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Adult Sheep Blood Metabolizes Dihydrotestosterone to 5α-Androstane-3α,17β-diol and 5α-Androstane-3β,17β-diol

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Abstract

The metabolism of 5α-dihydrotestosterone by adult sheep blood was investigated. Erythrocytes contain 3α- and 3β-hydroxysteroid dehydrogenase activities. The mean rate of reduction of 5α-dihydrotestosterone by erythrocytes established in 15-min incubations was $0{\cdot}66 \pm 0{\cdot}36$ (s.d.) μmol ml^{-1} erythrocytes h^{-1} and at equilibrium after a 60-min incubation, $90{\cdot}6 \pm 5{\cdot}1\%$ of the substrate was reduced. The reduction of 5α-dihydrotestosterone was shown to be dependent upon extracellular glucose and the intracellular cofactor NADPH. The proportion of the two reduction products was determined at equilibrium after separation by paper partition chromatography and favoured 5α-androstane-3α, 17β-diol (96·0%) to 5α-androstane-3β,17β-diol (4·0%). The identities and proportions of the two products were confirmed by recrystallization procedures.

The fact that erythrocytes can significantly metabolize the androgen 5α-dihydrotestosterone is evidence for the recognition of blood as a major component of steroid endocrine homeostasis in sheep.

Introduction

The possibility that blood enzymes might be responsible for maintaining relative concentrations of circulating oxidized and reduced steroid hormones has been long recognized. Axelrod and Werthesson (1961) in discussing cortisol, oestrone and testosterone metabolism by blood, suggested that 'conversions which occur in blood, therefore, may be of equal if not greater significance to homeostasis than those which occur in other organs'. 17β-Hydroxysteroid dehydrogenase (17β-HSD; EC 1.1.1.51) activity has been described in human blood (Migeon *et al.* 1962; Jacobsohn and Hochberg 1968; Mulder *et al.* 1972*a*, 1972*b*) and rat blood (Portius and Repke 1960; Mulder *et al.* 1972*b*; Heyns and de Moor 1974). 17β-HSD (Findlay and Seamark 1969) and 20α-HSD (EC 1.1.1.62) (Nancarrow and Seamark 1968) enzymes have also been found in erythrocytes from sheep fetal blood. More recent work has shown that the activity of 20α-HSD in whole sheep blood decreases during the last 30 days of gestation and reaches relatively low adult levels around 5 weeks after birth (Nancarrow 1983; Nancarrow *et al.* 1984). In both fetal and adult blood, low activities of 3α-HSD (EC 1.1.1.50) were also found when progesterone was used as a substrate (Seamark *et al.* 1970). In contrast, Nancarrow *et al.* (1981) have shown that 3β-HSD (EC 1.1.1.51) activity which converts 5α-dihydrotestosterone (DHT) is present in fetal calf erythrocytes and may share the same catalytic site as 20α-HSD activity (Sharaf and Sweet 1982).

Because experimental evidence suggested that adult sheep blood reduces DHT at the 3-oxo position, a study was carried out *in vitro* to measure the metabolism of DHT to 5α-androstanediols in whole blood and its main cellular component.

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Materials and Methods

Enzyme Assays

Methods used for collecting and handling blood and for estimating both the rate of 20α-HSD activity (v) in reducing progesterone and the apparent equilibrium position of the reaction (Nancarrow 1983; Nancarrow *et al.* 1984) were followed in these experiments. DHT was used as the substrate and the products were found to result from both 3α-HSD and 3β-HSD activities. Thus the measurements made refer to the reduction of DHT rather than to the formation of any one product.

