Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Steroid Biochemistry & Molecular Biology 91 (2004) 49–58
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Expression of cytochrome P450c17 and other steroid-converting enzymes in the rat kidney throughout the life-span

Luisa Dalla Valle*, Vania Toffolo, Silvia Vianello, Paola Belvedere, Lorenzo Colombo

Comparative Endocrinology Laboratory, Department of Biology, University of Padova, Via U. Bassi 58/B, 35131 Padova, Italy

Received 15 September 2003; accepted 21 January 2004

Abstract

We have investigated the metabolism of [^{14}C]-labelled progesterone (P4) and dehydroepiandrosterone (DHEA) by kidney tissues of newborn and 7-, 15-, 30-, 60- and 365-day-old rats of both sexes. The following enzymes were revealed at all ages by radiochemical identification of the corresponding products: 5α-reductase, cytochromes P450c17 and P450c21, 3β-hydroxysteroid dehydrogenase (HSD)/Δ^5–Δ^4 isomerase, and 17β- and 20α-HSDs, catalyzing reductive reactions. The major P4 metabolites were 5α-reduced C_{21} steroids, whose formation was almost completely suppressed by the 5α-reductase 4-azasteroid inhibitor, PNU 156765. Androstenedione and testosterone were also formed via 17α-hydroxyprogesterone, together with 11-deoxycorticosterone and 20α-dihydroprogesterone. DHEA was mainly converted to androst-5-ene-3β,17β-diol, with smaller amounts of the above androgens.

Cytochrome P450c17 mRNA and protein were demonstrated by Northern blotting and Western blotting analyses, respectively. P450c17 mRNA, assessed by Northern blotting, protein and catalytic activity all peaked in the kidney samples at 15 days of life and declined thereafter. Cytochrome P450arom was below the level of detection of semi-quantitative RT-PCR. Since the rat kidney has been previously shown to contain cytochrome P450scc as well as androgen and estrogen receptors, it is suggested that it is capable of autonomous hormonal steroidogenesis and that renal steroids, or nephrosteroids, may act locally, in a paracrine or autocrine fashion.

Keywords: Cytochrome P450c17; Extraglandular steroidogenesis; Rat kidney

1. Introduction

In recent years, growing evidence has been collected regarding the presence and activity of key steroid hormone-biosynthetic enzymes in peripheral tissues of the rat and other mammalian species. The extraglandular tissues appear to contain a variety of steroidogenic enzymes, including both steroid hydroxysteroid dehydrogenases (HSDs), such as 3β- and 17β-HSDs, which are heterogeneous and often present as different molecular forms with respect to their gonadal and adrenocortical counterparts, and steroid cytochromes P450s, including P450scc, P450c17 and P450arom required for progestogen, androgen and estrogen synthesis, respectively, which are essentially monomorphic [1].

In the rat, extraglandular steroidogenic cytochromes show frequently developmentally regulated patterns of expression and assortment. In the liver, mRNA, protein and enzymatic activity of P450c17, as evidenced by Northern and immunoblot analyses and radiochemistry, respectively, are detectable at birth at a level comparable to that of the neonatal testis, reach a peak at day 8 with a specific activity four-fold higher than that of the adult testis, and declines thereafter to disappear entirely after puberty. Since the hepatic peak of P450c17 corresponds to a depression of the same enzymatic activity in the neonatal testis, it is suggested that the liver is involved in the conversion of circulating progestogens into androgens to support gonadal aromatization during Sertoli and granulosa cell proliferation [2].

In the rat stomach and duodenum, P450c17 expression occurs mainly after the weaning period [2], and its localization is restricted to the parietal cells of the gastric mucosa [3] and the deep glandular portion of the duodenal mucosa [4], where it was suggested to be involved in the production of androgens for their regenerative turnover. More recently, Ueyama et al. [5] demonstrated that gastric parietal cells of male and female adult rats express also the P450arom enzyme and, by secreting estrogen, may play a potent endocrine role through a gastro-hepatic axis.

In the brain of the postnatal rat, P450scc mRNA expression is relatively constant throughout the organ and during

* Corresponding author. Tel.: +39 049 8276188; fax: +39 049 8276199.
E-mail address: luisadv@mail.bio.unipd.it (L. Dalla Valle).

0960-0760/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2004.01.008

neonatal life (3 and 10 days), but tends to decrease at adulthood (61 days of age), whereas the expression of 3β-HSD or P450c17 mRNAs seems to be more age- or region-dependent [6].

We have recently demonstrated that the rat kidney expresses cytochrome P450scc with a localization restricted to the cortical distal tubules, as evidenced by immunohistochemistry, and at higher levels during the first days of life [35]. A preliminary report concerning the presence and activities of cytochrome P450c17, together with some HSDs, such as 3β- and 17β-HSDs, in the rat kidney of aged male rats has been previously published by our laboratory [7]. The activities of the same steroidogenic enzymes have been recently reported by Quinkler et al. [8] also in the human kidney.

In the present study, we have investigated the metabolism of progesterone (P4) and dehydroepiandrosterone (DHEA) by the kidneys of male and female rats throughout the life-span in order to elucidate the steroidogenic pathways present in this organ and to verify, with the aid of biomolecular techniques, the possibility of a temporal pattern of expression of the enzymatic set involved in steroid production.

2. Materials and methods

2.1. Animals and tissue preparation

Male and female rats (Wistar strain; 0-, 7-, 15-, 30-, 60-day-old and 1-year-old) were obtained from Morini (Italy). Animals were killed by cervical dislocation after CO_2-induced asphyxia and kidneys were immediately dissected out. Tissues were minced and pooled separately from male and female donors. For enzymatic assays and microsomal fractioning, tissues were used immediately, whereas, for RNA extraction, they were frozen in liquid N_2 and stored at −80 °C until use.

2.2. Chemicals

Precursor [4-^{14}C]steroids (sp. act. 1.8 GBq/mmol) were obtained from Amersham Pharmacia (Italy), while [^{3}H]-labelled tracers (sp. act. 3.1 TBq/mmol) were purchased from New England Nuclear Co. (Italy). All radioactive steroids were purified by two-dimensional TLC prior to use. Reference steroids were obtained from Steraloids Inc. (Pawling, NY, USA) or Sigma–Aldrich (Italy). Plastic sheets pre-coated with silica gel 60 F_{254} (20 cm × 20 cm × 0.2 mm thickness) were from Merck (Italy). No-screen medical X-ray safety films from Kodak (Sigma–Aldrich) were utilized for autoradiography. The 5α-reductase inhibitor, PNU 156765, previously named FCE 28260, [(22*RS*)-*N*-(1,1,1-trifluoro-2-phenylprop-2-yl)-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide], was kindly provided by Pharmacia and Upjohn, Nerviano (MI), Italy [9].

