THEMATIC RESEARCH

SULFATION PATHWAYS

Formation and hydrolysis of sulfonated estrogens in the porcine testis and epididymis

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Abstract

Boars exhibit high concentrations of sulfonated estrogens (SE) mainly originating from the testicular-epididymal compartment. Intriguingly, in porcine Leydig cells, sulfonation of estrogens is colocalized with aromatase and steroid sulfatase (STS), indicating that *de novo* synthesis of unconjugated estrogens (UE), their sulfonation and hydrolysis of SE occur within the same cell type. So far in boars no plausible concept concerning the role of SE has been put forward. To obtain new information on SE formation and hydrolysis, the porcine testicular-epididymal compartment was screened for the expression of the estrogen-specific sulfotransferase SULT1E1 and STS applying real-time RT-qPCR, Western blot and immunohistochemistry. The epididymal head was identified as the major site of SULT1E1 expression, whereas in the testis, it was virtually undetectable. However, SE tissue concentrations are clearly consistent with the testis as the predominant site of estrogen sulfonation. Results from measurements of estrogen sulfotransferase activity indicate that in the epididymis, SULT1E1 is the relevant enzyme, whereas in the testis, estrogens are sulfonated by a different sulfotransferase with a considerably lower affinity. STS expression and activity was high in the testis (Leydig cells, rete testis epithelium) but also present throughout the epididymis. In the epididymis, SULT1E1 and STS were colocalized in the ductal epithelium, and there was evidence for their apocrine secretion into the ductal lumen. The results suggest that in porcine Leydig cells, SE may be produced as a reservoir to support the levels of bioactive UE via the sulfatase pathway during periods of low activity of the pulsatile testicular steroidogenesis.

Key Words

- ▶ boar
- ▶ testis
- ▶ epididymis
- ▶ SULT1E1
- ▶ steroid sulfatase
- ▶ estrogen

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Introduction

Estrogens are now recognized as important regulators of reproductive functions not only in females but also in males. However, information so far available, e.g. on estrogen concentrations and testicular expression patterns of aromatase and estrogen receptors, point to significant differences between mammalian species concerning the

roles of estrogens for male reproductive functions (for review see [Cooke et al. 2017](#)). The expression of estrogen receptors in porcine germ cells, Sertoli cells and Leydig cells ([Mutembei et al. 2005](#)) indicates that estrogens are important regulators of testicular functions also in boars, and there is evidence for a role in postnatal testicular

development (At-Taras *et al.* 2006, Berger *et al.* 2013) and the control of spermatogenesis (Wagner *et al.* 2006).

With the exception of the horse (Seamans *et al.* 1991, Hoffmann & Landeck 1999), the pig is the only species identified so far exhibiting an abundant production of sulfonated estrogens (SE) in the testicular-epididymal compartment (Claus & Hoffmann 1980, Setchell *et al.* 1983, Hoffmann *et al.* 2010, Schuler *et al.* 2014, 2018). Maximum concentrations measured in our previous studies (Schuler *et al.* 2014, 2018) in the systemic circulation of postpubertal boars were about 50 ng/mL for estrone sulfate (E1S) and 10 ng/mL for 17 β -estradiol-3-sulfate (E2S). Historically sulfonated steroids have been primarily regarded as inactivated metabolites destined for excretion. However, they are now also recognized as a pool of precursors for the local production of active unconjugated steroids in specific steroid-responsive cells characterized by the expression of steroid sulfatase (STS). This sulfatase pathway has been identified as the favored route of estrogen production in human hormone-dependent breast cancer tissue in comparison to their *de novo* synthesis (Santner *et al.* 1984, Reed *et al.* 2005, Mueller *et al.* 2015). However, information on the relevance of sulfatase pathways in physiological settings is only sparse.

Although the occurrence of high levels of conjugated estrogens in boars has been known for decades (Claus & Hoffmann 1980), important questions concerning their synthesis are still open and their function is not yet been elucidated. Data from measurements in testicular venous blood or in lymph fluid of the spermatic cord (Setchell *et al.* 1983, Hoffmann *et al.* 2010, Schuler *et al.* 2014) clearly identified the testicular-epididymal compartment as the predominant source of SE in boars. *In vitro* experiments using purified Leydig cells suggested that they are the main producers of SE in the adult porcine testis (Raeside & Renaud 1983). However, in our previous study, estrogen sulfotransferase (EST) activity in testicular homogenates was, if at all, only marginal in comparison to epididymal samples (Hoffmann *et al.* 2010). This observation challenges the concept of the testis as the only significant source of SE in boars. A mere role of SE produced in Leydig cell as inactivated hormones destined for elimination is implausible, as in the adult porcine testis by immunohistochemistry aromatase is exclusively detectable in Leydig cells (Fraczek *et al.* 2001, Mutembei *et al.* 2005). This observation provides clear evidence that in the porcine testis, *de novo* synthesis of estrogens from cholesterol and their sulfonation are colocalized in the same cell type. However, the control of the output of a steroidogenic cell by product inactivation seems

