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Evidence that the androgen receptor mediates sexual differentiation of mouse renal cytochrome *P*450 expression

Colin J. HENDERSON and C. Roland WOLF*
Imperial Cancer Research Fund, Molecular Pharmacology Group, Hugh Robson Building, George Square, Edinburgh EH8 9XD, U.K.

We have previously shown that sexual dimorphism in the expression of mouse renal cytochrome *P*450s is mediated by androgens, probably at a transcriptional level [Henderson, Scott, Yang & Wolf (1990), Biochem. J. **266**, 675–681]. In the present study we show that this effect is already observed for most isoenzymes at only 2–3 weeks of age, as is the ability to induce or suppress expression with exogenous testosterone. The testosterone responsiveness did, however, exhibit age- as well as dose-dependency. Intriguingly, the effects of androgen took up to 8 days to become maximized, and the dose of testosterone needed to convert the female into the male phenotype was much higher than the circulating levels normally found in males. Studies using testicular feminized (Tfm) male mice, which carry an androgen receptor defect, showed them to have the female kidney cytochrome *P*450 phenotype, and these animals were not responsive to testosterone treatment. These data demonstrate the involvement of the androgen receptor in the regulation process. Taken together, our results indicate that the androgen receptor does not interact directly with the *P*450 genes, but initiates a cascade of events leading to the changes in cytochrome *P*450 gene expression. Significant differences were observed in the degree of sexual dimorphism in kidney *P*450 expression in other mammalian species. The significance of these findings in relation to the observed sexual dimorphism in other species is discussed.

INTRODUCTION

Cytochrome *P*450s represent a large group of mono-oxygenases which are involved in hormone, bile acid and fatty acid metabolism, as well as the metabolism of drugs and other xenobiotics. *P*450s represents a gene superfamily which has been divided into families and subfamilies on the basis of sequence similarity (Nebert *et al.*, 1991).

In addition to their role in Phase I detoxification reactions, *P*450s may also employ the same oxygenation reactions to activate procarcinogens and other molecules to toxic, carcinogenic and mutagenic compounds (Guengerich, 1988). It is thus of central importance to establish the regulatory factors controlling the expression of *P*450 genes and proteins.

Although it is well established that *P*450s may be induced by a host of foreign chemicals, endogenous factors also play a role in determining cellular enzyme levels. For example, growth hormone (GH) has been extensively studied as both an inducer and a repressor of hepatic *P*450 expression (Skett, 1987). GH appears to operate through sexually dimorphic patterns of secretion. In male rats, GH secretion is episodic, whereas in the female it is more continuous (Eden, 1979; Zaphiropoulos *et al.*, 1989). The male pattern of secretion is believed to be 'imprinted' by neonatal exposure to androgen (Jansson *et al.*, 1985; Waxman *et al.*, 1988). More recent work has shown that this effect may be partly mediated through transcriptional activation, although there is also a post-transcriptional component to the regulatory process (Mode *et al.*, 1989).

We have recently demonstrated extensive sexual dimorphism in the expression of mouse kidney *P*450s, and showed that testosterone, not GH, mediates this effect (Henderson *et al.*, 1990). In the present study we demonstrate that testosterone is acting through the androgen receptor. The time course and dose-dependency of the testosterone effects indicate that a cascade of events is involved in the regulation process.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma, Poole, Dorset, U.K., or BDH, Glasgow, U.K., unless otherwise stated, and were of the highest purity available. Nitrocellulose was obtained from Schleicher und Schüll, Dassel, Germany. ^{125}I-Protein A, [α-^{32}P]ATP and the Enhanced Chemiluminescence (ECL) kit were purchased from Amersham International, Amersham, Bucks., U.K.

Animals

Male and female C57BL/6 mice (8 weeks old), Wistar rats (8 weeks old) and New Zealand White rabbits (12–18 months old), all sexually mature, were obtained from Banton and Kingman, Hull, U.K. Testicular feminized (Tfm) mice (Ohno & Lyon, 1970; Lyon & Hawkes, 1970) were obtained from the M.R.C. Radiobiology Unit, Chilton, Didcot, U.K. The Tfm mice (8 weeks old) used were: (1) genotype $++blo/Y$; male controls with 'blotchy' coat; and (2) genotype $Tfm++/Y$, male mice, phenotypically female. These mice are feminized because of a defective androgen receptor. Adult (2 years old) male and female Beagle dog kidney tissue was obtained from the Huntingdon Research Centre, Huntingdon, Cambs., U.K. All animals were maintained under standard animal house conditions and fed *ad libitum* on standard animal diet.

