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Expression and Characterization of Isoforms of 3β-Hydroxysteroid Dehydrogenase/$\Delta^{5\rightarrow4}$-Isomerase in the Hamster

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The enzyme 3β-hydroxysteroid dehydrogenase/$\Delta^{5\rightarrow4}$-isomerase (3β-HSD) is essential for the production of all classes of steroid hormones. Multiple isozymes of this enzyme have been demonstrated in the kidney and liver of both the rat and the mouse, although the function of the enzyme in these tissues is unknown. We have characterized three isozymes of 3β-HSD expressed in various tissues of the hamster. Both western and northern blot analyses demonstrated very high levels of 3β-HSD in the adrenal, kidney and male liver. Conversely, there were extremely low levels of enzyme expression in the female liver. cDNA libraries prepared from RNA isolated from hamster adrenal, kidney and liver were screened with a full-length cDNA encoding human type 1 3β-HSD. Separate cDNAs encoding three isoforms of 3β-HSD were isolated from these libraries. To examine the properties of the isoforms, the cDNAs were ligated into expression vectors for over-expression in 293 human fetal kidney cells. The type 1 isoform, isolated from an adrenal cDNA library, was identified as a high-affinity 3β-hydroxysteroid dehydrogenase. A separate isoform, designated type 2, was isolated from the kidney, and this was also a high-affinity dehydrogenase/isomerase. Two cDNAs were isolated from the liver, one identical in sequence to type 2 of the kidney, and a distinct cDNA encoding an isoform designated type 3. The type 3 3β-HSD possessed no steroid dehydrogenase activity but was found to function as a 3-ketosteroid reductase. Thus male hamster liver expresses a high-affinity 3β-HSD (type 2) and a 3-ketosteroid reductase (type 3), whereas the kidney of both sexes express the type 2 3β-HSD isoform. These differ from the type 1 3β-HSD expressed in the adrenal cortex.

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INTRODUCTION

The enzyme 3β-hydroxysteroid dehydrogenase/$\Delta^{5\rightarrow4}$-isomerase (3β-HSD) is essential for the production of all classes of steroid hormones, being responsible for the formation of a 3-keto-4-ene conformation essential for activity of mineralocorticoids, glucocorticoids, androgens and estrogens. The enzyme is expressed in the adrenal cortex and in steroidogenic cells of the gonads, consistent with this role. However, 3β-HSD is also expressed in other tissues, such as the liver and kidney, where its function is unknown. Most of the work characterizing 3β-HSD in liver and kidney has been performed in the rat and mouse. In the rat, two isoforms of 3β-HSD have been identified in the liver, a low affinity dehydrogenase [1] and a structurally related 3-ketosteroid reductase that possesses no dehydrogenase activity [2], whereas the kidney expresses a high affinity dehydrogenase identical to that expressed in the adrenal and gonad [3, 4]. In the mouse both a high [5] and low affinity [6] dehydrogenase have been described in the liver. In contrast to the rat, there is a 3-ketosteroid reductase that is expressed exclusively in the kidney [7]. An important feature of 3β-HSD in the liver and kidney is the sex-specific expression of different isoforms in these two tissues. In the rat, both the

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dehydrogenase and reductase enzymes are expressed at much higher levels in the adult male liver than in female liver [1, 8]. This evidence is based on studies based on northern blot RNA analysis, western immunoblotting and activity determinations. The expression of both isozymes in the liver appear to be regulated directly by the pattern of growth hormone secretion, and indirectly by testosterone and estrogen acting to change the nature of growth hormone release [1, 8]. In the mouse, the sex-specific expression of isozymes of 3β-HSD in both the liver and kidney has been demonstrated by western blotting [6].

Although the rat and mouse have been much used as models for the study of steroidogenesis, they produce corticosterone in the adrenal as their principal glucocorticoid and do not synthesize cortisol, a feature of species such as human [9]. The hamster is, however, a rodent species in which cortisol is the principal corticosteroid [10], and a study on the regulation of adrenal steroidogenic enzymes has suggested that the hamster is a good model for human steroidogenesis [11]. In this study, we cloned and characterized a cDNA encoding hamster adrenal 3β-HSD and then proceeded to determine the nature of 3β-HSD(s) expressed in hamster liver and kidney.

