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ONTOGENY OF TESTICULAR STEROID DEHYDROGENASE ENZYMES IN PIG (3α/β-, 20α- AND 20β-): EVIDENCE FOR TWO FORMS OF 3α/β-HYDROXYSTEROID DEHYDROGENASE

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Summary—Three enzymatic activities (3α/β-hydroxysteroid dehydrogenase, 20β- and 20α-hydroxysteroid dehydrogenases) were measured in testes of pigs as a function of age. Earlier studies reported a highly purified 20β-hydroxysteroid dehydrogenase from neonatal pig testes that also showed strong 3α/β-hydroxysteroid dehydrogenase activity [Ohno *et al.*, *J. Steroid Biochem. Molec. Biol.* **38** (1991) 787–794]. We report here that neonatal pig testis is rich in 3α/β- and 20β-hydroxysteroid dehydrogenase activities, both of which fall to low levels (measured as specific activity) at 60 days. Thereafter the activity of 3α/β-reduction rises to high levels whereas 20β-reduction remains low. Activity of 20α-reduction is of intermediate level in the neonate, falls to a nadir at 60 days and rises to high levels in the mature animal. Western blots of cytosolic proteins show that the bifunctional enzyme (3α/β-plus 20β-hydroxysteroid dehydrogenase) is high in neonatal testes and falls to low levels at maturity. It is proposed that the neonatal testis possesses the bifunctional enzyme which is replaced by a second enzyme at maturity, that is a 3α/β-hydroxysteroid dehydrogenase without 20β-reductase activity. The possible functional significance of these changes is considered.

INTRODUCTION

Although the synthesis of steroid hormones by the steroid-forming organs proceeds by defined pathways involving well characterized enzymes, some steroidogenic enzymes are capable of catalyzing more than one reaction and the sequence in which the enzymes act varies with the species (reviewed in Ref. [1]). It has also been reported that in some cases a given reaction may be catalyzed by two different enzymes, each of which uses a different intermediate in the pathway e.g. a C_{21} as opposed to a C_{19} steroid. In some cases these confusing issues have not been clearly resolved. For example, a purified 20β-hydroxysteroid dehydrogenase (20β-HSD) from neonatal pig testis [2] showed vigorous 3α- and 3β-HSD activity with 5α-androstan-17β-ol-3-one (5α-DHT) as substrate [3]. A similar bifunctional enzyme (i.e. capable of reducing ketones at C_{20} and C_3) has been reported in human placenta [4]. On the other hand, a number of investigations have reported the presence of 3α- and 3β-HSD activities in immature testes of rat and mouse with progesterone as substrate [5–9].

In order to investigate these confusing observations we examined the 3α- and 3β-HSD activities of neonatal and mature porcine testes and at two intermediate ages. Our findings demonstrate the presence of two distinct enzymes only one of which, that of the neonatal porcine testis, shows 20β-HSD activity in addition to reductase activity of C_3.

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Abbreviations: 17α-hydroxyprogesterone, 17α-hydroxypregn-4-ene-3,20-dione; 5α-dihydrotestosterone (5α-DHT), 5α-androstan-17β-ol-3-one; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; BSA, bovine serum albumin; DAB, 3,3′-diaminobenzidine tetrahydrochloride; EDTA, ethylenediaminetetraacetic acid; KPB, potassium phosphate buffer; HSD, hydroxysteroid dehydrogenase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TLC, thin layer chromatography.

EXPERIMENTAL

Material

[4-^{14}C]17α-hydroxyprogesterone (1.96 GBq/mmol) and [4-^{14}C]5α-DHT were purchased from New England Nuclear Corporation (Boston, MA, U.S.A.) and were purified by thin layer

chromatography on Kodak 13181, silica gel with fluorescent indicator, using benzene–acetone (8:2) before use in these studies. Western blot kit was purchased from Amersham Int. (Bucks., England). NADPH was obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Crystalline bovine serum albumin, fraction V (BSA) was from the Armour Pharmaceutical Co. (Kankakee, IL, U.S.A.). Tween 20 and *N,O*-bis (trimethylsilyl)acetamide were from Tokyo Kasei Kogyo Co. (Tokyo, Japan). 3,3′-Diaminobenzidine tetrahydrochloride (DAB) was from Wako Pure Chemicals Industries (Osaka, Japan). Other reagents used were of the best grade available and obtained from Iwai chemicals (Tokyo, Japan).

