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Genetic Regulation of Testosterone 15α-Hydroxylase (Cytochrome P-450$_{15\alpha}$) in Renal Microsomes of Female Mice†

E. James Squires and Masahiko Negishi*
Laboratory of Pharmacology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709
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ABSTRACT: P-450$_{15\alpha}$ is a form of cytochrome P-450 purified from liver microsomes of female 129/J mice that is specific for oxidation of testosterone to its 15α-hydroxylated product. Testosterone 15α-hydroxylase activity that was inhibited by anti-P-450$_{15\alpha}$ antibody was approximately 50 times higher in renal microsomes from 129/J than in BALB/cJ females. Western blots of renal microsomes using anti-P-450$_{15\alpha}$ antibody showed the presence of immunoreactive protein with a molecular weight identical with that of hepatic P-450$_{15\alpha}$ in 129/J but not in BALB/cJ female mice. To investigate the genetic basis for the strain differences in this activity, the distribution of P-450$_{15\alpha}$-dependent testosterone 15α-hydroxylase activity in renal microsomes from individual females of 129/J and BALB/cJ, of F1 offspring of these strains, and of F1 back-crosses to the progenitor strains were determined. The results were consistent with a sex-related autosomal dominant regulation of the higher activity in 129/J females by a single locus, designated Rsh (regulation of steroid hydroxylase). The amounts of immunochemically cross-reactive P-450$_{15\alpha}$ protein were linearly correlated with testosterone 15α-hydroxylase activities in renal microsomes from Rsh heterozygotes and homozygotes. At least twice as much mRNA, which hybridized with the cDNA clone for hepatic P-450$_{15\alpha}$, was detected in 129/J and 129CF1/J compared to BALB/cJ female kidneys. The evidence suggests a pretranslational regulation of the P-450$_{15\alpha}$ isozyme in the female mouse kidney by the Rsh locus. More than 90% of the renal testosterone 15α-hydroxylase activity in the female mice was inhibited by anti-P-450$_{15\alpha}$ while the activity in renal microsomes from 129/J and BALB/cJ males was decreased in the presence of the antibody only to approximately 40% of the control levels. This indicates the presence of another isozyme besides P-450$_{15\alpha}$ in the kidney of male mice. The total and P-450$_{15\alpha}$-dependent testosterone 15α-hydroxylase activities in renal microsomes were higher in male than in female mice; the opposite is true in hepatic microsomes from the same mice.

As with several other liver enzymes and proteins such as drug oxidases, monoamine oxidase, prolactin receptor, and major mouse urinary protein (MUP),[1] steroid hydroxylase activities in microsomes exhibit a marked sexual dimorphism is rodents (Roy & Chatterjee, 1983; Colby, 1980). For instance, testosterone 16α-hydroxylase activities are predominant in males while testosterone 15β- and 15α-hydroxylase activities are predominant in females (Einarsson et al., 1973; Levin et al., 1975; Gustafsson & Ingelman-Sundberg, 1974; Harada & Negishi, 1984a,b). These hydroxylase activities are catalyzed by specific forms of cytochrome P-450, the terminal oxidase of a membrane-bound monooxygenase system which also consists of NADPH-cytochrome P-450 reductase, cytochrome b_5, and NADH-cytochrome b_5 reductase (Sato & Omura, 1978).

It has been reported that hepatic testosterone 15α-hydroxylase activity is severalfold higher in females than in males of some mouse strains such as 129/J and AKR/J, but not in BALB/cJ (Ford et al., 1979; Hawke & Neims, 1983; Harada & Negishi, 1984a). A form of cytochrome P-450 specific for hydroxylation of testosterone at the 15α-position (P-450$_{15\alpha}$) was recently purified from liver microsomes of untreated female 129/J mice by this laboratory (Harada & Negishi, 1984a,b). A specific inhibitory antibody elicited against the purified P-450$_{15\alpha}$ in rabbits was used to elucidate the female-predominant expression of this isozyme in mouse liver. Furthermore, cDNAs encoding P-450$_{15\alpha}$ were cloned and utilized to demonstrate that the hepatic level of P-450$_{15\alpha}$ mRNA was 6 times higher in female than in male 129/J mice (Burkhart et al., 1985).

Alcohol dehydrogenase, β-glucuronidase, and ornithine decarboxylase are known to be expressed predominantly in kidneys of male rather than female rats (Paigen et al., 1975;

† E.J.S. is the recipient of a postdoctoral fellowship from the Natural Science and Engineering Research Council of Canada.
* Address correspondence to this author.