2.3. Incubations with [4-^{14}C]-labelled precursors

Minced renal tissues (0.25 g wet weight) were separately incubated in 10 ml of medium containing 18.5 kBq (0.9 μM) of [4-^{14}C]P4 or [4-^{14}C]DHEA, previously dissolved in 100 μl of propylene glycol. Incubations were carried out in a Dubnoff metabolic shaker for 4 h at 37 °C under carboxygen (95% O_2–5% CO_2). Metabolism was terminated with 2 volumes of ethanol/ethyl acetate (3:1) and incubates were stored at −20 °C until processed. Boiled tissues were incubated as controls.

2.4. Inhibition of 5α-reductase by PNU 156765

For the experiments with PNU 156765, tissues were homogenized in a balanced salt medium (100 mM KC1,16 mM K_2HPO_4, 4 mM KH_2PO_4, 1 mM dithiothreitol, 1 mM EDTA, and 2 μg/ml each of leupeptin and pepstatin A, pH 7.4) and the homogenates were centrifuged four times at 1000 × *g* for 15 min at 4 °C. Proteins in the last supernatant were measured by the BCA protein assay kit (Pierce, Celbio, Italy) with BSA as a standard. To evaluate PNU 156765 inhibition of kidney 5α-reductase activity, the inhibitor (500 nM) was added to renal homogenates from 15-day-old male and female rats and incubated with [4-^{14}C]P4 (9 kBq, 2 μM), dissolved in 20 μl of propylene glycol, for 2 h at 37 °C. Incubations were performed in a final volume of 2 ml containing 1.8 ml of renal homogenate (10 mg protein) in presence of 200 μl of an NADPH-generating system (final concentrations: 0.1 mM NADPH, 1 mM $NADP^+$, 10 mM glucose-6-phosphate, and 2 IU glucose-6-phosphate dehydrogenase). The enzyme activity was expressed as pmol/h/mg protein.

2.5. Extraction and chromatography

Incubates were extracted four times with 1 volume of 95% ethanol which was centrifuged at 1000 × *g* for 10 min to precipitate the tissue and suspended matter. One-fifth of each extract was first analyzed to establish the content of metabolites. Appropriate carriers (3 μg) and [^{3}H]-tracer steroids were then added to the remainder in a known dpm quantity for recovery control and metabolite identification by recrystallization to constant isotope ratio or constant specific activity. The ethanol extracts were concentrated to 2.5 ml under air at 40 °C and the aqueous residues, diluted to 5 ml with distilled water, were extracted four times with 2 volumes of ethyl acetate to obtain the free steroid fraction. The water-soluble metabolite fraction was calculated from the difference between the radioactivities of the ethanol and ethyl acetate extracts.

Metabolites were separated by two-dimensional TLC with the solvent systems cyclohexane/ethyl acetate (95:5, v/v; five to seven runs) in the first direction for defatting, and toluene/acetone (8:2, v/v; two runs) in the second direction

for steroid separation which was completed with cyclohexane/ethyl acetate (1:1, v/v; two runs) again in the first direction. Chromatoplates were then autoradiographed for 5 days and the spots corresponding to the labelled carriers were removed, eluted in 20 ml of acetone and counted in a Packard Tri-Carb 1500 liquid scintillation analyzer with a dual-label program.

2.6. Identification of steroid metabolites

Radiochemical identity and homogeneity of [^{14}C]metabolites were inferred from their isopolarity and isomorphicity with authentic [^{3}H]compounds diluted with carriers. Isopolarity was demonstrated by the constancy of the [^{3}H]/[^{14}C] ratio after acetylation or CrO_3 oxidation of the metabolite [10]. Coincidence between the transformed carrier and [^{14}C]radioactivity after chromatography was also checked by autoradiography.

For each incubate, conclusive identification of labelled metabolites mixed with a tracer was based on recrystallization in acetone–water or pyridine–acetone–water to constant [^{3}H]/[^{14}C] ratio of the unchanged compounds or their acetylated derivatives. Isomorphycity was established when, after recrystallization, the [^{3}H]/[^{14}C] ratios of three consecutive crystal crops and of the last mother liquor were within ±5% of the mean. When radioactivity was low (<1% conversion), pooled samples were recrystallized. The residual precursor was identified only by its mobility in the first two-dimensional chromatogram. Percent conversions were calculated from the last [^{3}H]/[^{14}C] ratio in the purification procedure of individual steroids and were thus corrected for losses. For lack of corresponding [^{3}H]tracers, yields of 5α-pregnan-3β-ol-20-one were not corrected for losses and these compounds were recrystallized as their mono-acetates to constant [^{14}C]dpm mg^{-1} carrier ratio.

2.7. Northern blotting analysis of cytochrome P450c17

Total RNA was extracted from pools of frozen kidneys of newborn and 7-, 15-, 30- and 60-day-old male rats using the commercial product RNAzol B (Celbio) according to the manufacturer's instructions. Testicular RNA was used as a positive control. The RNA was enriched in polyadenylated mRNA utilizing the NucleoTrap mRNA kit (M-Medical, Italy).

The RNAs were electrophoresed through 1.1% formaldehyde-denaturing gel, blotted onto a nylon membrane (Hybond-N+, Amersham) and baked for 2 h at 80 °C. The DIG-labelled RNA molecular weight marker II (Roche Applied Science, Italy) was used as a size standard. Membranes were hybridized overnight at 68 °C using a DIG-labelled cRNA probe corresponding to a fragment of the coding region of P450c17 (nucleotides +450/+1242) in 5× SSC containing 50% formamide, 0.02% SDS, 0.1% lauroylsarcosine, 1% blocking reagent and 100 μg/ml of transfer RNA. After incubation with an anti-DIG antibody, signals were detected using a DIG-nucleotide detection kit (Roche Applied Science) according to the manufacturer's instructions. The signals were revealed by exposing the membranes to an X-ray film for 1 h. Following hybridization and detection of P450c17 mRNA transcripts, the membrane blots were stripped and then rehybridized with antisense mouse β-actin DIG-labelled cRNA probe, as a control for mRNA integrity. The analysis was performed twice.

2.8. RT-PCR of cytochrome P450arom

To analyze the possibility of renal P450arom mRNA expression in male rat, a semi-quantitative RT-PCR was conducted as described previously, using the 18S rRNA gene as an internal standard [11]. Briefly, RT-PCR was performed using the Superscript One-Step RT-PCR system (Invitrogen, Italy) and the P450arom primer pair (forward: 5′-ATC TTC CAT ACC AGG TCC TGG CTA-3′; reverse: 5′-GCG TGT TAG AAG TGT CCA GCA TGA-3′). To maintain product accumulation in the linear range, the number of cycles was established at 38 in preliminary experiments with testicular RNA. The amplification procedure consisted of 2 min at 95 °C followed by 38 cycles at 95 °C (30 s), 60 °C (30 s), and 72 °C (30 s). The extension phase of the last cycle was prolonged by 10 min. The analysis was performed using total RNA extracted from kidney tissues of newborn and 7-, 15-, 30- and 60-day-old male rats. Ovary and testis were used as positive controls and spleen as a negative control. Analyses were carried out with different quantities of total RNA, ranging from 200 ng for gonads to 1 μg for kidney and spleen.

