Equine 5α-reductase activity and expression in epididymis

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Abstract

The 5α-reductase enzymes play an important role during male sexual differentiation, and in pregnant females, especially equine species where maintenance relies on 5α-reduced progesterone, 5α-dihydroprogesterone (DHP). Epididymis expresses 5α-reductases but was not studied elaborately in horses. Epididymis from younger and older postpubertal stallions was divided into caput, corpus and cauda and examined for 5α-reductase activity and expression of type 1 and 2 isoforms by quantitative real-time polymerase chain reaction (qPCR). Metabolism of progesterone and testosterone to DHP and dihydrotestosterone (DHT), respectively, by epididymal microsomal protein was examined by thin-layer chromatography and verified by liquid chromatography tandem mass spectrometry (LC-MS/MS). Relative inhibitory potencies of finasteride and dutasteride toward equine 5α-reductase activity were investigated. Pregnenolone was investigated as an additional potential substrate for 5α-reductase, suggested previously from *in vivo* studies in mares but never directly examined. No regional gradient of 5α-reductase expression was observed by either enzyme activity or transcript analysis. Results of PCR experiments suggested that type 1 isoform predominates in equine epididymis. Primers for the type 2 isoform were unable to amplify product from any samples examined. Progesterone and testosterone were readily reduced to DHP and DHT, and activity was effectively inhibited by both inhibitors. Using epididymis as an enzyme source, no experimental evidence was obtained supporting the notion that pregnenolone could be directly metabolized by equine 5α-reductases as has been suggested by previous investigators speculating on alternative metabolic pathways leading to DHP synthesis in placenta during equine pregnancies.

Key Words
- progesterone
- testosterone
- steroidogenesis
- epididymis
- pregnancy
- equine
- reductase

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Introduction

The specific short-chain dehydrogenase reductase enzymes known as 5α-reductases (types 1 and 2) are best known for their role in masculinization of the male reproductive tract (Wilson *et al*. 1995) but are also recognized to play a role in pregnancy and parturition in some species (Mahendroo & Russell 1999, Scholtz *et al*. 2014). These isozymes are expressed in various male reproductive tissues including the epididymis

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(Robaire *et al*. 1977), where they reduce testosterone to the more biopotent agonist, dihydrotestosterone (DHT), significantly amplifying the androgenic response. However, progesterone is an equally good substrate for 5α-reductases in several species (Levy *et al*. 1995), and there is increasing recognition of important physiological effects of 5α-reduced pregnanes in female reproduction. Dihydroprogesterone (DHP) is the immediate product of the 5α-reduction of progesterone, and it is as potent an agonist as progesterone in some species. For instance, we demonstrated recently that DHP is able to maintain pregnancy in mares in the absence of progesterone and activates the equine progesterone receptor as potently as progesterone (Scholtz *et al*. 2014). In addition, DHP itself is a substrate for further metabolism by 3α-reduction in the synthesis of allopregnanolone, a potent neurosteroid that can both allosterically regulate and directly activate γ-aminobutyric acid (GABA) receptors. Activation of GABA receptors may be of importance in both male and female reproduction centrally (Henderson 2007, Brunton *et al*. 2014) and perhaps also peripherally. For example, sperm membranes express GABA receptors that are involved in initiating the acrosome reaction (Meizel 1997, Vigil *et al*. 2011). With respect to female physiology, there is extensive metabolism of DHP to allopregnanolone during equine (Legacki *et al*. 2016) and human pregnancy (Hill *et al*. 2007), and allopregnanolone has been implicated in affective mood disorders in pregnant and cyclic women (Schiller *et al*. 2014). In addition, allopregnanolone may influence neonatal physiology because it has been shown to induce obtundation in newborn foals (Madigan *et al*. 2012), mimicking symptoms often accompanying maladjustment syndrome (Rossdale *et al*. 1995). Thus, 5α-reductase enzymes play important roles both in male and female reproductive functions but were not extensively studied in horses.

The 5α-reductase enzymes catalyze reduction of Δ4 steroids, testosterone and progesterone (Fig. 1) being the more physiologically significant known substrates (Russell & Wilson 1994), and this is believed to be the case for all mammals. However, these enzymes are hard to study; to date, none of the isozymes of any species has been purified to homogeneity due to protein instability (Thareja *et al*. 2015), making kinetic analysis and modeling additionally challenging. While not considered a substrate, the possibility has been proposed from studies in horses (Silver 1994) that the Δ5 steroid, pregnenolone might be directly reduced and subsequently oxidized to form DHP by an alternative metabolic pathway in equine placenta, bypassing the synthesis of progesterone (Fig. 1). Indeed, alternative (essentially redundant), active metabolic pathways exist for the synthesis of DHT in other species (Auchus 2004) and may exist similarly for DHP in the horse. Experimental support for the suggestion that pregnenolone might be directly reduced by 5α-reductase resulted from observations made of steroids produced in pregnant mares infused with pregnenolone (Schutzer & Holtan 1996, Schutzer *et al*. 1996). However, such studies are not only difficult to conduct, but they are difficult to interpret because multiple competing metabolic pathways can function in parallel especially *in vivo* where different tissue compartments can contribute to steroids monitored in blood. Clarifying which pathways are active in using available substrate might be obviated using inhibitors of specific enzymes, but some like 3β-hydroxysteroid dehydrogenase (3β-HSD) can be difficult to inhibit in mares *in vivo* (Fowden & Silver 1987). Inferring metabolic routes for the synthesis of particular steroids is particularly challenging under such circumstances. The equine epididymis is unlikely to express 3β-HSD (Amann 1987) but is very likely to exhibit robust 5α-reductase activity (Ganjam *et al*. 1991), features that might considerably simplify testing the hypothesis that pregnenolone is a substrate for 5α-reductase enzymes in the horse.