[1] Abbreviations: MUP, mouse urinary protein; Rsh, regulation of steroid hydroxylase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

Goldstone et al., 1982; Ohno et al., 1970). Sex-dependent differences in testosterone hydroxylase activities in mouse renal microsomes were also reported by Hawke (Hawke et al., 1983). Of interest to us, testosterone 15α-hydroxylase activity was higher in renal microsomes of male than of female mice, which is reversed from the situation in the liver. Consequently, we examined the sex-dependent regulation of renal testosterone 15α-hydroxylase (P-450$_{15\alpha}$) using a specific antibody and cDNA clone. We found that the strain difference in renal testosterone 15α-hydroxylase activity between 129/J and BALB/cJ was due to regulation by a single locus.

Experimental Procedures

Animals. Inbred 129/J and BALB/cJ mice (5–11 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME). The F1 offspring 129CF1/J (129/J female × BALB/cJ male) and C129F1/J (BALB/cJ female × 129/J male), F2 offspring (129CF1/J × 129CF1/J), and back-cross to progenitors (129CF1/J female × 129/J male or BALB/cJ male) offspring were produced by the Comparative Medicine Branch, National Institute of Environmental Health Sciences. New Zealand White rabbits were purchased from Charles River Laboratories (NJ).

Chemicals. [4-^{14}C]Testosterone (50–60 mCi/mmol) was obtained from New England Nuclear (Beverly, MA), and [^{32}P]dCTP (3000 Ci/mmol) and the nick-translation kit were from Amersham (Arlington Heights, IL). Collaborative Research (Lexington, MA) supplied the oligo(dT)–cellulose (type 2). Nitrocellulose paper and ABM paper were purchased from Schliecher & Schuell (Keene, NH); silica gel plates GHL were obtained from Analtech (Newark, DE). Goat anti-rabbit IgG and rabbit peroxidase-anti-peroxidase conjugate were purchased from Cappel Laboratories (Cochraville, PA). 3,3′-Diaminobenzidine was obtained from Sigma (St. Louis, MO) and Coomassie blue protein assay kit from Bio-Rad (Rockville Centre, NY). All other laboratory chemicals were of the highest purity available from major commercial suppliers.

Preparation of Kidney Microsomes. Mouse kidneys were homogenized with 10 mM Tris-HCl buffer, pH 7.5, containing 150 mM KCl and 10 mM EDTA. Kidney microsomes were prepared from the homogenates by the procedure described previously (Harada & Negishi, 1984a). For the assay of testosterone hydroxylases, the kidney microsomes were resuspended in 100 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol and 1 mM dithiothreitol.

Purification of P-450$_{15\alpha}$ and Preparation of Its Antibody. Mouse testosterone 15α-hydroxylase (P-450$_{15\alpha}$) was purified as previously described (Harada & Negishi, 1984a). The antibody to the purified P-450$_{15\alpha}$ was raised in rabbits and purified by antigen-affinity chromatography (Harada & Negishi, 1984b).

Other Analytical Methods. SDS–polyacrylamide gel electrophoresis was performed by the method of Laemmli (Laemmli & Faire, 1970). Western blots of microsomal proteins were performed as described by Towbin (Towbin et al., 1979), and the immunoreactive proteins were visualized by peroxidase immunostaining (Domin et al., 1984). Total cytochrome P-450 contents and protein concentrations were determined by the methods of Omura and Sato (1964) and Bradford (1976), respectively. Testosterone 15α-hydroxylase activity was measured as described previously (Harada & Negishi, 1984a).

Isolation of Poly(A+) RNA and Estimation of mRNA Content. Total kidney RNA was extracted with guanidine hydrochloride (Cox, 1968), and poly(A+) RNA was enriched by oligo(dT)–cellulose chromatography (Aviv & Leder, 1972). To estimate P-450$_{15\alpha}$ mRNA, various amounts of poly(A+) RNA were dotted on DBM or nitrocellulose paper (Norstedt & Palmiter, 1984) and hybridized with nick-translated ^{32}P-labeled p15α-29 cDNA (Burkhart et al., 1985) under the conditions previously reported (Tukey et al., 1981). After exposure to X-ray films, the appropriate areas of the hybridized paper were cut out, and the radioactivity was estimated by liquid scintillation counting.

