11 β -Hydroxysteroid dehydrogenase enzymes in the testis and male reproductive tract of the boar (*Sus scrofa domestica*) indicate local roles for glucocorticoids in male reproductive physiology

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Abstract

11 β -Hydroxysteroid dehydrogenase (11 β HSD) enzymes modulate the target cell actions of corticosteroids by catalysing metabolism of the physiological glucocorticoid (GC), cortisol, to inert cortisone. Recent studies have implicated GCs in boar sperm apoptosis. Hence, the objective of this study was to characterise 11 β HSD enzyme expression and activities in the boar testis and reproductive tract. Although 11 β HSD1 and 11 β HSD2 mRNA transcripts and proteins were co-expressed in all tissues, cortisol–cortisone interconversion was undetectable in the corpus and cauda epididymides, vas deferens, vesicular and prostate glands, irrespective of nucleotide cofactors. In contrast, homogenates of boar testis, caput epididymidis and bulbourethral gland all displayed pronounced 11 β HSD activities in the presence of NADPH/NADP⁺ and NAD⁺, and the penile urethra exhibited NAD⁺-dependent 11 β -dehydrogenase activity. In kinetic studies, homogenates of boar testis, caput epididymidis and bulbourethral gland oxidised cortisol with K_m values of 237–443 and 154–226 nmol/l in the presence of NADP⁺ and NAD⁺ respectively. Maximal rates of NADP⁺-dependent cortisol oxidation were 7.4- to 28.5-fold greater than the V_{max} for NADPH-dependent reduction of cortisone, but were comparable with the rates of NAD⁺-dependent cortisol metabolism. The relatively low K_m estimates for NADP⁺-dependent cortisol oxidation suggest that either the affinity of 11 β HSD1 has been increased or the cortisol inactivation is catalysed by a novel NADP⁺-dependent 11 β HSD enzyme in these tissues. We conclude that in the boar testis, caput epididymidis and bulbourethral gland, NADP⁺- and NAD⁺-dependent 11 β HSD enzymes catalyse net inactivation of cortisol, consistent with a physiological role in limiting any local actions of GCs within these reproductive tissues.

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Introduction

Physiological glucocorticoids (GCs) exert a number of deleterious effects on Leydig cells, including inhibition of testosterone biosynthesis, suppression of luteinising hormone receptor expression and induction of Leydig cell apoptosis (Bambino & Hsueh 1981, Monder *et al.* 1994b, Gao *et al.* 1997). It has also been speculated that GCs might regulate ion and fluid transport across the epithelia of the epididymidis and vas deferens, and affect erectile function in the rat (Waddell *et al.* 2003). Although GCs have recently been reported to induce apoptosis in boar spermatogonia (Claus *et al.* 2005), the physiological relevance of GCs in the boar testis and reproductive tract remains poorly understood at present.

In potential target tissues, the physiological GCs, cortisol and corticosterone, can be reversibly converted to their inactive 11-ketosteroid metabolites (cortisone and 11-dehydrocorticosterone respectively) by 11 β -hydroxysteroid dehydrogenase (11 β HSD) enzymes (Bush *et al.* 1968). Although 11 β HSD1 is a bidirectional enzyme in cell homogenates, this enzyme generally acts predominantly as an 11-ketosteroid reductase (11KSR) in intact cells, such that its primary role appears to be to regenerate cortisol from cortisone (Seckl & Walker 2001, Michael *et al.* 2003). The 11 β HSD1 enzyme has a higher affinity for cortisone (K_m =300 nmol/l) than it does for cortisol (K_m =17–27 μ mol/l) and preferentially utilises NADP(H) as its nucleotide co-substrate. In most tissues (including

liver), hexose-6-phosphate acts in the lumen of the smooth endoplasmic reticulum to maintain a high NADPH:NADP⁺ ratio, which favours the reductase action of 11 β HSD1 (Draper *et al.* 2003, Atanasov *et al.* 2004, Banhegyi *et al.* 2004, Bujalska *et al.* 2005, McCormick *et al.* 2006). However, in steroidogenic gonadal cells (e.g. rat testis Leydig cells, human granulosa–lutein cells, bovine and porcine granulosa cells), 11 β HSD1 exhibits predominantly 11 β -dehydrogenase (11 β DH) activity (Phillips *et al.* 1989, Ge *et al.* 1997, Michael *et al.* 1997, Ge & Hardy 2000, Sunak *et al.* 2007, Thurston *et al.* 2007). This has been attributed to the preferential usage of NADPH for steroid biosynthesis, which could alter the NADPH:-NADP⁺ ratio in favour of the 11 β DH activity of 11 β HSD1 (Michael *et al.* 2003, Ge *et al.* 2005). In contrast to the relatively low affinity, NADP(H)-dependent, bidirectional 11 β HSD1 enzyme, 11 β HSD2 has a relatively high affinity for cortisol (K_m =40–60 nmol/l), only exhibits 11 β DH activity and relies solely on NAD⁺ as its oxidant co-substrate. 11 β HSD2 is expressed at its highest levels in mineralocorticoid target tissues, such as the kidney, colon and parotid salivary gland (Edwards *et al.* 1988, Mercer & Krozowski 1992, Walker *et al.* 1992, Agarwal *et al.* 1994, Albiston *et al.* 1994, Whorwood *et al.* 1995).