Given the importance of 5α-reductases in both male and female physiology, the following studies were conducted to better characterize equine 5α-reductase enzyme activity using epididymal tissues from castrated stallions. The levels of expression and activity of 5α-reductase in equine epididymis were determined in the caput, corpus and cauda regions because activities

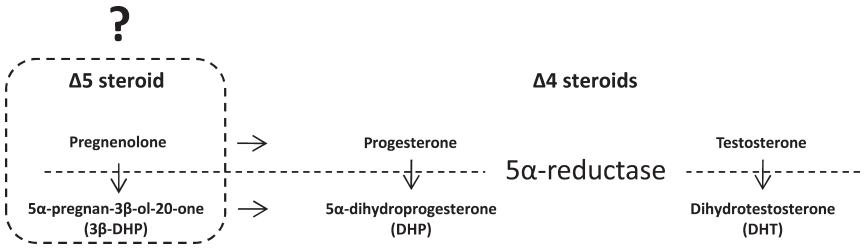

Figure 1
Schematic showing known (progesterone and testosterone, Δ4 steroids) and previously hypothesized substrates (pregnenolone, Δ5 steroid, '**?**') of equine 5α-reductase activity, and their known (5α-DHP and dihydrotestosterone (DHT)) and hypothesized (5α-pregnan-3β-ol-20-one (3β-DHP)) products. No evidence of direct metabolism of pregnenolone by 5α-reductase was found in the studies conducted and reported herein.

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differ significantly by region in some livestock species (Kelce *et al*. 1990), perhaps also stallions (Ganjam *et al*. 1991). Subsequent experiments explored the relative inhibitor potencies of two common therapeutics, hypothesizing that dutasteride would be a more potent inhibitor of enzyme activity than finasteride. Second, the hypothesis that pregnenolone is directly metabolized by 5α-reductases was also tested.

Materials and methods

Tissues

Epididymal tissues were dissected from testes after routine castration of normal healthy untreated stallions ($n=14$). Procedures were reviewed and approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC: 2013–1088). Briefly, 11 (postpubertal but not mature, 2-year-old) stallions (mixed breed ponies) that had not received any treatments (they were controls in another study) were anesthetized (xylazine HCl, butorphanol tartrate, ketamine HCl) for castration. Recovered tissues were placed immediately on ice and transported (10 min) to the laboratory for dissection. Epididymides were dissected free from surrounding mesentery and divided into caput, corpus and cauda epididymal (Fouchecourt *et al*. 1999) tissues. Aliquots of each epididymal region were snap frozen in liquid nitrogen or preserved in RNAlater (5°C overnight) followed by storage at −80°C until processed. Testes were examined histologically from these stallions to confirm the onset of spermatogenesis (mature sperm in seminiferous tubules were verified to be present in all, data not shown). Subsequent to completion of studies on 2-year-old males, caput, corpus and cauda epididymis from another three normal, but more mature, stallions (6, 7 and 13-year-old) were collected similarly and used for qPCR analysis that was run along with a subset of the samples from the 2-year-old group.

Reagents

Steroids (progesterone, pregnenolone, testosterone, dehydroepiandrosterone (DHEA) and DHP) were purchased from Steraloids (Newport, RI, USA). Enzyme inhibitors were purchased from the following sources: trilostane, Sanofi Research Division, Great Valley, PA, USA; finasteride, Sigma Chemical Co, St Louis, MO, USA; dutasteride, Aurum Pharmatech, Howell, NJ, USA. Methylene chloride (HPLC grade) and diethyl ether were purchased from Fisher (Fair Lawn, NJ, USA) and ethyl acetate (chromatography grade) from EMD Millipore.

Microsomal enrichment

Tissues were homogenized in 0.1 M KPO4 pH 7.4, 20% glycerol, 5 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride and 1 μg/μL aprotinin, and then briefly sonicated. Homogenate was centrifuged at 15,000***g*** for 10 min. Supernatant was transferred to a new tube and spun for 1 h at 100,000***g***, and the resultant microsomal pellet was resuspended in buffer. Protein concentrations were determined using the Pierce BCA Protein Reagent (Thermo Scientific). Microsomal preparations were stored at −80°C.

