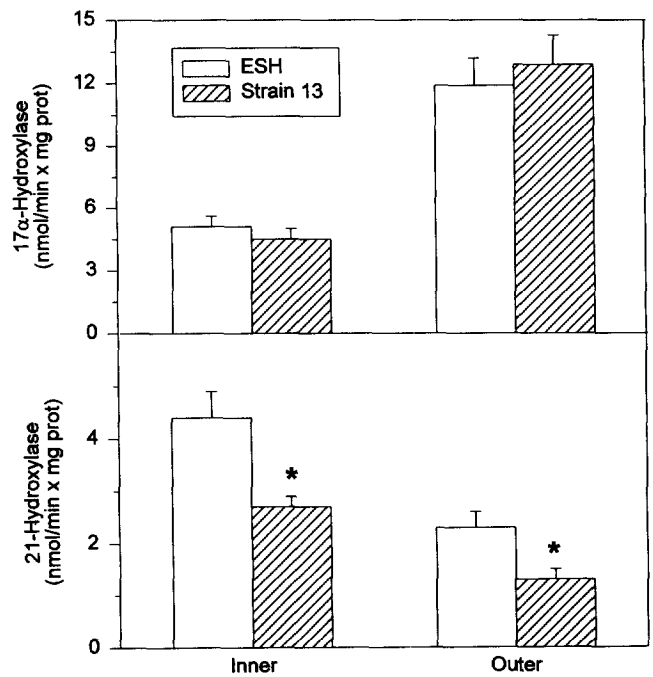

Fig. 1. Steroid 17α-hydroxylase and 21-hydroxylase activities in adrenal inner and outer zone microsomal preparations from English Short-Hair (ESH) and Strain 13 guinea pigs. Enzyme activities were determined as described in Section 2. Values are means ± S.E.M. of 3–6 animals in each group. *$p < 0.05$ (vs corresponding ESH value).

