Biosynthesis of androgens and pheromonal steroids in neonatal porcine testicular preparations

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The biosynthesis of testosterone and 4-androstene-3,17-dione and some 16-androstenes has been studied in homogenates or subcellular fractions of testes from 3-week-old Landrace piglets. Pregnenolone was converted into 5,16-androstadien-3β-ol, 4,16-androstadien-3-one, 5α-androst-16-en-3-one and 5α-androst-16-en-3α- and 3β-ols, but the quantities were some 50 times less than those formed in the mature boar testis. Androgens were also formed in the microsomal fractions but the quantities of 4-androstene-3,17-dione (from side-chain cleavage of 17-hydroxyprogesterone) and of testosterone (from reduction of 4-androstene-3,17-dione) were 50–70 times lower than in the adult animal. The kinetic parameters and cofactor preference of the 3α- and 3β-hydroxysteroid dehydrogenases were determined in the cytosolic, microsomal and mitochondrial fractions of neonatal porcine testes.

Porcine *Neonate* *Testis* *Androgen* *16-Androstene* *3-Hydroxysteroid oxidoreductase*

1. INTRODUCTION

The biosynthesis of 16-androstenes in boar testis has been studied using a large number of experiments both in vitro [1] and in vivo [2–4]. It has been shown that large yields are obtained from pregnenolone in incubations of boar testis preparations [5–8]. In view of the important primer [9,10] and releaser [11] effects of two 16-androstenes, namely, 5α-androstenone and 5α-androst-16-en-3α-ol (an-α), it seems particularly appropriate that the boar testis has this high capacity for forming these compounds. It has also become clear that the 16-androstenes are formed to a greater extent in this tissue than the androgens [4,6].

Using analytical techniques, it has been shown that the quantities of endogenous 16-androstenes in porcine testis vary with the age of the animal [12]. In general, concentrations increase from the foetal stage (84 days gestation) to reach a maximum around the time of birth to 6 weeks of age, and then decrease sharply by the age of 12–18 weeks. Thereafter, the concentrations of 5α-androstenone, an-α and 5α-androst-16-en-3β-ol (an-β) but not of androstadienone increase. This is especially marked in the case of the two alcohols, an-α and an-β.

It is of interest that, during the perinatal stage, an-α predominates over an-β, whereas the reverse

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Abbreviations: androstenedione, 4-androstene-3,17-dione; pregnenolone, 3β-hydroxy-5-pregnen-20-one; 17-hydroxyprogesterone, 17-hydroxy-4-pregnene-3,20-dione; 16-dehydropregnenolone, 3β-hydroxy-5,16-pregnadien-20-one; testosterone, 17β-hydroxy-4-androsten-3-one; 5α-DHT, 17β-hydroxy-5α-androstan-3-one; andien-β, 5,16-androstadien-3β-ol; androstadienone, 4,16-androstadien-3-one; 5α-androstenone, 5α-androst-16-en-3-one; an-α(β), 5α-androst-16-en-3α(β)-ol

Enzyme abbreviations: C-17,20-lyase, 17-hydroxy-C_{21} steroid C-17,20-lyase; 3α(β)-HSDHs, 3α(β)-hydroxysteroid dehydrogenases [3α(β)-hydroxysteroid:NAD(P)+-oxidoreductases, EC 1.1.1.50 and 51]; 17β-HSDH, 17β-hydroxysteroid:NADP+-17-oxidoreductase, EC 1.1.1.64

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is true after approx. 18 weeks of age. A ratio of an-β:an-α in the testes of 2-year-old boars of approximately 3:1 has been established consistently using both in vitro [6,13] and in vivo [4] techniques.

Recent studies using neonatal porcine testis microsomes [14,15] have revealed that pregnenolone can be metabolized to some C_{19} steroids, including 5,16-androstadien-3β-ol (andien-β) and that 16-dehydropregnenolone is an intermediate in the latter conversion, as proposed earlier [16]. A cytochrome P-450 has been isolated from neonatal porcine testis [17] that catalyses the 17-hydroxylation of C_{21} steroids and their subsequent side-chain cleavage into C_{19} steroids. To our knowledge, however, information is not available for the overall biosynthesis of the 16-androstenes or of the androgens. Thus, here, we have studied 16-androstene formation from pregnenolone in homogenates of testes taken from 3-week-old Landrace piglets and we present data on two enzymes involved in androgen synthesis. Further, the conversion of 5α-androstenone to an-α and an-β has been studied in detail.