METHODS

Western blot analysis

Western blotting was performed as described previously [1]. Briefly, Syrian Golden hamster tissues were homogenized in lysing buffer containing 1% sodium cholate and 0.1% sodium dodecyl sulfate to solubilize membrane-bound protein. One-dimensional electrophoresis was performed in an 8% polyacrylamide gel. Protein was electroblotted on to Immobilon-PVDF membranes for 1 h at 100 V. The filters were probed with polyclonal antibodies (5 μg immunoglobulin G/ml) raised against human placental 3β-HSD [12]. After washing, the bound antibody was detected by incubation with approx. 10,000 cpm/ml [^{125}I]-protein A (ICN, Irvine, CA, U.S.A.) and exposing the filter to X-ray film.

Northern blot analysis

Northern blot analysis was performed as described previously [1]. Briefly, total RNA was isolated from tissues using the method of Chomczynski and Sacchi [13]. Total RNA (15 μg) was electrophoresed in a 1.0% denaturing agarose gel. The RNA was then electroblotted on to Immobilon-N nylon membranes and fixed on to the membrane by UV irradiation. The filters were hybridized overnight at 42°C with a full-length human 3β-HSD type I cDNA labelled with [^{32}P]dCTP by the random primer method. The blots were washed with 1 × SSC (0.15 M NaCl, 0.015 M sodium citrate) + 0.1% SDS and exposed to X-ray film.

Screening of cDNA libraries

Lambda-ZAP cDNA libraries were synthesized by Stratagene Inc. (La Jolla, CA, U.S.A.) from RNA isolated from hamster adult male adrenal, kidney and liver. The libraries were screened with a full-length cDNA encoding human type I 3β-HSD [14], labelled with [^{32}P]dCTP by the random primer method. Positive clones were isolated and the Bluescript SK/KS plasmid, containing the cDNA of interest, was excised from the phagmid as per manufacturer's instructions and transformed into SOL-R competent *E. coli* cells. Both strands of DNA were sequenced by the dideoxy chain termination method [15] using Sequenase (United States Biochemicals) and [^{35}S]dATP.

Construction of 3β-HSD expression vectors and transfection into 293 human fetal kidney cells

Hamster 3β-HSD types 1 and 3 were ligated into the pcDNA 3 vector (Invitrogen Corp., San Diego, CA, U.S.A.). XhoI–NotI digests were made to excise the full-length clone from the vector, which was purified from an agarose gel by the freeze–squeeze method [16] and ligated into the pcDNA 3 vector digested the same way. For hamster 3β-HSD type 2, constructs made in this way possessed no activity. It was decided to PCR amplify the coding region of the 3β-HSD type 2 cDNA, using the upstream primer GAATTCGCCATGCCTGGATGGAGC, which placed an Eco RI site on the end of nucleotides −3 to +15, and the downstream primer TCACATTCACTGAGCCTTTGT, complementary to the 3′ end of the coding region, +1108 to +1128. This PCR product was ligated directly into the expression vector pCR3, and sequenced to ensure there was no mis-incorporation of nucleotides into the sequence during amplification.

293 cells were grown in Dulbecco's modified Eagle's high glucose medium supplemented with Hepes (10 mM), calf bovine serum (10%) and antibiotics/antimycotics. The cells were stably transfected using Lipofectin reagent (Gibco-BRL, Gaithersburg, MD, U.S.A.) according to the protocol supplied by the manufacturer. Geneticin (0.5 mg/ml) was added to the culture medium 48 h after transfection to facilitate the selection of cells expressing the neomycin resistance gene. Initial characterization of 3β-HSD activity of intact cells was conducted after addition of tritium-labelled pregnenolone (1 μM) to the culture medium for a period of 3 h. The medium was then analyzed for the conversion of pregnenolone to progesterone as described below.

Measurement of 3β-HSD activity

The kinetics of the conversion of pregnenolone to progesterone and dehydroepiandrosterone (DHEA) to androstenedione, were measured by adding substrate to the cultured cells in a concentration range of 0.3–30 μM. The kinetics of 5α-dihydrotestosterone

(5α-DHT) metabolism were not examined. Steroids were extracted from the culture medium by the addition of dichloromethane (10 vol). The aqueous layer was removed and the organic solvent evaporated. The reactions were analyzed by thin layer chromatography (TLC). For the separation of pregnenolone and progesterone, a solvent system of chloroform : ethyl acetate (v/v, 8:2) was used. For the separation of 5α-dihydrotestosterone and 5α-androstan-3β,17β-diol (5α-Adiol) were resolved using two developments in chloroform:ethyl acetate (v/v, 9:1). The plates were analyzed using a radioanalyzer and the results expressed as pmol product formed/min · ml medium. Double reciprocal Lineweaver–Burk plots were generated to derive the K_m values for the substrates.