very inefficient and in 'classical' steroidogenic organs such as the gonads and the adrenal cortex steroidogenic activity is primarily regulated by the provision of the original substrate cholesterol to the inner mitochondrial membrane, the expression of steroidogenic enzymes and factors modulating their activities (Miller & Auchus 2011). Also considering a role of SE as substrates for the local production of active estrogens via the sulfatase pathway does not readily lead to a sound hypothesis concerning the function of testicular SE in boars. Firstly, in the systemic circulation of adult boars, unconjugated estradiol-17 β and estrone circulate at significant concentrations (Wagner *et al.* 2006, Hoffmann *et al.* 2010, Schuler *et al.* 2014), questioning the need for a sulfate pathway in estrogen-responsive tissues. Moreover, in the porcine testis STS is highly and specifically expressed in Leydig cells (Mutembei *et al.* 2009), which—as mentioned earlier—obviously possess a high capacity to produce unconjugated estrogens (UE) *de novo*. Accordingly, due to these seemingly inconsistent observations, so far no plausible concept has been put forward concerning the biological role of SE produced in the porcine testicular-epididymal compartment.

Thus, to get further insights into the production and hydrolysis of SE in boars as a basis to understand their functions, the principal aims of this study were (a) to characterize the expression of mRNA specific for STS, the highly estrogen-specific sulfotransferase *SULT1E1* and the phenol sulfotransferase *SULT1A1* in defined segments of the epididymis in comparison to their expression in the testis; (b) to comprehensively characterize STS and *SULT1E1* expression in the porcine testicular-epididymal compartment on a cellular level using immunohistochemistry (IHC); (c) to investigate the sulfonation of UE and the hydrolysis of SE in defined segments of the epididymis in comparison to the testis and (d) to determine the concentrations of UE and SE in testicular and epididymal tissues.

Material and methods

Collection and preparation of tissue samples

Animal experiments were in accordance with the relevant regulations (Regierungspraesidium Giessen, permit No. V54-19c-20-15(I) Gi 18/14-No. 32/2010). Tissue samples were collected from a total number of 8 postpubertal domestic crossbreed boars (*Sus scrofa domestica*, German Landrace $\times$ Pietrain) aged 9.7–15.6 (mean: 11.9 $\pm$ 2.0) months under general anesthesia as previously described (Schuler *et al.* 2014). For WB analysis and for measurements

of mRNA expression and enzyme activities, tissue samples were prepared from defined localizations (Fig. 1): upper and lower half of the testis (TE1, TE2), rete testis (RT1: central part, RT2: transition part towards the epididymal head), proximal and distal part of epididymal head (EH1, EH2), four segments of the epididymal body (EB1-4, from proximal to distal), proximal and distal part of epididymal tail (ET1, ET2) and deferent duct (DD). They were wrapped with aluminum foil and stored at -80°C until further analysis. Correspondingly, for IHC, tissue samples were collected from the same localizations and conserved for 20–24 h in 10% neutral phosphate-buffered formalin.

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; RT1, rete testis in the central mediastinum; RT2, ductules of the rete testis adjacent to the epididymal head; TE1, TE2, testicular parenchyma in the upper/lower half of testis.

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

Sample sets of five boars were analyzed. Pieces of frozen tissues were pulverized with a pestle in a mortar 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), RNA was reverse transcribed with GeneAmp RNA PCR Kit (Perkin Elmer). Real-time RT-qPCR was performed in a CFX96 Real-time Thermal Cycler by means of the SYBR green method using SYBR Green I Master Mix (both from Bio-Rad). The primer pair specific for *STS* was adopted from a previous publication (Mutembei et al. 2009; for information on primer pairs see Table 1). Primer pairs for *SULT1E1* and *SULT1A1* were designed and purchased from Eurogentec (Cologne, Germany). 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. Melting curves of the PCR product were generated to test for the occurrence of nonspecific products and primer dimers. Correct size of PCR products was confirmed by agarose gel electrophoresis. In order to further validate the real-time RT-PCR methods, standard curves were generated by serial dilutions of the cDNA according to the standard protocol provided by the supplier of thermocycler. The slope of the standard curve was used to calculate the efficiencies of the PCR reactions. Relative gene expression (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 reference genes, hypoxanthine-guanine phosphoribosyltransferase (*HPRT1*) and TATA-binding protein (*TBP*).

As RGE levels varied substantially between individual animals and to make the tissue distribution patterns more recognizable, for each animal, the expression level in the tissue exhibiting the highest expression the RGE value was set to 1 and the expression levels in the remaining tissues were calculated relative to 1 (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 all animals investigated, they were transformed by the arc

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

Primer	Sequence (5'→3')		Product length (bp)	Reference or sequence information
	Forward	Reverse		
<i>SULT1A1</i>	GCCGTCTCTATTACCAC	GGCGTTGTTCTTCATCTC	303	Lin et al. (2004)
<i>SULT1E1</i>	TGAGGCAAGACCAGATGAC	AGGTGGCAGATGAGACTTC	232	NM_213992
<i>STS</i>	GAAGACAGGATCATTGACG	AGAACTTGGGTGTGAAGAAG	172	Mutembei et al. (2009)
<i>HPRT1</i>	GCATTCTGAACTCTTTATTGCA	TTCACGAGTCAAACAACAATCCA	80	NM_001032376
<i>TBP</i>	TGGTACATTTAATGGTGGTGTGTC	CCTAAACCGAACGTCCATCCT	65	DQ845178.1

F, forward; R, reverse.

sine function and presented as re-transformed values of arithmetic mean $\pm$ standard deviation ($\bar{x} \pm$ s.d.) of the arc sine transformed values (modified $\bar{x} \pm$ s.d. range).

