THEMATIC RESEARCH

SULFATION PATHWAYS

Expression of *SULT2A1*, *SULT2B1* and *HSD3B1* in the porcine testis and epididymis

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Abstract

In the porcine testis, in addition to estrogen sulfates, the formation of numerous sulfonated neutral hydroxysteroids has been observed. However, their functions and the underlying synthetic pathways are still widely unclear. To obtain further information on their formation in postpubertal boars, the expression of sulfotransferases considered relevant for neutral hydroxysteroids (*SULT2A1*, *SULT2B1*) was investigated in the testis and defined segments of the epididymis applying real-time RT-qPCR, Western blot and immunohistochemistry (IHC). Sulfotransferase activities were assessed in tissue homogenates or cytosolic preparations applying dehydroepiandrosterone and pregnenolone as substrates. A high *SULT2A1* expression was confirmed in the testis and localized in Leydig cells by IHC. In the epididymis, *SULT2A1* expression was virtually confined to the body. *SULT2B1* expression was absent or low in the testis but increased significantly along the epididymis. Immunohistochemical observations indicate that both enzymes are secreted into the ductal lumen via an apocrine mechanism. The results from the characterization of expression patterns and activity measurements suggest that *SULT2A1* is the prevailing enzyme for the sulfonation of hydroxysteroids in the testis, whereas *SULT2B1* may catalyze the formation of sterol sulfates in the epididymis. In order to obtain information on the overall steroidogenic capacity of the porcine epididymis, the expression of important steroidogenic enzymes (*CYP11A1*, *CYP17A1*, *CYP19*, *HSD3B1*, *HSD17B3*, *SRD5A2*) was monitored in the defined epididymal segments applying real-time RT-qPCR. Surprisingly, in addition to a high expression of *SRD5A2* in the epididymal head, a substantial expression of *HSD3B1* was detected, which increased along the organ.

Key Words

- ▶ pig
- ▶ testis
- ▶ epididymis
- ▶ *SULT2A1*
- ▶ *SULT2B1*
- ▶ *HSD3B1*

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Introduction

Historically, sulfonation of steroids has been mainly regarded as an important pathway to limit their effects and to facilitate elimination, rendering to steroid sulfates a role of inactive metabolic end products. However, more recently, the concept of sulfonated steroids has become

much more complex, and their role as substrates for the local intra-tissue production of bioactive steroids and functions distinct from unconjugated counterparts have attracted increasing attention (for review see Dawson 2012, Mueller et al. 2015). An intriguing feature of boars among

the males of domestic mammalian animal species is their unusually broad spectrum of sulfonated steroids, which partly circulate at remarkably high concentrations (Baulieu *et al.* 1967, Claus & Hoffmann 1980, Schwarzenberger *et al.* 1993, Raeside *et al.* 2006, Schuler *et al.* 2014, 2018a). For example, in our previous study (Schuler *et al.* 2018a), highest concentrations found in the systemic circulation for sulfonated epiandrosterone, 5-androstene-3 β ,17 β -diol, epitestosterone, dehydroepiandrosterone and estrone were 236, 131, 78, 63 and 43 ng/mL, respectively. However, concentrations varied significantly between individual animals and exhibited considerable diurnal fluctuations. For the majority of sulfonated steroids, data from measurements in testicular vein (Baulieu *et al.* 1967, Gower *et al.* 1970, Raeside & Howells 1971, Setchell *et al.* 1983, Raeside *et al.* 2006, Schuler *et al.* 2014, 2018a) or in lymph fluid of the spermatic cord (Setchell *et al.* 1983) clearly point to the testicular-epididymal compartment as their predominant source. Many of the sulfonated steroids occurring in boars have been measured at high concentrations in testicular tissue (Ruokonen & Vihko 1974). This observation provides clear evidence for the testis as the predominant site where sulfonation takes place and observations from *in vitro* experiments using purified Leydig cells indicate that they are the main producers of sulfonated estrogens and several sulfonated C19-steroids (Raeside & Renaud 1983, Orava *et al.* 1985, Raeside *et al.* 1992, Sinclair *et al.* 2005). The biological role of steroid sulfonation in the porcine testis is widely unclear, and it is still elusive why the porcine testis inactivates bioactive steroids (estrogens, steroidal pheromones) immediately after their production in Leydig cells and why it removes important intermediates of steroidogenesis such as pregnenolone (P5), dehydroepiandrosterone (DHEA) or 5-androstene-3 β ,17 β -diol from the pool of unconjugated steroids.

Sulfonated steroids arise from the activities of several members of the large supergene family of cytosolic sulfotransferases (SULTs), which catalyze the sulfonation of small endogenous molecules and xenobiotics. In humans, SULTs 2A1 and 2B1 have been identified as the relevant sulfotransferases for neutral hydroxysteroids. SULT2A1 exhibits a broad substrate spectrum. In addition to its prototypical substrate DHEA and other 3 β -hydroxysteroids, it is also capable of sulfonating 3 α -hydroxysteroids, estrogens and xenobiotics. For human SULT2B1, P5 (isoform 2B1a) and cholesterol (isoform 2B1b) are regarded as preferential steroidal substrates (Falany *et al.* 1995, Strott 2002, Gamage *et al.* 2006, Dawson 2012, Pasqualini 2009, Falany & Rohn-Glowacki 2013, Mueller *et al.* 2015). Information on the substrate specificities and organization

of SULTs 2A1 and 2B1 in other species is still sparse, but observations made so far point to considerable species differences (Kohjitani *et al.* 2006, Falany & Rohn-Glowacki 2013, Mueller *et al.* 2015). Despite some information on steroid sulfonation in the porcine testis from studies on enzyme activities in testicular tissue (Cooke *et al.* 1983, Hobkirk *et al.* 1989, Sinclair *et al.* 2006) and the formation of sulfonated steroids in Leydig cell culture (Raeside & Renaud 1983, Orava *et al.* 1985, Raeside *et al.* 1992, Sinclair *et al.* 2005), many questions are still open concerning the involvement of individual SULTs during steroid sulfonation in the porcine testis. Moreover, in previous studies a considerable estrogen sulfotransferase activity was detected in the porcine epididymis (Hoffmann *et al.* 2010, Zdunczyk *et al.* 2012, Schuler *et al.* 2018b), pointing to this organ as another relevant site of steroid sulfonation in boars. Thus, in order to obtain new insights into the sulfonation of neutral hydroxysteroids in the porcine testicular-epididymal compartment, the major aims of this study were to characterize the expression of SULTs 2A1 and 2B1 in the porcine testis and defined segments of the epididymis and to assess the sulfonation of DHEA and P5 in corresponding tissue samples. Moreover, to obtain a more holistic view on steroid production and metabolism in the porcine testicular-epididymal compartment, the defined segments of the epididymis were also screened for the expression of steroidogenic enzymes catalyzing important conversions of free steroids.