In adult rat Leydig cells, the 11 β DH activity of 11 β HSD1 predominates, which coincides with increasing Leydig cell numbers and testosterone production, suggesting protection by 11 β HSD1 from GC-mediated inhibition of steroidogenesis (Phillips *et al.* 1989, Monder *et al.* 1994a, Ge & Hardy 2000). Recent reports in the rat suggest that 11 β HSD2 may work with 11 β HSD1 to inactivate GCs in Leydig cells (Ge *et al.* 2005). In the reproductive tract of the adult male rat, 11 β HSD1 has been localised to the epithelium of the caput epididymidis, vas deferens, vesicular gland and penile urethra (Waddell *et al.* 2003), consistent with a role for 11 β HSD1 in modulating GC actions within these regions. However, 11 β HSD1 knockout mice are fertile, indicating that 11 β HSD1 in the epididymidis cannot be critical for sperm maturation in the mouse (Seckl & Walker 2001). It has been suggested that in the rat epididymidis, high NAD⁺-dependent 11 β DH activities prior to puberty may enable aldosterone to activate the mineralocorticoid receptor and regulate ion and fluid transport (Pearce *et al.* 1986), which could also be the case in post-pubertal animals. 11 β HSD2 has also been identified in the epididymal epithelium and corpus cavernosum of the adult rat penis (Waddell *et al.* 2003).

To date, there has been a single report showing that 11 β HSD enzymes can catalyse cortisol oxidation in adult boar testicular homogenates (Claus *et al.* 2005), but no studies of enzyme expression or activities in the boar reproductive tract. Therefore, the aims of the current study were to characterise the expression and activities of

11 β HSD1 and 11 β HSD2 in the boar testis and throughout the male reproductive tract to assess the region-specific pattern of GC metabolism in these tissues.

Materials and Methods

Tissue collection and storage

Boar liver, kidneys, testes and reproductive tracts were obtained from commercial boars (crosses of White Pietrain, Large White, Landrace, and Duroc breeds) at slaughter. All boars were proven breeders (>22 weeks of age) and were being culled for meat products in accordance with the requirements of the UK Meat and Livestock Commission. (Boar age was confirmed by the abattoir operator and by the assessment of testis size). Liver, kidneys, testes and reproductive tracts were transported on ice to the laboratory where each individual tissue was dissected out, aliquoted into 1 cm³ segments, and either used fresh or snap frozen and stored at –80 °C until use. All studies were conducted on tissues from at least three independent boars.

PCR

Total RNA was extracted from ~30 mg wet weight of each tissue using the RNeasy Mini Kit (Qiagen Ltd) 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 then reverse transcribed using an oligo-dT primer, and 5 μ l first-strand cDNA was used as a template in a PCR using primers specific for porcine *hsd11b1* and *hsd11b2* (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; Table 1). The 18S oligonucleotide primers were designed based on the human 18S sequence (accession number M10098) using nucleotide sequences known to be fully conserved among human, rat, mouse and rabbit (Table 1).

PCR was performed using the Taq PCR Core Kit (Qiagen). 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. Ten microlitres of each PCR were run on a 1.6% (w/v) agarose gel to visualise PCR products. PCR products from kidney, liver and testis were isolated and subjected to the dideoxy-DNA sequencing method to confirm specificity.