Quantification after agarose gel electrophoresis was carried out by measuring the relative intensity of the bands stained by ethidium bromide, using the Quantity One Quantitation software (Bio-Rad, Italy). Analyses were performed twice. For each RT-PCR, a negative control was prepared by using all reagents except RNA solution, that was substituted with an equivalent volume of sterile water to check for cross-contamination.

2.9. Western blotting with an anti-P450c17 antibody

The kidney, testis and spleen from male rats were minced and homogenized in an Ultraturax homogenizer at 4 °C in a homogenization buffer (100 mM potassium phosphate buffer, pH 7.4, 250 mM sucrose, 1 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride, 4 mM $MgCl_2$, and 2 ng/ml each of leupeptin, pepstatin A and aprotin). After the removal of nuclei and debris by three centrifugations at 1000 × g for 15 min at 4 °C, the mitochondrial fractions were pelletted at 10,000 × g for 45 min at 4 °C. The supernatant fractions were centrifuged at 100,000 × g for 60 min to obtain a microsomal pellet. The pellets were suspended in the same buffer and centrifugation was repeated. Finally, the

microsomal pellets were resuspended in a 100 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 20% glycerol, 0.5 mM phenylmethylsulfonylfluoride, and 2 ng/ml each of leupeptin, pepstatin A and aprotin.

The presence of the P450c17 enzyme in the kidney microsomal proteins was examined by Western immunoblot analysis using an anti-rat P450c17 polyclonal antibody kindly offered by Dr. M. Waterman (Nashville, TE, USA). The testis and spleen were used as positive and negative control tissues, respectively. Forty micrograms of renal and splenic microsomal proteins and 2–4 μg of corresponding testicular proteins were loaded onto 10% SDS-PAGE (Nupage, Invitrogen) and transferred onto nitrocellulose membranes (Roche Applied Science). After an overnight blocking step at 4 °C with 5% BSA in TBS-T buffer (20 mM Tris–HCl, pH 7.6, 137 mM NaCl and 0.1% Tween-20), filters were hybridized with the antibody against rat P450c17 (1/3000 dilution) in TBS-T for 2 h at room temperature. Filters were then washed (3 × 5 min; 1 × 15 min) with TBS-T and further incubated for 1 h at room temperature with goat horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1/35,000 dilution) (Pierce) in TBS-T. Membranes were washed as described above and HRP activity was detected using the SuperSignal West-Dura Chemiluminescent kit (Pierce), according to the manufacturer's protocol.

3. Results

3.1. Incubations of rat kidney with [4-^{14}C]P4

After correction for losses, an average of 99% (range 96–101%) of initial precursor radioactivity was recovered in the total steroid fraction (ethanol extract) of all incubates. Water-soluble metabolites (presumably steroid conjugates) were found in detectable amounts in all samples with an average of 18% of the total radioactivity (range 11.3–24.1%). In all incubates, P4 was actively metabolized, as its recovery ranged from an average of 31% (30-, 60- and 365-day-old rats of both sexes) to only 8% (0-, 7- and 15-day-old rats of both sexes). The following enzymes were present in male and female renal tissues: 5α-reductase, cytochromes P450c17 and P450c21 and 20α-HSD. The main metabolites found in both sexes were 5α-reduced C_{21} steroids, whose sum amounted to more than 50% of incubated radioactivity at all ages, except with 60-day-old rats in which it was lower than 30%. The major product of 5α-reductase activity in both sexes and at all ages was 5α-pregnane-3,20-dione (5α-DHP), followed by 5α-pregnan-3β-ol-20-one (5α-DHP-3β) and 5α-androstane-3,17-dione (5α-A-dione) (Table 1 and Fig. 1). Two compounds (marked as 1 and 2 in Table 1 and Fig. 1) were also 5α-reduced products because, after oxidation, were isopolar with 5α-DHP, but could not be completely identified. These metabolites are likely 5α-reduced steroids hydroxylated at the 3α position instead of 3β: the activity of 3α-HSD has been in fact demonstrated in the rat kidney [12].

The activity of cytochrome P450c17 was measured as the sum of the yields of 17α-hydroxyprogesterone (17α-P4, range 0.01–0.14%), androstenedione (A-dione, range 0.03–0.68%), testosterone (T, range 0.01–0.13%), and 5α-A-dione (range 0.03–1.1%) (Table 1 and Fig. 1). The P450c17 activity reached a peak at 15 days in both sexes and was generally higher in males (Fig. 2). Low levels of 11-deoxycorticosterone (DOC, range 0.03–0.32%)

Table 1
Percent of unchanged precursor and percent yields of steroid metabolites after incubation of renal tissues from male and females rats at different ages with [4-^{14}C]P4

	P4	5α-DHP-3β	5α-DHP	1	2	20α-P4	DOC	17α-P4	A-dione	T	5α-A-dione
M (0 day)	7.77	16.67[a]	38.57[b]	6.66[c]	1.86[c]	0.31[a]	0.19[a,d]	0.07	0.27[b]	0.01[a,d]	0.33
F (0 day)	10.00	17.66[a]	35.70[b]	7.10[c]	1.40[c]	0.30[a]	0.14[a,d]	0.07	0.27[b]	0.01[a,d]	0.30
M (7 days)	2.77	11.71[a,e]	26.95[b]	3.62[c]	0.96[c]	0.05[a]	0.03[a,d]	0.02	0.13[b]	0.01[a,d]	0.47
F (7 days)	3.27	12.83[a,e]	26.49[b]	6.53[c]	1.37[c]	0.11[a]	0.03[a,d]	0.02	0.10[b]	0.01[a,d]	0.31
M (15 days)	10.49	8.10[a,e]	39.42	5.24[c]	1.14[c]	0.33[a]	0.11[a,d]	0.13[d]	0.68[b]	0.02[a,d]	1.10[b]
F (15 days)	11.74	5.59[a,e]	39.98	8.25[c]	1.08[c]	0.44[a]	0.06[a,d]	0.06[d]	0.32[b]	0.02[a,d]	0.64[b]
M (30 days)	24.30	5.65[a]	50.56	1.83[c]	0.51[c]	0.38[a]	0.32[a,d]	0.14	0.24[b]	0.01[a,d]	0.08
F (30 days)	25.43	4.76[a]	49.44	2.00[c]	0.46[c]	0.30[a]	0.21[a,d]	0.08	0.15[b]	0.01[a,d]	0.06
M (60 days)	36.83	1.93[a]	12.10	3.25[c]	0.50[c]	1.75[a]	0.07[a,d]	0.08	0.06[b]	0.01[a,d]	0.03
F (60 days)	21.78	3.10[a]	18.40	7.26[c]	0.40[c]	0.22[a]	0.06[a,d]	0.01	0.03[b]	0.01[a,d]	0.03
M (365 days)	31.32	2.60[a]	32.59	8.69[c]	0.75[c]	2.00[a]		0.03	0.11	0.05[a,d]	0.07
F (365 days)	13.77	3.79[a]	35.46	14.40[c]	0.25[c]	0.14[a]		0.01	0.09	0.13[a,d]	0.11

Steroid abbreviations: P4 = progesterone; 17α-P4 = 17α-hydroxyprogesterone; 20α-P4 = 20α-dihydroprogesterone; 5α-DHP = 5α-pregnane-3,20-dione; 5α-DHP-3β = 5α-pregnan-3β-ol-20-one; DOC = 11-deoxycorticosterone; A-dione = androstenedione; T = testosterone; 5α-A-dione = 5α-androstane-3,17-dione; 1 and 2 = unknown.