Materials and methods

Collection and preparation of tissue samples

All animal experiments were in accordance with the relevant regulations (permit No. V54-19c-20-15(I) Gi 18/14-No. 32/2010; Regierungspraesidium Giessen). Tissue samples were collected from a total number of ten postpubertal crossbreed boars (nine German Landrace $\times$ Pietrain, one Large White) aged between 10 and 16 months. The testes with epididymis and spermatic cord were removed under general anesthesia (2 mg/kg BW azaperone, Stresnil – Janssen Animal Health, Neuss and 20 mg/kg BW ketamine, Ursotamin 100 mg/kg – Serumwerk Bernburg AG, Bernburg) and transported on ice to the laboratory within 5 min. For Western blot (WB) analysis and for measurements of relative gene expression (RGE) and enzyme activities, tissue samples were prepared from the following localizations (Fig. 1): upper and lower half of the testis (TE1, TE2), proximal and distal part of epididymal head (EH1, EH2), four

Figure 1

Localizations of tissue sampling. DD, deferent duct; EB1-4, segments of epididymal body, from proximal to distal; EH1, EH2, proximal/distal segment of epididymal head; ET1, ET2, proximal/distal segment of epididymal tail; TE1, TE2: testicular parenchyma in the upper/lower half of testis.

segments of the epididymal body (EB1-4, from proximal to distal), proximal and distal part of epididymal tail (ET1, ET2) and the deferent duct (DD); they were stored at -80°C until further analysis. Correspondingly, for immunohistochemistry (IHC) tissue samples collected from the same localizations were fixed for 20–24 h in 10% neutral phosphate-buffered formalin.

Measurement of relative target gene mRNA levels by real-time RT-qPCR

Pieces of frozen tissue samples (-80°C) were pulverized in a pre-chilled mortar with a pestle under liquid

nitrogen. Total RNA was isolated from 100 mg of powdered tissue using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. After DNase treatment (Roche Diagnostics GmbH), a total of 30 ng RNA were reverse transcribed with GeneAmp RNA PCR Kit (Perkin Elmer) using random hexamers. Real-time qPCR was performed in a CFX96 Real-time Thermal Cycler (Biorad) applying the CYBR green method. The primer pairs applied are specified in Table 1. PCR was performed in a volume of 20 μL using 10 μL SYBR Green, 300 nM of each primer and 1 μL cDNA. The cycling conditions for amplification included an initial denaturation for 10 min at 95°C , followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. All samples were run in duplicates. In order to validate the qPCR methods, standard curves were generated by serial dilutions of the cDNA, and their slopes were used to calculate the efficiencies of the PCR reactions, which were between 88.2% and 106.8% for the target genes and the two housekeeping genes hypoxanthine-guanine phosphoribosyltransferase (HPRT1) and TATA-binding protein (TBP). RGE values were calculated using the comparative CT method ($\Delta\Delta\text{CT}$ method) and reported as n-fold differences in comparison to the sample with the lowest amount of the respective target gene transcripts (calibrator) after correction for the efficiency of the PCR reaction and normalizing the samples referring to the two housekeeping genes. For further validation of the qPCR methods, after completion of the amplification a melting curve of the PCR product was generated to test for the occurrence of nonspecific products and primer dimers. Moreover, correct size of PCR products was confirmed by agarose gel electrophoresis.

Depending on the target gene investigated, in part, RGE levels varied extensively between individual animals (Supplementary Table 1, see section on supplementary data given at the end of this article). Thus, in order to demonstrate tissue distribution patterns more recognizably, for each animal and target gene the highest RGE value measured was set to 1, and the expression levels measured in the remaining tissues of an individual animal are presented relative to the tissue sample exhibiting maximal expression (RGE levels scaled to 1). Data from this type of evaluation are inherently limited by the borders 0 and 1. To avoid error bars exceeding 1 or falling below 0 when combining the data from individual animals, data were transformed by the arc sine function and presented as re-transformed values of mean $\pm$ s.d. of the arc sine transformed values (modified mean $\pm$ s.d. range).

Table 1 List of primers used in real-time RT-qPCR.

Primer	Sequence (5'→3')		Product length (bp)	Reference or sequence information
	Forward	Reverse		
CYP11A1	CATCAGGGAAGACCTGTTTCG	GGCGCTCTCCAAATATGACATT	65	Favreau et al. (2009)
CYP17	GCTCTTGGTTTCTTCTTGC	ATTCTTGCCCTTCTTGAG	269	M63507.1
CYP19*	GATTCCATCACCAAGCACCT	GATCCCCAGGAAGAGGTTGT	123	NM_214430.1
HSD3B1	ATCCACACCAGCAGCATAG	CCAGCACAGCCTTCTCAG	142	AF232699.2
HSD17B3	AGTTTCGCCAAGGTCTTTC	TCCCTATCGTCCACTCAATC	182	NM_001244790.1
SRD5A2	GCCTTTCCAGTTGTATTCC	ACCACTCAGCAGGGTATTC	107	AF008440.2
SULT2A1	ACCTCCCCATCCAACCTTTC	CTTCTCGTGTCCGTTTCAG	283	NM_001037150.1
SULT2B1	TTGTCAGGCGAATACTTCC	CTTGGGGTAGGTGATGATG	129	NM_001243697.1
HPRT1	GCATTCTGAACCTTTATTGCA	TTCACGAGTCAAACAACATCCA	80	NM_001032376.2
TBP	TGGTACATTAATGGTGGTGTGTG	CCTAAACCGAACGTCATCCT	65	DQ845178.1

*Designed to amplify all three porcine CYP19 isoforms (Conley et al. 2009).