2. MATERIALS AND METHODS

[4-^{14}C]Pregnenolone (spec. radioact. 58.2 mCi/mmol), [4-^{14}C]androstenedione (spec. radioact. 58 mCi/mmol), 17-hydroxy[4-^{14}C]progesterone (spec. radioact. 60 mCi/mmol) and [1,2,6,7-^{3}H]-androstenedione (spec. radioact. 82 Ci/mmol) were purchased from Amersham International, England. Their radiochemical purities were checked before use by thin layer chromatography (TLC) in benzene/acetone (8:1, v/v), run twice followed by radioscanning and/or radioautography. 5α-[5α,6α-^{3}H]Androstenone (spec. radioact. 25 Ci/mmol) was obtained from Isocommerz, Kontor Dresden, DDR, and its radiochemical purity was examined by radioscanning after TLC in benzene/diethyl ether (9:1, v/v). It was then purified by alumina column chromatography using a similar method as in [3].

2.1. *Preparation of subcellular fractions*

Subcellular fractions were obtained from testes of 3-week-old Landrace piglets using differential centrifugation [14,18]. The purity of the mitochondrial, microsomal and cytosolic preparations was checked by electron microscopy and by 'marker-enzyme' assays. These assays included succinate-INT-reductase [19]; NADPH cytochrome *c* reductase [20,21] with the inclusion of rotenone; glucose 6-phosphatase [22] and lactate dehydrogenase [23]. The small degree of cross-contamination between fractions [18] was considered to be acceptable in the light of earlier work [24]. The protein content of fractions was estimated as in [25].

2.2. *Enzyme assays*

2.2.1. 3α- and 3β-Hydroxysteroid dehydrogenases (3α- and 3β-HSDHs) in subcellular fractions

Aliquots of microsomal preparations (1 ml, protein conc. 300 μg/ml) obtained from pooled 12 testes were incubated for 1.5 min at 37°C with a fixed amount of 5α-[^{3}H]androstenone (16 nmol/l) and increasing concentrations of unlabelled 5α-androstenone, viz. 0, 5, 20, 60, 200, 400 and 600 μmol/l. The reaction was initiated by the addition of NADPH (1.2 mmol/l) and NADH (1.4 mmol/l). After terminating the reaction with ethyl acetate (4 ml), carrier steroids, an-α and an-β (100 μg of each), were added before extraction. The labelled products, an-α and an-β, after drying over anhydrous sodium sulphate, were purified by TLC in benzene/diethyl ether (9:1, v/v), run twice. For the control experiments, boiled (5 min) microsomes were used and with the omission of unlabelled 5α-androstenone. The labelled metabolites were examined by radiochromatogram scanning and quantification was by gas-liquid chromatography (GLC) and liquid scintillation spectrometry. Further purification and characterization of metabolites was performed as described earlier [26].

The activity of the 3-HSDHs in mitochondrial and cytosolic fractions was determined in a similar way, except that unlabelled 5α-androstenone was added in the concentration ranges 0–200 and 0–120 μmol/l, respectively.

For determination of cofactor requirements, cytosolic and microsomal fractions (1 ml each of protein conc. 300 μg/ml) were incubated separately with 5α-[^{3}H]androstenone (25.91 pmol) in the presence of NADH (0.7 mmol/l), NADPH (0.6 mmol/l) or no cofactor for 30 min at 37°C.