[a] Acetylated.

[b] Recrystallized in acetone–water or pyridine–acetone–water to constant isotope ratio of the unchanged compounds or their acetylated derivatives.

[c] Oxidized.

[d] Recrystallized as a pool.

[e] Recrystallized as mono-acetates to constant specific activity.

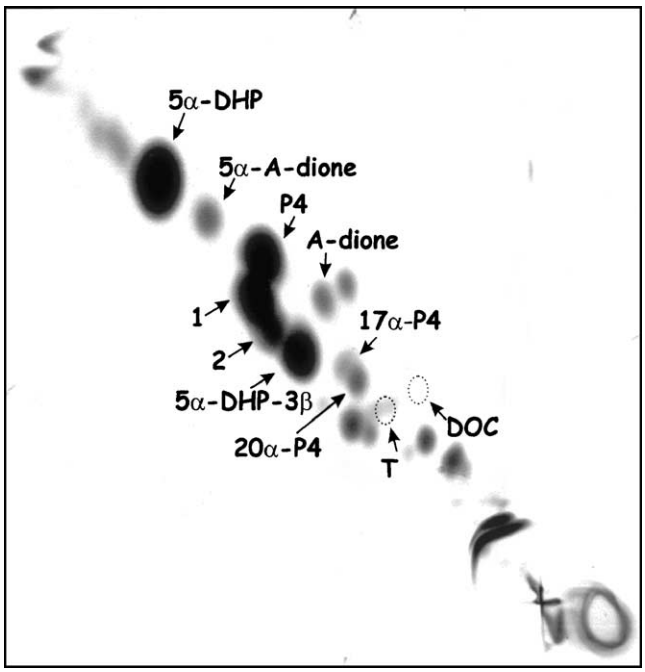

Fig. 1. Representative autoradiograph of the thin-layer chromatogram of the free steroids extracted from the incubate of 0.25 g of kidney tissue of 15-day-old female rats with 0.9 μM [4-^{14}C]P4. Arrows indicate identified metabolites.

were identified in all tissues, except aged rats of both sexes (Table 1 and Fig. 1).

All tissues reduced progesterone to 20α-dihydroprogesterone (20α-P4, range 0.05–2%). The high level of 20α-HSD activity measured in aged male rats is in agreement with the results of a previous incubation performed with [4-^{14}C]pregnenolone [7]. Recrystallization data are not shown to save space.

3.2. Incubations of rat kidney with [4-^{14}C]P4 and the 5α-reductase inhibitor PNU 156765

The incubations with [4-^{14}C]P4 alone or with PNU 156765 were performed with renal homogenates from 15-day-old male and female rats because, at this age, a high 5α-reductase activity was measured together with significant P450c17 and 20α-HSD activities. As shown in Table 2, the enzyme 5α-reductase was almost completely inhibited by the 4-azasteroid compound. The inhibition was specific to 5α-reductase because the other steroidogenic enzymatic activities were not affected by PNU 156765 treatment: on the contrary, in both sexes, the overall products of P450c17 were approximately doubled and the product of 20α-HSD was 5 and 10 times higher in male and female rats, respectively, in the presence of the inhibitor.

3.3. Incubations of rat kidney with [4-^{14}C]DHEA

All tissues consumed about half of [4-^{14}C]DHEA, except for aged rat, which reached only 30%. Formation of water-soluble compounds was less pronounced than that obtained with P4 and averaged 7% of initial radioactivity (range 1–16.7%).

With DHEA as a precursor, the most active enzyme was 17β-HSD with the formation of androst-5-ene-3β,17β-diol (A-diol) (range 0.7–20.83%). Its production increased by 15 days to the adulthood, particularly in the male. Moreover, all tissues contained 3β-HSD/Δ^5–Δ^4-isomerase activity, as revealed by the synthesis of A-dione (range 0.9–8.1%) and T (range 0.01–0.5%), and 5α-reductase activity, as indicated by 5α-A-dione (range 0.3–2.33) (Table 3 and Fig. 3).

3.4. Northern blotting analysis of cytochrome P450c17

Hybridization of rat testicular, digoxigenin-labelled P450c17 cRNA probe to poly(A+)-enriched RNA from male rat kidneys at different ages is shown in Fig. 4 (upper panel). The mRNA extracted from adult testis was used as a positive control. A strong and unique signal of about 2 kb was present in the testis, whereas a faint signal was detected in kidney samples up to 15 days from birth. Checking of RNA integrity and loading was established by the subsequent hybridization with a rat β-actin cRNA probe (Fig. 4, lower panel).

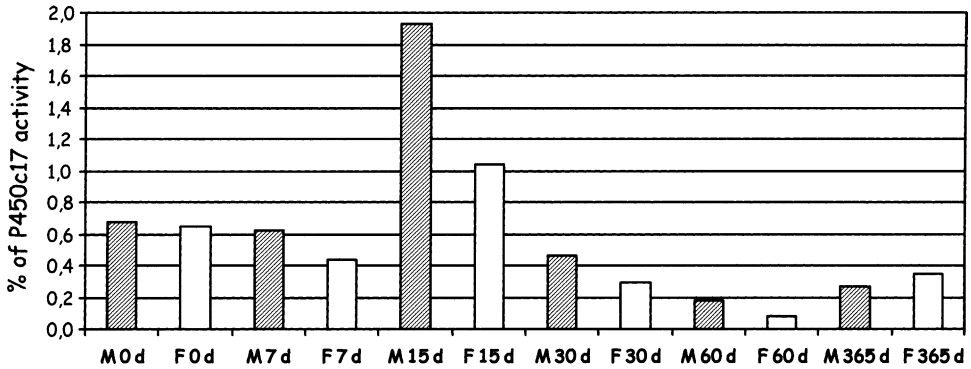

Fig. 2. P450c17 activities (expressed as the sum of the % yields of 17α-P4, A-dione, T and 5α-A-dione) from the incubates of kidney tissues from newborn and 7-, 15-, 30-, 60-day-old and aged male and female rats with [4-^{14}C]P4. M = male; F = female; d = days from birth.