Western blot analysis

All tissues were lysed on ice in radioimmunoprecipitation buffer containing 50 mmol/l Trizma and 154 mmol/l NaCl (pH 7.4) with a protease inhibitor cocktail (Mini-

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 (bp)	Product size (bp)
11 β HSD1	Forward 5'-CGCTCTGTATCCTCGGTCTC-3' Reverse 5'-GTGTAGCGTAGAGTGTTCGA-3'	709–720 1102–1082	394
11 β HSD2	Forward 5'-CCAGCAGGAGATATGCCATT-3' Reverse 5'-CTCGACGATGTCCGGATACC-3'	723–743 952–932	221
18S	Forward 5'-CGATGCTCTAGCTGAGTGT-3' Reverse 5'-AGTCTATGGCAGCATCAAGG-3'	862–881 1176–1157	315

complete protease inhibitor, Roche). Protein concentrations were determined using the NanoDrop ND-1000 full spectrum UV/Vis spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). Protein from boar liver, kidney, testis and reproductive tract was diluted with sample buffer to give a final loading concentration of 25 μ g total protein per 25 μ l and separated by SDS-PAGE on a 12.5% (w/v) polyacrylamide gel before being transferred to a polyvinylidene difluoride membrane using a TE22 Mighty Small transphor tank wet transfer unit (Hoefer, San Francisco, CA, USA).

The membranes were incubated overnight at 4 °C in a 1/1000 dilution of sheep anti-human 11 β HSD1 or sheep anti-human 11 β HSD2 polyclonal antibody each in PBS-T containing 5% (w/v) non-fat milk. 11 β HSD antibodies were raised commercially against the human 11 β HSD1 protein sequence (amino acids 19–33) and the human 11 β HSD2 protein sequence (amino acids 137–160 and 334–358; The Binding Site Ltd, Birmingham, UK). Sequence alignment confirmed that the human peptide sequences against which the 11 β HSD1 and 11 β HSD2 antibodies were directed shared 100, 82.6 and 92% amino acid identities with the corresponding regions of porcine 11 β HSD1 and 11 β HSD2 respectively. 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. 11 β HSD proteins were visualised by incubating with ECL detection reagents (Amersham Biosciences) and exposed onto Hyperfilm ECL. To confirm integrity of protein transfer, membranes were stripped and re-probed for β -actin using a polyclonal β -actin antibody (Abcam) at a dilution of 1/5000.

In order to confirm the number of protein bands within each lane exhibiting 11 β HSD activity, samples were also resolved under non-denaturing, non-reducing conditions, such that proteins remained in a native polymerised state. Protein preparations from boar liver, kidney, testis and reproductive tract were each diluted with a non-reducing sample buffer to a final loading concentration of 25 μ g total protein per 25 μ l. Proteins were then resolved on a non-reducing, 12.5% (w/v) polyacrylamide gel. Resolved gels were incubated for up to 24 h at room temperature with a reaction mixture comprising 0.01 mol/l sodium phosphate buffer (pH 7.4)

containing cortisol (0.007 mg/ml; Sigma), nitroblue tetrazolium (NBT; 0.147 mg/ml; Sigma), nicotinamide (0.234 mg/ml; Sigma) and either NADP⁺ or NAD⁺ (each at a final concentration of 1.055 mg/ml). The presence of functional 11 β HSD protein was localised within each lane by the deposition of purple formazan bands, formed by the sequential transfer of reducing equivalents from the cortisol to the NBT via the pyridine dinucleotide cofactor (NADP⁺/NAD⁺).

Immunohistochemistry (IHC)

The concurrent assessments of enzyme activity (described below) revealed the highest 11 β HSD enzyme activities in boar testis, caput epididymidis and bulbourethral gland. Hence, only these three tissues were subjected to IHC to localise the expression of 11 β HSD1 and 11 β HSD2 proteins. Freshly isolated biopsies (1 cm³) of boar testis and reproductive tract tissues were fixed in BDH Gurr neutral buffered formalin (VWR International, Poole, UK) for 1 month. Each biopsy was embedded in a paraffin block and a ribbon (approximately six sections) of 6–7 μ m sections was cut on a microtome. Prior to use, the paraffin-embedded sections mounted on Polysine slides (VWR) were dewaxed and rehydrated by successively placing the slides in 100% (v/v) xylene, 100% (v/v) ethanol, 70% (v/v) ethanol and ddH₂O to complete rehydration. Endogenous peroxidase activity was then inhibited by washing with 0.1 mol/l sodium phosphate buffer (Na₂HPO₄·2H₂O and NaH₂PO₄·2H₂O; Fluka, Biochemica, Germany) containing 20% (v/v) methanol, 0.3% (v/v) Triton X-100 Sigma-Ultra (Sigma) and 1% (v/v) hydrogen peroxide (Sigma). Non-specific binding was blocked by a 2-h incubation in blocking buffer (0.1 mol/l sodium phosphate buffer, 0.3% (v/v) Triton X-100 and 1% (w/v) BSA fraction V $\geq$ 96% (Sigma)). The sections were incubated overnight at 4 °C with primary antibody diluted to a working titre of 1/250 with blocking buffer. On day 2, the sections were incubated for 2 h at room temperature with fluorescent secondary antibody in blocking buffer before a 5-min incubation in the dark with 4',6-diamidino-2-phenylindole, diluted to a working titre of 1/5000 with 0.1 mol/l phosphate buffer. Coverslips were mounted

with the use of Vectorshield (Vector Laboratories Inc., Burlingame, CA, USA) and all sections were stored at 4 °C in the dark until visualisation.