RESULTS

Northern blot analysis

The results of northern blot analysis are shown in Fig. 1. The size of the mRNA for 3β-HSD in the hamster is 2.1 kb. High levels of mRNA are found in both the male and female adrenal. Moderate levels are found in the kidney, and low levels in the testis and ovary. In the liver, there is sexually dimorphic expression of 3β-HSD; high levels are observed in the male liver, but are almost undetectable in the female liver. The ovarian status of the female donor hamsters were not established. Because the level of hybridizable ovarian 3β-HSD mRNA species was low, it is likely that the animals were in the follicular stage of their cycle.

Western blot analysis

The results of the western blot analysis (Fig. 2) are consistent with the northern blot results. High levels of 3β-HSD expression are apparent in the adrenal, moderate levels in the kidney and ovary, and low levels in the whole testis. Also consistent is the very high expression of protein in the male liver compared to almost undetectable levels in the female liver.

Screening of cDNA libraries

From the adrenal library, 9 positive clones were isolated, 5 of which contained the entire coding region, as compared to the sequences of 3β-HSD from other species. All clones gave identical sequence. This will be referred to as type 1 in future. The cDNA codes for a protein of 373 amino acids, with a calculated molecular weight of 41,753. From the kidney library, 5 positive clones were isolated, four of which contained the entire coding region. All clones gave identical sequence, but it was distinct from the adrenal sequence and this clone was named type 2. This clone also codes for a protein of 373 amino acids, with a calculated molecular weight of 41,903. From the liver library, 13 positive clones were isolated. Twelve of these had identical sequence to type 2. The remaining clone has a sequence distinct from both types 1 and 2 (Fig. 3) and is referred to as type 3. This isozyme also consists of 373 amino acids, with a calculated molecular weight of 41,817. The respective percentage identities and similarities between these three hamster 3β-HSDs are 92.2 and 95.2% (types 1 and 2), 83.2 and 90.4% (types 1 and 3) and 79.7 and 88.2% (types 2 and 3).

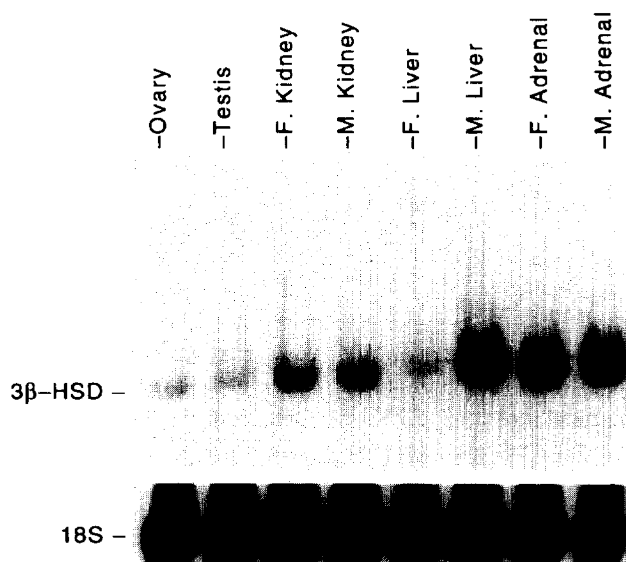

Fig. 1. Northern blot analysis of 3β-HSD mRNA levels in total RNA (15 μg loaded per lane) isolated from adult male (M) and female (F) hamster tissues, including ovary, testis, kidney, liver and adrenal. The size of the 3β-HSD mRNA species was estimated to be 2.1 kb. The membrane was reprobed for 18S RNA to establish the consistency of loading.