Preparation of cytosolic fractions and homogenates

For WB analysis and measurements of enzyme activities cytosolic fractions were prepared. Pieces of frozen tissue with a total weight of 0.2 g were placed with 1 g ceramic beads (Lysing Matrix D, MP-Biomedicals, Eschwege, Germany) and 1 mL homogenization medium (0.1 M potassium phosphate buffer pH 7.4 including 5 mM β -mercaptoethanol and 20% (v/v) glycerol) in 2 mL Micro Tubes (Sarstedt AG & Co, Nuembrecht, Germany) and shredded by 2×20 s bursts using a Fast Prep-24 homogenizer (MP-Biomedicals, Eschwege, Germany). The resulting suspension was centrifuged for 10 min at 15000g and 4°C to remove remaining particles of connective tissue, cellular debris, nuclei and the beads. The supernatants were centrifuged at 100,000g for 1 h at 4°C (L-70 Optima Ultracentrifuge, Beckman Coulter GmbH, Krefeld, Germany). The supernatant (cytosolic fraction) was decanted and protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Fischer Scientific GmbH). Finally, the cytosols were stored at –20°C until further analysis.

As an alternative to cytosols, sulfotransferase activities were also determined in tissue homogenates. Homogenization of tissue samples was performed as described earlier for the preparation of cytosols. However, Ringer solution/50 mmol/L HEPES pH 7.0 was used as a homogenization medium. The resulting crude homogenates were centrifuged at 4°C for 35 min at 20,000g (Biofuge 28 RS, Heraeus, Osterode, Germany) and the supernatants were stored at –20°C until further use.

Detection of SULT2A1 and SULT2B1 in cytosolic fractions using Western blot

Cytosolic samples were separated on a 10% polyacrylamide gel under reducing conditions and subsequently

electroblotted to nitrocellulose membranes (Optitran BA-S85, Schleicher & Schüll, Dassel, Germany). For blocking, membranes were incubated overnight in PBS-T (PBS with 0.05% Tween-20) with 5% nonfat dry milk. They were then washed in PBS-T and incubated for 60 min with the respective primary antibody (SULT2A1: rabbit polyclonal antibody against a synthetic peptide corresponding to amino acids 253–285 of human SULT2A1, PA5-12243, Fisher Scientific, Schwerte, Germany, dilution: 1:500; SULT2B1: rabbit polyclonal antibody generated against a fragment corresponding to a region within amino acids 20–387 of human SULT2B1; GTX114812, GeneTex, Irvine, CA, USA, dilution 1:2000; alignments of human and porcine amino acid sequences for SULT2A1 and SULT2B1 are presented in Supplementary Figs. 1 and 2, respectively). After washing in PBS-T, they were incubated for 30 min with the horseradish peroxidase-linked secondary goat-anti-rabbit immunoglobulin antibody (Dianova, Hamburg, Germany) at a dilution of 1:50,000 in PBS-T. For visualization of the immunoreaction, a chemiluminescence-based method was applied (AceGlow Ultrasensitive Chemiluminescence Substrate, detection of immunoreaction and documentation of results with Fusion Solo VilberLourmat, both from PEQLAB Biotechnologie GmbH, Erlangen, Germany).

Determination of DHEA sulfotransferase (DHEA-ST) and pregnenolone sulfotransferase (P5-ST) activities in cytosolic fractions and tissue homogenates

Measurement of DHEA-ST and P5-ST activities were performed in sample sets prepared from three boars. Cytosolic fractions (50 μ L) or tissue homogenates (50 μ L), 250 μ mol/L 3'-phosphoadenosine-5'-phosphosulfate (PAPS, Santa Cruz Biotechnology), 7.5 mmol/L $MgCl_2$, 16 μ mol/L of the steroid sulfatase inhibitor STX64

(Sigma-Aldrich GmbH and 0.8 $\mu\text{mol/L}$ of the respective substrate were incubated at 37°C for 20 min in a total volume of 200 μL applying Ringer solution/50 mmol/L HEPES buffer pH 7.0 as an incubation medium. In order to facilitate the determination of substrate conversion and product formation, in addition to the unlabeled substrate, the incubations included a small amount of the corresponding tritiated substrate (7.5 nmol/L 6,7- ^3H (N)-DHEA, Hartmann Analytic GmbH, Braunschweig, Germany or 15 nmol/L 7- ^3H (N)-P5, PerkinElmer). In order to assess substrate conversions unrelated to sulfotransferases, for each sample, parallel incubations were performed in which PAPS was replaced by incubation buffer. Following incubation, the reaction was stopped by addition of 50 μL acetonitrile (ACN) and placing the samples on ice. After centrifugation (20,000 g , 10 min, 4°C), a 50 μL aliquot of the supernatant was separated on a reversed phase column (150 $\times$ 4 mm Eurospher II 100-5 C18 column) in an HPLC system (Smartline Manager 5050 and Pump 150, all HPLC equipment from Knauer, Berlin, Germany) with an ACN/water gradient (30–70% during 14 min) and a flow rate of 0.5 mL/min. The eluate was collected in 0.5 mL fractions, in which ^3H -activities were measured (Tri-Carb 2810TR, PerkinElmer, Rodgau, Germany) after addition of liquid scintillation fluid (Aquasafe 300 plus, Zinnser Analytic, Frankfurt, Germany). Identification of substrate and metabolites was based on a comparison of the retention times with authentic tritiated standards (Fig. 2A and B for exemplary chromatograms). Conversion of substrate was calculated from the distribution of ^3H -activity over the peaks of the unconjugated and sulfonated steroid after subtraction of the technical background. Due to the specific features of the method applied for the assessment of enzyme activities, low substrate concentrations relative to enzyme activity could result in virtually complete substrate depletion, whereas high concentrations could lead to product peaks disappearing in the technical background. Thus, the substrate concentration applied was established in preliminary experiments to ensure a sufficient differentiation between the enzyme activities present in the different tissue samples (Fig. 2C).