Enzyme activity assays

All incubations were performed with epididymal microsomes in buffer (50 mM K_3PO_4, 1 mM EDTA pH 7.4) with a generating system consisting of 17 mM glucose-6-phosphate, 1 mM NADPH, 2 mM NADP and 1 unit of glucose-6-phosphate dehydrogenase (Sigma) for 2 h at 37°C after the evaporation of the inhibitor, if used, in the tube. Reactions were stopped by extraction with 10 volumes methylene chloride, and the organic phase was evaporated to dryness for subsequent analysis by thin-layer chromatography (unlabeled plus radiolabeled substrates) or liquid chromatography tandem mass spectrometry (unlabeled substrates only). In initial studies, epididymal microsomes (12 μg) were incubated with increasing concentrations (50 nM–500 μM) of substrate (progesterone and [1,2,6,7-^{3}H]-progesterone, 98.0 Ci/mmol, PerkinElmer, or testosterone and [1β, 2β-^{3}H]-testosterone, 40.3 Ci/mmol, American Radiolabeled Chemicals, St Louis, MO, USA) for 2 h at 37°C to determine concentration dependence of 5α-reduced product formation (DHP and DHT), respectively. Subsequent experiments utilized 3 μM [4-^{14}C]-progesterone (54 mCi/mmol, American Radiolabeled Chemicals) to assess relative levels of 5α-reductase activity in different regions (caput, corpus and cauda) of equine epididymis. Additional incubations were conducted to determine the relative inhibitory potencies of two 5α-reductase inhibitors, finasteride and dutasteride (1–100 μM), using 3 μM [4-^{14}C]-progesterone. Reactions conducted with unlabeled substrates were used for analysis by mass spectrometry. Reactions were performed with or without finasteride (30 μM) or trilostane (30 μM), a specific inhibitor

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of 3β-HSD as follows: 3 μM progesterone (± finasteride), pregnenolone (± trilostane), testosterone (± finasteride) or dehydroepiandrosterone (± trilostane).

Thin-layer chromatography

Samples were resuspended in 25 μL ethyl acetate, spotted onto silica gel plates (Whatman, Maidstone, Kent, UK) along with the appropriate cold standards including progesterone, DHP, testosterone or DHT and developed in methylene chloride: diethyl ether (5:2, v:v) (progesterone) or 100% ethyl acetate (testosterone). Progesterone and testosterone standards were visualized with handheld UV lamp; then plates were sprayed with 50% sulfuric acid and baked for 5 min at 100°C in order to visualize DHP or DHT bands. Spots were isolated in approximately 1 × 1 cm^2, steroid eluted in 6 mL ethyl acetate into glass scintillation vials. Ethyl acetate was evaporated and radioactivity was quantified by scintillation counting in 10 mL EcoLite (MP Biomedicals, Solon, OH, USA).

Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Standards and solutions: The analytes used in this study were purchased from Steraloids: 5α-DHP, 5α-pregnen-3β-ol-20-one (3β-DHP), 17α-hydroxyprogesterone (17-OHP), 20α-hydroxyprogesterone (20α-OHP), allopregnanolone (AlloP), androstenedione (A4), dehydroepiandrosterone (DHEA), d7-androstendione (A4-d7), pregnenolone (P5), progesterone (P4) and d9-progesterone (P4-d9). The remaining analytes were purchased from Cerilliant (Round Rock, TX, USA): 5α-DHT, testosterone and d3-testosterone (T-d3). Analytes were in powder form and a 1 mg/mL reference solution was prepared in methanol for each. Dilutions were made at 10, 1, 0.1 and 0.01 ng/mL in methanol. Methanol and water were of HPLC grade and obtained from Burdick and Jackson (Muskegon, MI, USA). Formic acid and methyl *tert*-butyl ether were of ACS grade and obtained from EMD (Gibbstown, NJ, USA).

Sample preparation

Extracted samples were reconstituted with 200 μL of 50:50 water and methanol. Calibration standards were run at the beginning and at the end of each sample set; QC samples were run at the beginning of each sample set. Quantitation of analytes was determined by linear regression analysis of the ratio of the area of analyte to internal standards, which were chosen for each analyte based on structure and chromatographic retention time. Tandem mass spectral detection was accomplished using a Bruker EVOQ triple quadrupole Mass Spectrometer (Billerica, MA, USA). Calculations were done using the Bruker software (Billerica, MA, USA). A minimum of a six-point calibration curve and maximum of ten points were used depending on the concentration range of each analyte.