Preparation of testicular cytosol

Testes from pigs 3 to 90 days of age were obtained at castration and those from mature pigs (1-year-old) from a slaughter house. Testes were kept ice cold in 0.1 mM EDTA in KCl 0.15 M. Testes were decapsulated, weighed and cut into small pieces which were homogenized in 4.5 vol of the above solution using a Physcotron homogenizer at 15,000 rpm for 30 s. The homogenate was centrifuged at 9000 *g* for 30 min. The supernatant was centrifuged at 105,000 *g* for 60 min. These steps were performed at ice cold temperature.

Enzyme assays

Measurement of 20α- and 20β-reductase activity was based upon conversion of [4-^{14}C]17α-hydroxyprogesterone to the corresponding 20α- and 20β-hydroxysteroids, respectively. Measurement of 3α/β-reductase activity was measured by the conversion of [4-^{14}C]5α-DHT to the corresponding 3α- and 3β-hydroxysteroids. Cytosol was incubated with the appropriate steroid substrate added in ethanol (10 μl). An NADPH-generation system was prepared to contain $NADP^+$, 250 nmol; glucose-6-phosphate, 5 μmol; glucose-6-phosphate dehydrogenase, 1 U and $MgCl_2$, 0.5 μmol. The incubation mixture was made to a final volume of 1.0 ml with KPB pH 7.4. After incubation for 3 min at 37°C the reaction was started by addition of $NADP^+$ 250 nmol. Incubation was performed at 37°C for 30 min. After incubation, 10 ml of methylene dichloride was added to the incubation medium and the mixture was vigorously shaken. After removal of the aqueous phase, the organic phase was dried with anhydrous sodium sulfate and the methylene dichloride was removed from the sodium sulfate and taken to dryness under nitrogen. The residue was applied to thin layer plates of silica gel with fluorescent indicator (Kodak 13181). The chromatograms were developed in a benzene–acetone (8:2) or a benzene–ethyl acetate (2:1) to separate the various products and unused substrates, 17α-hydroxyprogesterone or 5α-DHT, respectively. After radioautography (Fuji X-ray film, Rx), the relevant spots and radioactive areas of the chromatogram were cut out and directly placed in scintillation vials. ^{14}C was measured in 10 ml of toluene containing 2,5-diphenyloxazole (0.4%, w/v) and 2,2′-*p*-phenylene-bis-(5-phenyl oxazole) (0.01%, w/v) with a liquid scintillation counter (Packer Tri-Carb 460). The enzyme activity was determined from the radioactivity of the area corresponding to an objective metabolite on the TLC plate.

For determination of 3α- and 3β-HSD activities, gas chromatography was employed for separation of 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol, which could not be separated by TLC. The mixture on the TLC plate, previously described, was eluted with methylene dichloride. After evaporation of the organic solvent, the mixture was trimethylsilylated with *N,O*-bis(trimethylsilyl)acetamide–pyridine (1:1) at room temperature for 30 min. Gas chromatography was performed with a Shimadzu GC-4CM PF using a column of OV-1 (1%) on Gaschrom Q (0.3 × 200 cm) at 240°C, flow rate, 40 ml/min, detector, FID. The metabolites were identified by comparing the retention times with standard steroids treated under the same conditions. Enzyme activities were calculated from the ratio of peak areas corresponding to 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol, and the 3α/β-HSD activity observed.