Immunolocalization of SULT2A1 and SULT2B1

After 20–24 h of fixation in 10% phosphate-buffered formalin and subsequent washing in 0.1 M phosphate buffer pH 7.2 for removal of the fixative, the tissue samples were dehydrated in an ascending ethanol series and embedded in paraffin. Sections (5 μm) were prepared and heated in 10 mM citrate buffer pH 6.0 for epitope retrieval

Figure 2

Determination of substrate sulfonation. (A) Exemplary chromatograms obtained after incubation (20 min) of 50 μL testicular homogenate (boar #10) with 0.8 $\mu\text{mol/L}$ DHEA as a substrate in the presence (+) or absence (–) of the sulfotransferase cosubstrate PAPS (250 $\mu\text{mol/L}$). (B) Exemplary chromatograms obtained after incubation (20 min) of 50 μL epididymal cytosol (sample TL1, boar #8) with 0.8 $\mu\text{mol/L}$ pregnenolone (P5) in the presence (+) or absence (–) of PAPS (250 $\mu\text{mol/L}$). Arrows indicate the retention time of authentic tritiated standards (C) Results from a preliminary experiment conducted to establish a suitable substrate concentration for the determination of hydroxysteroid sulfotransferase activities. Testicular homogenates from four boars were incubated for 20 min in the presence of PAPS (250 $\mu\text{mol/L}$) with a constant amount of ^3H -DHEA (7.5 nmol/L) and increasing amounts of unlabeled DHEA (0, 50, 200, 800 nmol/L). Based on the distribution of ^3H -activity in HPLC chromatograms over the peaks for the substrate and the sulfonated product (see table below the diagram) the reaction rate was calculated.

at temperatures between 98 and 100°C for 20 min. Indirect immunoperoxidase staining method were employed using the streptavidin-biotin technique for signal enhancement following standard procedures. Primary antisera were the same as specified for WB. Negative controls were set up replacing the specific antiserum with purified non-immunized rabbit IgG (Life Technologies). Blocking serum, biotinylated secondary antibody and avidin-biotin complex were taken from the Vectastain Elite ABC kit 6101 and used following the manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA). NovaRed substrate kit (Vector Laboratories) was applied to visualize the immunoreaction. To further corroborate the

specificity of immunohistochemical method for porcine SULT2B1, a preadsorption test was performed in which the primary antibody working solution was incubated with or without addition of human recombinant SULT2B1b (concentration: 8 µg/mL; GenWay Biotech catalog no. GWB-BSP311; distributed by BIOZOL Diagnostica, Eching, Germany) for 24 h before application to tissue sections prepared from testis and epididymal tail. Additionally, as a positive control, tissue porcine small intestine (ileum) has been used, which has been shown to highly express *SULT2B1* in mice and rats (Shimizu *et al.* 2003, Alnouti & Klaassen 2006, Kohjitani *et al.* 2006).

Results

Expression of *SULT2A1*

For *SULT2A1*-mRNA, the testis was the predominant site of expression (Fig. 3A; Supplementary Table 1). In two of five animals investigated, considerable expression levels were also measured in the middle part of the epididymal body. In the remaining three animals, expression in the epididymal body was substantially lower but still slightly higher compared to the epididymal head and tail. Consistently, when screening the testicular-epididymal compartment from three boars for the expression of the protein by WB applying cytosol specific bands exhibiting the expected molecular weight of 34 kDa were detected in the testis and epididymal samples EB2 and/or EB3 (Fig. 3B; see also Supplementary Fig. 3A). Sample B4 was omitted in these experiments to include liver as a positive control, in which a specific band of high intensity occurred. In an additional experiment, testicular samples from five boars were investigated in one analytical process also including the liver sample (Fig. 3C). Again, in the liver, a band of considerable intensity was found, whereas intensity in testicular samples was lower and exhibited a substantial variability between individual animals. By means of IHC, in porcine liver used as a positive control tissue, a distinct to intense cytoplasmic staining occurred in virtually all hepatocytes, whereas no considerable staining was observed in other cell types (Fig. 4A). In negative controls (Fig. 4B), no noteworthy staining was found. Complete sample sets from three boars were immunostained and visually evaluated. In the testis (Fig. 4C), a weak-to-distinct cytoplasmic staining occurred in practically all Leydig cells. Moreover, in a variable proportion of Leydig cells in addition to cytosolic staining, a weak to distinct nuclear staining was visible, which however was considered nonspecific (see Discussion section). Negative

Figure 3

Expression of *SULT2A1* in the porcine testicular-epididymal compartment. (A) *SULT2A1*-mRNA expression based on RGE levels measured in five sexually mature boars by semi-quantitative real-time RT-qPCR (Supplementary Table 1). For each animal, the expression level in the tissue sample with the highest RGE value was set to 1 and expression levels of the remaining samples were calculated relative to this sample. The data shown are presented as re-transformed $\bar{x} \pm s.d.$ calculated from arc sine transformed data (modified $\bar{x} \pm s.d.$ range). (B) WB analysis of *SULT2A1* expression in three individual boars. Sample EB4 was omitted to include porcine liver (L) as a positive control. In each lane, 30 µg cytosolic protein was loaded. (C) Comparison of *SULT2A1* expression between testicular tissue from five boars (TE2/1-TE2/5) and liver (L). In each lane, 20 µg cytosolic protein was loaded. Tissue samples: TE1,-2: upper/lower half of testis; EH1,-2: proximal and distal part of the epididymal head; EB1-4: segments of the epididymal body, from proximal to distal; ET1,-2: proximal and distal part of the epididymal tail; DD: deferent duct.

controls were devoid of noteworthy staining (Fig. 4D). Also in the epididymis (Fig. 4E and F), a weak-to-moderate nuclear staining considered nonspecific was found in the ductal epithelium throughout the organ with decreasing intensity from head to tail. Specific cytoplasmic staining

Figure 4

Immunolocalization of SULT2A1 in the porcine testis and epididymis. (A) Immunostaining in porcine liver used as a positive control. (B) Negative control (porcine liver). (C) Immunostaining in testis. (D) Negative control (testis). (E) Immunostaining in epididymal body (segment EB2). (F) Immunostaining in the distal epididymal body (segment EB4) with moderate nuclear staining of questionable specificity. (G) Negative control (epididymal body, segment EB3). dl, ductal lumen; e, epithelium; it, interstitial tissue; m, muscular layer; st, seminiferous tubules; sz, conglomerates of spermatozoa.

in the ductal epithelium was only weak or absent with the exception of the epididymal body (segments EB1-EB3), where a more pronounced staining occurred in clusters of epithelial cells (Fig. 4E). In addition to the ductal epithelium, immunostaining was observed in spherical particles situated in the ductal lumen and in vascular endothelial cells of the epididymal connective tissue (not shown). Negative controls prepared from epididymal tissue samples were devoid of noticeable staining (Fig. 4G).