[1,2,-^{3}H]DHT (2·07 TBq/mmol) and [4-^{14}C]DHT (2·07 GBq/mmol) were obtained from Amersham International plc, Bucks, U.K., and checked for purity on thin-layer chromatogram (TLC) sheets (No. 13179; Eastman Kodak Co., Rochester, New York, U.S.A.) developed with chloroform–acetone (185 : 15, v/v). The labelled DHT was diluted with non-labelled DHT to form solutions containing 0·2 μmol plus either 3·3 kBq of ^{3}H or 0·6 kBq of ^{14}C in 0·05 ml ethanol. In some instances undiluted labelled steroid only was incubated with blood. Separation of the substrate from the products was always achieved by TLC. Identification of the areas on the plates containing the steroids was made with a Model 7201 Radiochromatogram scanner (Packard Instrument Co. Inc., Downers Grove, Illinois, U.S.A.). Appropriate regions were cut out, placed in glass vials, dampened with ethanol, extracted into 7 ml scintillation cocktail and assessed for radioactivity. Calculations for v and the equilibrium position were made according to Nancarrow *et al.* (1984). Initial experiments were carried out to determine optimal conditions for estimation of v and the equilibrium position.

The steroids were extracted once only with 4 ml ethyl acetate which yielded 83% of DHT and 78% of 5α-dihydroandrostane-3α,17β-diol (3α-diol) when added to 1 ml blood diluted with 1 ml saline. In three cases a second extraction was tested and the proportions of substrate and products were found to be identical with those of the first extraction. The mean value for blank (no blood) incubations was 0·59% for 14 estimates with a coefficient of variation (c.v.) of 20·3%. These blank values were subtracted before calculations of enzyme activity were made.

Identification of Metabolites

The same two radioactive components from each of the incubations were detected in all TLC plates. These corresponded to the substrate DHT (R_F = 0·48), and the combined products 3α-diol and 5α-dihydroandrostane-3β,17β-diol (3β-diol) (R_F = 0·32 and 0·33). Authentic 3α-diol and 3β-diol (Steraloids Inc., Wilton, New Hampshire, U.S.A.) were added to some mixtures prior to chromatography, then extracted from the TLC plates with ethyl acetate, applied to 2·5 cm wide lanes of Whatman No. 1 chromatography paper and subjected to paper partition chromatography using the system light petroleum (80–100°C)–toluene–methanol–water (60 : 40 : 85 : 15 v/v) (Hodgins and Hay 1973) after overnight equilibration. The two products were separated after a run of 3·5 h and located with the radiochromatogram scanner or by development of a blue reaction to phosphomolybdic acid (10% w/v in ethanol) when heated at 80°C. The proportions of 3α-diol and 3β-diol were estimated by scintillation counting. The products of some [4-^{14}C]DHT incubations were eluted from the TLC plates, halved and each portion added to approximately 15 mg of one or other of the authentic steroids previously prepared by mixing approximately 200 mg steroid with 70 kBq (2·07 TBq/mmol, Amersham) of [1,2-^{3}H]steroid and recrystallizing the 3α-diol twice from ethyl acetate–ethanol (1 : 1 v/v) and the 3β-diol twice from ethyl acetate–cyclohexane (1 : 1 v/v). These mixtures were then recrystallized from ethyl acetate–cyclohexane (1 : 1 v/v) until a constant ^{14}C : ^{3}H ratio was obtained.

Variability of DHT Reduction

Both v and the equilibrium position were estimated for a group of 17 Merino ewes and 11 Merino rams. Three sheep were sampled two to five times over a 3-month period to obtain an estimate of the 'within animal' variability of the measurements. During this period the sheep were maintained indoors on a daily ration of 600 g (ewes) or 800 g (rams) of a pelleted 60 : 40 mixture of lucerne chaff and crushed oats.

Energy Requirements of Erythrocytes for DHT Reduction

Blood was obtained from two rams and v estimated on diluted whole blood and washed cells after storage overnight at 4°C. A second sample of washed cells was lysed (Nancarrow *et al.* 1984) before glucose or the cofactors NADH and NADPH were tested for their ability to restore the enzyme activity of both washed cells (glucose) and lysed cells (cofactors).