Measurement of 20β-HSD protein by Western blotting

Samples of cytosol were subjected to SDS–PAGE by the method of Laemmli [10] on 12% acrylamide gel (10 × 14 cm, 0.1 cm thick, pH 8.8), 25 mA, 150 min. Proteins were electrotransferred to nitrocellulose membrane (Hibond-C, Amersham) in 0.1 M Tris base, 0.192 M glycine, 0.02% SDS and 20% methanol at 200 mA for 1 h. The membrane was then incubated for 1 h at room temperature in TBS (10 mM Tris–HCl pH 7.4, 150 mM NaCl) containing 2% BSA and for 1 h in TBS in the presence of the specific 20β-HSD antibody at

the desired dilution, then washed four times for 5 min each with TBST (0.05% Tween 20 added to TBS), and then incubated for 1 h at room temperature in TBS containing 0.2% BSA solution which contained a 1:1000 diluted anti-rabbit IgG horse radish peroxidase conjugate (Amersham) with gentle rocking. The membrane was then washed four times for 5 min each with TBST. The color reaction was carried out in TBS containing 0.4 mg/ml DAB–0.009% H_2O_2 as the substrate of peroxidase. The color reaction was stopped by several washes in water and then the membrane was dried.

Measurement of protein

Protein was measured by the method of Lowry *et al.* [11] with crystalline BSA as standard.

RESULTS

The specific enzyme activities of porcine testes are shown as a function of age (Fig. 1). The activity of 20β-HSD (reductase) was high from 7 to 30 days of age and then fell to low levels as the animals reached maturity. At approx. 1 year of age (maturity) values were < 10 pmol/min/mg. The activity of 3α/β-HSD was biphasic with respect to age being high in the first 10 days of life, falling to very low levels between 40 and 80 days and rising thereafter to high levels at maturity. The two ordinate scales in Fig. 1 should be noted because high levels of the 3α/β-HSD are approximately twenty times higher than the corresponding value for 20β-reductase activity and more than twenty times values for 20α-reductase. The activity of 20α-reductase showed a biphasic change being of intermediate level in the neonatal period, falling to low levels between 40 and 80 days and finally rising to a maximum at maturity (Fig. 1). Total activities in the cytosol fraction per testis showed that 3α/β- and 20β-reductase activities reached a peak at 30 days and thereafter fell as maturity approached. The total activity of 20α-reductase showed a low, broad peak at 30 days (Fig. 2). Testicular weight increased as an approximately linear function of age (Fig. 2).

Western blots were performed on the same amounts of total cytosolic protein at different ages using antibodies (IgG) raised against highly purified 20β-HSD from porcine testis (Fig. 3). These blots show that the amount of this enzyme protein (20β-reductase) was highest at 30 days and fell to very low levels by 90 days. This protein was undetectable at maturity when 640 ng protein was used in SDS–PAGE before blotting (Fig. 3).