11 β HSD bioactivity

Porcine liver, kidney, testis and regions of reproductive tract were each homogenised separately in 18 ml hypotonic Tris–EDTA lysis buffer (0.6 g/l Trizma, 0.3 g/l MgCl₂, 0.6 g/l EDTA) followed by the addition of 2 ml potassium chloride (1.5 mmol/l) 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 that the final protein concentration for each tissue was <1500 μ g protein/ml. In pilot assays conducted using the three tissues with the highest 11 β HSD enzyme activities (testis, caput epididymidis and bulbourethral gland), 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 μ g 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 pyridine nucleotide co-substrates $\pm$ 10 mmol/l glucose-6-phosphate (G6P) as appropriate. Measurements of net 11KSR and net 11 β DH activities were initiated by the addition of 0.5 μ Ci (11.11 nmol/l) [1,2,(*n*)-³H]cortisone or 0.5 μ Ci (7.25 nmol/l) [1,2,6,7-³H]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 the extracts were evaporated to dryness at 45 °C under nitrogen. Steroid residues were resuspended in 30 μ l ethyl acetate containing 1 mmol/l cortisol and 1 mmol/l cortisone. [³H]cortisol and [³H]cortisone were resolved by thin layer chromatography in an atmosphere of 92:8 (v/v) chloroform:95% (v/v) ethanol, and 11 β HSD activities were quantified using a Bioscan System 200 radiochromatogramme scanner (Lablogic, Sheffield, UK).

11 β HSD enzyme kinetic analysis

The kinetics of cortisol–cortisone metabolism were assessed in homogenates of testis, caput epididymidis and bulbourethral gland from three boars 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 [³H]cortisone or [³H]cortisol, each at a final concentration of 100 nmol/l. Tissue homogenates were subsequently incubated for 2 h

at 37 °C in 1 ml PBS containing [³H]cortisone (12.5, 30, 60 and 100 nmol/l) plus 0.4 mmol/l NADPH and 10 mmol/l G6P, or with [³H]cortisol (6.8, 10, 30, 60, 100, 300 and 1000 nmol/l) plus 0.4 mmol/l NADP⁺ or NAD⁺.

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* Bonferroni multiple comparison test, where appropriate. A *P* value <0.05 was accepted as statistically significant in all tests.

Results

Expression of hsd11b1 and hsd11b2 mRNA

Both 11 β HSD1 and 11 β HSD2 mRNA transcripts were expressed in boar liver, kidney, testis and all reproductive tract tissues (Fig. 1). When sequenced, all PCR products were found to have 100% identity with previously published sequences for porcine 11 β HSD1 and 11 β HSD2.

Expression of hsd11b1 and hsd11b2 proteins

11 β HSD1 and 11 β HSD2 protein bands were detected in boar liver, kidney, testis and all regions of the male reproductive tract (Fig. 2). The 11 β HSD1 antibody recognised a major band at 32 kDa (the expected size) and a minor band at 44 kDa in boar liver and kidney. However, only the 44 kDa band was seen in boar testis and all reproductive tract tissues (Fig. 2C). The 11 β HSD2 antibody consistently recognised a single protein band at 44 kDa (the expected size) in boar testis and reproductive tract regions (Fig. 2B). For all tissues, a single enzymatic protein band was visualised by incubation of non-reducing gels with NBT plus either NADP⁺ or NAD⁺ (data not shown).

IHC localised 11 β HSD1 and 11 β HSD2 immunoreactivity to the interstitial tissues of the boar testis, caput epididymidis duct and bulbourethral gland (Fig. 3).