Table I: Total and P-450$_{15\alpha}$-Dependent Testosterone 15α-Hydroxylase Activity and Cytochrome P-450 Contents in Renal Microsomes[a]

strain	sex	testosterone 15α-hydroxylase activity [pmol min^{-1} (mg of protein)$^{-1}$] total activity	testosterone 15α-hydroxylase activity [pmol min^{-1} (mg of protein)$^{-1}$] P-450$_{15\alpha}$ dependent activity	cytochrome P-450 content (pmol/mg of protein)
129/J	female	52.1	47.0	45.3
	male	934.1	474.1	298.1
BALB/cJ	female	6.6	1.6	31.2
	male	760.3	408.3	323.4

[a] Renal microsomes were prepared from female and male 129/J and BALB/cJ mice as described under Experimental Procedures. Total testosterone 15α-hydroxylase activity in the microsomes was measured as follows. The microsomes (250 μg) were preincubated at 37 °C for 1 min in the reaction buffer containing [4-^{14}C]testosterone as described previously (Harada & Negishi, 1984b), and then the hydroxylase activity was initiated by the addition of NADPH. The reaction mixture was incubated at 37 °C for 10 min, and testosterone metabolites formed were extracted with dichloromethane and separated by one-dimensional thin-layer chromatography with chloroform–methanol (92:8) as the solvent. The thin-layer plate was exposed to X-ray film, and the area containing [^{14}C]-15α-hydroxytestosterone was scraped from the thin-layer plate and counted by liquid scintillation. In order to determine P-450$_{15\alpha}$-dependent 15α-hydroxylase activity, the renal microsomes (250 μg) were preincubated with 5 μg of anti-P-450$_{15\alpha}$ antibody at 37 °C for 1 min prior to initiation of the hydroxylase activity. The amount of the antibody used was enough to inhibit all of the P-450$_{15\alpha}$-dependent activity in the microsomes (Burkhart et al., 1985; Figure 1). The amount of [^{14}C]-15α-hydroxytestosterone formed in the presence of anti-P-450$_{15\alpha}$ was measured by liquid scintillation counting. The P-450$_{15\alpha}$-dependent activity was calculated by subtracting the activity remaining after incubation with the antibody from the total activity. Values reported are the mean of three experiments. Average error of the assays was less than 5% of the means.

Results

Male-Predominant Expression of Testosterone 15α-Hydroxylase Activity in Renal Microsomes. Testosterone 15α-hydroxylase activities and total cytochrome P-450 contents in renal microsomes prepared from male and female 129/J and BALB/cJ mice are summarized in Table I. A marked sexual dimorphism was found in both the hydroxylase activities and P-450 contents in both strains of mice. The total activity measured in the absence of anti-P-450$_{15\alpha}$ antibody was similar in male microsomes from both 129/J and BALB/cJ mice and 18 and 100 times higher than in the female microsomes from 129/J and BALB/cJ mice, respectively. The cytochrome P-450 contents of the microsomes were 6 to 10 times higher in the males than in the females. Testosterone 15α-hydroxylase activity catalyzed by P-450$_{15\alpha}$ was estimated by inhibition of this activity with antibody elicited against P-450$_{15\alpha}$ purified from mouse liver microsomes (Table I). There was no detectable P-450$_{15\alpha}$-dependent activity in kidney microsomes from BALB/cJ females while the activity in 129/J females was approximately 50 pmol min^{-1} (mg of protein)$^{-1}$. In renal microsomes from the male mice, P-450$_{15\alpha}$-dependent

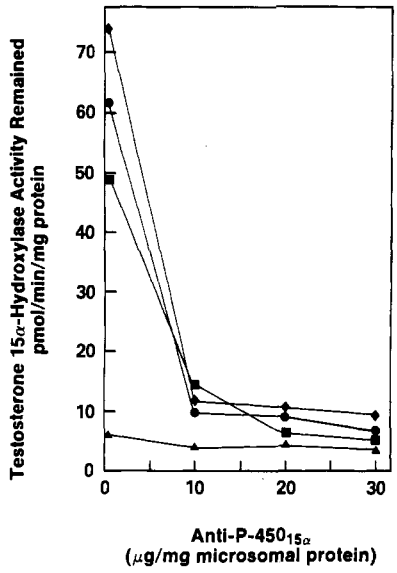

FIGURE 1: Inhibition of testosterone 15α-hydroxylase activity in renal microsomes from 129/J and BALB/cJ mice and their F1 offspring. Renal microsomes were isolated from five female 129/J (■), BALB/cJ (▲), 129CF1 (●), and C129F1/J (♦) mice as described under Experimental Procedures. Testosterone 15α-hydroxylase activities in these microsomes were measured as follows. The microsomes (250 μg) were preincubated with various amounts (0, 2.5, 5, and 7.5 μg) of anti-P-450$_{15\alpha}$ antibody for 3 min prior to the initiation of testosterone 15α-hydroxylase activity by the addition of NADPH. The reaction conditions were the same as described previously (Harada & Negishi, 1984a), and the enzyme assay procedure was as described in the legend for Table I.