Expression of *SULT2B1*

SULT2B1-mRNA expression was only minimal in the testis, increased slightly along the initial and middle parts of the epididymis and was considerably higher in the hind part of the epididymal body and in the epididymal tail (Fig. 5A; Supplementary Table 1). In all animals

Figure 5

Expression of SULT2B1 in the porcine testicular-epididymal compartment. (A) *SULT2B1*-mRNA expression based on the relative gene expression levels measured in five sexually mature boars by semi-quantitative real-time RT-qPCR (Supplementary Table 1). For each animal, the expression level in the tissue sample with the highest RGE value was set to 1 and expression levels of the remaining samples are presented relative to this sample. The data shown are presented as re-transformed $\bar{x} \pm s.d.$ calculated from arc sine transformed data (modified $\bar{x} \pm s.d.$ range). (B) WB analysis of SULT2B1 expression in three individual boars. In each lane, 30 μ g cytosolic protein was loaded. Tissue samples: TE1,-2: upper/lower half of testis; EH1,-2: proximal and distal part of the epididymal head; EB1-4: segments of the epididymal body, from proximal to distal; ET1,-2: proximal and distal part of the epididymal tail; DD: deferent duct.

investigated, *SULT2B1*-mRNA expression was highest in the distal part of the epididymal tail. Concordant with results from real-time RT-qPCR, in WB applying a primary antiserum against the human enzyme (Fig. 5B; see also Supplementary Fig. 3B) specific bands of variable intensity corresponding to the expected molecular weight of 38.4 kDa were consistently found in samples of the distal epididymal body and in the epididymal tail. In other tissue samples, they were only of weak intensity or absent. In IHC control experiments, a distinct staining was found in epithelial cells of porcine ileum (Fig. 6A) used as a positive control tissue and in ductal epithelial cells of the epididymal tail (Fig. 6B), which almost completely disappeared after preadsorption of the primary antiserum with recombinant human full-length SULT2B1b (Fig. 6C and D). No noteworthy staining was observed in negative

Figure 6

Immunolocalization of SULT2B1. (A, B, C and D) Results from a control experiment. Distinct staining in (A) epithelial cells of ileum (positive control tissue) and (B) in epididymal duct epithelium (sample ET1). (C and D) The same localization as shown in Fig. (A) and (B) when the primary antiserum was preadsorbed with recombinant human SULT2B1. (E) Porcine epididymis (segment ET1); distinct immunostaining of the high ductal epithelium including apical protrusions (arrowheads) and of spherical material situated in the ductal lumen (arrows) between conglomerated spermatozoa. Moderate immunostaining is also present in the muscular layer. dl, ductal lumen; e, epithelium; m, muscular layer; sz, conglomerates of spermatozoa.

controls in which the primary antiserum was replaced by nonspecific rabbit immunoglobulin (not shown). Immunolocalization of SULT2B1 in sample sets from four boars yielded a substantial variability in the overall staining intensity between individual animals. However, the staining pattern was consistent in that staining intensity in the cytoplasm of epididymal epithelial cells increased significantly from virtually negative to moderate in the epididymal head to distinct or intense in the tail (Fig. 6B and E). Frequently but not in all localizations superficial protrusions of epithelial cells exhibited

moderate-to-intense staining, and immunostained spherical particles were found in the epididymal lumen (Fig. 6E). Moreover, a weak to moderate staining was partly observed in muscular cells of the epididymal tail (Fig. 6E). Different from the ileum epithelium where staining was exclusively cytosolic (Fig. 6A), in the epididymal duct epithelium in addition to cytoplasmic staining in part also nuclear signals were observed (Fig. 6B and E). In testicular samples from all four animals investigated nuclear staining was present in the vast majority of germ cells with the exception of elongating spermatids and sperm cells (not shown), which was only weak in one animal but distinct to intense in the remaining three boars. However, nuclear staining in the testis and epididymis was considered as an artifact (see Discussion section). In liver, no reliable staining occurred (not shown).

Activities of DHEA-ST and P5-ST

In preliminary experiments, sulfonation of DHEA in testicular cytosols from five boars was, if at all, only minimal ($3.4 \pm 2.0\%$), and no conversion exceeding background levels was found in epididymal samples (not shown). Moreover, in testicular cytosols, no considerable sulfonation of DHEA was observed even after supplementation of the incubation medium with the 3β -hydroxysteroid dehydrogenase (3β -HSD) inhibitor trilostane (data not shown). When measuring DHEA-ST activity in tissue homogenates (Fig. 7A), a variable but significant sulfonation of DHEA was detected in testicular samples (see also Fig. 2A and C). In epididymal samples, DHEA-ST activities were low in the head and in the proximal part of the body. In the distal body and tail, activities were in a similar range as observed in testicular samples, again with a considerable variability between individual animals and also between different segments of the organ.

When measuring P5-ST activities in cytosols (Fig. 7B), conversion rates of the substrate was only marginal in testis, epididymal head and the initial segment of the epididymal body. They increased in the subsequent segments of the epididymal body and were maximal in the epididymal tail. When measuring the sulfonation of P5 in homogenates prepared from the testis and selected segments (EH1, ET1) of the epididymis (Fig. 7C), as in cytosols sulfonation activities were high in epididymal tail and only marginal in epididymal head. However, different from the results obtained with cytosols, in testicular samples, a substantial sulfonation of P5 was observed.