11 β HSD1 and 11 β HSD2 bioactivities

In initial screens for 11 β HSD enzyme activities, performed over 24 h at limiting substrate concentrations (to maximise the likelihood of observing steroid substrate metabolism), homogenates of boar testis, bulbourethral gland and penile urethra all demonstrated pronounced NADP(H)- and NAD⁺-dependent cortisol–cortisone

Figure 1 11 β HSD1 and 11 β HSD2 mRNA expression in the liver, kidney, testis and reproductive tract regions of boars. 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 shows typical RT-PCR product gels in which the tissue-specific mRNA expression profile was representative of three boars.

interconversion (Figs 4 and 5). The only other region of boar reproductive tract with a substantial 11 β HSD enzyme activity was the caput epididymidis, which exhibited 11KSR activity (Fig. 4). In all other regions of the boar reproductive tract, enzyme activities in these

Figure 2 11 β HSD1 and 11 β HSD2 protein expression in the liver, kidney, testis and reproductive tract regions of boars. Western blots were probed with antibodies raised against peptide sequences in 11 β HSD1 (panels A and C), 11 β HSD2 (panel B) and β -actin (panels A and B). Panel C shows the pattern of immunoreactive proteins recognised in tissue protein preparations by the polyclonal anti-11 β HSD1 antibody; the antibody recognised a major protein at 32 kDa in boar liver and kidney (with minor bands recognised between 32 and 44 kDa), whereas the same antibody recognised a single 44 kDa protein in the boar testis and regions of reproductive tract. Each panel shows typical western blots in which the tissue-specific protein expression profile was representative of three boars.

initial assays were at or below our assay detection limit (≤ 0.4 pmol product/mg protein.24 h) such that these regions did not merit further investigation (Figs 4 and 5).

In terms of positive control tissues, the highest level of NADPH-dependent 11KSR activity was observed in boar liver homogenates co-incubated with 10 mmol/l G6P (Fig. 4), which also showed relatively high NADP⁺-dependent 11 β DH activities (Fig. 5A). The major 11 β HSD activity in boar kidney homogenates was the NAD⁺-dependent oxidation of cortisol (Fig. 5B), although this tissue also displayed moderate levels of NADP⁺-dependent cortisol oxidation (Fig. 5A).

11 β HSD enzyme kinetics

In light of the results described above, all subsequent assessments of enzyme activity were performed in boar testis, caput epididymidis and bulbourethral gland. In order to enable valid comparisons of enzyme activity parameters between tissues, we conducted kinetic analyses of cortisol–cortisone interconversion under first-order kinetic conditions. The reciprocal rates of substrate metabolism (in pmol product/h) were plotted against the reciprocal of the substrate concentrations (in nmol/l) to derive a linear Lineweaver–Burk plot for each enzyme activity in each tissue (Figs 6 and 7), from which we were able to estimate the maximal enzyme velocities ($V_{\max}$) and the Michaelis–Menten constants (K_m ; the concentrations of steroid substrate at which half maximal velocity was attained; Tables 2 and 3). For each enzyme activity, estimates of $V_{\max}$ (the reciprocal of the y-axis intercept) and K_m (the negative reciprocal of the x-axis intercept) were derived by rearranging the equation $1/V = m.1/S + c$ (where V =velocity, m =gradient, S =the substrate concentration and c =the intercept on the y-axis). For a given pyridine nucleotide cofactor, each of the estimated enzyme parameters did not differ significantly between homogenates of boar testis, caput epididymides or bulbourethral glands. While the K_m estimates for all three enzyme activities were similar, ranging from 132 to 443 nmol/l, the $V_{\max}$ estimates for the rates of cortisol inactivation in the presence of NADP⁺ and NAD⁺ (12.2–19.0 pmol cortisone/h and 10.0–11.2 pmol cortisone/h respectively) were consistently higher than the maximal 11KSR enzyme velocities in the presence of NADPH (1.7–2.7 pmol cortisol/h; Tables 2 and 3).

Discussion

Endogenous GCs (cortisol and corticosterone) have established roles in reproductive physiology, which are modulated in several reproductive tissues by one or more of the cloned 11 β HSD enzymes (reviewed by Michael *et al.* 2003). In the male, GCs are known to exert a range of adverse effects on Leydig cell function (Bambino & Hsueh 1981, Monder *et al.* 1994b, Gao

Figure 3 11 β HSD1 and 11 β HSD2 protein localisation in boar testis, caput epididymidis and bulbourethral gland. Immunofluorescence was clearly seen in interstitial areas of the testis, in the tissue comprising the caput epididymidis duct, and in the epithelium of the bulbourethral glands when all tissues were probed for 11 β HSD1 (panel A) and 11 β HSD2 (panel B). As expected, 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 boars.

et al. 1997) and, with specific reference to the boar, they have recently been shown to induce apoptosis in spermatogonia (Claus *et al.* 2005). However, the basic understanding of GC metabolism and actions in the boar testis and reproductive tract is very limited. This study found that although 11 β HSD1 and 11 β HSD2 mRNA and protein were co-expressed in boar testis and all regions of the male reproductive tract, rates of cortisol–cortisone interconversion were at or below the enzyme activity assay detection limit in all tissues apart from the testis, caput epididymidis, bulbourethral gland and penile urethra. In these tissues, we have now characterised, for the first time, the balance and kinetics of cortisol–cortisone metabolism by NADP(H)- and NAD⁺-dependent 11 β HSD enzymes.