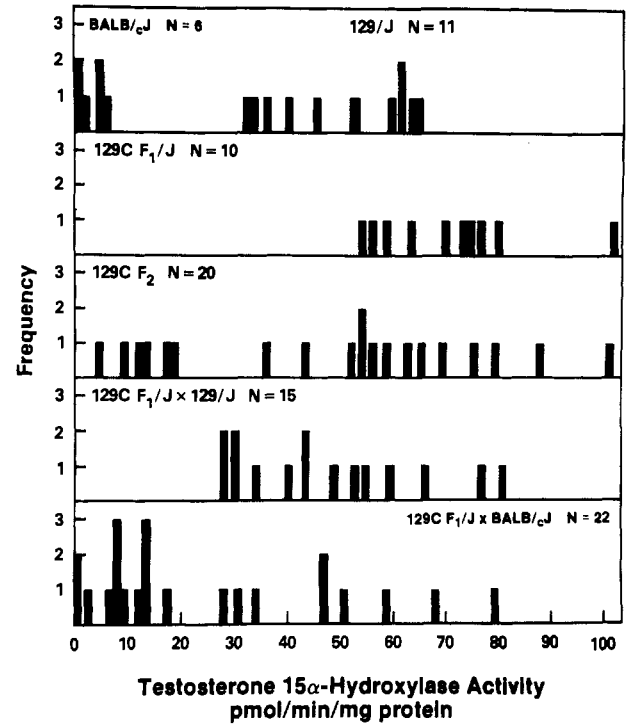

FIGURE 2: Distribution of testosterone 15α-hydroxylase activity in renal microsomes from individual females of 129/J, BALB/cJ, F1, F2, and F1 back-cross to 129/J and BALB/cJ mice. Renal microsomes were isolated from individual female mice of the two progenitors and the offspring. The testosterone 15α-hydroxylase activities in the renal microsomes were measured as described under Experimental Procedures.

activities were about 10 times higher than that seen in 129/J females. Thus, the male-predominant expression of testosterone 15α-hydroxylase activity in renal microsomes resulted from the occurrence of both a testosterone 15α-hydroxylase isozyme related immunochemically to hepatic P-450$_{15\alpha}$ and at least one more isozyme which were expressed predominantly in male mice. We have further investigated a genetic basis for strain differences in P-450$_{15\alpha}$-dependent activity in females of the two mouse strains.

Dominant Expression of P-450$_{15\alpha}$-Dependent Activities. Figure 1 shows the inhibition of testosterone 15α-hydroxylase activities in renal microsomes from 129/J, BALB/cJ, 129CF1/J, and C129F1/J females by anti-P-450$_{15\alpha}$ antibody. At least 85–90% of the activities in the microsomes from all of these strains were inhibited by the antibody, indicating that only P-450$_{15\alpha}$ is the major isozyme-catalyzing testosterone 15α-hydroxylase activity in the renal microsomes. Although the rates of testosterone 15α-hydroxylase activity in renal microsomes from female mice of both F1 offspring were higher than those of the progenitors, the results suggest dominant inheritance of P-450$_{15\alpha}$-dependent activity in this pair. Whether or not this dominant inheritance is regulated by a single locus was studied by taking advantage of the large differences in activity between 129/J and BALB/cJ female mice. Since the P-450$_{15\alpha}$ was the major isozyme in renal microsomes from 129/J, BALB/cJ, and F1 offspring, and the residual activity remaining after antibody inhibition was the same in all of these strains, the total testosterone 15α-hydroxylase activity were used as an accurate indicator of the genetic modulation of P-450$_{15\alpha}$-dependent activity in renal microsomes from these mice.