Mice treated with testosterone propionate received between 1 and 20 mg/kg body wt. intraperitioneally for between 5 and 8 days as detailed in the Figure legends. Control animals received the injection vehicle, corn oil.

All mice and rats were killed by cervical dislocation, followed by rapid removal of kidneys, which were rinsed with ice-cold sterile phosphate-buffered saline (Na_2HPO_4, 1.15 g/l; NaCl, 8 g/l; $MgCl_2,6H_2O$, 0.1 g/l; KH_2PO_4, 0.2 g/l; KCl, 0.2 g/l; $CaCl_2,2H_2O$, 0.132 g/l) and frozen on solid CO_2, before being

Abbreviations used: Tfm, testicular feminized; GH, growth hormone; ECL, enhanced chemiluminescence. The cytochrome *P*-450 nomenclature used in this paper is based on Nebert *et al.* (1991).

* To whom correspondence should be addressed.

stored at −70 °C prior to processing. The rabbits were terminally anaesthetized with CO_2 prior to removal of organs, as were the group of mice in the time-course experiment, to allow removal of blood by cardiac puncture, followed by cervical dislocation and removal of kidneys as described above. The blood was allowed to clot in plain tubes before being centrifuged to yield serum, which was stored at −70 °C prior to radioimmunoassay analysis for testosterone (see below).

Preparation of microsomes

Microsomal fractions were prepared from the mice, rat and rabbit organs by differential centrifugation as previously described (Henderson *et al.*, 1990). Microsomes were stored at −40 °C at a protein concentration of approx. 10–20 mg/ml. Protein content was determined according to the method of Lowry *et al.* (1951).

Immunoblotting

SDS/PAGE of microsomal samples, in 9% gels, was carried out as described by Laemmli (1970), as modified by Lewis *et al.* (1988). Immunoblotting with anti-(rat *P*450) sera has been previously described (Henderson *et al.*, 1990). Protein bands were visualized using ^{125}I-Protein A. For the CYP2E1 antibody, where the sensitivity of detection was particularly low, ECL was employed as an alternative detection system. The Amersham ECL kit was used according to the manufacturers' instructions.

Testosterone measurement

Measurement of circulating testosterone levels in male and female C57BL/6 mice was carried out by radioimmunoassay of the evaporated residue from ether-extracted sera, using ^{125}I-labelled tracer and a solid-phase second antibody separation. The maximum accurate level of detection in this assay was 250 nmol/litre.

RESULTS

Development of mouse renal sexual dimorphism and testosterone responsiveness

The expression of many hepatic cytochrome *P*450s is known to develop neonatally. This also was the case for mouse renal cytochrome *P*450s of the CYP2A, CYP2B (not shown) CYP2E, CYP3A and CYP4A gene families (Fig. 1). Certain isoenzymes, however, were expressed at maximum levels already at 3 weeks of age, e.g. CYP2A and CYP2C proteins. Interestingly, the expression of these proteins was already sexually dimorphic at this time point in a manner which could be at least partially reversed in females by administration of testosterone (Fig. 1). The expression of CYP2E1 was first detected in untreated male mice at 5 weeks of age and was not detected in females of any age. Administration of testosterone to males and females induced the expression of this protein at just 3 weeks of age. There did, however, appear to be an increased effect with increasing age. CYP3A proteins were first detected in male mice at 5 weeks of age, and these proteins could not be detected in females at any age without the administration of testosterone. Again, testosterone responsiveness was already observed at 3 weeks of age. The pattern of regulation of *P*450s in the CYP4A gene family was similar to the CYP2E1 family. However, one isoenzyme could be detected already at 3 weeks of age and was sexually dimorphic at 4 weeks. A marked increase in the level of this and a further isoenzyme was seen at the onset of puberty, where a very significant sexual differentiation was observed. Testosterone responsiveness was already evident at 3 weeks of age in both males and females, but this effect showed a marked age-dependency.