Preparation of subcellular fractions by differential centrifugation

For WB analysis and measurements of enzyme activities, cytosolic (EST) and microsomal fractions (STS) were prepared from frozen tissue samples by differential centrifugation following the method by Moran et al. (2002). Measurement of protein concentrations in subcellular fractions were performed using the Pierce BCA protein assay kit (purchased from Thermo Fischer Scientific GmbH).

Detection of steroid sulfatase and SULT1E1 in subcellular fractions using Western blot

Western blot analyses for the expression of STS and SULT1E1 were performed in sample sets from three boars. 20 μ g of microsomal (STS) or cytosolic protein (SULT1E1) were separated on a 10% polyacrylamide gel under reducing conditions and transferred to nitrocellulose membranes (Optitran BA-S85, Schleicher & Schüll, Dassel, Germany). For blocking, membranes were incubated in PBS-T (PBS with 0.05% Tween-20) with 5% nonfat dry milk overnight. Then, they were washed in PBS-T and incubated for 75 min with the respective primary polyclonal antiserum (Table 2). After washing in PBS-T, they were incubated with the horseradish peroxidase-linked secondary goat-anti-rabbit immunoglobulin antibody (Dianova, Hamburg, Germany) at a dilution of 1:50,000 in blocking

buffer. 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) following the protocol provided with the substrate kit.

Immunolocalization of steroid sulfatase and SULT1E1

Sections (5 μ m) were prepared from formalin-fixed, paraffin-embedded sample sets from three boars. For epitope retrieval, they were heated in 10mM citrate buffer pH 6.0 at 98–100°C for 20 min. Indirect immunoperoxidase staining methods were employed using the streptavidin-biotin technique for signal enhancement following standard procedures applying the primary antisera specified in Table 2. Negative controls were set up replacing the specific antisera 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 NovaRed substrate kit was applied to visualize the immunoreaction following the manufacturer's instructions (Vector Laboratories, Burlingame, USA).

Measurements of steroid sulfatase activities in microsomal fractions

STS activities were assayed in microsomal fractions prepared from sample sets from three boars based on the differential distribution of E1S and estrone (E1) between the aqueous and an organic phase (tertiary butyl methyl

Table 2 Primary antisera (host: rabbit) used for the detection of SULT1E1 and steroid sulfatase in Western blot and immunohistochemistry.

Target molecule	Name of antibody (source, reference)	Immunogen	Working dilution
SULT1E1	MM-0115-P (MediMabs, Montreal, Canada (Frenette et al. 2009))	Recombinant bovine SULT1E1	1:2000
Steroid sulfatase	Anti-human StS (Dibbelt & Kuss 1986, Dibbelt et al. 1989)	Human placental steroid sulfatase	1:2000

ether (tMBE)). Substrate (0.3 nmol/L [6,7- ^3H (N)] E1S plus 9.7 nmol/L non-labelled E1S; purchased from Perkin Elmer and MAKOR Chemicals, Jerusalem, Israel, respectively) and 200 μg microsomal protein were incubated in a shaking water bath at 37°C using Ringer-HEPES buffer pH 7.0 as an incubation medium in a total volume of 1 mL. Medium blanks were set up replacing microsomal protein by Ringer-HEPES buffer. Each reaction was run in duplicates. Following incubation (0, 30, 60 min), the samples were boiled for 15 min to stop the enzymatic reaction and centrifuged for 10 min at 3000g. For extraction of E1, an aliquot of 800 μL was transferred into an extraction tube, overlaid with 3 mL of tMBE, vortexed, shortly centrifuged (3000g) and the organic supernatant was decanted into a glass tube. This extraction step was repeated; the pooled extracts were evaporated to dryness (evaporator MicroDancer, Hettich AG, Bäch, Switzerland) and redissolved in scintillation fluid (Aquasafe, Zinsser Analytic, Frankfurt). The ^3H -activity was measured (Tri-Carb 2810TR, PerkinElmer Germany GmbH) and conversion of substrate was calculated from the increase of ^3H -activity in the organic phase after subtraction of the ^3H -activity measured in the medium blank.

To test for the substrate specificity of STS, corresponding experiments were performed using pregnenolone sulfate (P5S) and dehydroepiandrosterone sulfate (DHEAS) as a substrate and testicular microsomal protein as a source of the enzyme.