Table 2
Steroid metabolites produced from [4-^{14}C]P4 after incubation of 15-day-old male and female rat kidney homogenates with and without the 5α-reductase inhibitor PNU 156765

Steroid	Female		Male	
	Without inhibitor (pg/mg/h)	With inhibitor (pg/mg/h)	Without inhibitor (pg/mg/h)	With inhibitor (pg/mg/h)
A-dione	1.4	2.0	3.3	3.7
17α-P4	2.3	6.1	5.0	9.1
20α-P4	3.1	31.0	3.1	15.0
1	62.8	1.3	126.0	2.9
2	5.5	1.0	3.8	1.2
5α-DHP	22.7	1.0	73.2	2.7
5α-DHP-3β	2.6	1.4	12.6	3.1
5α-Red ($\Sigma_{5\alpha\text{-DHP, }5\alpha\text{-DHP-}3\beta\text{, }1\text{, }2}$)	93.6	4.7	215.6	9.9
P450c17 ($\Sigma_{17\alpha\text{-P4, A-dione}}$)	3.7	8.1	8.3	12.8

5α-Red = total 5α-reductase activity; P450c17 = total P450c17 activity. *Steroid abbreviations*: P = progesterone; 17α-P4 = 17α-hydroxyprogesterone; 20α-P4 = 20α-dihydroprogesterone; 5α-DHP = 5α-pregnane-3,20-dione; 5α-DHP-3P = 5α-pregnan-3β-ol-20-one; A-dione = androstenedione; 1 and 2 = unknown.

3.5. RT-PCR of cytochrome P450arom

When renal and gonadal total mRNAs were analyzed by a semi-quantitative RT-PCR, a unique amplified fragment of the correct size for P450arom cDNA was visualized on the ethidium bromide-stained gel in both testis and ovary, with approximately twenty times higher amplification in the ovary. No evidence of expression of the *CYP19* gene was observed in the kidney samples at any age examined (Fig. 5).

3.6. Western blotting analysis with an anti-P450c17 antibody

The presence of the P450c17 enzyme was assessed in male rat kidney by immunoblot analysis of renal microsomal proteins from newborn, 7-, 15-, 30- and 60-day-old animals using a polyclonal antibody against rat P450c17. Microsomes from adult rat testis were used as positive control. As illustrated in Fig. 6, a protein band derived from

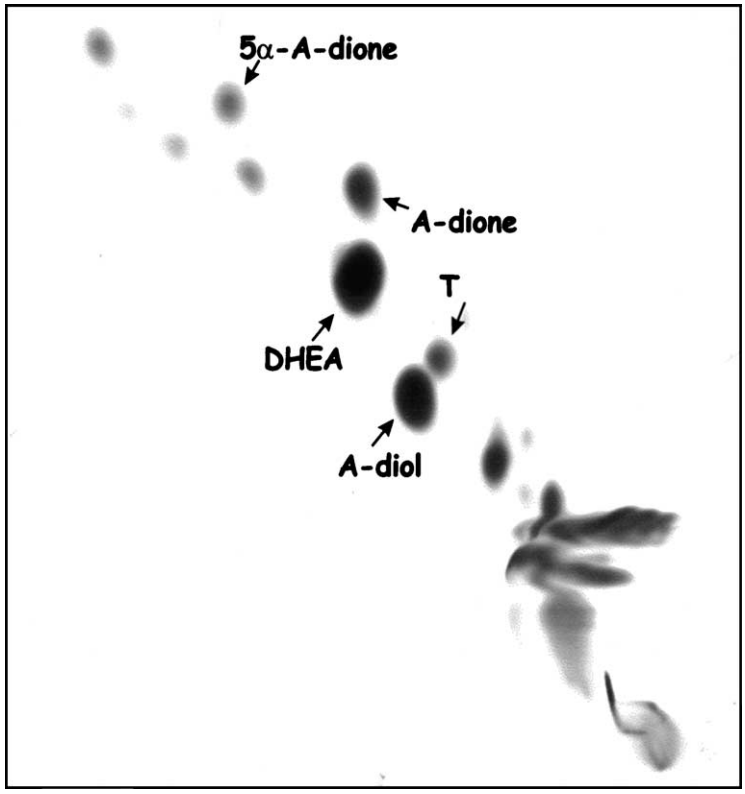

Fig. 3. Representative autoradiograph of the thin-layer chromatogram of the free steroids extracted from the incubate of 0.25 g of kidney tissue of 30-day-old male rats with 0.9 μM [4-^{14}C]DHEA. Arrows indicate identified metabolites.

Table 3
Percent of unchanged precursor and percent yields of steroid metabolites after incubation of renal tissues from male and females rats at different ages with [4-^{14}C]DHEA

	DHEA	A-dione	T	5α-A-dione	A-diol
M (0 day)	54.0	4.43[a]	0.04[b]	1.27[a]	2.39[b]
F (0 day)	42.8	8.10[a]	0.03[b]	2.33[a]	1.40[b]
M (7 days)	50.0	0.88	0.01[b]	1.37[a]	0.66[b]
F (7 days)	56.9	0.91	0.01[b]	1.29[a]	0.84[b]
M (15 days)	62.3	1.32	0.05[b]	0.66[a]	2.35[b]
F (15 days)	52.7	1.53	0.04[b]	1.11[a]	1.63[b]
M (30 days)	50.0	1.67	0.11[b]	0.63	6.13[a,b]
F (30 days)	64.5	1.89[a]	0.11[b]	0.73	4.54[a,b]
M (60 days)	47.4	0.98[a]	0.45[a,b]	0.31[a]	7.98[a,b]
F (60 days)	59.3	0.91	0.20[b]	0.60[a]	4.22[a,b]
M (365 days)	69.5	1.14	0.53[a,b]	0.48	20.83[b]
F (365 days)	73.4	2.36	0.22[b]	1.30	5.81[b]

Steroid abbreviations: DHEA = dehydroepiandrosterone; A-dione = androstenedione; T = testosterone; A-diol = 5-androstene-3β,17β-diol; 5α-A-dione = 5α-androstane-3,17-dione.

[a] Recrystallized in acetone–water or pyridine–acetone–water to constant isotope ratio of the unchanged compounds or their acetylated derivatives.

