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Expression and activities of 11βHSD enzymes in the testes and reproductive tracts of sexually immature male pigs

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ABSTRACT

In light of studies implicating glucocorticoids in the control of testicular steroidogenesis and/or spermatogenesis, the objective of this study was to characterise the expression and activities of the 11β-hydroxysteroid dehydrogenase (11βHSD) enzymes in the testis and reproductive tract of the pre-pubertal pig. Although 11βHSD1 and 11βHSD2 mRNA transcripts and proteins were co-expressed in all regions of the reproductive tract, cortisol–cortisone inter-conversion was detectable in the testis, caput epididymidis and bulbourethral glands only. In homogenates of these 3 tissues, the apparent K_m for $NADP^+$- and NAD^+-dependent 11β-dehydrogenase activities ranged between 152–883 and 47–479 nmol l^{-1}, respectively. Irrespective of the pyridine nucleotide co-substrate, estimates of V_{max} were consistently two orders of magnitude higher in the testis. Moreover, while, in each tissue, levels of cortisol oxidation were comparable in the presence of either $NADP^+$ or NAD^+, maximal rates of $NAD(P)^+$-dependent cortisol oxidation were up to 33-fold greater than the V_{max} for NADPH-dependent reduction of cortisone. We conclude that in the testis, caput epididymidis and bulbourethral gland of the immature pig, $NADP^+$- and NAD^+-dependent 11βHSD enzymes catalyse net inactivation of cortisol, suggesting a physiological role for these enzymes in limiting local actions of glucocorticoids within these male reproductive tissues prior to puberty.

1. Introduction

Stress is known to impair reproduction in a variety of domestic farm animal species [1]. In the adult male, elevated local concentrations of endogenous glucocorticoids (cortisol and corticosterone) are known to exert many deleterious effects on Leydig cell function, including the inhibition of testosterone biosynthesis, suppression of luteinising hormone receptor expression and the induction of Leydig cell apoptosis [2–4]. Boar spermatozoa are known to be highly responsive to the local environment and glucocorticoids have recently been implicated in the induction of apoptosis in boar spermatogonia [5]. However, the physiological roles of glucocorticoids within the developing immature testis and reproductive tract have not yet been reported.

In several reproductive tissues, the actions of glucocorticoids are modulated by one or more of the cloned 11β-hydroxysteroid dehydrogenase (11βHSD) enzymes (reviewed in [6]). 11βHSD1 is a bi-directional enzyme in cell homogenates. However, in intact cells this enzyme acts predominantly as an 11-ketosteroid reductase (11KSR), where its primary role is believed to be the regeneration of cortisol from cortisone [6,7]. The 11βHSD1 enzyme has a higher affinity for cortisone (K_m = 300 nmol l^{-1}) than for cortisol (K_m = 17–27 μmol l^{-1}) and it preferentially utilises NADP(H) as its nucleotide co-substrate. It is reported that hexose-6-phosphate dehydrogenase (H6PDH) activity maintains a high $NADPH:NADP^+$ ratio in the lumen of the smooth endoplasmic reticulum, thereby favouring the reductase action of 11βHSD1 [8–14]. However, 11βHSD1 exhibits predominantly 11β-dehydrogenase (11βDH) activity in steroidogenic gonadal cells (e.g. rat testis Leydig cells, human granulosa–lutein cells, bovine and porcine granulosa cells) [15–20], where the preferential usage of NADPH for steroid biosynthesis is thought to alter the $NADPH:NADP^+$ ratio in favour of 11βDH activity [6,21]. In contrast to the 11βHSD1 enzyme, 11βHSD2 relies solely on NAD^+ as its oxidant co-substrate and has a relatively high affinity for cortisol (K_m = 40–60 nmol l^{-1}), acting exclusively as a dehydrogenase to inactivate glucocorticoids in target cells. Evidence for a novel, low K_m, $NADP^+$-dependent isoform of 11βHSD has also been reported in sheep kidney [22] and Leydig cells of mice and rats [17,23].

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Within Leydig cells isolated from rat testes, 11βHSD1 acts predominantly as an 11β-dehydrogenase in the adult rat but predominantly as an 11KSR in immature rat Leydig cells [17]. This 11KSR activity in the pre-pubertal rat testis is proposed to be important for promoting positive effects of glucocorticoids on Leydig cell differentiation and in limiting testosterone synthesis. Boar testes were initially demonstrated to express both *hsd11b1* and *hsd11b2* mRNA transcripts through reverse transcriptase PCR [5]. Our laboratory recently reported that 11βHSD1 and 11βHSD2 mRNA and protein are co-expressed in the testis and throughout the reproductive tract of sexually mature boars. However, enzyme activities were only detectable in boar testis, caput epididymidis, bulbourethral glands and penile urethra, where the 11β-dehydrogenase activity was predominant [25]. This suggested a physiological need to decrease local concentrations of cortisol in these tissues, thereby altering the environment in which spermatozoa mature and reside. It has since been reported that during foetal development, 30% of the porcine testicular Leydig cells express glucocorticoid receptors (GR) and 11βHSD2 [24]. Moreover, foetal germ cells were also positive for GR and 11βHSD2, with increasing expression of both proteins coincident with gonocyte differentiation to spermatogonia. This suggests that the expression of 11βHSD enzymes may change between foetal life and adulthood and, in so doing, serve a functional role during the development of male reproductive cells and tissues. Hence, in view of our prior observations in sexually mature boars, the objectives of the current study were to characterise the expression and activities of both 11βHSD1 and 11βHSD2 in the testis and reproductive tract of pre-pubertal pigs.

2. Materials and methods

2.1. Tissue collection and storage

Pre-pubertal pig tissues were obtained from the Royal Veterinary College, London. All pigs were known to be 6–8 weeks of age and were humanely culled in accordance with the Animals (Scientific Procedures) Act 1986, under the terms of a UK Home Office Licence and with approval from the Ethics and Welfare Committee of the Royal Veterinary College. Liver, kidneys, testes and reproductive tracts were transported to the laboratory, where each individual tissue was dissected out, aliquoted into 1 cm^3 segments, and either used fresh or snap frozen and stored at −80 °C until use. Tissues intended for immunohistochemistry were dissected into 1 cm^3 segments and placed straight into fixative. All studies were conducted on tissues from at least 3 independent pigs.