In order to characterize the induction of renal *P*450 isoenzymes by testosterone propionate, the time course and effect of dose on *P*450 levels was investigated in female mice (Fig. 2). After 3 days of testosterone treatment, renal *P*450 expression had begun to change from the female pattern. This time-dependent change in

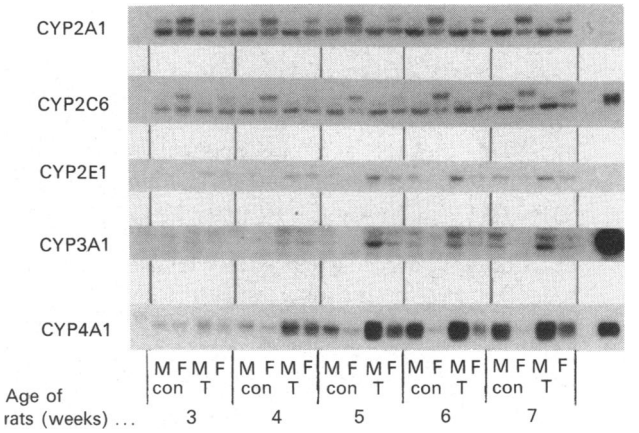

Fig. 1. Development of sexual dimorphism and testosterone responsiveness in mouse renal *P*450 expression

Male (M) and female (F) C57BL/6 mice, aged 3–7 weeks, were treated either as controls (con), or with 2 mg of testosterone propionate (T)/kg body wt. as outlined in the Materials and methods section. Kidney microsomes (30 μg) were separated on SDS/PAGE (9% gels), transferred to nitrocellulose and probed with various antisera to the rat liver *P*450s shown, as described in the Materials and methods section and by Henderson *et al.* (1990). Loading of all gels was checked by Coomassie Blue staining. *P*450 standards (0.8 pmol) were run in the right-hand lane. The M_r values of the immunoreactive bands recognized by the antisera are detailed in the legend to Fig. 2.

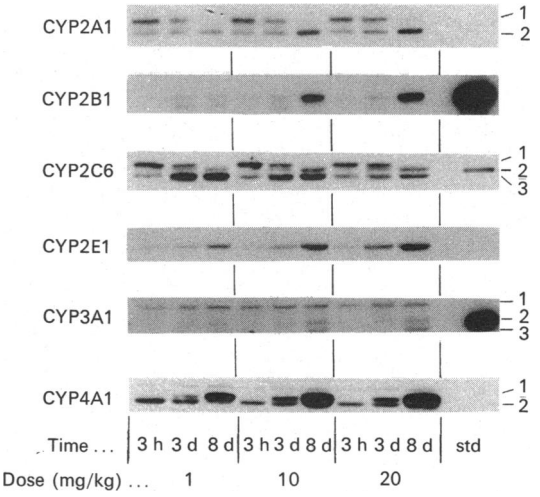

Fig. 2. Time course and dose-dependence of the effects of testosterone on mouse renal *P*450 expression

Adult female C57BL/6 mice were treated with 1, 10 or 20 mg of testosterone propionate/kg body wt., as outlined in the Materials and methods section. The animals were killed after 8 h, 3 days (d) or 8 days, and the kidney microsomal fraction was prepared. Microsomes (30 μg) were separated on SDS/PAGE (9% gels), transferred to nitrocellulose and probed with antisera to the rat liver *P*450s as described in the Materials and methods section. The M_r values of the immunoreactive bands recognized by the antisera are as follows. CYP2A1: 1, 55000; 2, 49500; CYP2B1: 51500; CYP2C6: 1, 56500; 2, 52500; 3, 48000; CYP2E1: 55500; CYP3A1: 1, 60000; 2, 53000; 3, 49000; CYP4A1: 1, 52000; 2, 50000. std, M_r standards.