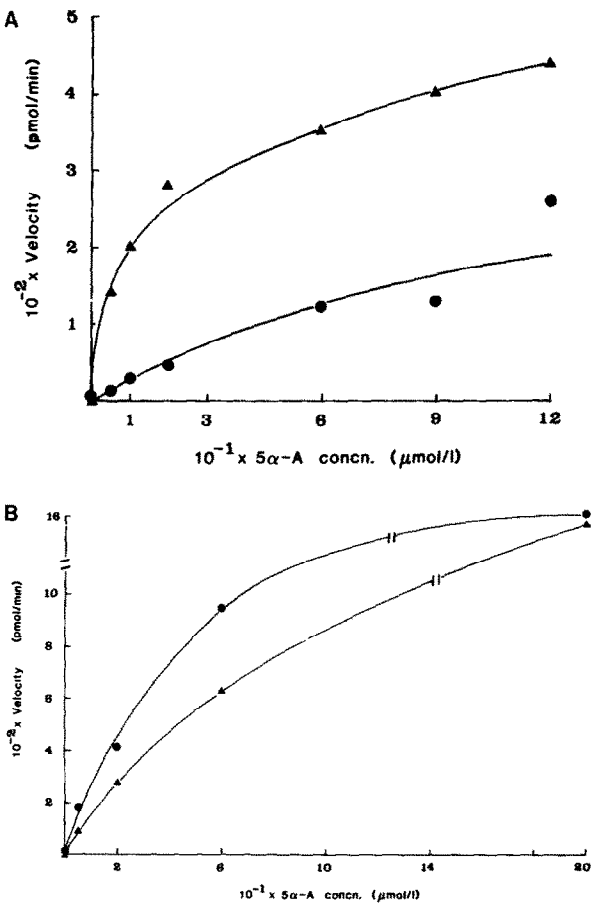
2.2.2. C-17,20-lyase

Microsomal suspensions (1 ml), obtained from pooled 6 testes and of protein concentration 200 μg/ml, were pre-incubated at 37°C for 5 min, with 0.23 μCi of 17-hydroxy[^{14}C]progesterone. The reaction was initiated by the addition of NADPH (0.6 mmol/l) and allowed to proceed for 10 min, then terminated as above. Carrier steroid, androstenedione (100 μg), was added before extraction. The dried extracts were subjected to TLC in benzene/acetone (8:1, v/v), run twice. Correction for analytical losses was made as above and further purification and quantification was as described earlier [26]. Control experiments were also set up with boiled (5 min) microsomal preparations and in the absence of NADPH.

2.2.3. 17β-Hydroxysteroid dehydrogenase (17β-HSDH)

For this enzyme study, microsomal preparations (1 ml) obtained from each of 4 testes of 3-week-old Landrace piglets and of protein concentration 200 μg/ml were pre-incubated separately with 0.21 μCi of [^{14}C]androstenedione at 37°C. The reaction was initiated by the addition of NADPH (0.6 mmol/l) and the incubation allowed to proceed for 10 min at 37°C and reaction terminated as above. Carrier steroid, testosterone (100 μg), was added before extraction. The dried extracts were purified by TLC in benzene/methanol (9:1, v/v), followed by benzene/acetone (6:1, v/v) and the radioactive steroids were analysed as above, but including radioautography. Control experiments were conducted with boiled (5 min) microsomes.

Assays were also performed, for comparison, with testicular microsomal suspensions (1 ml, protein conc. 200 μg/ml) obtained from young Large White boars of 20 weeks of age. In these experiments, [^{3}H]androstenedione (1.02 μCi) plus unlabelled androstenedione (4.34 nmol) were used as substrate in the presence of NADPH (1.2 mmol/l) and incubated for 10 min at 37°C. The tritium-labelled testosterone was isolated, detected by radiochromatogram scanning and quantified [26].



3. RESULTS

3.1. *Metabolism of pregnenolone and 5α-androstenone in porcine testicular homogenates*

When [^{14}C]pregnenolone (0.5 μCi) was incubated with homogenates of testes from 3-week-old piglets, andien-β, androstadienone, 5α-

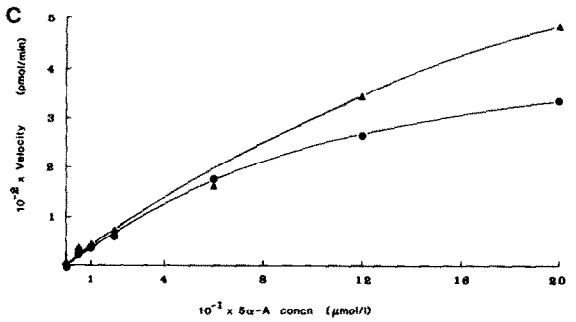


Fig.1. Michaelis-Menten plots of reduction of 5α-androstenone (5α-A) in (a) cytosolic, (b) microsomal and (c) mitochondrial fractions of neonatal porcine testis. ▲, an-α; ●, an-β. Each point represents the mean of duplicate determinations. For further details see section 2.2.