Measurements of estrogen sulfotransferase (EST) activities in cytosol

Determination of EST activity was performed in cytosolic fractions prepared from sample sets from three boars and was based on the same principle as described earlier for STS activity. Substrate (0.3 nmol/L [2,4,6,7- ^3H (N)] E1 plus 9.7 nmol/L non-labelled E1; purchased from Perkin Elmer and Paesel Lorei GmbH, Hanau, Germany, respectively), 400 nmol/L of the cosubstrate 3'-phosphoadenosine-5'-phosphosulfate (PAPS; Sigma-Aldrich Chemie GmbH) and 200 μg cytosolic protein were incubated in a total volume of 1 mL at 37°C for 0, 10 and 20 min using Ringer-HEPES buffer pH 7.0 as incubation medium. After extraction of E1, the E1S formed and dissolved in the aqueous phase was assessed as E1 after enzymatic hydrolysis as previously described (Hoffmann et al. 2001). Conversion of substrate was calculated from the increase of ^3H -activity in the aqueous fraction after subtraction of the ^3H -activity measured in the medium blank.

To confirm the specificity of this assay, an HPLC-based method was applied on selected tissue samples from an individual boar (TE, EH1, ET2). Following incubation (20 min) and after prepurification of the samples by solid phase extraction, E1 and E1S were 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 acetonitrile (ACN)/water gradient (30–95%) and a flow rate of 0.5 mL/min. The eluate was collected in 0.5 mL fractions, in which ^3H -activity was measured. Identification of substrate and metabolite was based on a comparison of the retention times with authentic tritiated standards. Conversion of substrate was calculated from the ^3H -activity corresponding to the peaks after subtraction of the technical background.

Measurements of estrogen sulfotransferase activities in testicular and epididymal homogenates as a function of substrate concentration

Small 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 Ringer solution/50 mmol/L HEPES pH 7.0 in 2 mL Micro Tubes (Sarstedt AG & Co, Nuembrecht, Germany) and shredded by 2 $\times$ 20 s bursts using a Fast Prep-24 homogenizer (MP-Biomedicals). The resulting crude homogenates were centrifuged at 4°C for 35 min at 20,000g to remove particles of connective tissue and cellular debris. The supernatants obtained from this centrifugation step were stored at –20°C until further use.

For the measurement of EST activity 50 μL homogenate, 125 $\mu\text{mol/L}$ PAPS and 16.2 $\mu\text{mol/L}$ of the steroid sulfatase inhibitor STX64 (Sigma-Aldrich Chemie GmbH) were incubated with a constant amount of ^3H -E1 (6.4 nmol/L) in the presence of increasing concentrations of unlabeled E1 (0, 0.05, 0.2, 0.8 and 2.0 $\mu\text{mol/L}$) in a total volume of 200 μL . Ringer solution/50 mmol/L HEPES buffer pH 7.0 was applied as an incubation medium, and the time of incubation was 20 min. For each sample parallel incubations were performed omitting PAPS as controls. All incubations were performed in duplicate. After incubation 50 μL ACN were added to the tubes to stop the reaction, followed by centrifugation (10 min at 20,000g). For analysis, a 20 μL aliquot of the supernatant was directly subjected to HPLC analysis applying the method described above. Calculation of substrate sulfonation was based on the distribution of ^3H -activity

over the peaks corresponding to the substrate and the sulfonated product, respectively.

Measurements of endogenous tissue concentrations of unconjugated and sulfonated estrogens and of testosterone in testis and epididymal head

UE and SE were determined in TE and EH2 of five boars. Tissue homogenates were prepared by mincing 200 µg tissue (FastPrep-24, MP Biomedicals) in 1 mL Ringer solution. In order to block hydrolysis of SE during the analytical procedure by endogenous STS, STX64 (160 µmol/L) was added to the homogenization buffer. E1 and E1S were determined by radioimmunoassay as described in detail for plasma (Hoffmann *et al.* 1996, 1997). The lower limit of detection was at 1 pmol/g tissue. Radioimmunological measurements of 17β-estradiol (E2) and E2S were performed as described above for E1 and E1S applying an antiserum exhibiting the following cross reactions: E2: 100%; E1: 1.3%, all tested non-phenolic steroids <0.01%. For reasons of comparison testosterone was measured in the same samples by radioimmunoassay (Ludwig *et al.* 2009).

Results

Expression and activity of steroid sulfatase

STS mRNA levels were higher in testis, epididymal head and tail and deferent duct compared to the epididymal body (Fig. 2A). However, a considerable variability was observed between individual animals (Supplementary Table 1, see section on supplementary data given at the end of this article). In WB, a specific band with a molecular weight of approx. 61 kDa was detected in all samples investigated (Fig. 2B). Band intensity was consistently high in the testis. In epididymal samples bands of variable intensities were found with no clear correlation between their intensity and individual epididymal segments. In testicular tissue IHC identified STS predominantly in Leydig cells (Fig. 3A), which exhibited a moderate to intense cytoplasmic staining. Moreover, a weak to moderate cytoplasmic staining was also present in the epithelium of rete testis ductules (Fig. 3C). No specific immunostaining was found in the tubular compartment and in peritubular myoid cells. In the epididymis immunostaining was practically restricted to the ductal epithelium and was predominantly cytoplasmic. Staining intensity was generally weak to moderate (Fig. 3F and G) and tended to be higher in the head and the distal part