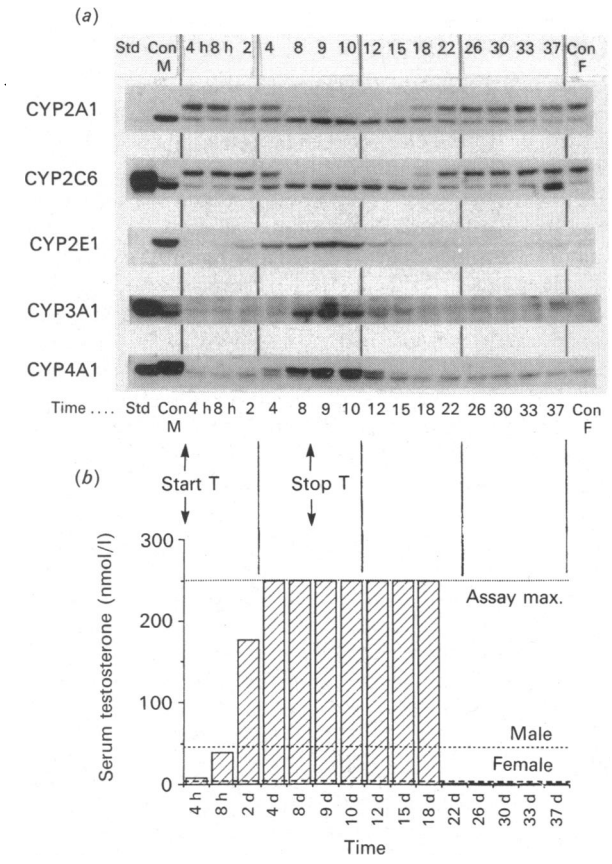

Fig. 3. Time course of androgen action on mouse renal *P*450 expression

(*a*) Adult female C57BL/6 mice were treated for 8 days with testosterone propionate (T) at 2 mg/kg body wt. as outlined in the Materials and methods section. Animals were killed at various time points during and after this treatment, and kidney microsomes were prepared. Microsomes (30 μg) were separated on SDS/PAGE (9% gels), transferred to nitrocellulose and probed with various anti-*P*450 sera to the rat liver isoenzymes shown, as described in the Materials and methods section. M_r values of the immunoreactive bands recognized by the antisera are detailed in the legend to Fig. 2. M and F are microsomal samples from untreated male and female animals respectively. Time points are shown above and below the tracks and are in days (d), unless otherwise stated. Con, control; std, standard. (*b*) Serum testosterone levels in adult male and female C57BL/6 mice treated as in (*a*), determined by radioimmunoassay as outlined in the Materials and methods section.

the isoenzyme profile towards the male pattern continued, and was most marked at 8 days after the commencement of treatment. As 8 days was the longest time period studied, it is feasible that even higher levels of expression for some isoenzymes would be obtained with continued testosterone administration. A dose of 1 mg of testosterone/kg was sufficient to convert the female renal *P*450 isoenzyme profile to that of the male. There did, however, appear to be a dose-dependent effect, and the expression of almost all of the proteins was higher at doses of 10 and 20 mg of testosterone propionate/kg body wt. The only exception was the expression of CYP2C proteins, where a higher dose of testosterone propionate led to a lowered level of renal expression in the male. It is interesting and perhaps important to note that, in spite of the fact that higher testosterone concentrations altered the magnitude of the effects, these doses did not alter the rate of change from the male to the female phenotype.

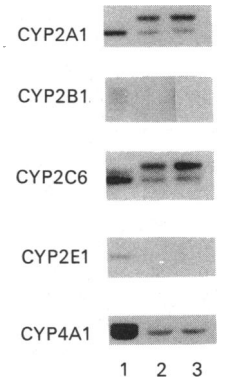

Fig. 4. Involvement of androgen receptor in the sexual dimorphism of mouse renal cytochrome *P*450 expression

Tfm+ + / *Y* and + + *Blo*/ *Y* adult mice were treated either as controls or with 4 mg of testosterone propionate/kg as outlined in Materials and methods section. Kidney microsomes (30 μg) were separated on SDS/PAGE (9% gels), transferred to nitrocellulose and probed with various anti-*P*450 sera as described in the Materials and methods section. M_r values of the immunoreactive bands recognized by the antisera are detailed in the legend to Fig. 2. Lane 1, control; lane 2, Tfm mice; lane 3, Tfm mice treated with testosterone.

Rates of synthesis and degradation of androgen-regulated *P*450 isoenzymes

The time scale over which testosterone propionate alters the female renal *P*450 profile is shown in Fig. 3.

Although some changes in expression were observed 2–3 days after treatment, maximal effects occurred between days 4 and 8. On cessation of testosterone treatment on day 8, it took several days before the female pattern of cytochrome *P*450 expression was re-established. In almost all cases this was approx. 1 week, although in some cases significant decreases in expression, e.g. of CYP2E and CYP4A proteins, were already seen after 3 days. At this time point testosterone levels were still very high (Fig. 3*b*), but they had dropped sharply by day 22.

Fig. 3(*b*) illustrates the serum testosterone levels at the same time points at which the protein expression was studied by immunoblotting. It is interesting to note that testosterone levels in the female mice very rapidly rise to male levels and beyond within 8 h of the start of the experiment. In contrast, the changes in the renal *P*450 profile were much slower, taking over 1 week to become evident. The testosterone levels remained high for over 2 weeks, before falling sharply to normal female values, with a concomitant reappearance of the female renal *P*450 isoenzyme profile.