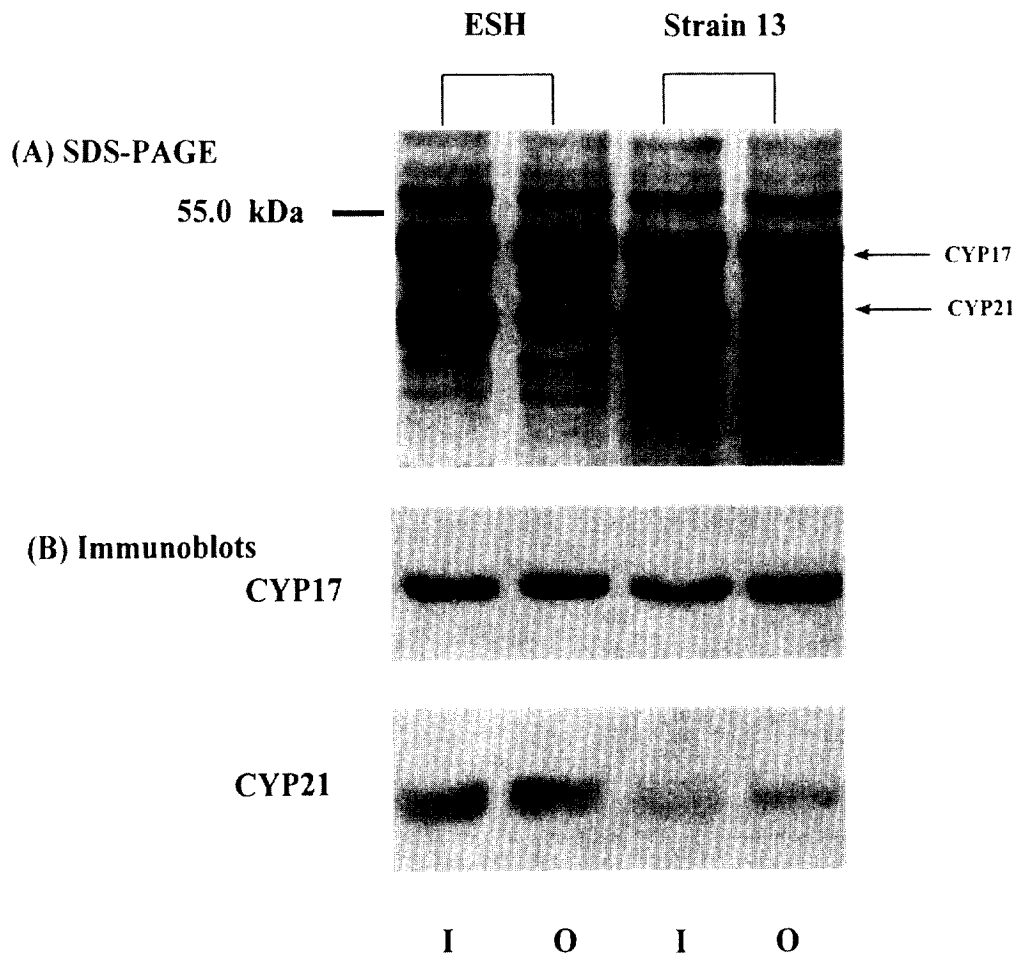

Fig. 2. SDS-PAGE and Western blot analyses of adrenal inner (I) and outer (O) zone microsomes from English Short-Hair (ESH) and Strain 13 guinea pigs. Electrophoresis was done with 10 μg microsomal protein loaded in each lane and Western blotting was done with anti-P450c17 and anti-P450c21, as described in Section 2.

film (Kodak X-OMAT-R) exposures at −70°C. Quantitation was done by video densitometry, as described above for Western blots.

Enzyme assays

Adrenal microsomal 17α-hydroxylase activity was determined as the rate of conversion of progesterone to 17α-hydroxyprogesterone plus 11-deoxycortisol, and 21-hydroxylase activity as the rate of conversion of 17α-hydroxyprogesterone to 11-deoxycortisol. Incubation conditions and HPLC analyses of metabolites were previously described in detail [18]. For all enzyme assays, conditions were established to ensure linearity of product formation with respect to protein concentrations and incubation times. Data are presented as means ± S.E.M. Statistical analyses of the differences between group means were done with the Newman–Keuls multiple-range test; $P < 0.05$ was considered significant.

RESULTS

In both English Short Hair (ESH) and Strain 13 guinea pigs, 17α-hydroxylase activities were greater in adrenal outer (zona fasciculata plus zona glomerulosa) than inner (zone reticularis) zone microsomal preparations (Fig. 1). In addition, there was no apparent strain difference in 17α-hydroxylase activity; enzyme activity in each zone was similar in Strain 13 and ESH animals. By contrast, 21-hydroxylase activities were greater in inner than outer adrenal zones in both strains of guinea pigs (Fig. 1) and in each zone, activities were greater in microsomes from ESH than in those from Strain 13 guinea pigs (Fig. 1).

To pursue the mechanisms responsible for the strain and zonal differences in steroid hydroxylase activities, CYP17 and CYP21 protein and mRNA analyses were done. As illustrated in Figs 2 and 3, Western blotting of adrenal microsomes with anti-CYP17 antiserum revealed that CYP17 protein concentrations were similar in the inner and outer zones, and did not differ between ESH and Strain 13 guinea pigs. When blotting of the same microsomal preparations was done with anti-CYP21, there were also no demonstrable zonal differences in protein concentrations (Figs 2 and 3). However, in both inner and outer zone microsomes, CYP21 protein levels were significantly greater in ESH than in Strain 13 guinea pigs, corresponding to the strain differences in 21-hydroxylase activities.

Northern analyses of adrenal RNA with a partial-length CYP17 cDNA as the probe (Fig. 4 and 5) indicated that CYP17 mRNA concentrations were similar in the inner and outer adrenal zones from both Strain 13 and ESH guinea pigs. In contrast, like CYP21 protein, CYP21 mRNA concentrations in both zones were greater in ESH than in Strain 13 adrenals, but there were no zonal differences within each strain (Figs 4 and 5).

DISCUSSION

The recent demonstration of strain differences in adrenal CYP2D16 expression in guinea pigs [12] led us to determine if the expression of microsomal steroid hydroxylases was also strain-dependent. Because steroid hydroxylase activities and P450 expression vary in the different zones of the adrenal cortex [1–3], we studied both inner and outer zone preparations from the guinea pig adrenal cortex [17, 18]. The results demonstrate a strain difference in 21-hydroxylase activities in both adrenal zones. The differences in enzyme activity probably originate at the transcriptional level since corresponding strain differences in CYP21 mRNA and protein concentrations were also found.