Migeon *et al.* (1962) have shown that glucose added to the incubation mixtures resulted in an increase in oestrone transformation to oestradiol in human cells. We examined the possibility that glucose was limiting in the assays by using four sheep. Each ewe was maintained for 12 days on a diet of 750 g of a mixture of crushed oats and lucerne hay (60 : 40) which was fed daily at 1400 h. Blood samples for plasma glucose estimations (Nancarrow *et al.* 1984) and DHT reduction assays were taken prior to feeding on the 10th, 11th and 12th day after initiation of the regime. The rate of DHT reduction was estimated in the presence of 0, 0·83, 1·67, 2·50 and 3·33 mmol glucose added per litre. The equilibrium value of the reduction was measured singly for two animals in the presence of both 0 and 3·33 mmol glucose l^{-1}.

Results

Conditions of Assay

A time course for v was carried out on blood from five sheep for the times 7·5, 15, 22·5, 30 and 60 min. The means ± s.e. for these times were 0·58 ± 0·35, 0·45 ± 0·11, 0·43 ± 0·13, 0·43 ± 0·12 and 0·30 ± 0·08 μmol ml^{-1} h^{-1} respectively. The initial velocity of DHT reduction cannot be reliably estimated as very short incubation times give v values with an unacceptably high c.v. (Nancarrow 1983). As a compromise, assays for v were standardized using 15-min incubations which resulted here in a reduction of DHT varying from 0·08 to 3·6% above background. Although this leads to an underestimation of the initial velocity, the v estimated after 15 min was a constant 77% (c.v. = 7·9%) of the rate calculated for 7·5 min incubations.

The equilibrium position reached in blood from four sheep was estimated by incubating 1 ml whole blood diluted 1 : 1 with saline, with undiluted labelled steroid for 0·25, 0·5, 0·75 and 1·0 h. Total DHT reductions obtained for these times were 87·5 ± 11·3% (s.d.), 93·7 ± 4·8%, 95·1 ± 2·5% and 96·1 ± 1·7% respectively. Aliquots (2 ml) of blood from one ewe incubated with 0·2 μmol steroid over 0·5, 1·0, 2·0 and 4·0 h metabolized DHT by 69·8, 90·7, 92·7 and 90·6% respectively to androstanediols. The latter protocol, with an incubation period of 1 h, was used in most experiments of this series. Comparative values for progesterone reduction by 20α-HSD (Nancarrow *et al.* 1984) in the blood of two sheep from this group were 12·0 and 8·9% by 5 h and 13·3 and 6·8% by 24 h.

Identification of Metabolites

The 3α-diol and 3β-diol were both obtained from DHT by enzyme activity in the blood of adult male and female sheep. Radioactive peaks co-migrated with internal standards during TLC and paper partition chromatography. Blood samples from five sheep gave mean values for v of 0·64 ± 0·25 (s.d.) μmol ml^{-1} h^{-1} and for the equilibrium conversion of 92·3 ± 1·6%. In these incubations the proportion of 3α-diol formed was 95·9 ± 2·4%. The proportions of 3α-diol to 3β-diol formed in individual blood samples from two sheep estimated from recrystallization techniques were 97·4 and 76·0% compared to the estimates of 98·1 and 79·3%, respectively, from paper chromatography. Separation by paper chromatography was sufficiently accurate and precise (c.v. of 2·3 and 0·9% for these duplicate estimates) to be used to estimate the proportion of 3α-diol formed in other incubation mixtures. No differences were observed between blood samples from male and female sheep.

Variability of DHT Reduction

Mean values and variances between animals for either v or the equilibrium position were similar for grouped rams, cyclic ewes or pregnant ewes (Table 1). The grand means for these parameters were 0·66 ± 0·36 (s.d.) μmol ml^{-1} h^{-1} and 90·6 ± 5·1% respectively. This table also shows the precision of a sequence of estimates for individuals sampled over a 3-month period. Coefficients of variation for several estimates of v made on individual sheep (<15%) were less than those for sheep within groups (50–60%), but fell within the range for duplicates. Coefficients of variation for equilibrium estimates were all <7%.