Involvement of the androgen receptor

Tfm mice were used to investigate the potential role of the androgen receptor in the regulation of mouse renal *P*450 expression (Fig. 4). In all cases, the + + *Blo*/ *Y* control male mice exhibited the expected male pattern of renal *P*450 expression. In contrast, the *Tfm*+ + / *Y* tfm 'male' mice treated with corn oil demonstrated the female pattern. In addition, this pattern of expression was not affected by testosterone treatment.

Renal *P*450 expression in other species

Fig. 5 illustrates renal *P*450 expression in adult male and female C57BL/6 mice, Wistar rats, New Zealand White rabbits and Beagle dogs. For all *P*450s, mouse and rat renal enzymes were sexually differentiated. In the rabbit, kidney proteins in the CYP2A gene family only appeared to be expressed at a very low level. Proteins in the CYP2B, CYP2E and CYP4A families were

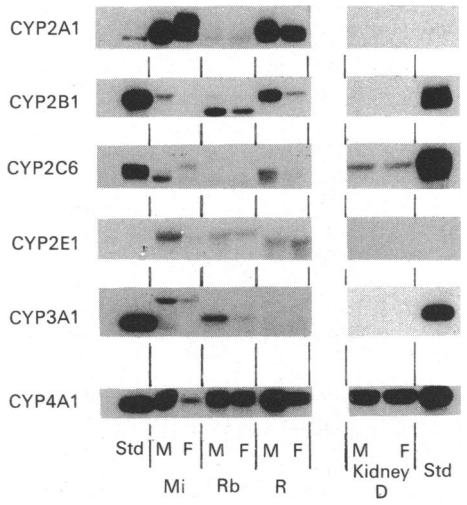

Fig. 5. Renal cytochrome *P*450 expression in different species

Kidney microsomes were prepared from adult male (M) and female (F) C57BL/6 mice (Mi), Wistar rats (R), New Zealand White rabbits (Rb) and Beagle dogs (D), and were separated (30 μg) on SDS/PAGE (9% gels), transferred to nitrocellulose and probed with the various anti-*P*450 sera shown, as described in the Materials and methods section. M_r values of the immunoreactive bands recognized by the antisera are detailed in the legend to Fig. 2.

only marginally sexually differentiated, if at all. A protein of the CYP3A gene family did, however, appear to be sexually dimorphic. In the Beagle dog, only *P*450s in the CYP2C and CYP4A gene families could be identified. These proteins did not exhibit any sex difference in their expression. In these untreated animals no *P*450s in the CYP1A gene family could be clearly identified in any of the species, when tested by either Northern or Western blot analysis.

DISCUSSION

We have previously reported (Henderson *et al.*, 1990) that gene expression in the mouse kidney is sexually differentiated to the extent that all *P*450 proteins detectable by Western blotting either are found to be present in males and absent in females (CYP2B1, CYP2E1) or a different isoenzyme pattern is seen between the sexes. For example, a single CYP2A protein in males migrates with the CYP2A1 standard, whereas in females the expression of this band is decreased, and another, higher-M_r, band is detected. These two proteins are probably the same as those described by Squires & Negishi (1988) as Type I and Type II respectively. With CYP2C6, three protein bands are seen (Fig. 2). The protein of intermediate M_r (band 2) is not expressed in females, the high-M_r protein (band 1) is female-predominant, and the low-M_r band is male-predominant. The anti-CYP3A1 antibody identifies two proteins in the male, expressed at very low levels, which are absent or greatly decreased in females. A higher-M_r protein present in both sexes was also identified at approximately equal levels; however, the identity of this protein is at present unclear. A single protein in the CYP4A gene family is expressed in female mice. This protein is expressed at much higher levels in males, together with a further higher-M_r male-specific protein.