Table 1

Kinetic parameters of 3α- and 3β-HSDHs in neonatal porcine testicular fractions

Enzyme	Apparent K_m (μmol/l)	Apparent V_{max} (nmol/min per mg protein)
3α-HSDH		
Microsomal	127	7.5
Mitochondrial	106	2.1
Cytosolic	13	1.5
3β-HSDH		
Microsomal	68	7.1
Mitochondrial	107	1.7
Cytosolic	176	1.5

For experimental details, see section 2.2

androstenone, an-α and an-β were all formed but only in small yields (total 16-androstenes 2.5 pmol/min per mg protein; mean value for 12 testes). When 5α-[^{3}H]androstenone (0.86 μCi) was incubated under similar conditions, the products were an-α and an-β in the approx. ratio of 7–9:1. These results indicated that the 16-androstenes could be formed in the testes of very young pigs, and prompted detailed studies of some of the enzymes involved in 16-androstene and androgen biosyntheses.

3.2. *Assay of some steroid-transforming enzymes*

3.2.1. 3α- and 3β-HSDHs

Fig.1 (a–c) shows plots of the reaction velocity (expressed as pmol an-α or an-β produced/min) as a function of the substrate concentration. The apparent median K_m and V_{max} values (table 1) were obtained by direct linear plot [27] using a computer. These data and fig.1 show that the cytosolic fraction of testes taken from 3-week-old piglets contains a 3α-HSDH, with an apparent K_m of 13 μmol/l and a slight preference for NADPH; NADH supported the reaction to the extent of 75% relative to NADPH (table 2). A less active microsomal 3α-HSDH was also present, which had a larger apparent K_m value of 127 μmol/l and a very marked preference for NADPH, NADH being a poor cofactor. In addition, a mitochondrial counterpart may occur, having an apparent K_m of 106 μmol/l, similar to that of the microsomal enzyme, but with a much lower V_{max} (2.1, compared with 7.5 nmol/min per mg protein, table 1).

In the case of the 3β-HSDH, activity was found in cytosolic, microsomal and mitochondrial fractions. The microsomal enzyme appeared to be the most active, as evidenced by the apparent K_m and V_{max} data (table 1), and had a preference for NADH as cofactor (table 2).

3.2.2. C-17,20-lyase

The net androstenedione production from 17-hydroxyprogesterone was 7.5 pmol/min per mg

Table 2

Effects of cofactors on the production of an-α and an-β in cytosolic and microsomal fractions of neonatal porcine testis

Tissue fraction	Cofactor	An-α[a]		An-β[a]	
		pmol	% yield[b]	pmol	% yield[b]
Soluble	None	1.58	6.10	0.10	0.39
Soluble	NADH	8.53	32.92	0.20	0.77
Soluble	NADPH	12.14	46.85	0.10	0.39
Microsomes	None	0.10	0.38	0.10	0.39
Microsomes	NADH	0.77	2.97	0.46	1.78
Microsomes	NADPH	8.38	32.34	0.27	1.04

[a] Total an-α or an-β formed from incubating cytosolic or microsomal preparations (each of protein conc. 300 μg/ml) with tritium-labelled 5α-androstenone for 30 min at 37°C (see section 2.2)

[b] Yield calculated as a percentage of the starting substrate, tritium-labelled 5α-androstenone (25.91 pmol)

protein (mean of duplicates which did not vary by more than 6%).

3.2.3. 17β-HSDH

The net testosterone production from androstenedione was 7.37 ± 1.4 (4) pmol/min per mg protein (mean ± SD for 4 testes). The corresponding value for 20-week-old Large White pigs was 5.07 ± 2.4 (9) pmol/min per mg protein, a value not significantly different ($p = 0.05$) from that obtained for the 3-week-old animals.