Energy Requirements of Erythrocytes for DHT Reduction

Around 90% of the activity of erythrocytes from two sheep was lost upon washing and storing overnight at 4°C (Table 2). More than 90% of the original activity was regained in the presence of 0·67 (No. 6528) or 0·17 (No. 6529) mmol glucose l^{-1}. Lysis of washed erythrocytes reduced the activity to ≃30% (Table 2) whereupon addition of 25 μmol l^{-1} of NADPH enhanced the activity to ≃160%. The addition of NADH up to 0·4 mmol l^{-1} resulted in a minor increase of activity in lysates of cells from the same two sheep to 44% of the original values (data not shown).

Table 1. Reduction of DHT by blood of different groups of sheep

Values are means ± s.d.

Animal group	N[A]	v (μmol ml^{-1} h^{-1})[B]	Coeff. of variation (%)		Reduction (%) at equilibrium	Coeff. of variation (%)	
			Between collections	Of duplicates		Between collections	Of duplicates
Rams	11	0·71 ± 0·36	50·6	13·5	93·0 ± 3·0	3·2	1·1
Ewes	10	0·63 ± 0·38	60·5	9·8	88·6 ± 5·4	6·1	3·3
Pregnant ewes (20–42 days)	7	0·63 ± 0·36	57·5	9·2	89·0 ± 5·4	6·1	6·8
Individuals[C]							
4468♂	2	0·50 ± 0·05	9·1	21·5	93·0 ± 2·6	2·8	1·2
6528♂	5	0·47 ± 0·06	13·7	4·9	92·3 ± 2·3[D]	2·5	0·4
8114♀	3	0·92 ± 0·14	14·7	7·0	–	–	–

[A] Number of animals or samples.
[B] Rate of reduction, measured as the sum of 3α- and 3β-diol products of DHT metabolism.
[C] Adult sheep sampled over a 3-month period.
[D] Mean values for only four samples.

Table 2. Effect of energy sources on reduction of DHT by washed or lysed erythrocytes

Volumes of resuspended washed cells or lysates equivalent to 0·1 ml whole blood were incubated for 15 min with 3·3 kBq, 0·2 μmol [1,2-^{3}H]DHT, 50 μl ethanol and phosphate-buffered saline, pH 7·2 (cells), or 1 μM phosphate buffer, pH 6·0 (lysate); the final volume was 2 ml. Values are means of duplicate estimates

Concn of glucose (mmol l^{-1})	v (μmol ml^{-1} h^{-1})[A] Sheep No. 6528	Sheep No. 6529	Concn of NADPH (μmol l^{-1})	v (μmol ml^{-1} h^{-1})[A] Sheep No. 6528	Sheep No. 6529
	Washed cells			**Lysed cells**	
	0·39 (100)	0·12 (100)		0·42 (100)	0·34 (100)
0	0·04 (9·3)	0·02 (13)	0	0·14 (33)	0·11 (32)
0·17	0·20 (52)	0·14 (111)	25	0·66 (157)	0·55 (162)
0·33	0·31 (80)	0·14 (112)	50	0·72 (171)	0·49 (144)
0·67	0·35 (89)	0·17 (139)	100	0·72 (171)	0·56 (165)
1·00	0·35 (91)	0·18 (142)	200	0·86 (205)	0·54 (159)

[A] Percentage of original activity given in parentheses.

The concentration of glucose was normal for all four sheep sampled prior to their daily feeding regimen (3·7 ± 0·3 s.d. mmol l^{-1}). The establishment of a range of glucose concentrations did not affect the rate of DHT reduction in unwashed erythrocytes, the grand means for v for each sheep being 0·74, 0·80, 0·36 and 0·30, c.v. range 8–19%). Likewise the addition of 3·33 mmol glucose l^{-1} to the 1 h incubation mixtures did not influence the proportion of DHT reduced at equilibrium (93·2 ± 1·5% v. 93·3 ± 2·3%).