2.2. RNA isolation and RT-PCR

Expression of *hsd11b1* and *hsd11b2* in pre-pubertal tissues was assessed as previously described [25]. Briefly, total RNA was extracted from approximately 30 mg wet weight of each tissue using the RNeasy Mini Kit (Qiagen Ltd., West Sussex, UK) according to the manufacturer's instructions. The integrity of the total RNA extracts was assessed in all samples by visualising and amplifying 18S rRNA transcripts. Total RNA was reverse transcribed using an oligo-dT primer and 5 μl first-strand cDNA was used as a template in a PCR using primers specific for porcine *hsd11b1* and *hsd11b2* (see Table 1). All primers were designed using Primer3 (http://frodo.wi.edu/cgi-bin/primer3) and sequences of porcine *hsd11b1* and *hsd11b2* obtained from Genbank (accession numbers NM 214248 and NM 213913, respectively). The 18S oligonucleotide primers were designed from nucleotide sequences (accession number M10098) known to be fully conserved between the human, rat, mouse and rabbit.

PCR was performed using the *Taq* PCR Core Kit (Qiagen, West Sussex, UK). Cycling parameters for PCR were as follows for all amplified cDNAs: an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C, and a final extension step of 5 min at 72 °C. 10 μl of each PCR reaction was run on a 1.6% (w/v) agarose gel to visualise PCR products. PCR products from pre-pubertal pig kidney, liver and testis were isolated and subjected to the dideoxy-DNA sequencing method to confirm specificity.

2.3. Western blot analysis

All tissues were lysed on ice in radioimmunoprecipitation (RIPA) buffer containing 50 mmol l^{-1} Trizma and 154 mmol l^{-1} NaCl (pH 7.4) with a protease inhibitor cocktail (Mini-complete protease inhibitor, Roche, Germany). Protein concentrations were determined by using the NanoDrop® ND-1000 full spectrum UV/Vis spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Protein from pre-pubertal pig liver, kidney, testis and reproductive tract was diluted with sample buffer to give a final loading concentration of 25 μg total protein per 25 μl and separated by SDS-PAGE on a 12.5% (w/v) polyacrylamide gel before being transferred to a polyvinylidine difluoride (PVDF) membrane using a TE22 Mighty Small transphor tank wet transfer unit (Hoefer, San Francisco, USA).

The membranes were incubated overnight at 4 °C in a 1/1000 dilution of sheep anti-human 11βHSD1 or sheep anti-human 11βHSD2 polyclonal antibody each in PBS-T containing 5% (w/v) non-fat milk (as previously described in [25]). Membranes were incubated with a 1/10,000 dilution of rabbit anti-sheep IgG secondary antibody conjugated to horseradish peroxidase (HRP) (Abcam, Cambridge, UK) in PBS-T containing 5% (w/v) non-fat milk. 11βHSD proteins were visualised by incubating with enhanced chemiluminescence (ECL) detection reagents (Amersham Biosciences, UK) and exposed onto Hyperfilm ECL®. To confirm efficiency of protein transfer, membranes were stripped and reprobed for β-actin using a polyclonal β-actin antibody (Abcam, UK) at a dilution of 1/5000.

To confirm the number of protein bands within each lane that exhibited 11βHSD activity, samples were also resolved under non-denaturing, non-reducing conditions, such that proteins remained in a native, polymerised state. Protein preparations from pre-pubertal pig liver, kidney, testis and reproductive tract were each diluted with a non-reducing sample buffer to a final loading concentration of 25 μg total protein per 25 μl. Proteins were then resolved on a non-reducing, 12.5% (w/v) polyacrylamide gel. Resolved gels

Table 1
Details of the PCR primer sequences, regions of the target genes that each set of primers will amplify and the expected product sizes for the PCR amplicons.

Primer	Sequence	Region	Product size
11βHSD1	Forward 5′-CGCTCTGTATCCTCGGTCTC-3′ Reverse 5′-GTGTAGCGTAGAGTGTTCGA-3′	709–720 bp 1102–1082 bp	394 bp
11βHSD2	Forward 5′-CCAGCAGGAGATATGCCATT-3′ Reverse 5′-CTCGACGATGTCCGGATACC-3′	723–743 bp 952–932 bp	221 bp
18S	Forward 5′-CGATGCTCTTAGCTGAGTGT-3′ Reverse 5′-AGTCTATGGCAGCATCAAGG-3′	862–881 bp 1176–1157 bp	315 bp

were incubated for up to 24 h at room temperature with a reaction mixture comprised of 0.01 mol l^{-1} sodium phosphate buffer (pH 7.4) containing cortisol (20 μmol l^{-1}) (Sigma, Dorset, UK), nitroblue tetrazolium (NBT) (0.18 mmol l^{-1}) (Sigma, UK), nicotinamide (1.9 mmol l^{-1}) (Sigma, UK) and either $NADP^+$ or NAD^+ (each at a final concentration of 1.5 mmol l^{-1}). The presence of functional 11βHSD protein was localised within each lane by the deposition of purple formazan bands, formed by the sequential transfer of reducing equivalents from cortisol to NBT via the pyridine dinucleotide cofactor ($NADP^+$/NAD^+).

2.4. Immunohistochemistry (IHC)

Concurrent assessments of enzyme activity (described below) revealed that the highest 11βHSD enzyme activities were in pre-pubertal pig testis, caput epididymidis and bulbourethral glands. Hence, IHC was conducted only in these three tissues to localise the expression of 11βHSD1 and 11βHSD2 proteins. Freshly isolated biopsies (1 cm^3) of pre-pubertal testis and reproductive tract tissues were fixed in BDH Gurr® neutral buffered formalin (VWR International, UK) for 1 month. Prior to use, the paraffin embedded sections mounted on Polysine slides (VWR, UK) were dewaxed and rehydrated. Endogenous peroxidase activity was then inhibited by washing with 0.1 mol l^{-1} sodium phosphate buffer ($Na_2HPO_4{\cdot}2H_2O$ and $NaH_2PO_4{\cdot}2H_2O$, Fluka, Biochemica, Germany) containing 20% (v/v) methanol, 0.3% (v/v) Triton-X-100 Sigma-Ultra (Sigma, UK) and 1% (v/v) hydrogen peroxide (Sigma, UK). Non-specific binding was blocked by a 2 h incubation in blocking buffer (0.1 mol l^{-1} sodium phosphate buffer, 0.3% (v/v) Triton-X-100 and 1% (w/v) BSA fraction $V \geq 96\%$ (Sigma, UK)). The sections were incubated overnight at 4 °C with primary antibody (1/250). On day two, the sections were incubated for 2 h at room temperature with fluorescent secondary antibody (1/250) before a 5 min incubation in the dark with 4′,6-diamidino-2-phenylindole (1/5000). Coverslips were mounted with the use of Vectorshield (Vector Laboratories Inc., UK) and all sections were stored at 4 °C in the dark until visualisation.