Figure 2

Expression of steroid sulfatase (STS) in the porcine testicular-epididymal compartment on the mRNA- and protein level. (A) Relative gene expression (RGE) levels for STS mRNA as measured by real-time RT-qPCR in five boars. For each of the animals the expression level in the sample exhibiting the highest expression was set to 1. Data are presented as re-transformed values of $\bar{x} \pm \text{s.d.}$ of arc sine transformed data (modified $\bar{x} \pm \text{s.d.}$ range). (B) Qualitative detection of STS by Western blot in three individual boars. In each lane 20 µg microsomal protein was loaded. Tissue samples (Fig. 1): DD, deferent duct; EB1-4, segments of epididymal body, from proximal to distal; EH1,-2, proximal/distal segment of epididymal head; ET1,-2, proximal/distal segment of epididymal tail; TE1,-2, testicular parenchyma in the upper/lower half of testis.

of the tail compared to the segments located in between (Supplementary Table 2). Occasionally distinct staining of protrusions from the epithelial surface into the lumen of the epididymal duct (Fig. 3G) was observed. Only sporadically a weak to moderate immunostaining was also found in the muscular layer and in the vascular system.

Using E1S as a substrate, STS activity of microsomal protein was consistently high in the testis (Fig. 4A). In most of the epididymal samples, it was only slightly above background levels. However, in individual samples of the epididymal head and body considerable STS activity was present. When incubating testicular microsomal protein with PSS and DHEAS as substrates, hydrolysis was only minimal in comparison to E1S (Fig. 4B).

Figure 3

Immunolocalization of steroid sulfatase in the porcine testis and epididymis (A) Distinct specific staining in Leydig cells. (B) Negative control (testis) (C) Moderate specific staining in epithelial cells of rete testis. (D) Negative control (rete testis) (E) Negative control (epididymal head) (F) Distinct immunostaining in duct epithelium of the epididymal head. (G) Specific staining in superficial protrusions (arrows) from epithelial cells of the epididymal head. ct, epididymal connective tissue; ed, lumen of epididymal duct, ml, muscular layer of epididymal duct, mt, connective tissue of mediastinum testis, rt, ductule of rete testis, st, seminiferous tubules, sz, agglomerates of spermatozoa in epididymal duct lumen. A full colour version of this figure is available at <https://doi.org/10.1530/JME-17-0245>.

Expression of *SULT1E1* and estrogen sulfotransferase (EST) activity

SULT1E1-mRNA expression profiles were relatively consistent between the individual animals (Fig. 5A and Supplementary Table 1). Epididymal head and the initial segment of the epididymal body were clearly identified as the major site of *SULT1E1*-mRNA expression. In the testis, *SULT1E1*-mRNA was qualitatively detectable but RGE levels were extremely low in comparison to the epididymis.

With WB specific bands of approximately 35 kDa were found in all segments of the epididymis (Fig. 5B) but not in testicular samples. Their intensity was consistently weak in the epididymal tail; bands of moderate intensity were found in the remaining parts of the epididymis.

Figure 4

Steroid sulfatase activity in the porcine testis and epididymis. (A) Hydrolysis of E1S (10 nmol/L) by 200 µg microsomal protein prepared from different tissues of the testicular-epididymal compartment from three boars. The results are presented as re-transformed $\bar{x} \pm s.d.$ calculated from arc sine transformed data (modified $\bar{x} \pm s.d.$ range). EB1-4, segments of the epididymal body (from proximal to distal); EH1, -2, proximal/distal segment of epididymal head; ET1, -2, proximal/distal segment of epididymal tail; TE, testis. (B) Hydrolysis of E1S, DHEAS and P5S by 200 µg microsomal protein prepared from the testes from three boars. The results are presented as re-transformed $\bar{x} \pm s.d.$ calculated from arc sine transformed data (modified $\bar{x} \pm s.d.$ range). Substrate concentration was 10 nmol/L.

When screening the testis for *SULT1E1* expression by IHC (Fig. 6), spermatogenic tubules and the interstitial tissue were virtually devoid of signals, only occasionally a weak ubiquitous staining was observed (Fig. 6A) which was thus considered nonspecific. A weak to moderate immunostaining was observed in the epithelium of rete testis. However, this was restricted to ductules connecting the central part of the rete testis to the epididymal head (Fig. 6C; sample RT2 in Fig. 1). In the epididymis (Fig. 6E and F and Supplementary Table 3) a moderate to distinct staining occurred in the samples EH1, EH2 and

Figure 5

Expression of SULT1E1 in the porcine testicular-epididymal compartment on the mRNA- and protein level. (A) Relative gene expression levels (RGE) for *SULT1E1* mRNA as measured by real-time RT-qPCR in five boars. For each of the animals the expression level in the sample exhibiting the highest expression was set to 1. Data are presented as re-transformed values of $\bar{x} \pm s.d.$ of arc sine transformed data (modified $\bar{x} \pm s.d.$ range). (B) Western blot analysis of SULT1E1 expression in three individual boars. In each lane 20 μ g cytosolic protein was loaded. Tissue samples (Fig. 1): DD, deferent duct; EB1-4, segments of epididymal body, from proximal to distal; EH1, EH2, proximal/distal segment of epididymal head; ET1-2, proximal/distal segment of epididymal tail; TE1-2, testicular parenchyma in the upper/lower half of testis.