Discussion

Blood from adult sheep rapidly metabolizes the androgenic hormone, DHT, to each of its two possible epimeric products, 3α-diol and 3β-diol, with 3α-diol being the major product. Thus the presence of the 3α-HSD and 3β-HSD activities has been unequivocally demonstrated in erythrocytes from adult sheep. Both of these activities are also present in leucocytes, but at $<10\%$ of the values for an equal volume of erythrocytes (C. D. Nancarrow and P. J. Connell, unpublished observations) and are therefore considered negligible in whole blood.

The mean value for the rate of DHT reduction was 0·66 μmol ml^{-1} h^{-1}; blood from some sheep gave values of v above 1 μmol ml^{-1} h^{-1}. Equivalent values for 20α-reduction of progesterone were 2·31 for fetal blood (Nancarrow *et al.* 1984) decreasing to 0·10 μmol ml^{-1} h^{-1} for adult blood (Nancarrow 1983) as the proportion of fetal erythrocytes declines. The variations inherent in the DHT-reduction assay, both between samples and due to duplication were similar to those reported for the 20α-HSD assay. The underestimation of v by at least 23% was necessary to reduce excessive assay variability as the blank values were approached. This underestimate was similar to that for fetal 20α-HSD (32%) (Nancarrow 1983).

Similar quantitative estimates of v of erythrocytic HSD's have not been given in other publications but some comparisons can be made where sufficient data have been presented. For example, Migeon *et al.* (1962) obtained only 5–7% conversion of oestrone to oestradiol-17β in 1·55 ml 'whole' human blood in 24 h which represented a rate of 30 pmol ml^{-1} h^{-1}. Estimates of 17β-HSD activity can be made from the data of Jacobsohn and Hochberg (1968). For reduction of oestrone they obtained a value of 1·2 μmol ml^{-1} h^{-1} and for the oxidation reaction values of 7–10 nmol ml^{-1} h^{-1} were estimated. Reduction of dehydroepiandrosterone to androst-5-ene-3β,17β-diol by rat erythrocyte 17β-HSD was about 20 nmol ml^{-1} h^{-1} compared to human activity of 0·2 nmol ml^{-1} h^{-1} (Mulder *et al.* 1972*b*). Even when allowances are made for variation among the data due to long incubation conditions, low substrate levels, different enzymes and different substrates, the steroid-reducing enzymes in sheep blood, particularly 3α-HSD of adult and 20α-HSD of fetal (Nancarrow *et al.* 1984) are the most active types of HSD's found in mammalian erythrocytes.

Glucose provides the energy for DHT reduction in erythrocytes and maintains the cofactor, NADPH in a reduced state. Although washing and storage of erythrocytes decreased the rate of DHT reduction by about 90%, the 20-fold dilution of plasma glucose in the standard assay did not result in lower measured rates. Glucose added to simulate physiological concentrations did not increase v. Thus *in vivo* or *in vitro* variations below the normal adult plasma glucose concentrations of 3–6 mmol l^{-1} (Lindsay and Leat 1975) would not limit the activity of HSD's in erythrocytes. This was not so for human blood as glucose increased 17β-HSD activity markedly *in vitro* (Migeon *et al.* 1962). NADPH dependency has previously been shown for 17β-HSD and 20α-HSD from fetal ovine erythrocytes (Findlay and Seamark 1969; Nancarrow *et al.* 1984), 17β-HSD from adult human erythrocytes (Migeon *et al.* 1962; Jacobsohn and Hochberg 1968; Mulder *et al.* 1972*a*) and 3β-HSD from rat erythrocytes (Heyns and de Moor 1974). The fact that lysed erythrocytes from two sheep regained 160–200% of their original activities in the presence of NADPH, in contrast to the 56% of 20α-HSD activity regained in lysed fetal cells (Nancarrow *et al.* 1984), suggests that the maximum activity of 3α- and 3β-HSD is restricted not by plasma glucose concentrations, but by the relative impermeability of adult blood cells to glucose (Widdas 1961). Fetal erythrocytes maintain an intracellular concentration of glucose about twice that of the plasma (Lindsay and Leat 1975). Despite the suggestions by Widdas (1961) that sufficient glucose could still enter adult cells to maintain the metabolic profiles and by Nancarrow *et al.* (1984) that the restricted entry of glucose is not responsible for the periparturient decline in 20α-HSD activity, the possibility remains that endogenous NADPH production in adult erythrocytes is limited by factors regulating glucose entry or metabolism.