Exact mass analysis

The identity of analytes derived from epididymal metabolism of testosterone, progesterone, pregnenolone and dehydroepiandrosterone was confirmed by exact mass determination by LC-MS/MS using a Linear Ion Trap Orbitrap XL mass spectrometer (LTQ OrbiTrap XL, Thermo Scientific) coupled to an Acquity UPLC chromatography system (Waters Corporation, Milford, CA, USA). Two methods were employed to analyze metabolites. For 5α-reductase metabolites, spray voltage was set at 4300 V, sheath gas and auxiliary gas were 40 and 20, respectively (arbitrary units), and the capillary temperature was 350°C. Chromatography employed an ACE 3 C18 10 cm × 2.1 mm column (Mac-Mod Analytical, Chadds Ford, PA, USA) and a linear gradient of acetonitrile (ACN) in water, both with 0.2% formic acid, at a flow rate of 0.350 mL min^{-1}. The initial ACN concentration was held at 30% for 0.4 min, ramped to 90% over 10.6 min and held at that concentration for 1 min before re-equilibrating for 4 min at initial conditions. Products of 3β HSD metabolites were evaluated using a spray voltage setting of 4300 V, sheath gas and auxiliary gas were 45 and 10, respectively (arbitrary units), and the capillary temperature was 350°C. Chromatography employed an ACE 3 C18 10 cm × 2.1 mm column (Mac-Mod Analytical) and a linear gradient of acetonitrile (ACN) in water, both with 0.2% formic acid, at a flow rate of 0.350 mL min^{-1}. The initial ACN concentration was held at 5% for 0.4 min, ramped to 90% over 13.6 min and held at that concentration for 1 min before re-equilibrating for 4 min at initial conditions. For both methods, product masses and collision energies were optimized using injected standards; exact mass was used to verify the individual metabolites.

Quantitative analysis

Quantification of mass was performed on an EVOQ triple quadrupole mass spectrometer using a

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reverse-phase gradient separation on an Advance UHPLC (Bruker) C18 analytical column (1.8 µM particle size, 2.1 × 50 mm). The injection volume was 20 µL and the oven temperature was set to 40°C. Mobile phases A and B were: A – water with 0.2% formic acid and B – methanol and was delivered at 0.4 mL/min. An elution gradient was held at 40% B for the first 0.2 min, 40–69% B from 0.2 to 1 min, 60–78% B from 1 to 6 min, 80–90% B from 6.0 to 6.1 min, held at 90% B from 6.1 to 7.1 min, 90–40% from 7.1 to 7.2 min and held at 40% B until 10.2 min with an atmospheric-pressure chemical ionization (APCI) source. APCI conditions for cone gas flow, probe gas flow and nebulizer gas flow were held at 25, 25 and 50 (respectively) arbitrary units of dry nitrogen gas. The cone and heated probe temperatures were 300 and 450°C, respectively. Argon was used as a collision gas and set to 1.5 arbitrary units. Resolution parameters were set with Q1 and Q3 both at 0.7 *m/z*. Detection and quantitation of all analytes was accomplished using multiple reaction monitoring with a minimum of two transitions per analyte. Inter- and intra-accuracy and precision was assessed at four QC levels for all analytes at 0.6, 1.5, 20 and 80 ng/mL (Table 1).

Transcript analysis

Total cellular RNA was extracted from epididymal (caput, corpus and cauda) samples using TRIzol Reagent (Thermo Fisher Scientific) according to the manufacturer's recommendation. The RNA was precipitated as described previously (Ball *et al*. 2013). RNA was quantified via spectrophotometry (NanoDrop 2000; Thermo Fisher Scientific), and samples with a 260/280 ratio of 1.95 or greater and a 260/230 ratio of 2.0 or greater were used for analysis. RNA samples (1 µg/reaction) were treated with rDNase I (Thermo Fisher Scientific) for 30 min at 37°C, followed by treatment with DNase Inactivation Reagent (room temperature for 2 min), RNA was then reverse transcribed using the TaqMan Reverse Transcription Reagents (Thermo Fisher Scientific).

The mRNA expression of 5α-reductases types 1 and 2 (*SRD5A1* and *SRD5A2*, respectively) were quantified by qPCR in epididymal samples. Primers (Table 2) were designed based on sequences as reported previously for equine tissues (Klein *et al*. 2011, Scholtz *et al*. 2014). qPCR of duplicate samples was performed using the ViiA 7 Real-Time PCR System (Thermo Fisher Scientific). Reactions contained a mixture of cDNA (5 ng), primers (25 ng each) and a SYBR Green Master Mix (Thermo Fisher Scientific).

Table 1 Accuracy and precision values for LC-MS/MS analysis of progesterone, pregnenolone, testosterone, dehydroepiandrosterone (DHEA), 5α-dihydroprogesterone (DHP) and 5α-dihydrotestosterone (DHT) in equine incubation matrix.