While the 11 β HSD2 antibody recognised a single immunoreactive protein in all tissues which migrated at the anticipated size of 44 kDa (Lange *et al.* 2003), the anti-11 β HSD1 antibody recognised two protein bands (at 32 and 44 kDa) in boar liver and kidney, and only the 44 kDa protein in boar testis and all reproductive tract tissues. This finding accords with a previous study which found that anti-11 β HSD1 antibodies recognised proteins that are 14 kDa larger than anticipated in the male rat reproductive tract (Waddell *et al.* 2003). The larger size products in the rat were suggested to represent glycosylated forms of the 11 β HSD1 protein (Waddell *et al.* 2003). Certainly, the 44 kDa band observed in the current study could result from post-translational modifications of porcine 11 β HSD1. More importantly, when we assessed functional 11 β HSD protein bands under non-reducing, non-denaturing conditions, we observed

a single band of NADP⁺-dependent 11 β HSD bioactivity in proteins prepared from each tissue.

Published studies of the rat testis have localised the cloned 11 β HSD enzymes to the interstitial Leydig cells of the testis (Phillips *et al.* 1989, Ge *et al.* 2005). In the current immunohistochemical studies, both 11 β HSD1

Figure 4 11KSR activities in the liver, testis and reproductive tract tissues of boars. Using homogenates of each tissue, net 11KSR activities were measured in the presence of 0.4 mmol/l NADPH either in the absence (open bars) or in the presence (closed bars) of 10 mmol/l G6P. All data are presented as the mean $\pm$ s.e.m. enzyme activities in tissue homogenates from three independent boars, expressed as a percentage of the 11KSR activity measured in parallel in homogenates of boar liver incubated with 0.4 mmol/l NADPH (but without 10 mmol/l G6P). The reference mean hepatic 11KSR activity across these three assays equated to 5.2 pmol cortisol/mg protein.24 h.

Figure 5 11 β -Dehydrogenase activities in the liver, kidneys, testis and reproductive tract tissues of boars. Using homogenates of each tissue, net 11 β -dehydrogenase activities were measured in the presence of either 0.4 mmol/l NADP⁺ (panel A) or 0.4 mmol/l NAD⁺ (panel B). All data are presented as the mean $\pm$ S.E.M. enzyme activities in tissue homogenates from five independent boars, expressed as a percentage of the 11 β DH activities measured in boar liver homogenates incubated with NADP⁺ (panel A) or in boar kidney homogenates incubated with NAD⁺ (panel B). The reference mean 11 β DH activities across these five assays equated to 12.4 pmol cortisone/mg protein.24 h and 16.2 pmol cortisone/mg protein.24 h for panels A and B respectively.

and 11 β HSD2 proteins localised to the interstitial tissue of the boar testis, consistent with expression in the steroidogenic interstitial Leydig cells. In terms of the reproductive tract, both 11 β HSD1 and 11 β HSD2 proteins were also co-expressed in the caput epididymidis duct and bulbourethral gland. The bulbourethral gland consists of mucus-secreting epithelium that lines the acini of this gland. While we observed localisation of both 11 β HSD1 and 11 β HSD2 to this epithelium, the mucus itself appeared to be devoid of staining.

Kinetic analysis of the 11 β HSD enzyme activities in the boar testis, caput epididymidis and bulbourethral gland

Figure 6 Kinetic analysis of 11KSR activity in a boar testis homogenate. A representative Lineweaver-Burk plot for NADPH-dependent 11KSR activity in one boar 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 homogenates of testis, caput epididymidis and bulbourethral gland from three boars.

generated K_m values for the boar enzymes, which were noticeably different from those previously published for the rat and human 11 β HSD enzymes. Specifically, the K_m estimates for the NADP⁺-dependent oxidation of cortisol (237–443 nmol/l) were two orders of magnitude lower in all three tissues than published K_m values for the rat and human 11 β HSD1 enzymes. This relatively high-affinity NADP⁺-dependent activity could result from allosteric regulation and/or some other functional modification of the boar 11 β HSD1 protein, serving to increase the enzyme affinity for cortisol. A post-translational modification of the boar 11 β HSD1 protein would certainly be consistent with the increased mass of the 11 β HSD1 protein band in the Western blots. Alternatively, we cannot exclude the possibility of a novel high-affinity NADP⁺-dependent 11 β DH enzyme in the boar tract, given that the existence of such an enzyme has previously been suggested (Gomez-Sanchez *et al.* 1997).