Interestingly, the strain difference in CYP21 expression is opposite to that seen in CYP2D16 expression. The latter is expressed at higher levels in Strain 13 guinea pigs [12] whereas CYP21 expression is greater in the ESH strain. Thus, there appears to be independent regulation of these two adrenal P450 isozymes. The absence of any strain difference in CYP17 expression suggests the involvement of still other regulatory mechanisms that are distinct from

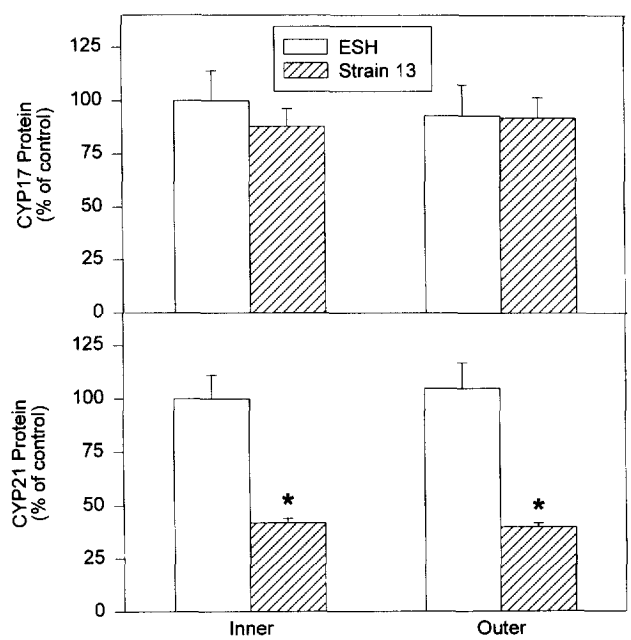

Fig. 3. Quantitative analyses of CYP17 and CYP21 protein concentrations in adrenal inner and outer zone microsomes from English Short-Hair (ESH) and Strain 13 guinea pigs. Protein levels were determined by densitometric analyses of Western blots as described in Section 2. Values are means of 3 different animals per group and are expressed as a percentage of the ESH inner zone value ± S.E.M. *$p < 0.05$ (vs corresponding inner zone value).