4. DISCUSSION

The results presented here indicate that the neonatal porcine testis has the ability to form 16-androstenes, two of which are pheromonal in pigs [9–11], as well as of androgens, such as androstenedione and testosterone. The total amount of 16-androstenes formed (2.5 pmol/min per mg protein) is very small compared with amounts formed in the testis of mature boars [5–8]. In particular, the amount of andien-β produced from pregnenolone [26] was 113 pmol/min per mg protein, a value some 50-times greater than in the neonatal animal. The activities of the 17β-HSDH and C-17,20-lyase were also much lower than the corresponding values in the mature animal. Cooke and Gower [26] found that testosterone formation from androstenedione was 492 pmol/min per mg protein (compare 7.4 pmol/min per mg protein in the present work) and that androstenedione formation from 17-hydroxyprogesterone was 395 pmol/min per mg protein (compare 7.5 pmol/min per mg protein in the neonatal animals). These increases in enzyme activities with age seem to be consistent with the increasing quantities of 16-androstenes and of androgens found earlier by analytical techniques. Unfortunately, it is not possible to make meaningful comparisons with regard to testosterone production from androstenedione because the 20-week-old animals were Large White, Landrace boars of this age not being available.

Earlier work [18] showed how rapidly reductions of 5α-androstenone to an-α and an-β occurred in homogenates of neonatal porcine testis. The present studies on the 3-HSDH activities in subcellular fractions were therefore carried out with an incubation time of 1.5 min, a period that fell within the linear region of the time-course curve [18]. Enzyme kinetic studies should preferably be performed at zero-order kinetics, using substrate concentrations above 10-times K_m. Owing to the very non-polar nature and limited solubility of 5α-androstenone, it was not possible to use such high substrate concentrations. Nevertheless, the kinetic parameters determined by direct linear plots [27] (table 1) and cofactor dependencies (table 2) suggest heterogeneity of the 3α- and 3β-HSDHs in neonatal porcine testis. Such a phenomenon has been reported for several other tissues, including liver, kidney and pituitary in a number of species (review [28]).

It is of interest that, in the cytosolic and mitochondrial (fig.1a,c) fractions, the production of an-α occurred at a faster rate than that of an-β, although the reverse is true of the microsomal fraction (fig.1b). In studies with neonatal porcine testicular homogenates here and elsewhere [18], the overall effect of the 3α- and 3β-HSDHs is the preferential formation of an-α, the ratio to an-β being 7–9:1 (section 3.1). This is in keeping with analytical data [12] but is in contrast to the lower 3α-HSDH activity in the adult boar testis where an-β:an-α ratios are 3–4:1 [4,6,13]. The reason for this marked shift in reductive enzyme activities is unknown at present but it is conceivable that hormonal mechanisms must be involved in any proposed explanation.

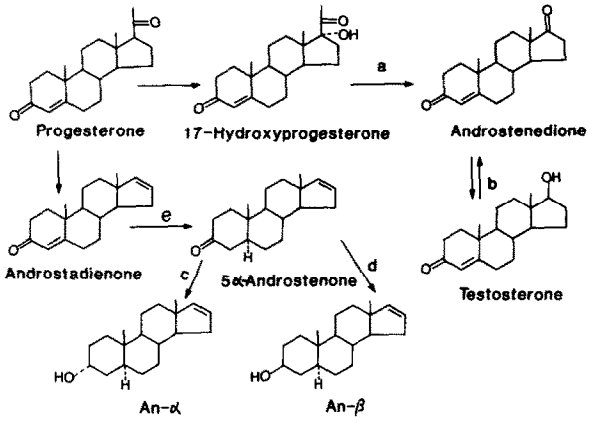


Fig.2. Scheme showing some of the enzymes involved in androgen and 16-androstene biosyntheses in neonatal porcine testis. a, C-17,20-lyase; b, 17β-HSDH; c and d, 3α- and 3β-HSDHs; e, 4-ene-5α-reductase.

The present work has not focused on the 4-ene-5α-reductase required for the formation of 5α-androstenone from its precursor androstadienone (fig.2), or for the formation of 5α-DHT from testosterone. Recent studies [29], using testosterone as substrate, have revealed that 5α-DHT production increases in the immature porcine testis from 3–6-weeks of age but is markedly less than in the adult animal. Further studies are in progress using androstadienone as substrate, but if the formation of 5α-androstenone is lower than in the mature animal, this would be in keeping with the suggestion that boar 'taint' (caused by 5α-androstenone and an-α, see [30]) is not significant in pigs younger than 14 weeks of age [31].

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