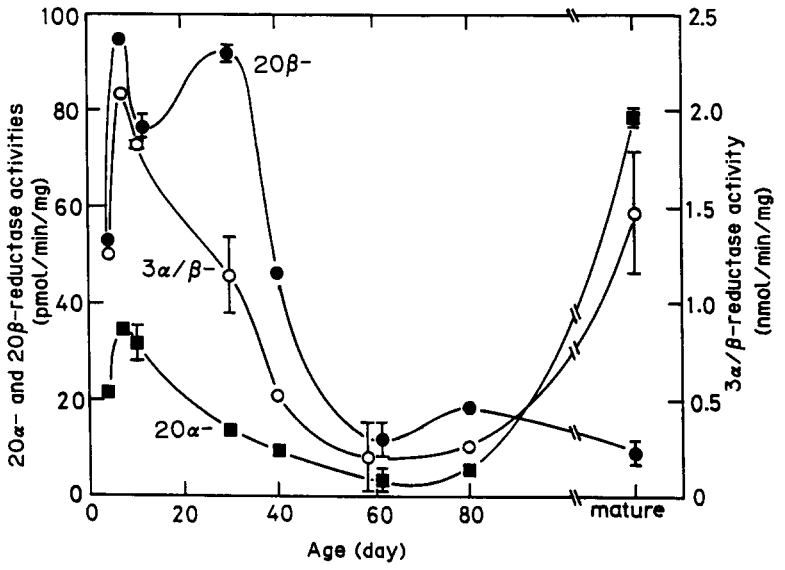

Fig. 1. Activities of steroid reductase enzymes of pig testes as a function of age. Cytosol (0.81 to 1.33 mg protein per flask) was prepared from testes of pigs at the ages shown. The cytosol was incubated with [4-^{14}C]17α-hydroxyprogesterone (20 nmol, 370 Bq) for measurement of 20α- (—■—) and 20β-reductase (—●—) activity in a volume of 1.0 ml including 50 mM KPB and an NADPH-generating system (see Experimental). For the measurement of 3α/β-reductase (—○—) activity, [4-^{14}C]5α-DHT (50 nmol, 370 Bq) was used as substrate. Values shown represent the means and SEM from two or four different experiments i.e. different testes. Each sample was measured in duplicate. The numbers of testes used were as follows: 10 days $n = 4$; 30 and 60 days and mature pigs $n = 2$. In all other cases $n = 1$.

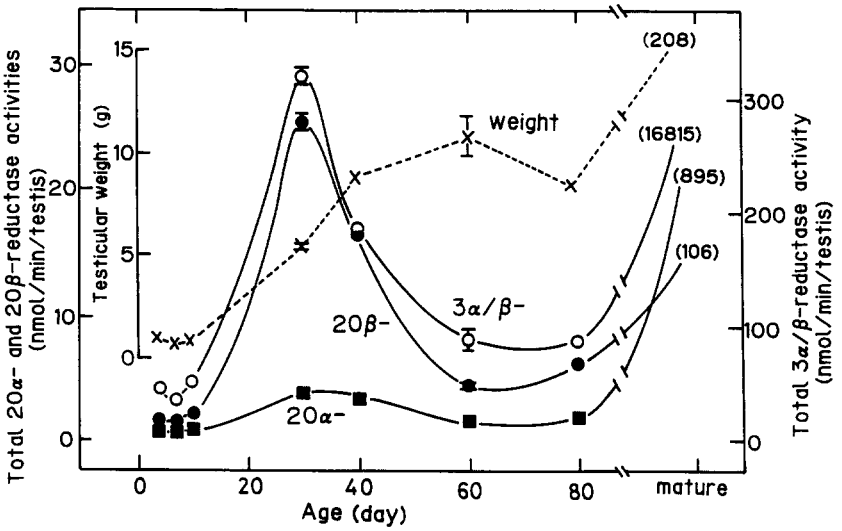

Fig. 2. Total cytosolic enzyme activities of reductase enzymes from pig testes as a function of age. Symbols represent 3α/β-reductase (—○—), 20α-reductase (—■—), 20β-reductase (—●—) and testicular weight (— × —). Symbols of enzyme activities represent means of duplicate determinations from a single testis. The bars at 30 and 60 days represent SEM from two separate experiments (i.e. two different animals). Values in parentheses are those for off-scale readings.

DISCUSSION

The present studies reveal that whereas 20β-HSD is of high specific activity in the neonatal porcine testis, this activity falls to low levels in the mature organ (Fig. 1). On the other hand, values for the specific activity of 3α/β-HSD as a function of age shows a biphasic change—being high in the neonatal testis, low at 60 days, rising again to high levels in the mature organ (Fig. 1). The changes in the amount of 20β-HSD protein determined by Western blotting changes with age like the activity of this enzyme i.e. high in the neonate and falling during maturation. Moreover our earlier studies showed that the purified enzyme 20β-HSD also catalyzed 3α- and 3β-dehydrogenation to a high degree [3]. However, since the 20β-reductase activity and the amount of this protein are low in the mature pig, the high levels of 3α/β-reductase activity in the mature animal cannot be attributed to the expression of the second activity of the 20β-reductase (i.e. 3α/β-reductase). On the other hand, the high levels of both 3 and 20β-reductase activities in the neonate are presumably due to the presence of the bifunctional enzyme at that stage of development.