Steroid	Concentration (ng/mL)	Intraday accuracy (% nominal concentration)	Intraday precision (% relative S.D.)	Interday accuracy (% nominal concentration)	Interday precision (% relative S.D.)
Progesterone	0.6	102.8	2.7	94	7.3
	1.5	92	5.2	87	8.2
	20	104	1.8	101	5.4
	80	98	3.7	103	7.4
Pregnenolone	0.6	103	9.4	108	8.8
	1.5	99	7.7	95	10
	20	102	7.6	101	7.1
	80	97	1.1	98	1.5
Testosterone	0.6	99	2.4	96	4.6
	1.5	99	5.5	98	5.8
	20	96	6.7	101	8.5
	80	96	4.6	100	9.5
DHEA	0.6	97	10	101	11
	1.5	96	5.8	92	9.3
	20	95	2.4	91	8.2
	80	101	4.3	100	7.6
DHP	0.6	94	6.3	83	13
	1.5	97	13	88	3.3
	20	103	2.4	96	8.6
	80	99	7.2	103	8.5
DHT	0.6	89	2.1	84	7.8
	1.5	87	2.9	80	5.1
	20	98	2.7	93	8.3
	80	99	6.9	107	6.1

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Table 2 Forward and reverse primers used for amplification of equine 5α±-reductase type 1 (SRD5A1) and equine 5α-reductase type 2 (SRD5A2) by qPCR.

Gene	GenBank accession #	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
SRD5A1	XM_014734978.1	GCTTTTTATTCACCAGAGCACA	TCCTGAACTTCGGATAATCTTCA
SRD5A2	XM_001501522.2	GCACTTGCATTTGCATTTTTC	AACCTATGGTGGTGAAAAGCTC
B2M	NM_001082502	GTGTTCCGAAGGTTCAGGTT	ATTTCAATCTCAGGCGGATG
GAPDH	NM_001163856	AGAAGGAGAAAGGCCCTCAC	GGAAACTGTGGAGGTCAGGA

Cycle parameters of polymerase chain reaction were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, and then a dissociation step of 95°C for 15 s. Melting curves for each sample were assessed to evaluate the specificity of the reaction. PCR efficiencies were calculated using LinRegPCR (version 2013.0) (Ruijter *et al*. 2009). All reactions were pipetted using the epMotion Automated Pipetting Systems (Eppendorf, Hauppauge, NY, USA). Negative controls (deionized water blanks) were included in the analysis.

The ΔC_T for each gene of interest was calculated by subtracting the geometric mean of the C_T for the two housekeeping genes from the C_T of the gene of interest. Using Normfinder software, the most stable reference transcripts in epididymal samples were obtained by combining the housekeeping genes β-2 microglobulin (*B2M*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Andersen *et al*. 2004). Gene expression data are presented as relative quantification values (ΔC_T) (Livak & Schmittgen 2001).

Statistical analysis

Enzyme activities in epididymal regions were analyzed by ANOVA using Proc Mixed in SAS (SAS Statistical Software, SAS Institute Inc, Cary, NC, USA). Rates of metabolism as a function of substrate concentration (DHP produced from progesterone; DHT produced from testosterone) were analyzed by linear regression, as were the concentration-dependent effects of 5α-reductase inhibitors (finasteride and dutasteride). The effects of finasteride on the products, progesterone and testosterone metabolism, were subjected to ANOVA using Proc Mixed in SAS and presented as mean ± S.E.M. Differences in relative expression of *SRD5A1* between epididymal regions of the 2-year-old stallions were analyzed using a Kruskal–Wallis test, and graphed to represent the nonparametric analysis conducted. Potential effects of sexual maturity and region of the epididymis were similarly assessed as main effects using data from 2-year-old stallions with those from more mature males. Data were reported as significant when $P<0.05$.

Results

Equine epididymis exhibited easily detectable and, in some samples, robust 5α-reductase activity. Under the conditions used to measure enzyme activity, increasing progesterone or testosterone substrate concentration increased 5α-reduced (DHP and DHT) product formation in a linear fashion ($P<0.001$, not shown). This activity was also inhibited by both finasteride and dutasteride in a concentration-dependent manner ($P<0.01$; Fig. 2). While dutasteride may have inhibited activity more potently at lower concentrations, maximum inhibition reached a plateau similar to that seen with finasteride. Though finasteride appeared to achieve greater maximum inhibition at the higher concentrations, no statistical difference was detected in inhibition induced by finasteride or dutasteride at the highest concentrations used.