2.5. 11βHSD bioactivity

Pre-pubertal liver, kidney, testis and regions of reproductive tract were each homogenized separately in 18 ml hypotonic Tris–EDTA lysis buffer (Trizma 0.6 g/l, $MgCl_2$ 0.3 g/l, EDTA 0.6 g/l) followed by the addition of 2 ml potassium chloride (1.5 mmol l^{-1}) to restore isotonicity. Homogenates were centrifuged at 1000 × *g* for 20 min at 4 °C and 1 ml volumes of supernatant were aliquoted for storage at −20 °C. Protein concentrations for each homogenate were determined as above. Prior to assay, tissue homogenates were diluted (using lysis buffer and KCl) to ensure the final protein concentration for each tissue was less than 1500 μg protein/ml. In pilot assays conducted using the three tissues with the highest 11βHSD enzyme activities (testis, caput epididymidis and bulbourethral glands), we had confirmed that at the selected substrate concentrations, the levels of substrate metabolism over 24 h increased linearly in proportion to protein concentration across the range of 0–1500 μg protein/ml.

Each enzyme activity was assayed in triplicate in a final volume of 1 ml PBS per tube containing 10% (v/v) tissue homogenate and 0.4 mmol l^{-1} pyridine nucleotide co-substrates ± 10 mmol l^{-1} glucose-6-phosphate (G6P) as appropriate. Measurements of net 11KSR and net 11βDH activities were initiated by the addition of 0.5 μCi (11.11 nmol l^{-1}) [1,2,(n)-^{3}H]-cortisone or 0.5 μCi (7.25 nmol l^{-1}) [1,2,6,7-^{3}H]-cortisol, respectively.

Following a 24 h incubation in a shaking water bath at 37 °C, 2 ml ice cold chloroform was added to each tube. Tubes were vortexed and subsequently centrifuged at 3000 × *g* for 20 min at 4 °C. The aqueous phase was aspirated and extracts were evaporated to dryness at 45 °C under nitrogen. Steroid residues were resuspended in 30 μl ethyl acetate containing 1 mmol l^{-1} cortisol and 1 mmol l^{-1} cortisone. [^{3}H]-cortisol and [^{3}H]-cortisone were resolved by thin layer chromatography (TLC) in an atmosphere of 92:8 (v/v) chloroform:95% (v/v) ethanol and 11βHSD activities were quantified using a Bioscan System 200 radiochromatogramme scanner (Lablogic, Sheffield, UK).

2.6. 11βHSD enzyme kinetic analysis

The kinetics of cortisol–cortisone metabolism was assessed in homogenates of testis, caput epididymidis and bulbourethral gland from 3 pre-pubertal pigs using radiometric conversion assays as described above. Initial time course assays confirmed linear rates of generation of products over time up to 4 h using either [^{3}H]-cortisone or [^{3}H]-cortisol, each at a final concentration of 100 nmol l^{-1}. Tissue homogenates were subsequently incubated for 2 h at 37 °C in 1 ml PBS containing [^{3}H]-cortisone (12.5, 30, 60 and 100 nmol l^{-1}) plus 0.4 mmol l^{-1} NADPH and 10 mmol l^{-1} glucose-6-phosphate, or with [^{3}H]-cortisol (6.8, 10, 30, 60, 100, 300 and 1000 nmol l^{-1}) plus 0.4 mmol l^{-1} $NADP^+$ or NAD^+.

To estimate kinetic parameters for each tissue, reaction velocity (pmol product formed per unit time) was plotted against substrate concentration for each pyridine nucleotide co-substrate. From this, the reciprocal rates of substrate metabolism (in pmol product/2 h) were plotted against the reciprocal of the substrate concentrations (in nmol l^{-1}) to derive a linear Lineweaver–Burk plot for each enzyme activity in each tissue (insets in Figs. 6–8 inclusive). Estimates of the maximal enzyme velocity (V_{max}) (the reciprocal of the *y*-axis intercept) and of the Michaelis–Menten constant (K_m) (the negative reciprocal of the *x*-axis intercept) were then derived for each enzyme activity by rearranging the equation $1/V = m \times 1/S + c$ (where V = velocity, m = gradient, S = the substrate concentration, and c = the intercept on the *y*-axis).

2.7. Statistical analyses of data

All statistical tests were performed using GraphPad Prism 4 statistical software, version 4.01 (GraphPad Inc., San Diego, CA, USA). Each data set was initially subjected to Kolmogorov–Smirnov tests to confirm that data conformed to Gaussian (normal) frequency distributions. For K_m and V_{max} estimates made under first order kinetic conditions, the estimates of each kinetic parameter were compared between tissues using one-way ANOVA followed by application of the *post hoc* Dunnett's multiple comparison test, where appropriate. $p < 0.05$ was accepted as statistically significant in all tests.

3. Results

3.1. Expression of hsd11b1 and hsd11b2 mRNA

11βHSD1 and 11βHSD2 mRNA transcripts were both expressed in pre-pubertal porcine liver and kidney, testicular and all reproductive tract tissues. However, even after 35 cycles, only very faint bands for both *hsd11b1* and *hsd11b2* were observed in the pre-pubertal caput epididymidis (Fig. 1). When sequenced, all PCR products were identical to the previously published cDNA sequences for porcine 11βHSD1 and 11βHSD2 which the primers were designed to amplify.