EB1, where SULT1E1 was predominantly localized in the epithelium, including apical protrusions. Mean epithelial staining intensity in the remaining part of the epididymis tended to be lower. Throughout the epididymis, variable immunostaining was observed in vascular endothelial cells. In the muscular layer of the epididymal duct only sporadically a weak to moderate immunostaining was found.

When EST activity was determined in cytosols prepared from testis and epididymal segments of three boars (Fig. 7A), it was readily detectable in the epididymis but at the limit of detection in the testis. In the epididymis, after highest levels in the initial segment of the head mean EST activity gradually decreased until the segment EB3. In segments EB4 and ET1 activity was highly variable between individual animals. In the hind part of the epididymal tail (ET2), it was consistently low. HPLC based analysis confirmed the specificity of the

Figure 6

Immunolocalization of SULT1E1 in the porcine testis and epididymis. (A) In testicular parenchyma, if at all, only a diffuse weak staining of questionable specificity occurred. (B) Negative control. (C) Immunostaining in rete testis duct epithelium located at the testicular-epididymal transition (sample RT2, Fig. 1). (D) The same localization as shown in (C) in a negative control. (E) Distinct staining in the epithelium of an epididymal duct which was attached immediately to the upper pole of the testis. The micrograph was taken from the same section as used for (A) enabling a direct comparison of signal intensities. (F) Staining pattern in epididymal head with specific staining in epithelial cells including superficial protrusions (arrows), endothelial cells of a blood vessel (bv) and in particles (arrowheads) located in the ductal lumen. ct, epididymal connective tissue; ed, lumen of epididymal duct; it, interstitial tissue; ml, muscular layer of epididymal duct; mt, connective tissue of mediastinum testis; rt, ductule of rete testis; st, seminiferous tubules. A full colour version of this figure is available at <https://doi.org/10.1530/JME-17-0245>.

method; no other metabolite than E1S could be detected (Supplementary Fig. 1).

In order to elucidate the contradiction between the clear evidence for a significant production of SE in the porcine testis and the absence of a corresponding EST activity in testicular samples a different *in vitro* incubation system was used to measure sulfonation of E1. In this alternative assay, homogenates prepared from TE and EH1 were incubated with different substrate

Figure 7

Estrogen sulfotransferase activity in the porcine testis and epididymis. (A) Sulfonation of estrone (10 nmol/L) by 200 µg cytosolic protein prepared from different tissues of the testicular-epididymal compartment from three postpubertal boars. The results are presented as re-transformed $\bar{x} \pm \text{s.d.}$ calculated from arc sine transformed data (modified $\bar{x} \pm \text{s.d.}$ range). EB1-4, segments of the epididymal body (from proximal to distal); EH1,-2, proximal/distal segment of epididymal head; ET1,-2, proximal/distal segment of epididymal tail; TE, testis. (B) Sulfonation of estrone by homogenates prepared from testis and epididymal head of a boar as a function of substrate concentration. Data are presented as $\bar{x} \pm \text{s.d.}$ from two independent experiments.

concentrations between 6.4 and 2006.4 nmol/L (Fig. 7B). In EH1 sulfonation of E1 was almost complete at substrate concentrations of 6.4 nmol/L ($88.7 \pm 4.0\%$ conversion) and 56.5 nmol/L ($87.5 \pm 1.9\%$). The reaction rate (pmol/h/mg tissue) was maximal at 206.4 (corresponding to $54 \pm 2.7\%$ conversion) and decreased with higher substrate concentrations. The percentage of sulfonated product was $9.8 \pm 1.1\%$ at 806.4 nmol/L and $3.1 \pm 1.2\%$ at 2006.4 nmol/L. In TE, the reaction rate increased continuously up to the highest substrate concentration tested. Percentage of sulfonated product was 8.9 ± 1.2 , 8.3 ± 0.5 , 6.2 ± 0.2 , 3.9 ± 0.2 and $2.9 \pm 0.2\%$ at the substrate concentrations applied, respectively. In control experiments without

PAPS, ^3H -activity corresponding to E1S did not exceed the technical background.

Tissue levels of unconjugated and sulfonated estrogens and of testosterone in testis and epididymal head

About equimolar concentrations of E1, E2 and their sulfonated forms were found in testicular tissue, with, however, distinct individual variations (Fig. 8). With the exception of E1S and E2S concentrations in boar no.1, estrogen concentrations in the epididymal head were clearly lower compared to the testis (Fig. 8B and C). Testicular testosterone concentrations were in a similar range in all five animals and considerably exceeded those in the epididymal head (Fig. 8D). However, in EH of boar no.1, similarly to SE, also testosterone concentrations were markedly higher compared to the remaining four animals.

Expression of *SULT1A1* mRNA

SULT1A1 mRNA was detected in all tissue samples investigated but expression patterns varied significantly between individual animals (Supplementary Table 1 and Fig. 2). Highest mean expression levels were observed in the initial segment of the epididymal head (EH1), followed by the deferent duct and the testis.