With regard to NAD⁺-dependent cortisol metabolism, the estimated K_m values in the present study (154–226 nmol/l) were slightly higher than anticipated based on the published values for the rat and human 11 β HSD2 enzymes (40–60 nmol/l). Hence, in these boar tissues, there may be a compound acting as a competitive inhibitor of 11 β HSD2, and so elevating the K_m for NAD⁺-dependent cortisol metabolism. In support of this suggestion, a number of physiological compounds have been reported to exert competitive inhibition of 11 β HSD2 activity in a variety of cell types (Souness *et al.* 1995, Ferrari *et al.* 1996, Gomez-Sanchez *et al.* 1996, Morita *et al.* 1996, Latif *et al.* 2005).

In the rat testis, GCs are known to inhibit testosterone biosynthesis and to induce Leydig cell apoptosis (Bambino & Hsueh 1981, Monder *et al.* 1994b, Gao *et al.* 1997). It has therefore been suggested that 11 β HSD1 acts as a predominant NADP⁺-dependent 11 β DH in adult rat Leydig cells as a mechanism to protect against the deleterious effects of GCs (Phillips *et al.* 1989, Ge & Hardy 2000). Recently, 11 β HSD2 has been shown to contribute to this protective system in the

Figure 7 Kinetic analysis of 11βDH activities in a boar testis homogenate. A representative Lineweaver–Burk plot for net oxidation of cortisol in the presence of 0.4 mmol/l NADP⁺ (panel A) or NAD⁺ (panel B) in one boar 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 testis, caput epididymidis and bulbourethral gland from three boars.

rat testis (Ge *et al.* 2005), although levels of NAD⁺-dependent 11βHSD2 activity appear to be <1% of the NADP⁺-dependent 11βHSD activity in adult rat Leydig cells. Following an initial report of NADP⁺-dependent inactivation of cortisol in the adult boar testis (Claus *et al.* 2005), we now report that boar testes actually co-express 11βHSD1 and 11βHSD2 mRNA transcripts and proteins, and that both of these enzymes appear to be operational in catalysing the interconversion of cortisol with its inert 11-ketosteroid metabolite, cortisone. While the co-expression of both cloned 11βHSD enzymes appears to be common to boar and rat testes, in contrast to the strong preference for NADP⁺ reported for rat Leydig cells (Ge *et al.* 1997, Ge *et al.* 2005), studies of enzyme

activities in boar testis homogenates established that the V_{max} for the NAD⁺-dependent oxidation of cortisol does not differ significantly from the V_{max} estimated in the presence of NADP⁺. This difference in enzyme activities between boar testis homogenates and rat Leydig cells could simply reflect morphological and/or physiological differences between the two species, and in this context, it may be relevant to note that in the boar testis, Leydig cells account for as much as 30% of the testis by volume (as compared with <10% in the rat testis). Alternatively, the overall balance of cortisol–cortisone metabolism in homogenates of boar testis may have been influenced by expression of 11βHSD enzymes in cells other than the Leydig cells (e.g. in peritubular myoid cells, Sertoli cells or spermatogonia/spermatocytes/spermatozoa at various developmental stages).

The caput epididymides are involved in rete testis fluid reabsorption. Hence, we would speculate that in this duct, the high NADP(H)-dependent 11βHSD activities, which we observed in this region, may be important in modulating the potential effects of GCs on ion and fluid transport. In light of the presence of sodium–proton co-transporters, any steroidal control of sodium flux within the caput epididymidis would be expected to alter the luminal pH and hence affect the final maturation of sperm in this duct (Pushkin *et al.* 2000, Phillips & Schultz 2002). Previous studies have also reported a predominant 11KSR activity in the rat cauda epididymidis (Waddell *et al.* 2003). Although the current study found 11βHSD1 and 11βHSD2 mRNA and protein to be co-expressed in the boar corpus and cauda epididymides, vas deferens, vesicular and prostate glands, our initial assessments of enzyme activities indicated that rates of cortisol–cortisone interconversion were barely detectable in these tissues. Therefore, biological roles for the 11βHSD enzymes seem unlikely in these regions of the porcine reproductive tract.