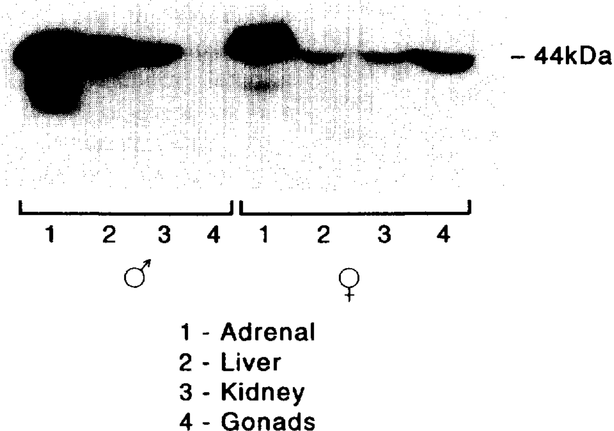

Fig. 2. Western blot analysis of 3β-HSD in adult male and female hamster tissues. Tissue homogenate samples (100 μg protein per lane) were electrophoresed on a 8% polyacrylamide gel and electroblotted on to a nylon membrane. The membrane was treated with a rabbit polyclonal antibody raised against human placental 3β-HSD and antibody binding was visualized using [^{125}I]-protein A and autoradiography.

Activity studies

Both the hamster type 1 and type 2 3β-HSDs are dehydrogenase/isomerases. In whole cells, the K_m values of type 1 for pregnenolone and DHEA were calculated as 5.5 and 2.4 μM, respectively. For the type 2 enzyme the calculated K_ms for pregnenolone and DHEA were 8.8 and 2.9 μM, respectively. For comparison, human type 1 [12] and type 2 3β-HSD cDNAs were transfected and assayed in intact cells at the same time as the hamster enzyme assays. The K_m values of type 1 human 3β-HSD for pregnenolone and DHEA were 5.6 and 3.9 μM, respectively, and for human type 2 they were 9.3 and 5.7 μM, respectively.

Both hamster type 1 and 2 enzymes are able to act as 3-keto-reductases reducing 5α-dihydrotestosterone to the corresponding 3β-Adiol (results not presented).

In contrast, type 3 did not act as a dehydrogenase/isomerase and acted exclusively as a 3-ketosteroid reductase. From the same batch of transfected cells, no conversion of pregnenolone to progesterone was observed, whereas a large conversion of 5α-DHT to 5α-Adiol was seen (Fig. 4).

DISCUSSION

In this paper we report the initial characterization of three separate isoforms of 3β-HSD in the hamster. Type 1 isoform was cloned from an adrenal cDNA library, type 2 was cloned from both liver and kidney libraries, and type 3 was cloned from the liver library. The types 1 and 2 isoforms are dehydrogenase/isomerases, whereas the type 3 enzyme is a 3-ketosteroid reductase.

The situation in the hamster is therefore similar to both the rat and the mouse. In both the rat and mouse, a high affinity dehydrogenase is expressed in the adrenal and gonad, consistent with the steroidogenic role of these tissues. Similar to the hamster, the mouse liver expresses a high affinity dehydrogenase, although in the rat liver a low affinity dehydrogenase has been described. In all three species, a reductase enzyme is expressed in the liver; in the mouse, though, a second reductase has been found that is expressed exclusively in the kidney.

Comparison of the amino acid sequences of the hamster and other rodent sequences reveals some interesting features. A major feature of the reductase enzymes in all species is their preference for NADPH as co-factor, whereas the reductase activity of the dehydrogenases uses NADH. Aspartate is located at the C-terminal side of the nucleotide binding domain of NAD/NADH preferring enzymes (e.g. codon 36 in hamster 3β-HSD types 1 and 2), whereas the corresponding residue in the NADPH-dependent 3-ketoreductases of the three species evaluated to date is phenylalanine (hamster, mouse) or tyrosine (rat). It has been previously demonstrated that aspartic acid 38 determines the co-factor specificity of Drosophila alcohol dehydrogenase [17].

There is sexually dimorphic expression of 3β-HSD in the hamster liver. Western and northern blot results show that the enzyme is expressed in very high levels in the male liver, equivalent to those in the adrenal, but there is very little expression in the female liver. This situation is also seen in the rat. The mouse is somewhat different; both male and female liver express a 47 kDa protein, whereas a 42 kDa protein is expressed exclusively in the male. The mouse also differs from both the hamster and rat in that there is also sexually dimorphic expression of 3β-HSD isoforms in the kidney and gonad [6]. This has not been observed in either the rat or hamster.