[b] Acetylated.

testis as well as kidney samples from all ages, except 30- and 60-day-old rats, was recognized by the antibody. The intensity of the immunoreaction in all positive kidney samples was markedly lower than in the testis, also considering that, for the testis samples, only 4 μg (TeA) or 2 μg (TeB) of microsomal proteins were loaded instead of 40 μg as in the kidney preparations. A higher expression of cytochrome P450c17 was found in kidney samples from 15-day-old

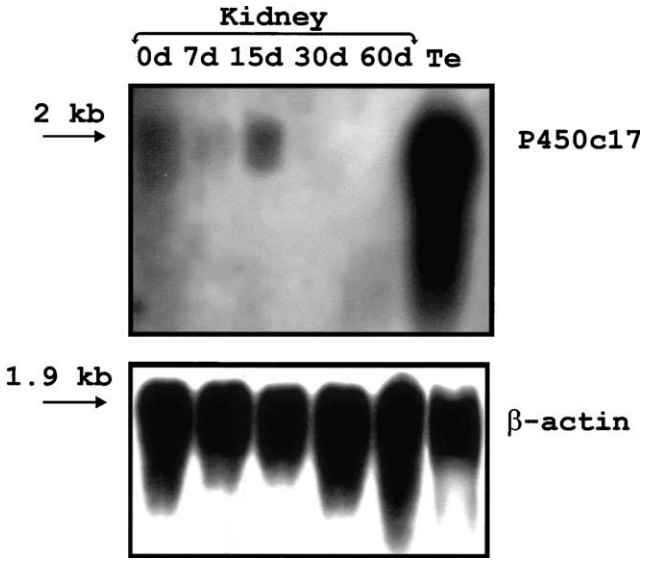

Fig. 4. Upper panel: representative Northern blot analysis of poly(A)+-enriched RNAs extracted from adult rat testis (Te) and kidney tissues of newborn and 7-, 15-, 30- and 60-day-old male rats with a rat cRNA P450c17 probe. Lower panel: Northern blot analysis of the same samples performed with a mouse cDNA β-actin probe; d = days from birth; Te = testis.

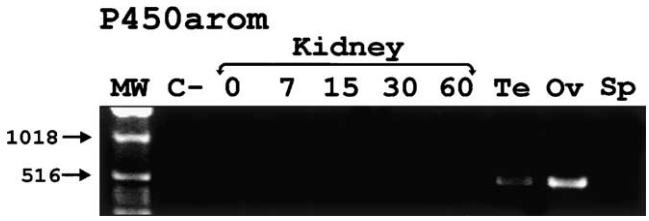

Fig. 5. Representative expression analysis of P450arom mRNAs in kidney tissues of newborn and 7-, 15-, 30- and 60-day-old male rats and in the testis (Te), ovary (Ov) and spleen (Sp) of adult rats, as determined by semi-quantitative RT-PCR in duplicate, using independently extracted RNA samples from different animals with equivalent results. MW = molecular weight; C− = negative control (water).

animals (Fig. 6). This pattern of expression correlates well with the temporal pattern of P450c17 mRNA expression and of P450c17 activity in male rat kidney. The enzyme could not be detected in spleen samples, confirming previous negative results obtained with RT-PCR analysis and Northern blotting [13,2].

4. Discussion

The renal expression of the cytochrome P450c17 has been previously demonstrated by radiochemistry in aged male rats [7] and by RT-PCR analysis and sequencing in neonatal and juvenile male rats [14]. Sequencing in both directions of amplified fragments of the coding region showed complete identity between renal and testicular amplificates, confirming the existence of a single gene encoding P450c17 mRNA in the rat. RT-PCR amplifications hinted at a decrement of expression of renal P450c17 mRNA after 15 days of age [14]. We have also demonstrated that the regulation of *CYP17* expression in rat peripheral tissues involves three different transcription start site (TSSs), two of which are used only in extraglandular tissues, such as the liver and gastrointestinal tract. Differently from these tissues, however, the rat kidney utilizes only the testicular TSS throughout the life-span [15]. This is at variance with the transcriptional control of the *CYP11A* gene in the rat kidney which is driven by a TSS located 76 bp upstream of that used in ovarian and testicular P450scc mRNA expression [35].

In a previous Northern blotting analysis, we obtained a negative result for P450c17 with total RNA extracted from the kidney of 15-day-old male rats [15]. The positive result obtained in the present work could be easily explained by the utilization of poly(A+)-enriched RNA instead of total RNA, with a consequent increase in assay sensitivity. At any rate, the renal occurrence of P450c17 mRNA appears to be considerably lower than the testicular one. The temporal pattern of expression correlates well with the results achieved with Western blotting analysis and radiochemical P450c17 study. In fact, P450c17 mRNA, protein and catalytic activity all peaked in the samples at 15 days of life and

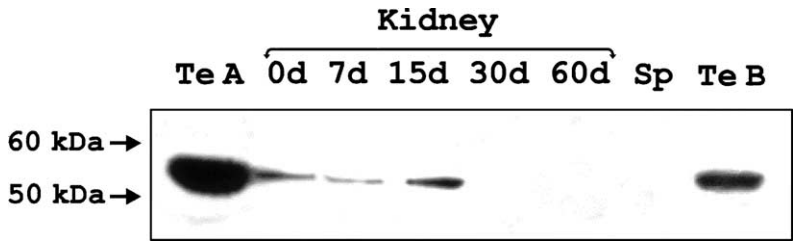

Fig. 6. Western immunoblot analysis of the P450c17 protein in the kidneys of newborn and 7-, 15-, 30- and 60-day-old rats. Testis (Te) and spleen (Sp) of adult rats were used as positive and negative controls, respectively. Each lane contained 40 μg of microsomal proteins of the renal and splenic tissues, while for the testis 4 μg (TeA) and 2 μg (TeB) of proteins were loaded. Electrophoresis was performed on 10% polyacrylamide gels.

declined thereafter. We also attempted to localize P450c17 in the rat kidney by immunohistochemistry using the same antibody showing good specificity in Western blotting analysis. However, results were inconclusive, probably because expression is widespread and tenuous. Instead, P450scc was easily revealed, being concentrated in the cells of cortical distal tubules.

The presence of P450c17, in addition to P450scc, indicates that the rat kidney has an autonomous steroidogenic capability, which cannot be due to strained adrenocortical cells entrapped within the kidney, since P450c17 is not expressed in the rat adrenal. The incubations with radioactive steroid precursors demonstrated that the following enzymes are present in male and female renal tissues: 5α-reductase, cytochromes P450c17 and P450c21, and 3β-, 17β- and 20α-HSDs.

With P4 as a precursor, the most active enzyme was the 5α-reductase, whose activity consumed about 50% of the substrate. With DHEA, the corresponding activity was much lower. This was clearly due to the fact that the enzyme saturates 4-ene-3-keto steroids and the activity of 3β-HSD/Δ^5–Δ^4-isomerase was rather low in all incubates, except in the case of newborns. Two isoforms of the 5α-reductase enzyme with different substrate affinities and tissue distribution have been sequenced in the rat: the type 1 isozyme that plays a largely catabolic role in steroid hormone metabolism, and the type 2 isozyme which is more important for hormonal activation, such as conversion of T to the more potent androgen, 5α-dihydrotestosterone (5α-DHT) [16]. The same authors reported that type 1 is more abundant than type 2 in the rat kidney. Actually, we did not observe the production of 5α-DHT in our incubates, an outcome that may be linked to the scarce accumulation of T from both P4 and DHEA. On the other hand, Matsuzaki et al. [17] demonstrated the synthesis of 5α-DHT from T by cultured cells from rat medullary collecting ducts. Interestingly, the addition to our incubates with P4 of PNU 156765, a 4-azasteroid that was reported to inhibit both human 5α-reductase isozymes [9], doubled androgen formation by renal P450c17. These results suggest the possibility that locally produced androgen might be further activated by saturation in the rat kidney.