Distribution of Activities in Females from Crosses of 129/J and BALB/cJ Mice. Renal microsomes were prepared from individual females of 129/J and BALB/cJ progenitors, F1 offspring (129CF1/J), the F2 offspring and offspring from 129CF1 back-crosses to 129/J and BALB/cJ progenitors. The testosterone 15α-hydroxylase activities in the microsomes were measured, and the data are shown in Figure 2. The BALB/cJ mice had an average activity 10 times lower than in the 129/J mice (top panel). The F1 offspring (second panel) all had activities as high as the 129/J progenitor and many had somewhat higher activities. The distribution of phenotypes in the F2 and back-cross offspring (third to fifth panels) were entirely consistent with a sex-limited autosomal-dominant regulation of the greater expression of activity in 129/J females by a single locus. In this experiment, we have designated testosterone 15α-hydroxylase activity less than 20 pmol min^{-1} (mg of protein)$^{-1}$ as low activity phenotype and testosterone 15α-hydroxylase activity greater than 28 pmol min^{-1} (mg of protein)$^{-1}$ as high activity phenotype. In the F2, the ratio of the low and high activity phenotypes was 6:14 which agrees well with the theoretical ratio of 1:3 ($p < 0.5$). The progeny from the back-cross to the 129/J progenitor all had the high activity phenotype, while the ratio of the low to high activity in the progeny from the back-cross to the BALB/cJ progenitor was 13:9 which was not significantly different from the expected ratio of 1:1 ($p < 0.1$).

Correlation of Testosterone 15α-Hydroxylase Activity with Amounts of P-450$_{15\alpha}$ Protein. Immunoblots of renal microsomal proteins with anti-P-450$_{15\alpha}$ antibody confirmed that the differences in testosterone 15α-hydroxylase activities in the offspring were due to the presence of different amounts of P-450$_{15\alpha}$ protein. Figure 3A shows a Western blot indicating the lower amount of P-450$_{15\alpha}$ protein in renal microsomes of BALB/cJ compared with 129/J females on the basis of the intensities of the band comigrating with hepatic P-450$_{15\alpha}$. The F1 offspring contained a similar amount of P-450$_{15\alpha}$ protein as the 129/J progenitor (data not shown). The anti-P-450$_{15\alpha}$ antibody bound to other bands on the immunoblots besides

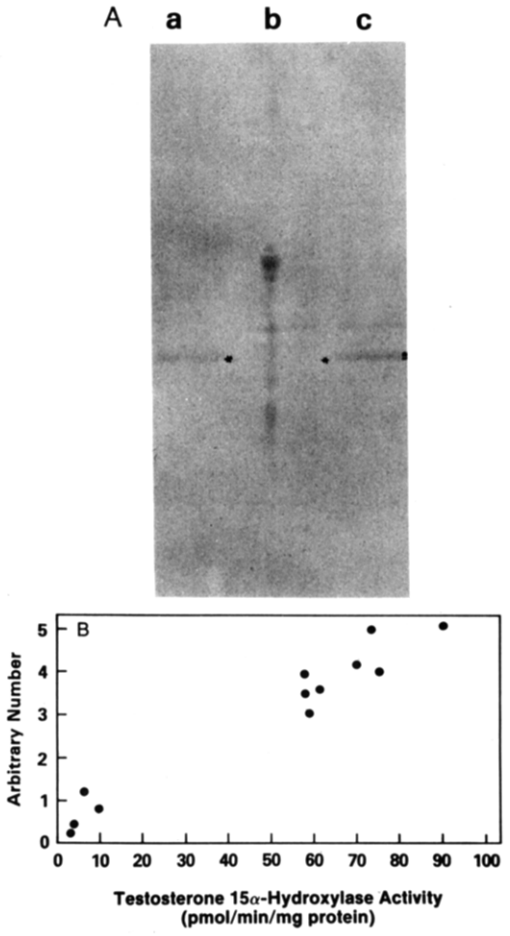

FIGURE 3: (A) Western immunoblot analysis of renal microsomal proteins with anti-P-$450_{15\alpha}$ antibody. Renal microsomes (5 μg) from female 129/J and BALB/cJ mice were electrophoresed on (9%) SDS–polyacrylamide gels and transferred to nitrocellulose paper. The protein bands that reacted with anti-P-$450_{15\alpha}$ antibody on the nitrocellulose paper were visualized by immunostaining with dimethybenzidine. "a" represents purified hepatic P-$450_{15\alpha}$ (10 ng); "b" and "c" represent renal microsomes from BALB/cJ and 129/J females, respectively. The asterisk indicates the protein band that comigrates with P-$450_{15\alpha}$. (B) Correlation between testosterone 15α-hydroxylase activity and amounts of P-$450_{15\alpha}$ detected immunochemically. Renal microsomes were prepared from four homozygotes and eight heterozygotes of the F2 offspring. The relative amounts of densitometry of the bands that comigrated with hepatic P-$450_{15\alpha}$ standard in Western blots were plotted as a function of the rates of testosterone 15α-hydroxylase activity in the same microsomes.

those corresponding to P-$450_{15\alpha}$, but there were no strain differences in the intensities of these bands.