The role of 3β-HSD in tissues such as the liver and kidney is unclear. Given the role of the liver in the metabolism and clearance of a wide variety of compounds, it is probable that 3β-HSD is involved with the metabolism of some biological compound. In the liver, it is possible that the role of the 3-ketosteroid reductase is in the inactivation of 5α-DHT, since this reaction is not reversible in the type 3 enzyme and would lead to the inactivation of this androgen. The much greater expression of the 3-ketosteroid reductase (3β-HSD type 3) in the male liver as opposed to the female liver would be consistent with this hypothesis, although in itself is not proof; many drug-metabolizing *P*450s in the liver are expressed in a sexually dimorphic manner, and only certain ones appear to be involved in the metabolism of sex steroids. Present evidence in various species is suggestive, however, that the 3β-HSD pathway may be more vital. The presence of high levels of the type 2 3β-HSD in the liver would suggest that any 5α-Adiol formed in the liver by the reductase would be oxidized back to 5α-DHT. The balance of the dehydrogenase situation would also depend on the environment within the cell as to which reaction, dehydrogenase or reductase, would predominate at a given time. Of course, it cannot be ruled out that 3β-HSD is involved in the metabolism of a compound, as yet unidentified, and not necessarily a steroid. There is a precedent for this in that a 3β-HSD must convert 7α-hydroxycholesterol to 7α-hydroxycholest-4-en-3-one in the bile acid biosynthetic pathway. Because of the sex-specific expression of the cloned liver 3β-HSD, however, it is unlikely that this isoform is the principal 3β-HSD involved in bile acid biosynthesis.

The reason for 3β-HSD expression in the kidney is also unclear. In the mouse it has been demonstrated that 3β-HSD is expressed in the kidney tubules. The kidney tubules are involved in fluid and electrolyte balance. In the adrenal, 3β-HSD is one enzyme involved in the synthesis of aldosterone, the key steroid hormone affecting tubule function. There is, however, no evidence of biosynthesis of aldosterone in the kidney tubules, nor for circulating levels of 3β-hydroxysteroid precursors that could be used as substrates to produce active aldosterone. Again, it is possible that there is

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                   10         20         30         40         50
TYPE 1     MPGWSCLVTG AGGFLGQRII RMLVQEKELQ EVRALDKVFR PETREEFCKL
TYPE 2     ********** ********** HL*****D*E ***L****** *******F**
TYPE 3     **A******* ********** ***A****** ***T*FRS*T *KH***LS**

                   60         70         80         90        100
           QTKTKVTVLE GDILDAQCLR RACQGISVVI HTAAAIDVFG AIPRQTIIDI
           ********** ********** ********** ********W* I*********
           ********** ********** ********** ********** ******V***

                  110        120        130        140        150
           NLKGTLNLLE ACVQASVPAF IYTSSIDVAG PNSYKEIVLN GHEEQQHEST
           *V******** ********** ********** ********** **********
           *****QH**D **IG*R**V* **S**VA*** *****V*IQ* *S**EN****

                  160        170        180        190        200
           WSDPYPYSKK MAEKAVLAAN GSSLKNGGTL HTCALRPMYI YGEKSPLISV
           *********M ********** **F******* ********** *****SIL*G
           *****A**** ********** **T**D**** ******LPF* *****KF***

                  210        220        230        240        250
           TIIRAVKNSG ILDVTGKFST VNPVYVNNAA WAHILAARGL QDPRKSPNIQ
           IM***I**N* **K******* ******S*** ********** ***K******
           *MD**L**N* LINGFSR**V ISS******* ***V****** ***K******

                  260        270        280        290        300
           GQFYYISDDT PHQSYDDLNY VLSKDWGLRP DSSWRPPVAL LYWLGFLLEL
           ********** *********N T***K***** ********** **********
           ********** ********C* T********* ****K***** ***F******

                  310        320        330        340        350
           VSFLLRPVYN YQPPFNRHLV TLSNTVFTFS YKKAQRDLGY EPLVGWEEAR
           *N******** *****T*Y** *I******** ********** **********
           ********** ********** **L*S***** ********** ****S*****

                  360        370
           ENTSEWIGSL VEQHKGTLNT KAQ
           ********** ********** ***
           *K******** *********I ***
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Fig. 3. Comparison of the amino acid sequences of hamster 3β-HSD types 1, 2 and 3, derived from the sequencing of corresponding cDNA clones.