In our initial enzyme activity studies, the boar bulbourethral glands displayed predominant NADP⁺-dependent 11βDH activities, whereas the penile urethra exhibited predominantly NAD⁺-dependent cortisol oxidation. In both of these tissues, the predominant direction of 11βHSD enzyme activity *in vitro* was to inactivate cortisol. The bulbourethral gland secretes

Table 2 Kinetic parameters for NADP(H)-dependent cortisol–cortisone metabolism.

	NADPH-dependent 11KSR activity		NADP ⁺ -dependent 11βDH activity		NADP ⁺ :NADPH
	K_m (nmol/l)	V_{max} (pmol cortisol/h)	K_m (nmol/l)	V_{max} (pmol cortisol/h)	
Testis	280±70	2.7±0.9	254±56	19.0±6.0	9.9±4.3
Caput epididymidis	132±42	1.7±0.4	237±46	12.2±1.7	7.4±0.8
Bulbourethral gland	219±155	2.1±1.6	443±158	18.1±6.2	28.5±14.2

11KSR activities were measured in the presence of 0.4 mmol/l NADPH plus 10 mmol/l G6P over 2 h; 11βDH activities were measured in the presence of 0.4 mmol/l NADP⁺ over 2 h. Data are presented as the mean±S.E.M. estimates of K_m/V_{max} with each cofactor in each tissue, where each value was estimated in triplicate for each of three independent boars. The mean±S.E.M. ratio of NADP⁺-dependent 11βDH activities to NADPH-dependent 11KSR activities (NADP⁺:NADPH) were estimated using the matched V_{max} estimates for these three boars. 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.

	NAD ⁺ -dependent 11 β DH activity	
	K_m (nmol/l)	V_{max} (pmol cortisone/h)
Testis	154 $\pm$ 69	10.0 $\pm$ 2.7
Caput epididymides	226 $\pm$ 125	11.2 $\pm$ 5.8
Bulbourethral glands	208 $\pm$ 35	10.2 $\pm$ 1.5

Net 11 β DH activities were measured in the presence of 0.4 mmol/l NAD⁺ over 2 h. Data are presented as the mean $\pm$ S.E.M. estimates of K_m/V_{max} with NAD⁺ in each tissue, where each value was estimated in triplicate for each of the three independent boars. Within each column, estimates did not differ significantly between tissues (one-way ANOVA, $P > 0.05$).

glycoproteins and antigens previously thought to help in the immune defence of the reproductive tract (Chughtai *et al.* 2005). Our current data raise the possibility that local expression and activity of 11 β HSD1 might also contribute to the protective role of the bulbourethral gland, protecting spermatozoa by decreasing cortisol concentrations in semen prior to ejaculation.

Turning finally to enzyme activities in the positive control tissues for our enzyme activity assays, previous studies have established that in the rat liver, 11 β HSD1 acts predominantly as an 11KSR enzyme (Krozowski & Funder 1983, Seckl & Walker 2001). However, in the current study, both the NADP⁺- and NAD⁺-dependent dehydrogenase activities in boar liver homogenates appeared to be higher than the NADPH-dependent oxo-reductase activity, despite the addition of an excess of exogenous pyridine dinucleotide co-substrates. In the rat kidney, NAD⁺-dependent 11 β DH activities have been reported with the expression of 11 β HSD2 mRNA and protein localised to the distal nephron and renal collecting ducts (Edwards *et al.* 1988, Mercer & Krozowski 1992, Walker *et al.* 1992, Whorwood *et al.* 1995). We now report that, both 11 β HSD1 and 11 β HSD2 mRNA and protein were co-expressed in pig kidneys, which not only exhibited relatively high rates of NAD⁺-dependent cortisol oxidation, but also displayed some NADP⁺-dependent 11 β DH activity.

In conclusion, we have demonstrated that 11 β HSD1 and 11 β HSD2 protein and mRNA are co-expressed in the boar testis and throughout the male reproductive tract. Homogenates of boar testis, caput epididymidis and bulbourethral gland each show predominant 11 β DH activities *in vitro*, with comparable V_{max} estimates using either NADP⁺ or NAD⁺ as the reaction co-substrates. Assuming that our *in vitro* measurements of enzyme activities reflect the balance of GC metabolism *in vivo*, we would speculate that the 11 β HSD enzymes could act in the testis and reproductive tract of the boar to limit the local actions of cortisol. Differences between the kinetics of cortisol oxidation in these boar tissues as compared with the cloned rat and human 11 β HSD enzymes suggest that either the porcine 11 β HSD enzymes have species-specific kinetic properties or the affinities of the boar

enzymes are locally modulated within the boar testis and male reproductive tract tissues.

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