The levels of P-$450_{15\alpha}$ protein in the microsomes of heterozygotes and homozygotes of the F2 offspring were estimated from the intensities of the 48.5K dalton bands on the blots and plotted as a function of the testosterone 15α-hydroxylase activities (Figure 3B). An excellent linear correlation ($r = 0.98$) was obtained between the amounts of P-$450_{15\alpha}$ protein and the rates of testosterone 15α-hydroxylase activity in the microsomes. We therefore concluded that the differences in the hydroxylase activities among the offspring were due to different amounts of P-$450_{15\alpha}$ protein present in microsomes.

Level of mRNA Hybridized with a cDNA for P-$450_{15\alpha}$. The relative levels of mRNA that hybridized with a specific cDNA probe for P-$450_{15\alpha}$ in the two progenitor strains and the F1 offspring were determined by dot blot analysis of total poly(A+) RNA prepared from kidney. The various amounts of hybridization of ^{32}P-labeled p15α-29 cDNA were estimated by comparing the slopes of the plots of the radioactivities recovered vs. the amounts of poly(A+) RNA dotted on DBM

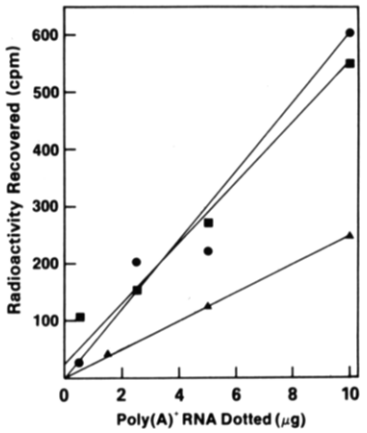

FIGURE 4: Quantitation of renal mRNA hybridized with cDNA for hepatic P-$450_{15\alpha}$. Various amounts (0.6, 1.25, 2.5, 5.0, and 10.0 μg) of poly(A+) RNA isolated from kidneys of 10 mice were dotted on DBM paper and hybridized with ^{32}P-labeled cDNA p15α-29. The hybridization was visualized by exposing the paper to X-ray film, and the radioactivity recovered from the areas of hybridized paper was plotted as a function of the amount of poly(A+) RNA dotted. (▲) BALB/cJ females; (■) 129/J females; (●) 129CF1/J offspring.

paper. The results (Figure 4) indicated that the levels of the P-$450_{15\alpha}$ mRNA in kidneys of 129/J and F1 female offspring were at least twice as high as those in kidneys of female BALB/cJ mice. This suggested that the mechanism of action of the Rsh locus is the pretranslational regulation of P-$450_{15\alpha}$ in female mouse kidney. However, the difference in the mRNA levels were not as great as the difference in the hydroxylase activity and contents of P-$450_{15\alpha}$ protein. This may be due to factors such as differences in the stabilities and translational efficiencies of mRNAs or different turnover rates of P-$450_{15\alpha}$ protein in the two mouse strains.

DISCUSSION

In this paper, we have demonstrated that the isozyme of testosterone 15α-hydroxylase, which is immunoreactive with anti-P-$450_{15\alpha}$ antibody and has the same molecular weight as the hepatic isozyme (P-$450_{15\alpha}$), is present in renal microsomes of mice. The total and the P-$450_{15\alpha}$ isozyme-dependent hydroxylase activities were male-predominant in the mouse kidney, while in the mouse liver the activities were female-predominant. The incomplete inhibition of the total testosterone 15α-hydroxylase activity in renal microsomes of male mice by anti-P-$450_{15\alpha}$ antibody implied the presence of more than one isozyme of testosterone 15α-hydroxylase. We have recently found that the expression of the activity that is not inhibited by anti-P-$450_{15\alpha}$ antibody is totally androgen dependent in sexually matured 129/J mice, while the P-$450_{15\alpha}$-dependent activity in renal microsomes is much less dependent on androgen for expression (unpublished observation). This provides further evidence for the presence of other isozyme(s) besides P-$450_{15\alpha}$ in renal microsomes of male mice. The immunochemical identity and the same apparent molecular weights of both the renal and hepatic P-$450_{15\alpha}$ do not prove that the two isozymes are structurally identical or a product from the same gene. We are currently isolating cDNA clones encoding renal P-$450_{15\alpha}$ for comparison to the nucleotide sequences of our cDNA clones for hepatic P-$450_{15\alpha}$.