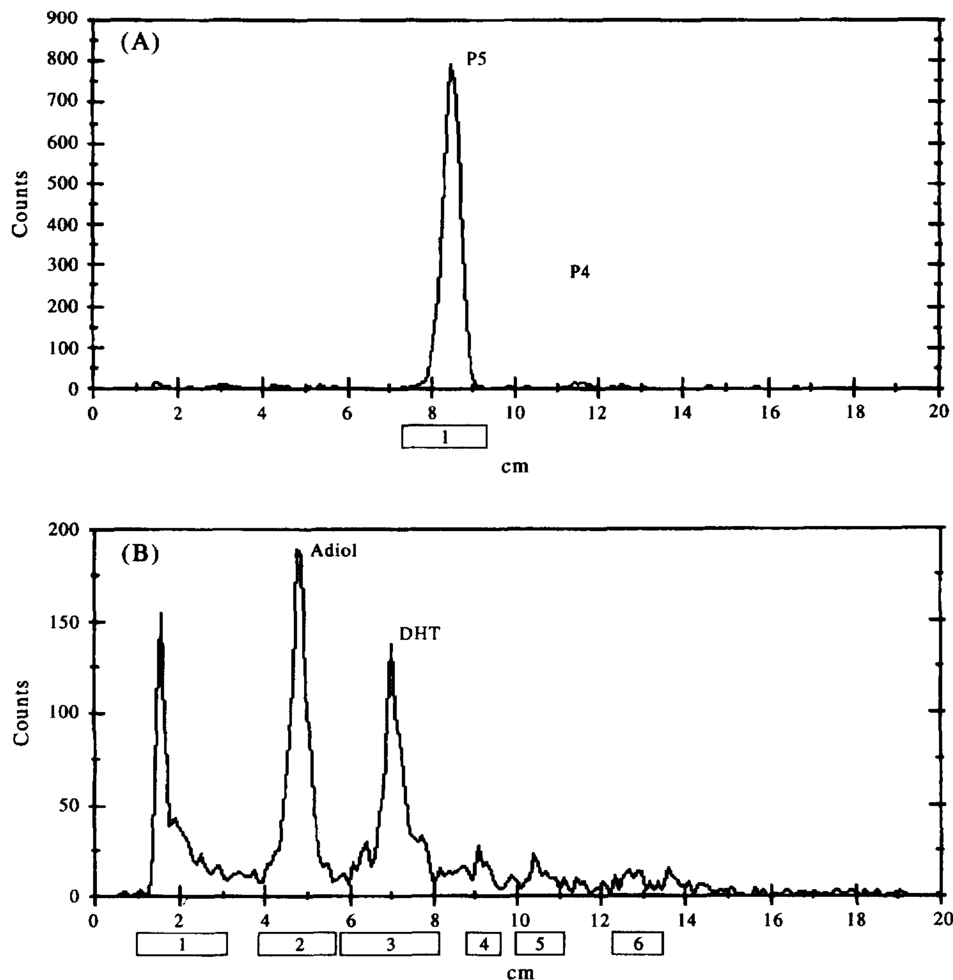

Fig. 4. Metabolism of pregnenolone (P5) and 5α-dihydrotestosterone (DHT) by 293 cells transfected with a cDNA encoding hamster type 3 3β-HSD ligated into the pcDNA3 expression vector. The cells were stably transfected using Lipofectin and geneticin selection. Cells were incubated with [^{3}H]-labelled steroid (1 μM) for 3 h; steroids were then extracted from the medium, separated by TLC and imaged on a RadioImage analyzer. (A) Metabolism of P5 to progesterone (P4). This minimal conversion was not different to that observed in mock-transfected cells. (B) Metabolism of DHT to 5α-androstane-3β,17β-diol (Adiol). The peak of polar product, close to the origin, was not identified. Mock-transfected cells exhibited a minimal conversion of DHT to Adiol.

another, as yet unidentified, substrate for 3β-HSD in the kidney.

It is clear that an understanding of the nature of the expression of the 3β-HSD family of enzymes in tissues such as kidney and liver requires much further endeavour.

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