Discussion

So far no plausible concept was available concerning the role of SE circulating in boars at intriguingly high concentrations. Observations from cell culture experiments (Raeside & Renaud 1983) and measurements of EST activities in purified porcine Leydig cells (Hobkirk et al. 1989) provided clear evidence that in boars testicular estrogens are sulfonated in Leydig cells immediately after their production from aromatizable precursors. However, the concept of the Leydig cell as the only significant source of SE in the testicular-epididymal compartment was challenged by our previous results of a considerable EST activity in homogenates prepared from epididymal tissue, whereas in testicular homogenates it was comparatively only marginal (Hoffmann et al. 2010). These previous results were confirmed in this study using cytosolic fractions instead of crude homogenates and applying HPLC as a more specific method for analysis (Fig. 7A and Supplementary Fig. 1). The conundrum of the relevant source of SE (Leydig cells vs the epididymis)

Figure 8

Concentrations of free and sulfonated estrogens and of testosterone in tissue homogenates prepared from testis (TE) and epididymal head (EH) of five individual boars. (A) Concentrations of 17β-estradiol (E2) and 17β-estradiol-3-sulfate (E2S) measured without addition of the steroid sulfatase inhibitor STX64 to the homogenization buffer. (B) E2 and E2S concentrations measured with addition of STX64 to the homogenization buffer. (C) Estrone (E1) and estrone sulfate (E1S) concentrations measured with addition of STX64 to the homogenization buffer. (D) Testosterone (T) concentrations.

was widely solved by the concerted characterization of SULT1E1 expression pattern, measurement of estrogen tissue concentrations and the application of a modified assay system for the measurement of EST activities.

Among the numerous members of the cytosolic sulfotransferase (SULT) family, SULT1E1 is the enzyme highly specific for estrogens with K_m values in the lower nanomolar range (Falany *et al.* 1995a, Zhang *et al.* 1998, Pasqualini 2009). So far in pigs no information was available on SULT1E1 expression patterns in reproductive organs. Consistent with results from our initial EST activity measurements (Hoffmann *et al.* 2010 and Fig. 7A in this study), a significant expression of SULT1E1 was only observed in the epididymis, whereas in the testis SULT1E1 expression was virtually undetectable apart from the rete testis ductules immediately adjacent to the testicular-epididymal transition. However, it is very unlikely that a moderate SULT1E1 expression restricted to a marginal part of the rete testis is the equivalent of the high SE concentrations measured in testicular tissue, which definitely corroborate the concept of the testis as the major source of SE, especially when considering the considerably higher mass of the porcine testis in comparison to the epididymis. When a modified assay system was applied for the measurement of EST activity using different substrate concentrations, in the epididymal head the reaction rate increased rapidly with increasing substrate concentration and the enzyme was saturated at rather low concentrations. However, in the testis the proportion of the substrate converted was low even at low

substrate concentrations, but the enzyme was not saturable at the highest concentration tested (Fig. 7B). Obviously in our initial experiments the capacity of testicular EST activity was underestimated due to the specific features of our methodological approach in combination with the use of a low substrate concentration. Although the data obtained from the subsequent experiments applying a broader range of substrate concentrations do not allow the calculation of exact K_m values, they clearly show that in epididymal samples a high affinity enzyme is active with a K_m value in the lower nanomolar range consistent with SULT1E1 (Falany *et al.* 1995a, Zhang *et al.* 1998, Pasqualini 2009), whereas the enzyme underlying the sulfonation of E1 in the testis shows a very different kinetic with a substantially higher K_m . These results obtained for testicular EST activity are consistent with observations by Hobkirk *et al.* (1989) who partially characterized EST activities in cytosol prepared from purified porcine Leydig cells towards E1. The isolated EST exhibited an apparent K_m of 4 μM, which is clearly different from the epididymal EST activity but in a similar range as the testicular EST activity observed in our study. Likely candidates for the SULT underlying testicular EST in boars are SULT1A1 or SULT2A1, which are both expressed in porcine testis (SULT1A1: Lin *et al.* 2004 and this study; SULT2A1: Sinclair *et al.* 2006). Currently no information is available on the utilization of estrogens as substrates by the porcine enzymes. However, in man they have been shown to sulfonate estrogens albeit at considerably higher substrate concentrations in the micromolar range

(Falany *et al.* 1995b, Pasqualini 2009). The involvement of different enzymes in the porcine testis and epididymis for the sulfonation of estrogens widely differing in their substrate affinities points to different physiological roles of estrogen sulfonation between the two organs. The high affinity enzyme SULT1E1 present in the porcine efferent ductules and the epididymis probably serves the most complete inactivation of estrogens entering the cells from the tubular fluid, in which the extensive fluid resorption (Hess *et al.* 2001) may not only lead to a concentration of sperm cells but also of solutes. The generally lower E1S or E2S tissue concentrations in the epididymal head compared to the testis despite a high SULT1E1 expression could result from a relatively low exposition of UE to the enzyme or by a rapid and efficient off-site transportation of SE via the blood or lymphatic system. The expression of a sulfotransferase in Leydig cells with a low affinity for estrogens may be important to allow a considerable proportion of UE to escape from sulfonation. This idea is corroborated by the high concentrations of UE measured in testicular tissue in the presence of an STS inhibitor at a concentration which was confirmed to completely prevent the hydrolysis of SE during sample processing (Fig. 8). Our result of a practically absent SULT1E1 expression in the porcine testis is obviously very different from the situation in mice, in which significant immunostaining was found in Leydig cells and SULT1E1 is readily detectable in testicular tissue with WB (Tong & Song 2002).