The genetic basis of the expression of microsomal testosterone 15α-hydroxylase activity in mouse kidney was investigated by utilizing the strain difference in this activity between 129/J and BALB/cJ females. The distribution of testosterone 15α-hydroxylase activity in renal microsomes prepared from individual female offspring of the various genetic crosses of 129/J, BALB/cJ, and the F1 is consistent with a sex-related,

single locus regulation. This regulation between 129/J and BALB/cJ pairs was autosomal-dominant in 129/J females. Here we propose that this murine locus be termed Rsh ("regulation of steroid hydroxylase"); Rsh^h/Rsh^h represents the genotype of 129/J females, and Rsh^l/Rsh^l represents that of BALB/cJ females.

The linear correlation between the rates of testosterone 15α-hydroxylase activity and the amounts of immunochemically detected P-$450_{15\alpha}$ protein indicates that the Rsh locus operates by modulating the amounts of P-$450_{15\alpha}$ protein in the renal microsomes. Furthermore, the higher level of P-$450_{15\alpha}$ mRNA in female 129/J than in female BALB/cJ kidney is indicative of regulation of the transcription of the P-$450_{15\alpha}$ gene(s) by the Rsh locus. It remains to be seen whether or not the Rsh locus represents cis-acting or trans-acting genetic element(s). The Ah locus is an example of a trans-acting genetic element which regulates polycyclic hydrocarbon-induced aryl hydrocarbon hydroxylase activity in liver microsomes (Gielen et al., 1972; Thomas et al., 1972). However, we have found the high level of P-$450_{15\alpha}$-dependent activity in renal microsomes from Ah-nonresponsive DBA/2J mice, indicating that the Rsh locus is distinct from the Ah locus (data not shown).

Many of the individual F1 offspring had renal testosterone 15α-hydroxylase activities that were somewhat higher than those of the 129/J progenitor. This phenomenon of increased expression of the activity of F1 heterozygotes may be due to minor modifying genes. These modifying genes may also contribute to making the simple mendelian genetics less clear in the F2 generation. The possible existence of these modifying gene(s) was previously discussed for the expression of phenobarbital-induced coumarin hydroxylase (a form of cytochrome P-450) regulated primarily by the murine Coh locus (Wood & Taylor, 1979). Genetic variations at the Coh locus result in structural mutation(s) of coumarin hydroxylase itself (Wood, 1979; Kaipainen et al., 1984).

Ford (Ford et al., 1979) and Pasleau (Pasleau et al., 1984) have reported the presence of a single murine locus regulating the constitutive expression of testosterone 16α-hydroxylase activity in liver microsomes of female mice. Noshiro and Negishi (unpublished observations) have recently studied the inheritance of the 16α-hydroxylase activity in 129/J and BALB/cJ pairs using the same individual female offspring used for the present experiments. It was confirmed that the expression of 15α- and 16α-hydroxylase activities is under the control of different loci.

The metabolic role of 15α-hydroxylation of steroids is unknown, but it may be an important first step in catabolism prior to conjugation and secretion. 15-Oxy steroids, including progesterone, are more potent mineralocorticoid antagonists than the parent steroids (Tweit & Kagawa, 1964). We have observed a 15α-hydroxylase activity of progesterone by P-$450_{15\alpha}$, but it has not yet been determined that 15α-hydroxyprogesterone is an intermediate in the formation of 15-oxyprogesterone.

The presence of testosterone 15α-hydroxylase activities in both liver and kidney raises interesting questions concerning tissue-specific and sex-dependent regulation of constitutive forms of cytochrome P-450 specific for hydroxylation of endogenous compounds. In this regard, it has recently been reported (Babany et al., 1985) that in situations where the total P-450 levels in liver are decreased, the total P-450 levels in kidney are reciprocally increased. It is, therefore, apparent that renal P-450's are metabolically important enzyme systems for the detoxification and metabolism of drugs and xenobiotics. The Rsh locus described here provides a suitable genetic model system to study the regulation of kidney P-$450_{15\alpha}$.