Figure 7

Assessment of DHEA and pregnenolone sulfotransferase activities in the porcine testis and epididymis. (A) Sulfonation of DHEA by homogenates (50 μ L) prepared from testis and epididymal segments of three individual boars (#4, #10, #12). (B) Sulfonation of pregnenolone by cytosols (50 μ L) prepared from testis and epididymal segments of three individual boars (#4, #8, #9). (C) Sulfonation of pregnenolone by homogenates (50 μ L) prepared from selected tissue samples of three individual boars (#4, #10, #12). In all experiments, initial substrate concentration was 0.8 μ mol/L and the time of incubation was 20 min. Hatched bars indicate substrate conversion in the absence of the sulfate donor PAPs. Tissue samples: TE2: testis (lower half); EH1,-2: proximal and distal part of the epididymal head; EB1-4: segments of the epididymal body, from proximal to distal; ET1,-2: proximal and distal part of the epididymal tail.

Relative expression levels of mRNA encoding important steroidogenic enzymes

RGE levels scaled to 1 are presented in Fig. 8, whereas information on original RGE levels is included in Supplementary Table 1. For *CYP11A1* (side-chain cleavage enzyme), *CYP17A1* (17 α -hydroxylase-C17,20-lyase), *CYP19* (aromatase; all three porcine isoforms) and *HSD17B3* (17 β -hydroxysteroid dehydrogenase isoform 3) from a

Figure 8

Expression levels of mRNA encoding important steroidogenic enzymes in the porcine testis, different segments of the epididymis and in the DD. The data shown are based on the relative gene expression (RGE) levels measured in 5–7 sexually mature boars by semi-quantitative real-time RT-qPCR (Supplementary Table 1). For each animal, the expression level in the tissue sample with the highest RGE value was set to 1 and expression levels of the remaining samples are presented relative to this sample. The data shown are presented as re-transformed $\bar{x} \pm$ s.d. calculated from arc sine transformed data (modified mean $\pm$ s.d. range). Tissue samples: TE1,-2: upper/lower half of testis; EH1,-2: proximal and distal part of the epididymal head; EB1-4: segments of the epididymal body, from proximal to distal; ET1,-2: proximal and distal part of the epididymal tail; DD: deferent duct.

quantitative point of view, the testis was clearly identified as the only significant tissue of expression. Expression levels of *HSD3B1* (3 β -hydroxysteroid dehydrogenase- Δ 5/4-isomerase) were relatively low in the testis, increased continuously along the epididymis and were maximal in the tail. RGE levels exhibited a considerable variability between individual animals as indicated by high deviation factors (Supplementary Table 1). *SDR5A2* (5 α -reductase isoform 2) was specifically expressed in the head and the initial segment of the epididymal body.

Discussion

SULTs 2A1 and 2B1 have been identified in humans (Falany et al. 1995, Strott 2002, Gamage et al. 2006, Dawson 2012, Pasqualini 2009, Mueller et al. 2015) and laboratory rodents (Shimizu et al. 2003, Kohjitani et al. 2006) as the relevant sulfotransferases for neutral

hydroxysteroids. Some studies have been conducted concerning the expression of these SULTs in the porcine testis and liver focusing on a putative role as genetic markers for selecting animals with low boar taint, however, with partly inconsistent results (Sinclair *et al.* 2006, Moe *et al.* 2007a,b, 2008, Grindflek *et al.* 2010, Rasmussen *et al.* 2012). So far, no information was available at all on the expression of *SULT2A1* and *SULT2B1* in the porcine epididymis and hydroxysteroid sulfotransferase activities in this organ, and no observations have been reported from immunohistochemistry for the porcine testis and epididymis with respect to the two enzymes.

Expression of *SULT2A1*

When screening the porcine testis for the expression of *SULT2A1*, consistent with earlier studies (Sinclair *et al.* 2006), a considerable expression was found on the mRNA level by real-time RT-qPCR and on the protein level by WB. With IHC in the testis, immunostaining was restricted to Leydig cells. However, different from hepatocytes where immunostaining was exclusively cytoplasmic, in a significant proportion of Leydig cells in addition to cytoplasmic staining nuclear signals of variable intensity occurred. As SULTs are generally considered as cytoplasmic enzymes, the authenticity of nuclear signals must be questioned although in humans, a nuclear localization has been described for *SULT2B1* in certain cell types (see Discussion below concerning *SULT2B1*). However, so far, no nuclear localization has been reported for *SULT2A1*. In the epididymis, with WB *SULT2A1* expression was exclusively detected in the mid-region of the epididymal body, which corresponded to higher RGE expression levels obtained with real-time RT-qPCR in comparison to other epididymal segments. Consistently, with IHC, a moderate-to-distinct cytoplasmic staining was observed specifically in the epididymal body, where the presence of immunostained spherical particles in the ductal lumen suggests that the enzyme was secreted into luminal compartment (see also Discussion below for *SULT2B1*). The role of *SULT2A1* expressed in a relatively restricted part of the epididymal ductal epithelium remains unclear given the broad spectrum of potential steroidal and nonsteroidal substrates. In addition to the cytoplasmic staining observed in the epididymal body, in the epithelium of the epididymal duct, a weak-to-moderate nuclear staining was present throughout the organ. However, as discussed earlier, it was considered nonspecific.