3.2. Expression of 11βHSD1 and 11βHSD2 proteins

11βHSD1 and 11βHSD2 immunoreactive protein bands were detected in porcine testis, and all regions of the male reproduc-

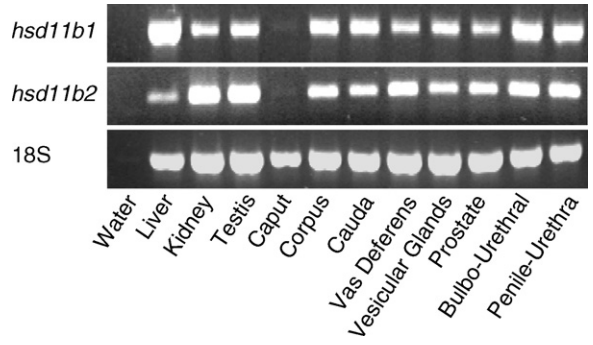

Fig. 1. 11βHSD1 and 11βHSD2 mRNA expression. Representative agarose gels to show 11βHSD1 and 11βHSD2 mRNA expression in the liver, kidney, testis and reproductive tract regions of pre-pubertal pigs. Amplicons were generated using primers specific for *hsd11b1*, *hsd11b2* and 18S cDNA and all bands observed in all tissues with each primer set are of the correct, predicted size; 394, 221 and 315 bp, respectively. The figure presents typical RT-PCR product gels in which the tissue-specific mRNA expression profile was representative of 3 pre-pubertal pigs.

tive tract including accessory sexual organs in pre-pubertal pigs (Fig. 2). In Western blots, the 11βHSD1 antibody recognised a major band at 32 kDa (the expected size) and a minor band at 44 kDa in pre-pubertal male porcine liver and kidney. However, when probing with the 11βHSD1 antibody, only the 44 kDa band was seen in both pre-pubertal porcine testis and all reproductive tract tissues (Fig. 2C). The 11βHSD2 antibody consistently recognised a single protein band at 44 kDa (the expected size) in pre-pubertal porcine liver, kidney, testis and reproductive tract regions (Fig. 2B). A single enzymatic protein band was visualised in porcine liver and kidney, testis and reproductive tract by incubation of gels with NBT plus $NADP^+$ (or NAD^+) (data not shown). Using IHC, 11βHSD1 and 11βHSD2 immunoreactivity were both localised to the interstitial

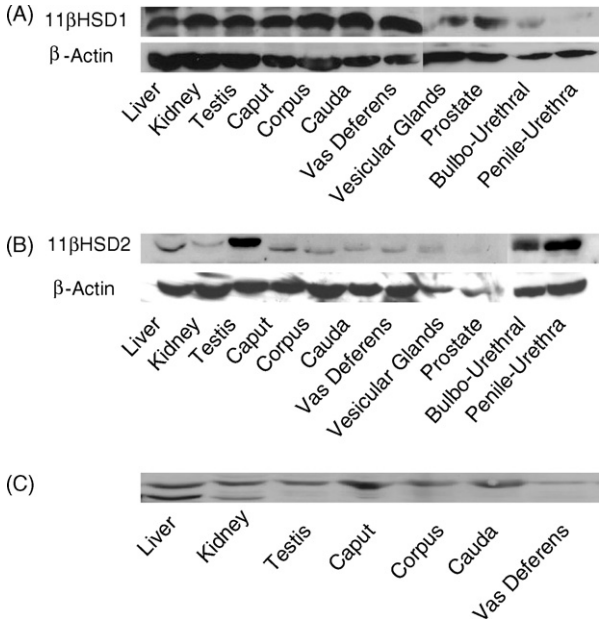

Fig. 2. 11βHSD1 and 11βHSD2 protein expression in pre-pubertal porcine tissues. 11βHSD1 (panel A) and 11βHSD2 (panel B) protein expression in the liver, kidney, testis and reproductive tract regions of pre-pubertal pigs. Western blots were probed with antibodies for 11βHSD1, 11βHSD2 and β-actin. Panel C shows the pattern of immunoreactive proteins recognised in tissue protein preparations by the 11βHSD1 antibody; the antibody recognised a major protein at 32 kDa in pre-pubertal porcine liver and kidney, whereas the same antibody recognised a single 44 kDa protein in the immature testis and reproductive tract tissues. Each panel presents typical Western blots in which the tissue-specific protein expression profile was representative of 3 pre-pubertal pigs.

tissues of the pre-pubertal porcine testis, caput epididymidis and bulbourethral gland (Fig. 3).

3.3. 11βHSD1 and 11βHSD2 bioactivities

Initial screens for 11βHSD enzyme activities were performed over 24 h at limiting substrate concentrations of [1,2,6,7-^{3}H]-cortisol or [1,2,(n)-^{3}H]-cortisone (final assay concentrations of 7.25 and 11.11 nmol l^{-1}, respectively) to maximise the likelihood of observing steroid substrate metabolism. Levels of NADP(H)- and NAD^+-dependent cortisol–cortisone inter-conversion were prominent in pig testis, bulbourethral glands and penile urethra (Figs. 4 and 5). The only other region of pre-pubertal reproductive tract with notable 11βHSD enzyme activity was the caput epididymidis which exhibited NAD^+-dependent activity (Fig. 5B).

In all other regions of the pre-pubertal male pig reproductive tract (corpus and cauda epididymides, vasa deferentia, vesicular and prostate glands), regardless of co-substrate or addition of G6P, the bi-directional 11βHSD enzyme activities in these initial assays were at or below the assay detection limit (≤0.5 pmol product/mg protein 24 h) such that these regions did not merit further investigation (Figs. 4 and 5). The highest level of NADPH-dependent 11KSR activity was observed in pre-pubertal liver homogenate (positive control) when co-incubated with 10 mmol l^{-1} G6P (Fig. 4). Liver homogenates also showed relatively high $NADP^+$-dependent 11βDH activities (Fig. 5A), whereas the major 11βHSD activity in pre-pubertal kidney homogenate was the NAD^+-dependent oxidation of cortisol (Fig. 5B).

3.4. 11βHSD enzyme kinetics

Based on the results described above, all subsequent assessments of enzyme activity were performed in pre-pubertal pig testis, caput epididymidis and bulbourethral glands. To enable valid comparisons of enzyme activities between tissues, all analyses of cortisol–cortisone inter-conversion were performed under first order kinetic conditions.

Estimates of the K_m and V_{max} values with each cofactor in the 3 tissues are tabulated in Tables 2 and 3. For a given pyridine nucleotide co-substrate, each of the estimated enzyme parameters did not differ significantly between homogenates of pre-pubertal pig testes, caput epididymides or bulbourethral glands (one-way ANOVA, $p > 0.05$; Tables 2 and 3). For each tissue, the V_{max} estimates for the rates of cortisol inactivation in the presence of both $NADP^+$ and NAD^+ were generally an order of magnitude higher than the maximal 11KSR enzyme velocities in the presence of NADPH (Figs. 6–8; Tables 2 and 3).