Adult blood was capable of rapidly reducing about 90% of DHT to 3α-diol and this is assumed to be a reasonable estimate of the equilibrium position. Similar reactions resulted in reduction of >70% progesterone by 20α-HSD (Nancarrow and Seamark 1968) and 80% oestrone by 17β-HSD (Findlay and Seamark 1969) in sheep fetal blood and >75% oestrone by 17β-HSD (Portius and Repke 1960) and 70% DHT by 3β-HSD (Heyns and de Moor 1974) in rat blood. We have also shown DHT reduction occurs in cattle and, to a lesser extent, pig blood with very little activity occurring in human blood (C. D. Nancarrow and P. J. Connell, unpublished observations). The equilibrium position rapidly achieved for DHT metabolism by sheep blood *in vitro* suggests that a ratio of 3α-diol : DHT between 10 : 1 and 20 : 1 should be found *in vivo*. Measurements of serum steroids of rams and bulls by radioimmunoassay following HPLC separation have shown this ratio to be much lower, varying around 1 : 1 (Cook *et al.* 1982). In rats much of the circulating 3α-diol is in the form of disulfate (Eckstein *et al.* 1981). Conjugated dehydroepiandrosterone is prevented from reaching the intracellular HSD by the cell membrane of rat erythrocytes (Mulder *et al.* 1972*b*), possibly in a similar manner to the exclusion of phosphorylated hexoses. However, this mechanism is not universal as human erythrocytes metabolize oestrogens and their sulfates similarly (Jacobsohn and Hochberg 1968). Thus the equilibrium established in the peripheral circulation between the free and conjugated forms of the steroids associated with differential tissue metabolism also influence the ratio. However, as the concentration of 100 μmol DHT l^{-1} used in our experiments was five orders of magnitude greater than the concentrations reported by Cook *et al.* (1982), there does remain a discrepancy which requires resolution.

The physiological consequences of rapid metabolism of DHT to the androstanediols in sheep blood cannot yet be resolved. It has been suggested that these various enzymes maintain hormonal homeostasis by balancing the proportions of oxidized and reduced steroids which reach the target organs (Jacobsohn and Hochberg 1968; Nancarrow and Seamark 1968; Mulder *et al.* 1972). Reduced metabolites of testosterone such as DHT exert their action mainly at the level of the external genitalia and accessory sex organs particularly during fetal development (see Wilson *et al.* 1983). Androstenedione and testosterone can also be converted in the rat brain to their 5α- and 3α-reduced metabolites (Denef *et al.* 1974; Martini 1976) and these steroids are certainly present in sheep serum (Cook *et al.* 1982). In this laboratory, Mattner (1980; personal communication) has shown that any one of testosterone, DHT, 3α-diol and 3α-hydroxy-5α-androstane-17-one, but not 3β-diol, act in concert with oestradiol to fully restore sexual behaviour in castrate rams. Thus, in contrast to the findings of Axelrod and Werthessen (1961), reductive metabolism of DHT in adult sheep blood results in the formation of a biologically active metabolite.

We consider that the blood of sheep, with its considerable capacity to modify progesterone and the androgenic steroids, acts as a secondary endocrine tissue which exerts a major function in maintaining endocrine homeostasis. Its importance in this capacity needs resolution. Experiments to determine the production rates and androgenic function of the components of the metabolic pathways are necessary for this understanding.

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