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Characterization of Native and Reconstituted Hydrogen Ion Pumping Adenosinetriphosphatase of Chromaffin Granules†

Gary E. Dean,‡ Pamlea J. Nelson, and Gary Rudnick*
Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510
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ABSTRACT: The ATP-dependent H^+ pump from adrenal chromaffin granules is, like the platelet-dense granule H^+ pump, essentially insensitive to the mitochondrial ATPase inhibitors sodium azide, efrapeptin, and oligomycin and also insensitive to vanadate and ouabain, agents that inhibit the Na^+,K^+-ATPase. The chromaffin granule H^+ pump is, however, sensitive to low concentrations of NEM (*N*-ethylmaleimide) and Nbd-Cl (7-chloro-4-nitro-2,1,3-benzoxadiazole). These transport ATPases may thus belong to a new class of ATP-dependent ion pumps distinct from F_1F_0- and phosphoenzyme-type ATPases. Comparisons of ATP hydrolysis with ATP-dependent serotonin transport suggest that approximately 80% of the ATPase activity in purified chromaffin granule membranes is coupled to H^+ pumping. Most of the remaining ATPase activity is due to contaminating mitochondrial ATPase and Na^+,K^+-ATPase. When extracted with cholate and octyl glucoside, the H^+ pump is solubilized in a monodisperse form that retains NEM-sensitive ATPase activity. When reconstituted into proteoliposomes with crude brain phospholipid, the extracted enzyme recovers ATP-dependent H^+ pumping, which shows the same inhibitor sensitivity and nucleotide dependence as the native pump. These data demonstrate that the predominant ATP hydrolase of chromaffin granule membrane is also responsible for ATP-driven amine transport and granule acidification in both native and reconstituted membranes.

Until recently, all ion-translocating ATPases were thought to fall into one of two classes: the F_1F_0 class, represented by mitochondrial ATPase, and the phosphoenzyme class of eukaryotic ATP-dependent ion pumps, represented by the Na^+,K^+-ATPase. It has now become apparent that a third class of transport ATPase exists in acidic intracellular vesicles such as lysosomes, endosomes, coated vesicles, and secretory granules (Rudnick, 1986a,b). All of these organelles are thought to participate in the movement of their content to or from the extracellular medium. In all cases examined, ATP drives H^+ influx into the organelle, and this pumping is insensitive to many inhibitors of previously characterized transport ATPases.

Two approaches have been used to demonstrate the uniqueness of secretory granule ATPase. Cidon and Nelson (1983) demonstrated that chromaffin granule membranes from which all immunoreactive F_1 β-subunit had been removed by sodium bromide extraction still retained 70% of their ATPase activity and that this remaining ATPase would reconstitute ATP-dependent H^+ influx (Cidon et al., 1983). Since the β-subunit is highly conserved in F_1F_0 ATPase from all known sources, it is extremely unlikely that this major chromaffin granule ATPase represents a typical F_1F_0 enzyme. It has never been conclusively demonstrated, however, that this major ATPase activity represents the H^+ pump that normally drives amine transport. Our laboratory has used a second approach in studying the H^+-pumping ATPase of platelet-dense granule membrane. Dean et al. (1984) demonstrated that the platelet granule H^+ pump is insensitive to concentrations of inhibitors, which maximally inhibit F_1F_0 ATPase or Na^+,K^+-ATPase.

In this work, we have turned our attention to the ATP-dependent H^+ pump of adrenal chromaffin granules. Chromaffin granule membranes may be prepared in higher yield and purity than those from other secretory granules. These properties make possible the comparison of ATPase and H^+-pumping properties of the membrane. To avoid complications from other ATP-dependent H^+ pumps that might contaminate the preparation, we employed a coupled assay for H^+ pumping. In this assay, the electrochemical H^+ potential ($\Delta\bar{\mu}_{H^+}$)[1] (interior acidic and positive) generated by H^+ pumping is measured by serotonin accumulation catalyzed by the granule amine transporter. The amine transported catalyzes the reserpine-sensitive exchange of intragranular H^+ with cytoplasmic biogenic amines. The assay, therefore, is sensitive only to those ATPases in the preparation that pump H^+ across the granule membrane.

To ultimately determine if the secretory granule ATPase belongs to a new class of H^+ pump, structural information on

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‡ Present address: Department of Microbiology and Molecular Genetics, University of Cincinnati College of Medicine, Cincinnati, OH 45267.

[1] Abbreviations: DCCD, *N*,*N*′-dicyclohexylcarbodiimide; Nbd-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride; $\Delta\bar{\mu}_{H^+}$, transmembrane electrochemical potential for H^+; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; HEPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; AMP-PNP, 5′-adenylyl imidodiphosphate.

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