An alternative route for the production of hormonally active androgens emerges from the observed metabolism of DHEA, in which 17β-HSD appears to play a prominent role with the formation of A-diol. Six isoforms of 17β-HSD are known to be coded in the rat genome, of which four types (I, II, IV and VI) were shown to be expressed in the kidney. Among these, only type I acts reductively on androgen and estrogen substrates, while the remaining three catalyze oxidative reactions [18–21]. Thus, formation of A-diol was likely due to type I activity, but we cannot exclude that the measured yields in the incubates were somehow lowered by the three enzymatic types controlling the opposite reaction. Hence, per cent conversions of A-diol must be regarded as minimal. The A-diol has been shown to interact agonistically with the estrogen receptor and to exert direct estrogenic effects at physiological concentrations [22]. More recently, A-diol, without conversion to T, was found to have specific androgenic activity by inducing androgen receptor-dependent gene transcription in human prostate cancer cells [23] and in human mammary cancer cells [24].

The low levels of 21-hydroxylase activity found in this work, are in agreement with those reported by others laboratories [25,26], although the biological significance of this extra-adrenal activity remains unknown.

Using semi-quantitative RT-PCR, no evidence of expression of the cytochrome P450arom was observed in the kidney samples at any age examined. However, with a different set of primers and greater amplification (40 cycles instead of the 38 adopted in this work), a low but positive signal was consistently obtained in kidney samples of rats of both sexes till 15 days of age, but not in those of 30- and 60 day-old animals, even after Southern blotting. P450arom mRNA was also detected in all glandular tissues, including the adult adrenal, and the fragment identity was confirmed by sequencing [14]. Although it is possible that traces of mRNA might be due to a leaky genome with no functional significance, it is noteworthy that P450arom was detected in the rat kidney only at the time of maximal P450c17 activity. Furthermore, P450arom has been demonstrated in the human fetal kidney both as mRNA [27] and, though at extremely low levels, as biochemical activity [28]. The expression and function of almost the same enzymes revealed in the present work have been recently reported also in

the human kidney by Quinkler et al. [8,29]. These authors interpreted this set of steroid conversions as a mechanism to counteract the mineralcorticoid antagonistic influence of progesterone, analogously to the renal cortisol inactivation by 11β-HSD type 2 [29]. This explanation is certainly interesting, but the complex steroidogenic pathways present in the postnatal rat kidney suggest an involvement not only in peripheral catabolism or inactivation of circulating steroids, but also in the autonomous synthesis of biological active steroids. As a matter of fact, the rat kidney also expresses the messengers of steroidogenic acute regulatory (StAR) and steroidogenic factor 1 (SF-1) proteins, which are normally required for hormone synthesis in steroidogenic endocrines [35]. Nevertheless, the fact that key enzymes, such as P450scc and P450c17, are expressed at low levels as compared to gonads and adrenals implies that renal steroids, or nephrosteroids, may act locally, in a paracrine or autocrine fashion, rather than being directed towards distal targets through the circulation.

This conclusion is supported by the occurrence of both androgen and estrogen receptors in the rat kidney [30]. Moreover, multiple effects of sex steroids have been demonstrated in the kidney: the regulation of transcription of numerous genes by androgen and estrogen [31,32]; the masculinization of the renal pattern of organic ion secretion after T treatment of female rats [33]; and the mitogenic action of androgens in human kidney cells, while estrogens display an anti-androgenic influence [34].

Acknowledgements

Work aided by 40 and 60% funding from the Ministry of the University and Scientific and Technological Research of Italy.