4. Discussion

This study demonstrates that both *hsd11b1* and *hsd11b2* mRNA transcripts are present before puberty in pig testis. This study has also examined tissues from pre-pubertal porcine reproductive tracts and reports for the first time that both *hsd11b1* and *hsd11b2* mRNA transcripts are expressed in caput, corpus and cauda epididymides, vasa deferentia, prostate, vesicular glands, bulbourethral glands and penile urethra. However, faint bands in the caput epididymidis for both *hsd11b1* and *hsd11b2* may suggest very low expression of both enzymes in this tissue prior to puberty in male pigs.

Western blotting showed that the 11βHSD1 antibody bound to a major protein band of the expected size for porcine 11βHSD1 (32 kDa) in pre-pubertal porcine liver and kidney homogenates, but also recognised a minor band at 44 kDa in these tissues. Moreover, when Western blots were probed with the anti-11βHSD1 antibody, pre-pubertal testis and reproductive tract tissues all showed

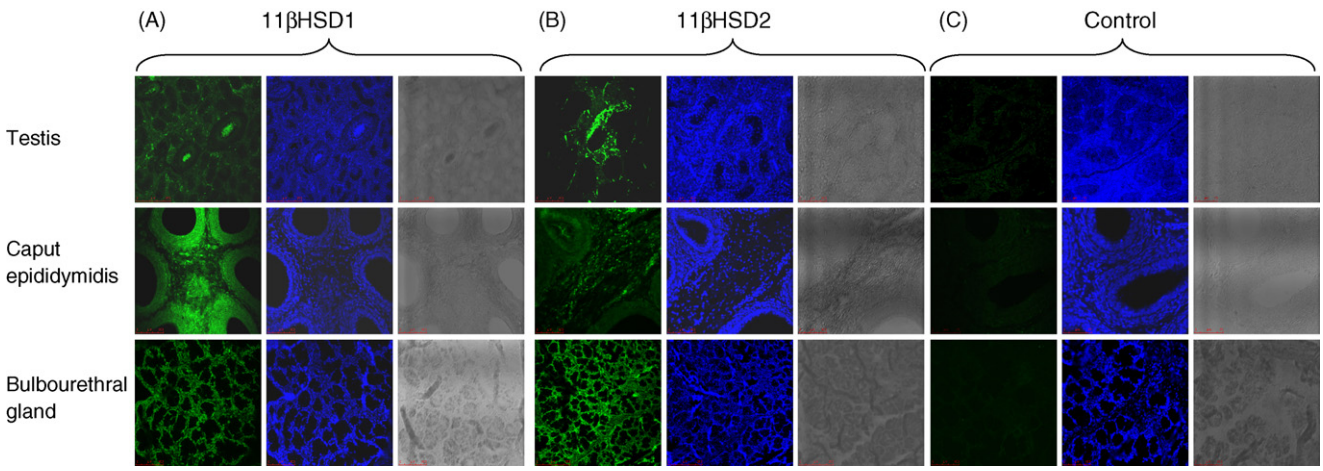

Fig. 3. 11βHSD1 and 11βHSD2 protein localisation in pre-pubertal pig testis, caput epididymidis and bulbourethral gland. Immunofluorescence was clearly seen in the interstitial areas of the testis, in the tissue comprising the caput epididymidis duct, and in the bulbourethral gland epithelium when each tissue was probed for 11βHSD1 (panel A) and 11βHSD2 (panel B). No fluorescence was observed when primary antibodies were omitted as a negative control (panel C). Each panel shows typical immunofluorescence in which the tissue-specific protein localisation is representative of three pre-pubertal pigs.

a single immunoreactive protein band migrating at a consistent size of 44 kDa. This finding is in agreement with our previous study of 11βHSD enzymes in the testis and reproductive tract of the sexually mature boar [25]. Furthermore, additional protein bands have been reported when probing for 11βHSD1 protein in the male rat reproductive tract [26], where immunoreactive bands for testis, caput and cauda epididymides were observed at both 34 kDa (the expected size for rat) and at 48 kDa. Adult rat testis reportedly showed a major band at 34 kDa, whereas the epididymal regions exhibited stronger signals at 48 kDa, suggested to be post-translationally modified forms of the 11βHSD1 enzyme [26]. The larger size products in the rat epididymidis were suggested to represent glycosylated forms of the 11βHSD1 protein [26]. Certainly the 44 kDa immunoreactive band observed in the current study could result from post-translational modifications of porcine 11βHSD1 in the testis and reproductive tract and it is possible that such post-translational modification could modulate the kinetics of the 11βHSD1 enzyme in these tissues. In the pre-pubertal porcine liver, kidney, testis and reproductive tissues, the 11βHSD2 antibody recognised a single immunoreactive protein which migrated as a

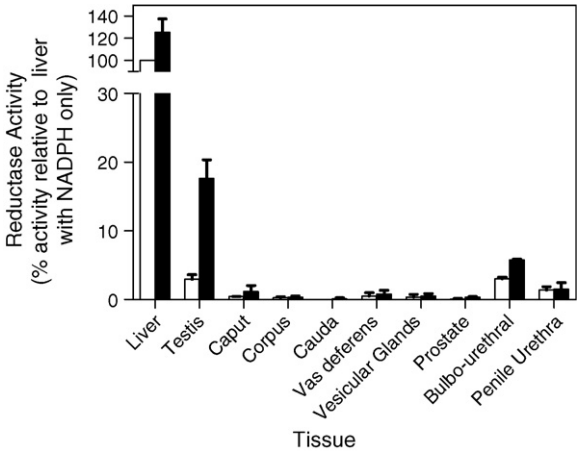

Fig. 4. 11KSR activities in the liver, testis and reproductive tract tissues of pre-pubertal pigs. All data are presented as the mean + SEM values for enzyme activities measured in triplicate within each assay for 3 independent pigs. Results are expressed as a percentage of the 11KSR activity in pre-pubertal pig liver homogenate incubated with 4 μmol l^{-1} NADPH (open bars) or with 4 μmol l^{-1} NADPH plus 10 mmol l^{-1} G6P (closed bars). (Reference hepatic 11KSR activities in the absence of G6P were 31.1 pmol cortisol/mg protein 24 h in pre-pubertal pig liver homogenates.)