Expression of *SULT2B1*

Expression pattern of *SULT2B1* in the porcine testicular-epididymal compartment was found to be clearly different from *SULT2A1*, which is consistent with the tissue selectivity for the expression of only one of the *SULT2* genes observed in humans (Falany & Rohn-Glowacki 2013). In boars, relative *SULT2B1*-mRNA expression was only minimal in testis and epididymal head, increased gradually in the initial segments of the epididymal body to reach maximum levels in the hind part of the epididymal body and in the tail. Corresponding results were obtained with WB. The fact that the molecular weight of the band detected was slightly below 40kDa provided clear evidence for the specificity of the antiserum applied for porcine *SULT2B1* in WB as this SULT has a clearly higher molecular weight compared to other structurally closely related members of the *SULT* superfamily. In humans, two *SULT2B1* isoforms have been identified on the mRNA level corresponding to 350 (*SULT2B1a*) and 365 amino acids (*SULT2B1b*). However, on the protein level, so far only the expression of *SULT2B1b* has been confirmed (Falany & Rohn-Glowacki 2013). In the pig, to date, only one *SULT2B1* isoform of 338 amino acids has been identified (GenBank accession no. NM_001243697.1; for alignment of amino acid sequences see Supplementary Fig. 2), which is consistent with the molecular weight observed in our WB experiments (Fig. 5B and Supplementary Fig. 3B). When screening the testicular-epididymal compartment for *SULT2B1* expression by IHC, a good correlation was found between cytoplasmic immunostaining and results from WB and real-time RT-qPCR. In the epididymis, occasionally immunostaining was especially prominent in the apical part of epithelial cells and their superficial protrusions consisting of an apical bleb connected to the cell surface with a cytoplasmic stalk. These apical blebs have been observed to detach into the ductal lumen without damage to the cell as a mechanism of apocrine secretion (Dacheux *et al.* 2005, Hughes & Berger 2015). Accordingly, at these localizations, immunostained spherical material with a diameter of up to approximately 5 µm could be found in the ductal lumen (Fig. 6E). Similar observations were made by Frenette *et al.* (2009) when characterizing the expression of *SULT1E1* in the bovine epididymis. Based on their observations, they suggested that *SULT1E1* is transferred to sperm cells via the inclusion into epididymosomes.

When screening the porcine testis for *SULT2B1* expression, depending on the individual animal, a weak-to-intense nuclear immunostaining occurred in the

majority of germ cells (not shown). Moreover, significant nuclear staining also occurred in individual epididymal samples. Human SULT2B1b is the only SULT for which, in selected tissues, a nuclear localization has been described, which has been so far only been reported in human syncytiotrophoblast and in normal and malignant breast tissue. It has been suggested that the nuclear localization of human SULT2B1 in specific tissues is mediated by its unique carboxy terminal tail, which is also involved in post-translational modification, protein interactions and enzyme stability (He *et al.* 2004, Falany *et al.* 2006, He & Falany 2006, Falany & Rohn-Glowacki 2013). However, although human and porcine SULT2B1 share a high overall identity (75%) and similarity (84%), the carboxy terminal tail of porcine SULT2B1 is considerably shorter compared to human SULT2B1 (for comparison of amino acid sequences see [Supplementary Fig. 2](#)). As stated on the datasheet provided by the supplier, the primary antiserum used in this study was generated by immunization against a recombinant protein encompassing a proprietary sequence within the center region of human SULT2B1 and was purified by affinity chromatography. Preadsorption of the antiserum with recombinant human full-length SULT2B1b not only abolished cytosolic staining in porcine ileum and epididymis ([Fig. 6A, B, C and D](#)) but also significantly reduced nuclear staining in germ cells (not shown) and epididymal duct epithelium ([Fig. 6D](#)). This result clearly shows that nuclear staining is related to the primary antibody. However, cross-reactivity with epitopes unrelated to the target molecule, which can be highly specific and limited to one tissue, has been observed even with monoclonal antibodies. Moreover, cross-reacting epitopes may be created during formalin fixation, which can result in diverging results in WB and IHC (Holmseth *et al.* 2012). The fact that partly intense nuclear staining without concomitant cytosolic signals occurred in a high proportion of germ cells in the presence of only marginal testicular *SULT2B1*-mRNA expression clearly challenges the authenticity of nuclear staining observed in the porcine testis and epididymis. However, further studies are needed to definitely clarify this issue.

Our observations of a marginal or absent expression of *SULT2B1* in the porcine liver and testis are clearly conflicting with results by Moe *et al.* (2007a), who applied a polyclonal antiserum against an epitope mapping at the C-terminus of human SULT2B1 to detect the enzyme in WB. When analyzing cytosolic preparations from porcine liver and testis, they found bands considered specific in both organs exhibiting a molecular weight of 45 kDa. However, our results are consistent with observations

in humans, rats and mice, where no quantitatively significant *SULT2B1* expression was detectable in these organs (Her *et al.* 1998, Javitt *et al.* 2001, Meloche & Falany 2001, Shimizu *et al.* 2003, Alnouti & Klaassen 2006, Kohjitani *et al.* 2006). Information on *SULT2B1* expression in epididymis is currently very sparse. Only one paper was found addressing epididymal *SULT2B1* expression demonstrating that in mice, the epididymis is among the organs exhibiting the highest expression levels (Sakakibara *et al.* 1998).

Hydroxysteroid sulfotransferase activities

Information on the activities of human SULTs 2A1 and 2B1 indicate overlapping substrate specificities concerning hydroxysteroids, with a clearly broader specificity of SULT2A1 and a specificity more restricted to 3 β -hydroxy-5-ene steroids of SULT2B1. Commonly DHEA is regarded as a prototypical substrate of SULT2A1, whereas P5 was shown to be an efficient substrate of both human SULT2B1 isoforms (Strott 2002). In order to characterize hydroxysteroid sulfotransferase activities in the testis and epididymis initially cytosolic preparations were incubated applying DHEA and P5 as substrates. Considerable sulfonation of P5 was detected in the hind part of the epididymal body and in the tail reminiscent of the expression pattern found for *SULT2B1* in real-time RT-qPCR and WB.

When starting preliminary experiments on DHEA-ST activities using cytosols, considerable activities were expected in testicular samples based on our results on *SULT2A1* expression from real-time RT-qPCR, WB and IHC and on previous observations by other researchers on DHEA sulfonation in porcine testicular cytosol (Cooke *et al.* 1983, Sinclair *et al.* 2006) or preparations obtained after chromatography of cytosol prepared from purified porcine Leydig cells (Hobkirk *et al.* 1989). However, ³H-activities associated with the DHEAS peak were only slightly above the background in the chromatograms. The reason for the unexpectedly low DHEA-ST activities in our cytosolic samples remains unclear. However, the detection of sulfotransferase activities in tissue samples is not trivial as indicated by the fact that it was the loss of enzyme activity during tissue processing or lysis of cells due to unidentified causes, which has significantly hampered the identification of *SULT2B1* in humans, and also for the rat and mouse SULT2B1 there are no reports of the isolation of enzymatically active tissue preparations (Falany *et al.* 2006, Falany & Rohn-Glowacki 2013). Although similar problems have not been reported on SULT2A1 activity, it

cannot be ruled out that DHEA-ST activity was widely lost due to the lack of factors stabilizing enzyme activity during tissue processing or the presence of molecules interfering with the active conformation of the enzyme, substrate binding or its interaction with the sulfate donor PAPS under the specific conditions applied. A possible reason for the higher activities found in tissue homogenates could be the application of a different buffer during tissue disruption.