References

[1] I. Hanukoglu, Steroidogenic enzymes: structure, function, and role in regulation of steroid hormone synthesis, J. Steroid Biochem. Mol. Biol. 43 (1992) 779–804.
[2] S. Vianello, M.R. Waterman, L. Dalla Valle, L. Colombo, Developmentally regulated expression and activity of 17α-hydroxylase/C-17,20 lyase cytochrome P450 (P450c17) in rat liver, Endocrinology 138 (1997) 3166–3174.
[3] C. Le Goascogne, N. Sananès, B. Eychenne, M. Gouézou, E.-E. Baulieu, P. Robel, Androgen biosynthesis in the stomach: expression of cytochrome P450 17α-hydroxylase/17,20-lyase messenger ribonucleic acid and protein, Endocrinology 136 (1995) 1744–1752.
[4] L. Dalla Valle, A. Ramina, S. Vianello, P. Belvedere, L. Colombo, Kinetic analysis of duodenal and testicular cytochrome P450c17 in the rat, J. Steroid Biochem. Mol. Biol. 58 (1996) 577–584.
[5] T. Ueyama, N. Shirasawa, M. Numazawa, K. Yamada, M. Shelangouski, T. Ito, Y. Tsuruo, Gastric parietal cells: potent endocrine role in secreting estrogen as a possible regulator of gastro-hepatic axis, Endocrinology 143 (2002) 3162–3170.
[6] C. Kohchi, K. Ukena, K. Tsutsui, Age- and region-specific expressions of the messenger RNAs encoding for steroidogenic enzymes P450scc, P450c17 and 3β-HSD in the postnatal rat brain, Brain Res. 801 (1998) 233–238.
[7] L. Dalla Valle, P. Belvedere, C. Simontacchi, L. Colombo, Extraglandular hormonal steroidogenesis in aged rats, J. Steroid Biochem. Mol. Biol. 43 (1992) 1095–1098.
[8] M. Quinkler, C. Bumke-Vogt, B. Meyer, V. Bahr, W. Oelkers, S. Diederich, The human kidney is a progesterone-metabolizing and androgen-producing organ, J. Clin. Endocrinol. Metab. 88 (2003) 2803–2809.
[9] D. Giudici, G. Briatico, C. Cominato, T. Zaccheo, C. Iehle, M. Nesi, A. Panzeri, E. di Salle, FCE 28260, a new 5α-reductase inhibitor: in vitro and in vivo effects, J. Steroid Biochem. Mol. Biol. 58 (1996) 299–305.
[10] L. Colombo, H.A. Bern, J. Pieperzyk, D.W. Johnson, Corticosteroidogenesis in vitro by the head kidney of *Tilapia mossambica* (Cichlidae, Teleostei), Endocrinology 91 (1972) 450–462.
[11] L. Dalla Valle, A. Ramina, S. Vianello, P. Belvedere, L. Colombo, Cloning of two mRNA variants of brain aromatase cytochrome P450 in rainbow trout (*Oncorhynchus mykiss* Walbaum), J. Steroid Biochem. Mol. Biol. 82 (2002) 19–32.
[12] R. Ghraf, E.R. Lax, H.G. Hoff, H. Schriefers, The role of the gonads and the hypophysis in the regulation of hydroxysteroid dehydrogenase activities in rat kidney, Hoppe Seylers Z. Physiol. Chem. 356 (1975) 135–142.
[13] L. Dalla Valle, J. Couet, Y. Labrie, J. Simard, P. Belvedere, C. Simontacchi, F. Labrie, L. Colombo, Occurrence of cytochrome P450c17 mRNA and dehydroepiandrosterone biosynthesis in the rat gastrointestinal tract, Mol. Cell. Endocrinol. 111 (1995) 83–92.
[14] L. Dalla Valle, A. Ramina, S. Vianello, P. Belvedere, L. Colombo, Expression of steroidogenic cytochromes P450 in the rat kidney at different ages, in: W. Roubous, S.E. Wendelaar Bonga, H. Vaudry, A. De Loof (Eds.), Recent Developments in Comparative Endocrinology and Neurobiology, Shaker Publishing, Maastricht, 1999, pp. 37–39.
[15] L. Dalla Valle, S. Vianello, P. Belvedere, L. Colombo, Rat cytochrome P450c17 gene transcription is initiated at different start sites in extraglandular and glandular tissues, J. Steroid Biochem. Mol. Biol. 82 (2002) 377–384.
[16] K. Normington, D.W. Russell, Tissue distribution and kinetic characteristics of rat steroid 5α-reductase isozymes. Evidence for distinct physiological functions, J. Biol. Chem. 267 (1992) 19548–19554.
[17] K. Matsuzaki, T. Arai, T. Inumaru, M. Mihori, T. Momose, M. Sano, K. Koide, N. Shimizu, Androgen metabolism in cultured rat renal inner medullary collecting duct (IMCD) cells, Steroids 63 (1998) 105–110.
[18] L.A. Akinola, M. Poutanen, R. Vihko, Cloning of rat 17β-hydroxysteroid dehydrogenase type 2 and characterization of tissue distribution and catalytic activity of rat type 1 and type 2 enzymes, Endocrinology 137 (1996) 1572–1579.
[19] T. Puranen, M. Poutanen, D. Ghosh, R. Vihko, P. Vihko, Origin of substrate specificity of human and rat 17β-hydroxysteroid dehydrogenase type 1, using chimeric enzymes and site-directed substitutions, Endocrinology 138 (1997) 3532–3539.
[20] L.Q. Fan, R.C. Cattley, J.C. Corton, Tissue-specific induction of 17β-hydroxysteroid dehydrogenase type IV by peroxisome proliferator chemicals is dependent on the peroxisome proliferator-activated receptor α, J. Endocrinol. 158 (1998) 237–246.
[21] M.G. Biswas, D.W. Russell, Expression cloning and characterization of oxidative 17β- and 3α-hydroxysteroid dehydrogenases from rat and human prostate, J. Biol. Chem. 272 (1997) 15959–15966.
[22] R. Poulin, F. Labrie, Stimulation of cell proliferation and estrogenic response by adrenal C19-Δ^5-steroids in the ZR-75-1 human breast cancer cell line, Cancer Res. 46 (1986) 4933–4937.
[23] H. Miyamoto, S. Yeh, H. Lardy, E. Messing, C. Chang, Δ^5-Androstenediol is a natural hormone with androgenic activity in human prostate cancer cells, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 11083–11088.

[24] R. Hackenberg, I. Turgetto, A. Filmer, K.D. Schulz, Estrogen and androgen receptor mediated stimulation and inhibition of proliferation by androst-5-ene-3β,17β-diol in human mammary cancer cells, J. Steroid Biochem. Mol. Biol. 46 (1993) 597–603.
[25] S.H. Mellon, W.L. Miller, Extraadrenal steroid 21-hydroxylation is not mediated by P450c21, J. Clin. Invest. 84 (1989) 1497–1502.
[26] S. Lajic, L. Eidsmo, M. Hoist, Steroid 21-hydroxylase in the kidney: demonstration of levels of messenger RNA which correlate with the level of activity, J. Steroid Biochem. Mol. Biol. 52 (1995) 181–186.
[27] T. Price, J. Aitken, E.R. Simpson, Relative expression of aromatase cytochrome P450 in human fetal tissues as determined by competitive polymerase chain reaction amplification, J. Clin. Endocrinol. Metab. 74 (1992) 879–883.
[28] K.J. Doody, B.R. Carr, Aromatase in human fetal tissues, Am. J. Obstet. Gynecol. 161 (1989) 1694–1697.
[29] M. Quinkler, S. Johanssen, C. Grossmann, V. Bahr, M. Muller, W. Oelkers, S. Diederich, Progesterone metabolism in the human kidney and inhibition of 11β-hydroxysteroid dehydrogenase type 2 by progesterone and its metabolites, J. Clin. Endocrinol. Metab. 84 (1999) 4165–4171.
[30] G. Pelletier, Localization of androgen and estrogen receptors in rat and primate tissues, Histol. Histopathol. 15 (2000) 1261–1270.
[31] A. Crozat, J.J. Palvimo, M. Julkunen, O.A. Janne, Comparison of androgen regulation of ornithine decarboxylase and S-adenosylmethionine decarboxylase gene expression in rodent kidney and accessory sex organs, Endocrinology 130 (1992) 1131–1144.
[32] S.A. Jelinsky, H.A. Harris, E.X. Brown, K. Flanagan, X. Zhang, C. Tunkey, K. Lai, M.V. Lane, D.K. Simcoe, M.J. Evans, Global transcription profiling of estrogen activity: estrogen receptor regulates gene expression in the kidney, Endocrinology 44 (2003) 701–710.
[33] J.L. Reyes, E. Melendez, A. Alegria, F. Jaramillo-Juarez, Influence of sex differences on the renal secretion of organic anions, Endocrinology 139 (1998) 1581–1587.
[34] S. Stefani, G.L. Aguiari, A. Bozza, I. Maestri, E. Magri, P. Cavazzini, R. Piva, L. del Senno, Androgen responsiveness and androgen receptor gene expression in human kidney cells in continuous culture, Biochem. Mol. Biol. Int. 32 (1994) 597–604.
[35] L. Dalla Valle, V. Toffolo, S. Vianello, P. Belvedere, L. Colombo, Expression of cytochrome P450scc mRNA and protein in the rat kidney from birth to adulthood, J. Steroid Biochem. Mol. Biol. 88 (1) (2004) 79–89.