An intriguing feature of porcine Leydig cells is their significant expression of STS (Mutembei *et al.* 2009, Hoffmann *et al.* 2010) in light of their high capacity to produce estrogens *de novo* (Fraczek *et al.* 2001, Mutembei *et al.* 2005) and to sulfonate estrogens (Raeside & Renaud 1983, Hobkirk *et al.* 1989; this study), all the more since SE are obviously the preferential substrate of porcine STS in comparison to DHEAS or P5S (Hobkirk *et al.* 1989; our own results, Fig. 4B). Thus, on the first view a sulfatase pathway for the production of active estrogens in the porcine testis seems redundant. However, as clearly obvious from diurnal testosterone profiles, porcine testicular steroidogenesis exhibits a pronounced pulsatile pattern with 3–5 relatively short activity spurts per day, separated by longer phases of low activity (Claus & Hoffmann 1980, Tan & Raeside 1980, Schuler *et al.* 2014, 2018). Thus, in porcine Leydig cells a low affinity EST and STS could cooperate to widely decouple the levels of active UE from the availability of aromatizable precursors. During the activity spurts of testicular steroidogenesis, excessive UE may be transferred into a reservoir in form of SE, which is tapped by STS during the phases of low *de novo* production

to maintain sufficient levels of active UE. Consistent with this hypothesis, the diurnal fluctuations of UE are clearly less pronounced in comparison to testosterone (Claus & Hoffmann 1980, Supplementary Fig. 3).

In the epididymis, the initial segments (EH1,-2, EB1) were identified as the tissues exhibiting clearly the highest SULT1E1 mRNA expression levels. When applying WB and in measurements of EST activity this expression pattern was less perceivable. As indicated by the pronounced immunostaining associated with apical blebs of epithelial cells and with cellular debris situated in the lumen of the epididymal duct (Fig. 6F), this discrepancy may be explained by significant secretion of SULT1E1 into the ductal lumen and subsequent transport into the more distal parts of the organ, thereby concealing the original expression pattern. Our observations concerning SULT1E1 protein expression in the epididymis and in the testis are very similar to the findings described by Frenette *et al.* (2009) in the bull. However, epididymal SULT1E1 expression in pig and cattle is clearly different from the mouse, where SULT1E1 is virtually absent in the epididymal head but is highly expressed in the epididymal body and tail (Song *et al.* 1997, Tong & Song 2002). Moreover, in the bovine epididymis Frenette *et al.* (2009) also observed considerable immunostaining for SULT1E1 in apical blebs of the ductal epithelium, which are considered as the morphological equivalent of apocrine secretion (Hughes & Berger 2015). These blebs detach from the cell and rapidly disintegrate to give rise to the formation of numerous small vesicles named epididymosomes, which are considered as components of highly selective transfer mechanisms enabling the directed targeting of epididymis-derived molecules to their final specific place of destination on or even in maturing sperm cells (Aumüller *et al.* 1999, Hermo & Jacks 2002, Girouard *et al.* 2011, Sullivan & Saez 2013). Frenette *et al.* (2009) supposed that in the bovine epididymis SULT1E1 associated with sperm cells serves the sulfonation of cholesterol or other sterols in the sperm membrane despite its comparatively low activity towards these substrates. Anyhow, the uniform SULT1E1 expression pattern between the boar, which exhibits intriguingly high levels of estrogens including sulfonated forms, and the bull with levels of unconjugated estradiol-17 β in the lower pg/mL-range (Henricks *et al.* 1988) and estrone sulfate concentrations below 0.2 ng/mL (our own unpublished data) is striking.

STS expression in the epididymis was widely restricted to the ductal epithelium, where it is co-localized with SULT1E1. The biological role of this co-localization remains unclear. However, a special consideration

deserves the observation that in epididymal epithelial cells immunostaining for STS was especially high in apical blebs, pointing to a secretion of the enzyme into the epididymal duct as suggested for SULT1E1.

In conclusion, the results concerning the formation and hydrolysis of SE in the porcine testis suggest that the co-localization of a low-affinity EST, STS and aromatase in Leydig cells serves the considerable decoupling of the production of bioactive UE from the availability of aromatizable precursors, which is obviously subjected to significant diurnal fluctuations due to the pulsatile activity of porcine testicular steroidogenesis. Immunohistochemical observations indicate that in the porcine epididymis, SULT1E1 and STS may have functions in the ductal lumen, in which they are obviously released from the ductal epithelium by an apocrine mechanism.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-17-0245>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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