The levels of 5α-reductase enzyme activity were examined in epididymal microsomes from five stallions using progesterone as a substrate (Fig. 3). DHP was synthesized from progesterone at an average rate of 6.95 ± 2.63 pmol/mg/2 h. There was no significant difference among regions of the epididymis ($P>0.05$), although two samples from different regions from two

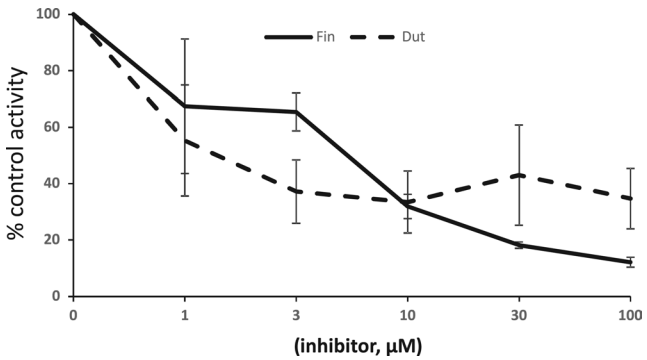

Figure 2
Concentration-dependent (1–100 μM) inhibition of 5α-reductase activity in equine epididymal microsomes by finasteride (solid line) and dutasteride (dashed line), shown as a percent of control (no inhibitor). Shown are the mean ± S.E.M. values of three independent experiments ($n=3$) performed on a pool of tissue from three different stallions.

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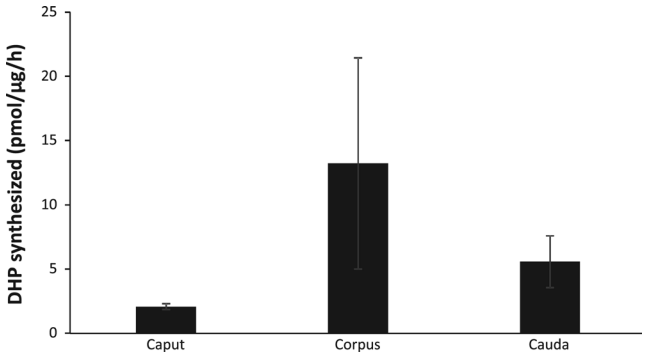

Figure 3
Activity of 5α-reductase in the caput, corpus and cauda regions of the equine epididymis based on conversion of progesterone to dihydroprogesterone (DHP) in incubations of epididymal microsomes as described in the 'Materials and methods' section. Shown are the mean ± S.E.M. values on microsomes from five different stallions ($n = 5$).

different stallions (corpus #71 and cauda #83) gave highly variable activities, 4- and 14-fold higher than the average of all other samples. Similar results were obtained when these assays were repeated. Even excluding these two samples from analysis, there was no obvious or consistent trend suggestive of major differences in 5α-reductase activity by epididymal region (Fig. 3). Epididymal tissues from an additional six 2-year-old and the three older stallions were used for RNA isolation and transcript analysis by real-time PCR. Expression was detected, but no differences in levels of expression of the type 1 isozyme were seen across the three epididymal regions in either the 2-year-old (Fig. 4; $P > 0.2$) or the three more mature stallions (data not shown), the only samples available from older males. In contrast to analysis for the type 1 isozyme, problems were encountered in analysis of the transcripts for the type 2 isozyme. Even after multiple attempts, and even after redesigning primers for the type 2 isozyme, little amplification of this transcript could be detected in any of the samples including epididymis, prostate and allantochorion (data not shown). As low as the signals were for type 2 reductase transcript, the values obtained with the two different primer sets were highly correlated with one another but not with type 1 isozyme expression, supporting the notion that the signals detected for the type 2 5α-reductase were likely real but very low.

The potential direct metabolism of pregnenolone by 5α-reductase to 3β-DHP (Fig. 1) was investigated using LC-MS/MS analysis of reaction products (Fig. 5). No 3β-DHP was detectable in any of the reactions with epididymal samples examined, using pregnenolone as a substrate, regardless of the level of 5α-reductase activity seen in the same microsomes when progesterone or testosterone were used as substrates instead.

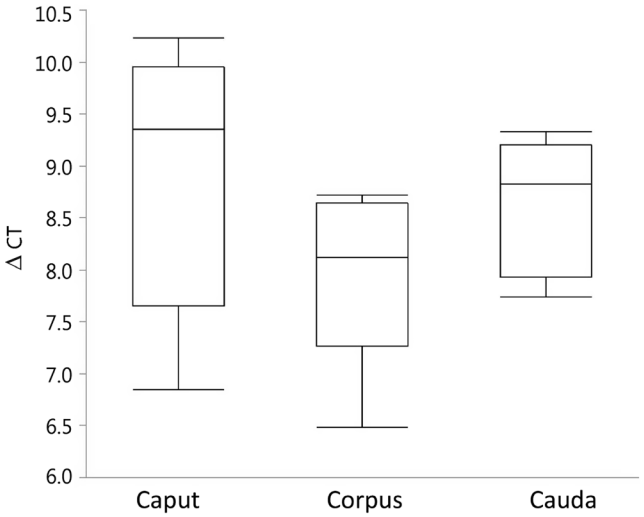

Figure 4
Relative expression of 5α-reductase type 1 (SDR5A1) in the caput, corpus and cauda regions of the equine epididymis determined by qPCR normalized to transcripts encoded by multiple housekeeping genes, as described in the 'Materials and methods' section. Data are shown as outlier boxplots (median and interquartile range) of expression in tissues from different stallions ($n = 6$).