In our earlier study, it was shown that both the induction and suppression of *P*450 isoenzyme expression in the kidney were mediated by testosterone. The mechanism of this effect was unclear, although measurement of mRNA levels indicated that in most cases it was at the transcriptional level. Tfm mice have been used here to investigate the role of the androgen receptor in mediating the testosterone response (Ohno & Lyon, 1970; Meseguer *et al.*, 1989). The control animals are both phenotypically and genotypically male, i.e. $++blo/Y$, and are distinguished by a 'blotchy' coat. The test animals were phenotypically female, but genotypically male, i.e. $Tfm++/Y$, with a deficient androgen receptor, and as a consequence are unable to respond to testosterone. These animals possess female external genitalia. Yarbrough *et al.* (1990) have recently shown that a single base mutation in the androgen receptor gene, resulting in a change of arginine-734 to a glutamine residue, is sufficient to result in the androgen-insensitivity displayed by the Tfm rat. The mechanism of inactivation of the androgen receptor in Tfm mice is not clear, but may involve similar mutations. The data presented in Fig. 4, where Tfm 'male' mice exhibit a female pattern of renal *P*450 expression which cannot be altered by testosterone administration, clearly demonstrate that the action of testosterone in determining the mouse renal *P*450 isoenzyme profile is mediated by the androgen receptor, and strengthens the case that transcriptional activation is involved. In all cases, with the exception of CYP4A, the sex difference in cytochrome *P*450 levels is reflected in a difference in mRNA levels. In agreement with this observation, preliminary nuclear run-on transcription experiments (results not shown) show an increase in transcription after 10 days of testosterone treatment. Rundlett *et al.* (1990), investigating the molecular basis of androgen action in the rat prostate, have recently demonstrated that androgens operating via the androgen receptor alter gene expression at the transcriptional level. Deletion of the *C*-terminal 250 amino acid residues of the androgen receptor converts the receptor into a constitutive initiator of transcription.

The time course of androgen action in the mouse kidney (Fig. 3) showed that it takes 8–10 days for testosterone to exert maximal effects on *P*450 protein expression on the renal *P*450 isoenzyme profile from female to male. This is very much slower than the time taken for the circulating testosterone levels to rise to male levels and beyond (approx. 8 h). This relatively long lag period has also recently been demonstrated by Watson & Paigen (1990), in relation to the induction of synthesis of other androgen-inducible mRNAs in the mouse kidney. These workers measured the rates of synthesis of, among others, mRNAs for alcohol dehydrogenase, β-glucuronidase, ornithine decarboxylase and kidney androgen-regulated protein. Watson & Paigen (1990) found that a period of 2–10 days, depending on the mRNA studied, was required before mRNA levels reached a maximum. This was clearly much slower than the time required (approx. 30 min) for the androgen receptor complex to migrate to the nucleus and exert its effects on transcription (Isomoa *et al.*, 1982; Catterall *et al.*, 1985).

The androgen-receptor-mediated regulation of *P*450 expression appears to be complex, and probably does not involve a direct interaction of the androgen receptor with a promoter in the *P*450 genes. This complexity is further demonstrated by the observation that the expression of the *P*450 isoenzymes could be further increased with testosterone doses above those expected to saturate the androgen receptor. Also, on removal of androgen the *P*450 level in females returned to the female profile before circulating levels of the hormone had returned to normal. The mechanism of these effects remains at present unclear. It is interesting to note that, whereas the expression of most *P*450s increased with increased testosterone dose, expression of members of the CYP2C gene family decreased, indicating negative feedback at high androgen concentrations.

The requirement for androgen and the androgen receptor for the sexual differentiation of renal cytochrome *P*450s is difficult to reconcile with the observation that, apart from a *P*450 isoenzyme

in the CYP4A gene family, all detectable *P*450s are already sexually differentiated at 3 weeks of age. It should be noted, however, that for some of the proteins both the level of expression and the difference in expression between the sexes was much less marked than at the onset of puberty. This was also the case for the effects of testosterone; thus it appears that in such cases there is a balance between testosterone effects and other hormonal influences. This could be explained if in adults testosterone is acting, via the androgen receptor, on a repressor of the male phenotype, and if this repressor is absent in pre-pubertal mice. This, however, would still not explain why 3-week-old mice can respond to treatment with androgen, unless other factors are involved.

The extent of sexual differentiation of renal *P*450s in other mammals showed that sexual differentiation of cytochrome *P*450 expression was very marked in rat and much less pronounced in rabbit. No sexual differentiation of *P*450 was observed in the dog kidney. The reasons why some species do not exhibit much profound sexual dimorphism in kidney gene expression is unclear, but may be related to hormone levels or the reproductive cycle. These findings are interesting in the light of the recent observation of Andersson (1990), who reported that male Rainbow trout have higher levels of cytochrome *P*450s and associated enzyme activities than females. Andersson (1990) found a regional distribution in trout renal *P*450 content, with total *P*450 levels 30-fold higher in males compared with females in the trunk region of the trout kidney, but the reverse in the head region. In addition, certain *P*450-dependent catalytic activities were found to be higher in females. It was concluded that, although *P*450 levels were usually higher in males, this was not due to a generalized increase in all *P*450s, but rather to an increased expression of specific isoenzymes. These data indicate that sexual differentiation of *P*450 expression has been conserved throughout evolution. Whether this is due to environmental or dietary factors, pheromone metabolism, etc., is open to speculation.

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