When using tissue homogenates, a considerable sulfonation of DHEA occurred in the testis and the distal segments of the epididymis. Moreover, with this assay system in testicular samples, a considerable sulfonation of P5 was observed, which was not the case with testicular cytosols. This observation points to SULT2A1 as the relevant enzyme for the sulfonation of P5 in the porcine testis. Taken together, the observations from the hydroxysteroid sulfotransferase assays suggest that in our assay system including homogenates both enzymes were active sulfonating both substrates, whereas the conditions of the assay system including cytosol only allowed the considerable sulfonation of P5 by SULT2B1. However, this interpretation needs further confirmation as to date the exact substrate specificities of porcine SULT2A1 and 2B1 are virtually unknown and contributions from other SULTs to the conversions observed cannot be excluded. However, the low substrate conversions in the absence of PAPS in control experiments provide clear evidence that the products formed in our activity assays were primarily sulfonated steroids.

The *SULT2B1* expression pattern found in the porcine testis and epididymis is very similar to the distribution of 'steroid sulfotransferase' described in the male hamster reproductive tract, which has been used as a model to study the biochemistry of sterol sulfates found at high concentrations in semen (Bouthillier *et al.* 1984, Roberts 1987). Consistent with our findings in boars, enzyme activity in hamsters, which was measured as sulfonation of DHEA or desmosterol, increased substantially along the epididymis, whereas it was minimal in the testis. Moreover, consistent with our immunohistochemical observations, also in hamster, the sulfotransferase appeared to be secreted into the luminal fluid (Bouthillier *et al.* 1984, Roberts 1987). Sterol sulfates avidly taken up by spermatozoa are considered as potent and specific inhibitors of acrosin providing protection against the premature release of acrosomal proteolytic activity (Roberts 1987, Keber *et al.* 2013). Accordingly, sulfonated cholesterol and desmosterol have been reported as potent inhibitors of porcine acrosin (Burck & Zimmerman 1980).

Our observations indicate that in boars, SULT2B1 could be the enzyme predominantly underlying this epididymal sulfotransferase activity.

Expression of mRNA encoding important steroidogenic enzymes in the epididymis

Investigations into epididymal steroid metabolism so far mainly focused on the conversion of testosterone into 5 α -dihydrotestosterone (DHT) by 5 α -reductase isoenzymes SRD5A1 and -2 (Pujol & Bayard 1978, Mahony *et al.* 1998, Robaire & Henderson 2006). Otherwise, there are only a very few reports in the literature addressing the steroidogenic capacity of the epididymis (Wiszniewska 2001, 2002, Gorowska *et al.* 2014). In porcine epididymis, RGE levels for *CYP11A1*, *CYP17A1*, *CYP19* and *HSD17B3* were only low in comparison to testis. Our results concerning the expression of *SRD5A2* in the epididymis are consistent with observations in the rat of a high expression in the epididymal head but considerably lower expression levels in the subsequent parts of the organ (Normington & Russel 1992, Robaire & Henderson 2006) but are clearly different from the equine epididymis where *SRD5A2* expression was virtually undetectable (Corbin *et al.* 2016). The low *SRD5A2* expression found in the porcine testis is in accordance with earlier observations (Robic *et al.* 2014).

A very intriguing finding was the considerable expression of *HSD3B1*-mRNA in the epididymis, which clearly exceeded the levels measured in testis. Identical results were obtained in preliminary experiments applying a different primer pair (data not shown). To our knowledge, no corresponding observations have been made so far in pigs or any other species. When in a pilot study, 3 β -HSD activity was measured in epididymal microsomes applying P5 as a substrate, a highly variable but significant formation of progesterone was found (data not shown). However, despite the low RGE levels found in testicular samples, enzyme activities in the testis were in a similar range compared to the epididymal tail, suggesting that the ratio between RGE levels and enzyme activity differs significantly between the two organs. Alternatively, different isoenzymes could be active in the testis and epididymis. Interestingly, in the porcine testis, evidence for separate 3 β -hydroxysteroid dehydrogenase- Δ 5/4-isomerases has been reported for the synthesis of androgens and Δ 16-androstenes (Cooke 1996). However, to date, in pigs, only *HSD3B1* and *HSD3B7* have been identified of which the latter is obviously unrelated to testicular steroidogenesis (Shea *et al.* 2007). The relevant

substrates and the role of HSD3B1 highly expressed in the porcine epididymis remain to be elucidated.

Conclusions

This study confirms that in the porcine testis *SULT2A1* is expressed in Leydig cells and shows that they are the only cell type of the organ exhibiting a detectable expression of this multispecific SULT. Observations on *SULT2A1* and *SULT2B1* expression suggest that in the porcine testis, *SULT2A1* is probably the relevant SULT for the synthesis of most, possibly all, sulfonated neutral steroids produced in the organ. The reason why in the porcine Leydig cells, numerous hydroxysteroids are removed from the pool of unconjugated steroids immediately after their synthesis remains to be elucidated. However, sulfonation may be a regulatory mechanism to maintain steroid homeostasis in the course of the substantial diurnal fluctuations of porcine testicular steroidogenesis (Schuler et al. 2018a, 2018b). *SULT2A1* and *SULT2B1* are both expressed in the porcine epididymis, with *SULT2A1* expression focusing on the epididymal body and *SULT2B1* expression on the tail, suggesting different functions. The substrates and the roles of *SULT2A1* and *SULT2B1* in the porcine epididymis are unclear. However, considering sporadic observations on epididymal steroid sulfotransferase activities in other species especially *SULT2B1* could serve the production of sterol sulfates, which have been proposed to act as membrane stabilizers and acrosin inhibitors in sperm cells. A surprising observation during this study was the high expression of *HSD3B1* in the porcine epididymis, which deserves further investigation.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-17-0277>.

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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