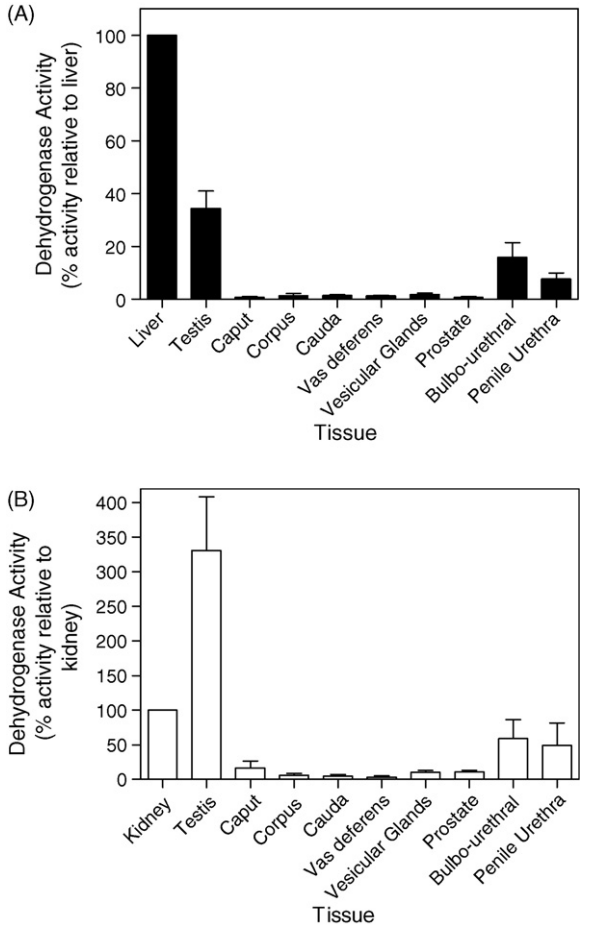

Fig. 5. 11β-Dehydrogenase activity in the liver, testis and reproductive tract tissues of pre-pubertal pigs. Net 11β-dehydrogenase activities were measured in the presence of 4 μmol l^{-1} $NADP^+$ (panel A) or 4 μmol l^{-1} NAD^+ (panel B). All data are presented as the mean + SEM values for enzyme activities measured in triplicate within each assay for 3 independent pigs for each panel. Results are expressed as a percentage of the 11βDH activities measured with $NADP^+$ in pre-pubertal pig liver or NAD^+ in pre-pubertal pig kidney homogenates (panels A and B). Reference 11βDH activities were 14.2 and 4.4 pmol cortisone/mg protein 24 h in pre-pubertal pig liver and kidney homogenates, respectively.

Table 2
Kinetic parameters for NADP(H)-dependent cortisol–cortisone metabolism in pre-pubertal porcine testis, caput epididymidis and bulbourethral gland.

	NADPH-dependent 11KSR activity		NADP⁺-dependent 11βDH activity	
	K_m (nmol l^{-1})	V_{max} (pmol cortisol/2h)	K_m (nmol l^{-1})	V_{max} (pmol cortisol/2h)
Testis	160 ± 86	10.3 ± 5.7	883 ± 425	148.9 ± 95.6
Caput epididymidis	161 ± 36	0.8 ± 0.3	167 ± 45	5.4 ± 2.8
Bulbourethral gland	16 ± 10	0.4 ± 0.2	152 ± 63	4.7 ± 3.7

11KSR activities were measured in the presence of 4 μmol l^{-1} NADPH plus 10 mmol l^{-1} G6P over 2 h; 11βDH activities were measured in the presence of 4 μmol l^{-1} NADP⁺ over 2 h. Data are presented as the mean ± SEM estimates for K_m and V_{max} with each co-substrate in each tissue, where each value was estimated in triplicate for each of 3 independent pre-pubertal pigs. The mean ± SEM ratio of NADP⁺-dependent 11βDH activities to NADPH-dependent 11KSR activities (11βDH:11KSR) were estimated using the matched V_{max} estimates for these 3 pigs. Within each column, estimates did not differ significantly between tissues (one-way ANOVA, $p > 0.05$).

Table 3
Kinetic parameters for NAD⁺-dependent cortisol oxidation in pre-pubertal porcine testis, caput epididymidis and bulbourethral gland.

	NAD⁺-dependent 11βDH activity	
	K_m (nmol l^{-1})	V_{max} (pmol cortisone/2h)
Testis	479 ± 366	182.5 ± 159.0
Caput epididymidis	109 ± 62	5.0 ± 2.0
Bulbourethral gland	47 ± 15	1.8 ± 0.4

Net 11βDH activities were measured in the presence of 4 μmol l^{-1} NAD⁺ over 2 h. Data are presented as the mean ± SEM estimates for K_m and V_{max} with NAD⁺ in each tissue, where each value was estimated in triplicate for each of the 3 independent pre-pubertal pigs. Within each column, estimates did not differ significantly between tissues (one-way ANOVA, $p > 0.05$).

44 kDa protein: the predicted size for porcine 11βHSD2 based on the primary sequence [27].

Published studies of enzyme expression in the adult rat testis have localised the cloned 11βHSD enzymes to the interstitial Leydig cells [18,21]. In the current immunohistochemical studies, both 11βHSD1 and 11βHSD2 proteins were localised to the interstitial tissue of the pre-pubertal porcine testis, consistent with expression in the steroidogenic, interstitial Leydig cells. This would be consistent with a protective role for the 11βHSD enzymes against the deleterious effects of physiological glucocorticoids on LH-induced testosterone production even in the pre-pubertal pig.