The dichotomy between the two activities in the mature animal i.e. low 20β-activity and high 3α/β-activity, suggests the presence of a second enzyme possessing only the 3α/β-reductase activity. In the neonatal pig the 3α/β-activity represents a second activity of 20β-HSD.

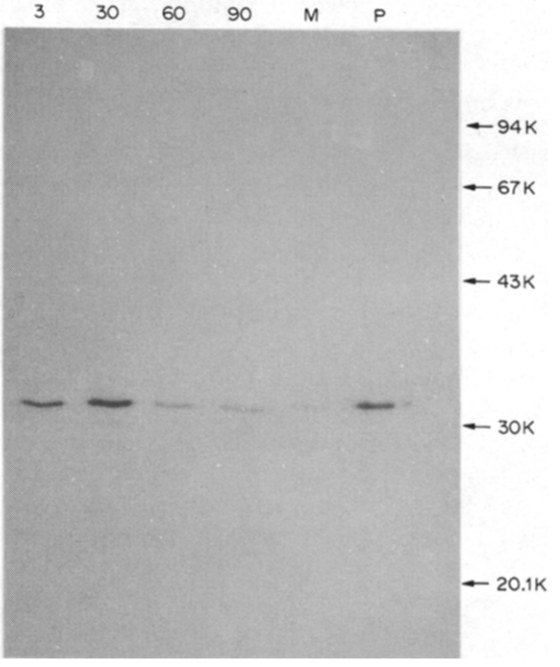

Fig. 3. Amounts of 20β-HSD in cytosol of porcine testes as a function of age. Cytosol protein (640 ng per lane) was prepared from porcine testes of various ages and run on SDS–PAGE followed by electrotransfer to membrane and detection of 20β-HSD by means of an antibody raised against the highly purified enzyme (see Experimental). Lanes are designated as follows: numbers refer to the age of pigs in days; M: mature; P: highly purified 20β-reductase from pig testes (14 ng). Positions of molecular weight markers are indicated as *K* values. Conditions are described in greater detail under Experimental.

A second possibility that seems less likely would be based upon inhibition of the 20β-reductase activity of the bifunctional enzyme at maturity.

A third enzyme activity (20α-HSD) shows yet another pattern of activity with age, namely moderate neonatal activity falling to very low levels at 60 days and rising to high values in the mature pig. There is at present no reason to believe that this enzyme is capable of other activities e.g. 3α/β-reduction. These changes in enzyme activities with age should be compared with total activities per testis which are however more difficult to interpret (Fig. 2). At about 30 days all three activities per testis reach their highest levels at a time when specific activities are still high although falling except 20β-HSD which falls soonafter. Both total and specific activities reach a nadir at 60 days. In the mature testis total and specific activities are high for 20α- and 3α/β-reductase but low for 20β-reductase. Evidently 20β-reductase is less important for the function of the mature testis than it is in the young animal.

The functional significance of these changes is not clear. It is known that various C_{21}, 20β-reduced steroids inhibit cytochrome *P*-450 C_{21SCC} (C_{17-20}-lyase) activity [12, 13]. This is true for both 4-ene and 5-ene steroids [13]. It has been proposed that 20β-reduction serves to divert progesterone from androgen synthesis in the immature animal [12] so that the decline in this activity during maturation permits the synthesis of androgens to increase. To sustain this hypothesis some explanation must be provided for the failure of high levels of 20α-reductase to inhibit the synthesis of androgens at puberty. So far no such explanation can be advanced. On the other hand, reduction of the ketone at C_3 is usually considered to be a disposal reaction for excess, unwanted steroid hormones. Perhaps the testis requires such a disposal pathway as well as the liver and other organs. Moreover this need will be greatest when steroidogenic activity is greatest i.e. in the neonate and in the mature pig.

The occurrence of a second 3α/β-HSD in porcine testis provides the opportunity to purify this enzyme preparatory to a detailed comparison of the two enzymes.

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