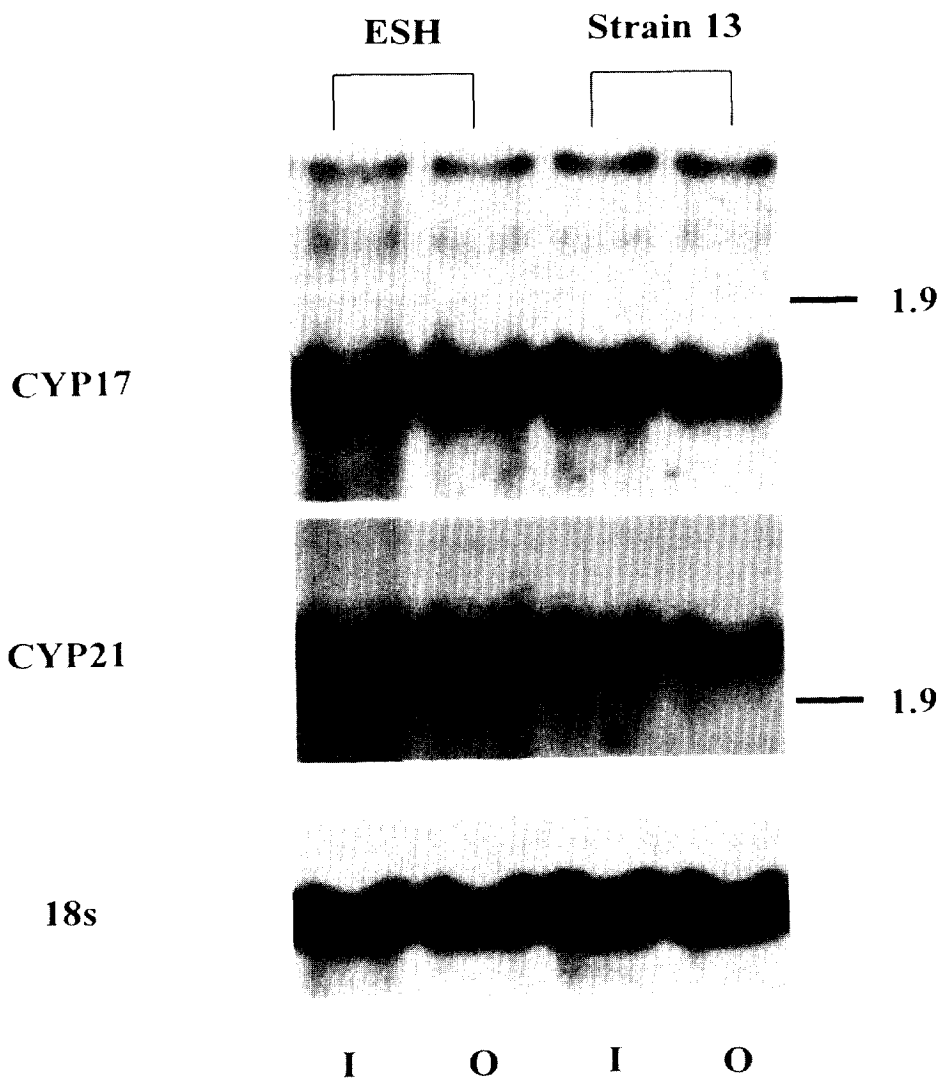

Fig. 4. Northern blot analyses of total RNA from adrenal inner (I) and outer (O) zones of English Short-Hair (ESH) and Strain 13 guinea pigs. Experiments were done with 10 μg total RNA loaded in each lane. Preparations were sequentially hybridized with a CYP21 cDNA, a CYP17 cDNA, and a rat 18 s ribosomal oligodeoxyribonucleotide, as described in Section 2.

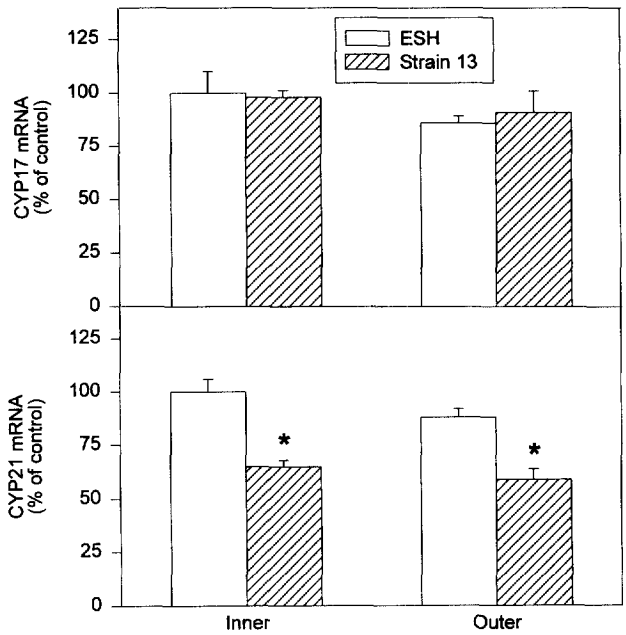

Fig. 5. Quantitative analyses of CYP17 and CYP21 mRNA levels in the adrenal inner and outer zones of English Short-Hair (ESH) and Strain 13 guinea pigs. mRNA levels were determined by densitometric analyses of Northern blots as described in Section 2. Values are means of 3 different animals per group and are expressed as a percentage of the ESH inner zone value ± S.E.M. *$p < 0.05$ (vs corresponding ESH value).

those for either CYP21 or CYP2D16. Studies are currently in progress on the mechanisms involved in the regulation of expression of each of these P450 genes.

Although the strain differences in adrenal 21-hydroxylase activities that we observed can apparently be explained by differences in CYP21 expression, the zonal differences in 21-hydroxylase and 17α-hydroxylase activities are not associated with any corresponding differences in enzyme mRNA or protein concentrations. These observations suggest that post-transcriptional processes may be of importance in controlling the activities of some steroidogenic enzymes. Other investigators have similarly noted a dissociation of adrenal steroid hydroxylase activities from enzyme protein and/or mRNA levels in the guinea pig [14, 23–25] as well as in other species [26, 27]). A number of mechanisms have been proposed to account for the apparent inconsistencies, but none has been definitively demonstrated. It is possible that Western blotting detects P450 apoprotein that is immunoreactive but nonfunctional. Similarly, denatured forms of P450 may also be immunoreactive with the antibody employed. Accordingly, zonal differences in P450 holoenzyme formation, via heme incorporation for example, and/or holoenzyme degradation could account for differences in enzyme activities despite similar amounts of measurable apoprotein. Differences in electron flow to cytochromes P450 or in membrane lipid composition could also influence enzyme activities independently of enzyme protein levels. Thus, there may be a variety of regulatory influences that interact to determine steroid hydroxylase activities. Further studies on the mechanisms involved should contribute to a fuller understanding of the factors affecting steroidogenesis.

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