In the caput epididymidis, rete testis fluid is reabsorbed. This process of fluid resorption and alteration of the luminal pH are driven by sodium flux which is known to be sensitive to the mineralocorticoid, aldosterone [28–31]. In the current study, we have identified expression of 11βHSD2 in the epithelium of the pre-pubertal caput epididymidis. By virtue of its high affinity dehydrogenase activity, 11βHSD2 is ideally located to restrict the access of cortisol to the mineralacorticoid receptor in this region of the reproductive tract. Given that 11βHSD1 was also expressed in the epithelial cells of the caput epididymidis and that this enzyme was found to act predominantly as a dehydrogenase in our activity studies, it may also be involved in modulating glucocorticoid

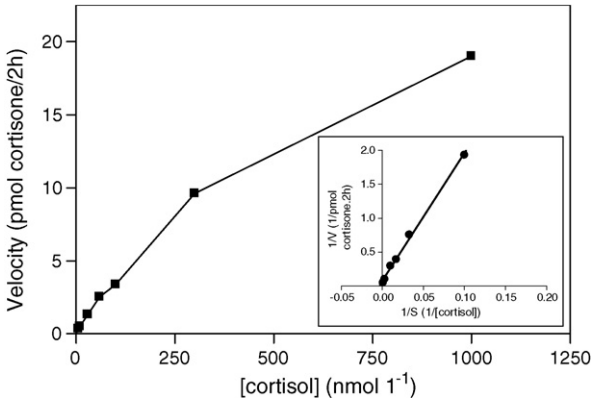

Fig. 7. Kinetic analysis of NADP⁺-dependent 11βDH activities in a pre-pubertal pig testis homogenate. A representative Michaelis–Menten kinetics plot with the corresponding Lineweaver–Burk plot (inset) for net oxidation of cortisol in the presence of 4 μmol l^{-1} NADP⁺ in one pig testis homogenate, from which K_m and V_{max} were estimated for that individual animal. This method of analysis was used to derive estimates of K_m and V_{max} in the presence of each pyridine nucleotide cofactor for homogenates of testes, caput epididymides and bulbourethral glands from 3 pigs in triplicate within each assay.

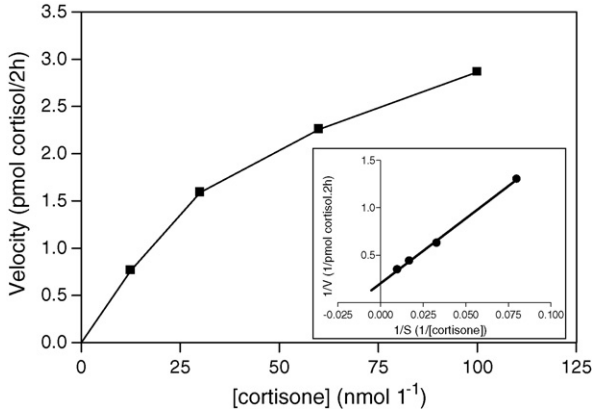

Fig. 6. Kinetic analysis of 11KSR activity in a pre-pubertal pig testis homogenate. A representative Michaelis–Menten kinetics plot with the corresponding Lineweaver–Burk plot (inset) for net reduction of cortisone in the presence of 4 μmol l^{-1} NADPH plus 10 mmol l^{-1} G6P in one pig testis homogenate, from which K_m and V_{max} were estimated for that individual animal. This method of analysis was used to derive estimates of K_m and V_{max} for NADPH-dependent 11KSR activities in homogenates of testes, caput epididymides and bulbourethral glands from 3 pigs in triplicate within each assay.

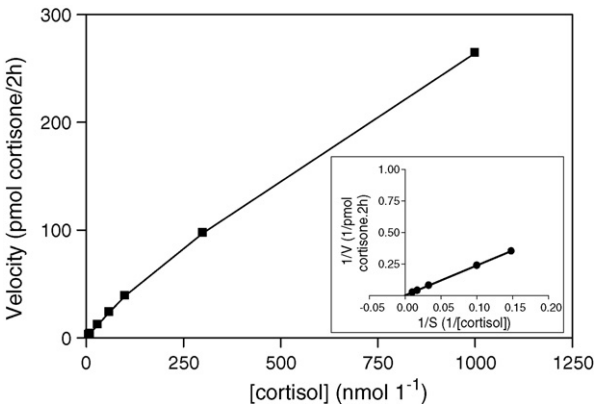

Fig. 8. Kinetic analysis of NAD⁺-dependent 11βDH activities in a pre-pubertal pig testis homogenate. A representative Michaelis–Menten kinetics plot with the corresponding Lineweaver–Burk plot (inset) for net oxidation of cortisol in the presence of 4 μmol l^{-1} NAD⁺ in one pig testis homogenate, from which K_m and V_{max} were estimated for that individual animal. This method of analysis was used to derive estimates of K_m and V_{max} in the presence of each pyridine nucleotide cofactor for homogenates of testes, caput epididymides and bulbourethral glands from 3 pigs in triplicate within each assay.

availability in this region. Both 11βHSD1 and 11βHSD2 immunoreactivities predominated in the mucus-secreting epithelium of the pre-pubertal bulbourethral gland but the mucus itself appeared to be devoid of staining. This localisation would allow the 11βHSD enzymes to modulate the cortisol content of the secretions that are passed into the seminal plasma from the bulbourethral gland.

Kinetic analysis of the 11βHSD enzyme activities in the pre-pubertal porcine testis, caput epididymidis and bulbourethral glands generated apparent K_m values for the porcine 11βHSD enzymes which were appreciably different from the previously published K_m values for the two cloned 11βHSD enzymes in the rat and human. Interestingly, species-specific differences in the active site of 11βHSD1 have been previously reported [32]. In our pre-pubertal pigs the estimated K_m values for the NADPH-dependent metabolism of cortisone were slightly lower than expected in the testis and caput epididymidis (~160 nmol l^{-1}) but were an order of magnitude lower in the bulbourethral glands (16 nmol l^{-1}) than the published K_m values for the 11KSR activity of rat and human 11βHSD1. However, this is unlikely to reflect a simple species difference since we recently reported that these same three tissues taken from post-pubertal boars exhibited similar K_m values for the reduction of cortisone to those previously published K_m values for rat and human 11βHSD1 [25]. Hence, it would appear that cortisone is metabolised to cortisol with higher affinity in pre-pubertal pig tissues than in the corresponding reproductive tissues from sexually mature boars.