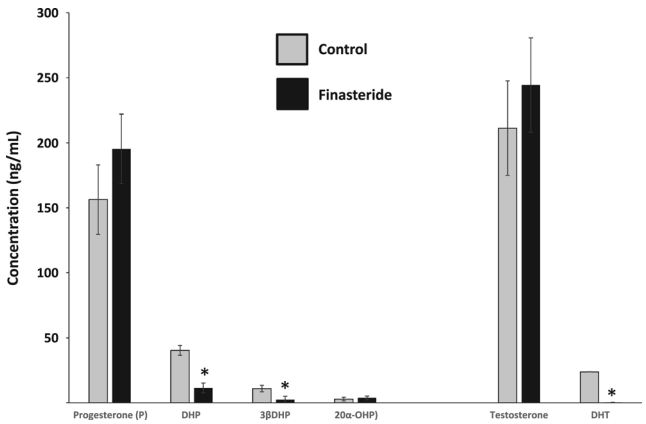

Figure 5
Metabolism of progesterone to dihydroprogesterone (DHP) and 5α-pregnan-3β-ol-20-one (3β-DHP) that is inhibited by finasteride, and metabolism of testosterone to dihydrotestosterone (DHT) also inhibited by finasteride, in incubations of equine epididymal microsomes analyzed by mass spectrometry. Also detected in incubations with progesterone was 20α-hydroxyprogesterone (20α-OHP) that was not inhibited by finasteride. Shown are the mean ± S.E.M. values of experiments on microsomes from epididymis of different stallions ($n = 5$). Note, when used as a substrate, pregnenolone was not directly reduced to 3β-DHP by 5α-reductase activity in equine epididymal microsomes. Significant inhibition of product by finasteride is shown (*$P < 0.01$). Note, despite active 5α-reduction of progesterone to DHP and testosterone to DHT, evidence for direct metabolism of pregnenolone to 3β-DHP by 5α-reduction was not detected in any reaction.

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However, 3β-DHP (ng/mL) was detected in reactions when progesterone was used as substrate. The concentrations of 3β-DHP were highly correlated with those of DHP ($r=+0.98$, $P<0.001$), but at about one quarter the concentrations. Moreover, the concentrations of 3β-DHP were inhibited by finasteride (from 10.9 ± 7.2 to 1.26 ± 1.26 ng/mL; $P<0.01$) as were those of DHP (from 40.4 ± 18.5 to 11.5 ± 3.8 ng/mL; $P<0.01$). Trace amounts of 20αOH-progesterone were detected in reactions conducted with progesterone as substrate, but concentrations were not affected by the presence of finasteride. Finasteride inhibited DHT when testosterone was used as substrate ($P<0.01$, Fig. 5). No 3β-HSD activity was detectable (metabolism of pregnenolone to progesterone or DHEA to androstenedione) and no effect of trilostane (30 μM) was observed (data not shown).

Discussion

The presence of 5α-reductase in epididymal tissues has been demonstrated in several species including ruminants and rodents (Aafjes & Vreeburg 1972, Robaire *et al*. 1977, Scheer & Robaire 1983, Amann 1987), but to our knowledge, only a single investigation has examined expression in equine epididymis (Ganjam *et al*. 1991). The current studies confirm the presence of 5α-reductase expression and activity in the equine epididymis that can metabolize both progesterone to DHP and testosterone to DHT. Although there were two different epididymal regions from two different stallions that showed far above the average enzyme activity of all the others, there was no obvious regional variance that was significant or predictable. On average, the estimates of activity in equine epididymal microsomes in this study were several fold higher than those reported previously for epididymal tissues collected from yearling (prepubertal) stallions (Ganjam *et al*. 1991), bucks (Kelce *et al*. 1990) and rats (Scheer & Robaire 1983). However, the studies conducted on equine and caprine epididymis used tissue homogenates that were not enriched microsomal fractions, as was done here. Microsomes exhibited several fold higher specific activity than did other subcellular fractions prepared from human, canine and rat prostate (Liang *et al*. 1985). Thus, it is not clear if the higher epididymal 5α-reductase activities reported here reflect differences in species or simply methodology. Based on the results from transcript analysis conducted with a small number of older stallions, it seems unlikely to be related to reproductive maturity, but the apparent lack of an age effect needs confirmation. The physiological significance of epididymal 5α-reductase expression is conjectural at this time. However, the current studies demonstrate that enzyme activity is sensitive to both of the clinically available inhibitors, finasteride and dutasteride. These data suggest that either of the inhibitors would be reasonable therapeutic candidates to test the impact of 5α-reductase activity on epididymal DHT synthesis and its physiological significance in stallions. Unlike other species tested (Liang *et al*. 1985), no significant difference was found in the potencies of these compound for inhibition of equine 5α-reductase activity, and their activity *in vivo* would be expected to depend more on pharmacokinetic behavior and metabolism.