The K_m estimates for the $NADP^+$-dependent oxidation of cortisol in pre-pubertal pigs (152–883 nmol l^{-1}) mirrors the pattern that we have previously reported for post-pubertal boars where K_m estimates were two orders of magnitude lower in boar testis, caput epididymidis and bulbourethral gland relative to the published K_m values for the oxidative activities of the rat and human 11βHSD1 enzymes [25]. It should be noted that with enzymes simultaneously running in both directions, the apparent K_m for the oxidation of cortisol will be increased by the opposing 11-oxoreduction of cortisone back to cortisol. Allosteric regulation and/or some other functional modification of the porcine 11βHSD1 protein could explain this relatively high affinity, $NADP^+$-dependent oxidation of cortisol. Molecular interactions between the porcine 11βHSD1 protein and other accessory proteins, such as H6PDH, could also account for these kinetic properties. As the known splice variants of 11βHSD1, 11βHSD1B and 11βHSD1C, have no biological activity [33–35], it is unlikely that these splice variants are capable of altering the kinetic properties of the 11βHSD1 enzyme in the current study. However, there may be novel splice variants of 11βHSD1 in the porcine testis and reproductive tract that are active and which can alter the kinetics of $NADP^+$-dependent cortisol oxidation in these tissues. Indeed, the possible presence of a novel, high affinity $NADP^+$-dependent 11βDH enzyme in both the pre-pubertal pig testis and reproductive tract cannot be excluded, given that the existence of such an enzyme has previously been suggested in the sheep kidney and in both rat and mouse Leydig cells [17,36,37]. The data reported herein has also shown that the NADP(H)-dependent isoform of 11βHSD acts primarily as a dehydrogenase in the pre-pubertal pig testis and reproductive tract tissues.

With regard to NAD^+-dependent cortisol metabolism, the estimated K_m values in the present study were higher in the pre-pubertal testis and caput epididymidis (109–479 nmol l^{-1}) than anticipated based on the published values for the rat and human 11βHSD2 enzymes (40–60 nmol l^{-1}). Our laboratory previously reported similarly increased K_m estimates for NAD^+-dependent oxidation of cortisol in post-pubertal boar testis, caput epididymidis and bulbourethral glands [25]. Hence, in these porcine tissues, there may be a compound acting as a competitive inhibitor of 11βHSD2 that elevates the apparent K_m for NAD^+-dependent cortisol metabolism. A number of physiological compounds have been reported to exert competitive inhibition of 11βHSD2 activity in a variety of cell types [38–42].

When the two cloned 11βHSD enzymes were initially characterised, it was widely accepted that given its low affinity for cortisol (K_m = 1–27 μmol l^{-1}), 11βHSD1 would be ineffective as an 11βDH enzyme. Instead, by virtue of its higher affinity (lower K_m) for cortisone, 11βHSD1 was thought to act primarily as an NADPH-dependent reductase to regenerate active steroid in target cells. In contrast, with a high affinity for cortisol, it was accepted that the NAD^+-dependent 11βHSD2 enzyme inactivates cortisol, particularly in mineralocorticoid target tissues. Over the past decade, these accepted views of the two cloned 11βHSD isoenzymes have been challenged in terms of both the net direction and physiological significance of enzyme activities. Recent studies have revealed that 11βHSD1 can act as either an 11KSR or as an 11βDH enzyme in a tissue- and context-specific fashion dependent on the redox state of NADP(H) in the lumen of the smooth endoplasmic reticulum [8–14]. In terms of kinetics, the affinity of 11βHSD2 is now recognised paradoxically to be too high; the low K_m of 40–60 nmol l^{-1} means that this isoenzyme is effectively saturated at the nadir cortisol concentration of 150 nmol l^{-1} and cannot increase its activity further as the physiological concentration of cortisol rises up to the circadian peak of 600 nmol l^{-1}. (This has led some researchers, such as J.W. Funder, to calculate that even at physiological concentrations, the enzymatic capacity of 11βHSD2 is exceeded such that the promiscuous mineralocorticoid receptors are activated by aldosterone and cortisol molecules in a 1:1 ratio.) Having dismissed the 11βDH activity of 11βHSD1 as physiologically irrelevant by virtue of its high K_m, it is now appreciated that low affinity enzymes usually have high capacity making them ideally placed to respond to an increase in substrate availability with a proportionate increase in their enzymatic activity. In the liver, it is the high capacity–low affinity nature of the GLUT1 glucose transporter, with a K_m for glucose transport far above the normal plasma glucose range, which allows this protein to increase hepatic uptake of glucose in direct proportion to the elevated postprandial glucose concentration delivered via the hepatic portal vein. By analogy, the high K_m of 11βHSD1 ensures that while this enzyme never experiences the high concentrations of cortisol required to attains its maximal velocity, it can moderate its rate of action across the physiological concentration range for cortisol (all be that below the enzyme's K_m), thereby ensuring that a constant proportion of glucocorticoid is inactivated within the cell irrespective of the cortisol concentration. In the present study, we have found that in the testes, caput epididymides and bulbourethral glands of pre-pubertal pigs, either in the presence of added $NADP^+$ or NAD^+, cortisol is inactivated with K_m values that approximate to the physiological concentration range for cortisol. It is this very concordance between the K_m values and the physiological cortisol concentrations which leads us to conclude that these enzymes play a physiological role in limiting the access of glucocorticoids to corticosteroid receptors within these reproductive tissues.

In Leydig cells from pre-pubertal rats, 11βHSD1 appears to act as a predominant 11KSR enzyme [17], and it has been suggested that the consequent increase in local glucocorticoid concentrations may promote Leydig cell differentiation while limiting testosterone output [43]. In the current study of pre-pubertal pig tissues, predominant $NADP^+$-dependent 11βDH activities were observed suggesting that these activities could protect the porcine testis against the adverse effects of glucocorticoids even at this early stage, by decreasing local concentrations of cortisol and allowing testosterone levels to increase. More importantly, in the pig, there are no major differences in glucocorticoid metabolism between pre- versus post-pubertal testis and regions of the reproductive tract. Therefore, the model invoking a role for altered glucocorticoid metabolism in rat puberty, advanced by Hardy et al. [44], does

not appear to apply to puberty in male pigs, and possibly other mammals.

In conclusion, this study has demonstrated that 11βHSD1 and 11βHSD2 enzymes are expressed and are functional in the pig testis and throughout the male reproductive tract prior to puberty. The patterns of expression are consistent with a requirement to modulate the concentration of active glucocorticoids in the interstitial cells of the testis, epithelial cells of the caput epididymidis and the mucus-secreting epithelium of the bulbourethral glands in pre-pubertal pigs. Given the similarities in enzyme kinetics between the current study of pre-pubertal tissues versus our previous findings in corresponding tissues from sexually mature boars [25], this suggests the sexual maturation of male pigs does not require substantial changes in glucocorticoid metabolism by the 11βHSD enzymes within the testis and reproductive tract.

Disclosure

The authors have no conflicts of interest to disclose with respect to this study.

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