Results of transcript analysis were far less variable and suggested that there was no gradient of 5α-reductase expression down the equine epididymis. Nor have others found differences in either testosterone concentrations or androgen receptor expression by region of the equine epididymis (Parlevliet *et al*. 2006), suggesting that DHT itself is likely present at similar concentrations from caput to cauda. In contrast, studies in bull, boar (Aafjes & Vreeburg 1972) and rat (Robaire *et al*. 1977) indicate that DHT and 5α-reductase activity is highest in the caput of the epididymis, decreasing toward the cauda. More surprising perhaps, in terms of apparent species differences, is the lack of apparent type 2, and predominant type 1, 5α-reductase transcript expression in equine epididymis. Although there is a preponderance of type 2 expression in primate epididymis (Mahony *et al*. 1997, 1998), both isozymes are still present, and men with a deficiency of the type 2 enzyme develop epididymis (Thigpen *et al*. 1992). Similarly, both isozymes are expressed in rat epididymis (Berman & Russell 1993, Viger & Robaire 1996). Further studies investigating isozyme expression of 5α-reductases in other accessory sex glands are necessary to determine the patterns and relative levels in the male reproductive tract of the horse. This information may help in distinguishing among or interpreting the phenotypes and genetic defects associated with male pseudohermaphroditism in horses, which have been far less extensively described or properly characterized (Lear & McGee 2012) than have karyotypic abnormalities or cases of sex reversal (Villagomez *et al*. 2011, Anaya *et al*. 2014, Demyda-Peyras *et al*. 2014). Defects in the androgen receptor gene associated with suspected androgen insensitivity syndrome have been reported in stallions (Revay *et al*. 2012), which presumably lack mesonephric duct-derived tubular or glandular accessory tissue, although phenotypes remain poorly defined with respect to internal genitalia. Other equine cases resembling human 5α-reductase deficiency have been described with female external genital anatomy,

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but prostate, bulbo-urethral glands, ampullae and tubular tract internally. Though suspected to suffer genetic defects in the type 2 enzyme (Knobbe *et al*. 2011) similar to the human condition, the present data would suggest a deficiency of the type 1 gene is at least possible, perhaps more likely.

An additional goal of the current studies was to examine the ability of equine 5α-reductase to directly metabolize pregnenolone, which has been proposed based on substrate infusion studies conducted in pregnant and nonpregnant mares (Silver 1994, Schutzer & Holtan 1996, Schutzer *et al*. 1996). It is not entirely clear how those data provide reliable support for that possibility. Peak starting substrate concentrations were not reported, so the efficiency, or fractional rate, of metabolism was impossible to assess (Schutzer & Holtan 1996). It is equally unclear how the introduction of pregnenolone experimentally into mares would compare in terms of emerging product profiles to pregnenolone supplied from the fetal circulation, as the major source physiologically (Ousey *et al*. 2003). Substrate infusion studies conducted *in vivo* are complicated by multiple tissue compartments and competing enzymes, most notably 3β-HSD, where pregnenolone is concerned in studies using placenta (Chavatte *et al*. 1995). Even in the simplest of cell systems, compartmental expression of enzymes can markedly alter the secreted steroid products in unexpected counterintuitive ways (Conley *et al*. 2011). Directly testing this hypothesis in other less complex experiments is difficult for many reasons, not least of which is the lack of any purified preparation of mammalian 5α-reductase. However, the data reported here demonstrate that equine epididymis expresses abundant 5α-reductase activity in the absence of any detectable 3β-HSD; neither progesterone nor androstenedione was detected as products in reactions in which pregnenolone and DHEA, respectively, were provided as substrates. Despite robust rates of 5α-reduction of progesterone and testosterone, no evidence was found that pregnenolone was metabolized to 3β-DHP (5α-pregnan-3β-ol-20-one), the expected product if it was directly reduced by the equine 5α-reductases. Moreover, 3β-DHP was a detected product in reactions using progesterone as substrate, and was inhibited in the presence of finasteride. This suggests that 3β-DHP is detectable as an indirect product of DHP metabolism by 3α-reduction, and is consistent with the presence of 3α-oxidoreductase activity in the epididymis (Robaire *et al*. 1977). It seems highly unlikely, therefore, that direct synthesis of 3β-DHP from pregnenolone (Fig. 1) would be missed if it were synthesized, given abundant 5α-reductase activity and the apparent absence of competing metabolic enzymes. The authors are unaware of any data supporting the speculation that pregnenolone is a substrate for any mammalian 5α-reductases.

In conclusion, the postpubertal equine epididymis expresses active 5α-reductase enzyme activity fairly uniformly from caput to cauda, consistent with reported androgen receptor expression profiles. This activity is sensitive to both finasteride and dutasteride, which may prove to be useful in studies investigating the significance of 5α-reduced pregnanes in equine reproduction. Finally, despite earlier speculation that pregnenolone may be a substrate for direct reduction by 5α-reductase, the results presented here argue strongly that it is not.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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