11β-Hydroxysteroid dehydrogenase expression and activities in bovine granulosa cells and corpora lutea implicate corticosteroids in bovine ovarian physiology

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Abstract

Cortisol–cortisone metabolism is catalysed by the bi-directional NADP(H)-dependent type 1 11β-hydroxysteroid dehydrogenase (11βHSD1) enzyme and the oxidative NAD^+-dependent type 2 11βHSD (11βHSD2). This study related the expression of 11βHSD1 and 11βHSD2 enzymes (mRNA and protein) to net 11-ketosteroid reductase and 11β-dehydrogenase (11β-DH) activities in bovine follicular granulosa and luteal cells. Granulosa cells were isolated from follicles of <4, 4–8, >8 and >12 mm in diameter in either the follicular or luteal phase of the ovarian cycle. Luteal cells were obtained from corpora lutea (CL) in the early non-pregnant luteal phase. Enzyme expression was assessed by reverse transcription-PCR and western blotting, while enzyme activities were measured over 1 h in cell homogenates using radiometric conversion assays with 100 nM [^{3}H]cortisone or [^{3}H]cortisol and pyridine dinucleotide cofactors. Irrespective of follicle diameter, the expression of 11βHSD2 and NAD^+-dependent oxidation of cortisol predominated in granulosa cells harvested in the follicular phase. In contrast, in granulosa cells obtained from luteal phase follicles and in bovine luteal cells, expression of 11βHSD1 exceeded that of 11βHSD2 and the major enzyme activity was $NADP^+$-dependent cortisol oxidation. Increasing follicular diameter was associated with progressive increases in expression and activities of 11βHSD2 and 11βHSD1 in follicular and luteal phase granulosa cells respectively. In follicular phase granulosa cells from antral follicles <12 mm, 11βHSD1 migrated with a molecular mass of 34 kDa, whereas in the dominant follicle, CL and all luteal phase granulosa cells, a second protein band of 68 kDa was consistently detected. In all samples, 11βHSD2 had a molecular mass of 48 kDa, but in large antral follicles (>8 mm), there was an additional immunoreactive band at 50 kDa. We conclude that 11βHSD2 is the predominant functional 11βHSD enzyme expressed in follicular phase granulosa cells from growing bovine antral follicles. In contrast, in bovine granulosa cells from dominant or luteal phase follicles, and in bovine luteal cells from early non-pregnant CL, 11βHSD1 is the major glucocorticoid-metabolising enzyme. The increasing levels of cortisol inactivation by the combined $NADP^+$- and NAD^+-dependent 11β-DH activities suggest a need to restrict cortisol access to corticosteroid receptors in the final stages of follicle development.

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Introduction

The current decline in the fertility of domestic livestock has major financial implications for the farming industry (Lamming *et al.* 1998), resulting in a need to investigate aspects of the reproductive system that could be manipulated to increase productivity. For many years, it has been recognised that increased output of the glucocorticoid cortisol from the cortex of the adrenal gland is associated with impaired gonadal function and decreased fertility. This endocrine interaction between the adrenal glands and the gonads is particularly well documented in conditions such as stress-related infertility (Michael & Cooke 1994). At ovulation, the levels of cortisol within follicular fluid rise due, at least in part, to displacement of cortisol from corticosteroid-binding globulin by progesterone (Andersen 1990). It has been proposed that this periovulatory rise in ovarian glucocorticoids may directly influence the oocyte quality and control the inflammatory process of ovulation (Hillier & Tetsuka 1998, Andersen *et al.* 1999).

In a diverse range of tissues, cortisol is metabolised to its inert 11-ketosteroid metabolite cortisone by isoforms of the enzyme 11β-hydroxysteroid dehydrogenase (11βHSD; reviewed by White *et al.* 1997, Kotelevtsev *et al.* 1999, Seckl & Walker 2001, Tomlinson *et al.* 2004, Draper & Stewart 2005). To date, two 11βHSD enzymes have been

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cloned. Type 1 11βHSD, which is ubiquitously expressed, is a relatively low-affinity enzyme (K_m for cortisol = 27 μM) that appears to act predominantly as an NADPH-dependent 11-ketosteroid reductase (11KSR) catalysing the reduction of cortisone to cortisol (Lakshmi & Monder 1988, Agarwal *et al.* 1989, Tannin *et al.* 1991). In contrast, type 2 11βHSD is an NAD^+-dependent high-affinity dehydrogenase enzyme (K_m for cortisol = 30–60 nM) that catalyses the inactivation of cortisol (Brown *et al.* 1993, Rusvai & Naray-Fejes-Toth 1993, Agarwal *et al.* 1994, Albiston *et al.* 1994).

Previous studies have reported the expression of mRNA encoding both of the cloned 11βHSD enzymes in human, rat and bovine ovaries (Waddell *et al.* 1996, Michael *et al.* 1997, Tetsuka *et al.* 1997, 1999, 2003, Ricketts *et al.* 1998, Thurston *et al.* 2003). Molecular studies indicate that the major 11βHSD isoenzyme expressed in the ovary depends on the prevalent hormonal milieu and/or functional phenotype of the cells. In the ovaries of rats and humans, the granulosa cells lining either preantral or immature antral follicles exclusively express 11βHSD2 (Tetsuka *et al.* 1997, 1999, Ricketts *et al.* 1998). However, following exposure to an ovulatory concentration of luteinizing hormone, these luteinising granulosa cells cease to express 11βHSD2 and switch to the expression of 11βHSD1 (Michael *et al.* 1997, Tetsuka *et al.* 1997, 1999, Thurston *et al.* 2003). As human granulosa cells luteinise in culture, the increased expression of 11βHSD1 mRNA is mirrored by a progressive increase in 11βHSD1 protein expression (Thurston *et al.* 2003). Consistent with this switch from 11βHSD2 to 11βHSD1 expression, luteinised human granulosa cells exhibit increased 11KSR activity (Michael *et al.* 1997, Tetsuka *et al.* 1997).

The cow offers a model system in which to study changes in the expression and/or activities of the cloned 11βHSD enzymes in a mono-ovulatory species across the ovarian cycle. Moreover, this model affords an opportunity to assess hormonal regulation of the 11βHSD enzyme by comparing glucocorticoid metabolism between cells from size-matched bovine follicles in the follicular phase (exposed to high concentrations of oestradiol and inhibin) versus luteal phase (high local concentrations of progesterone and oxytocin). To date, the only published information regarding the bovine ovary is limited to a report of mRNA expression (Tetsuka *et al.* 2003). While this prior report noted differences in the level of expression of 11βHSD1 mRNA between healthy 'oestrogen active' and atretic 'oestrogen inactive' granulosa cells, the primary focus of the study was a comparison of 11βHSD transcript levels between the early, mid and late bovine corpora lutea (CL). Therefore, the aim of the present study was to investigate whether the expression of the cloned 11βHSD enzymes and, more importantly, the corresponding enzyme activities change in bovine granulosa cells during follicle development and in the formation of the early CL.

Materials and Methods

Isolation of granulosa and luteal cells from the bovine ovary

Pairs of bovine ovaries were obtained from a local abattoir within 2 h of slaughter. On collection, each pair of ovaries was placed into a separate bag to ensure that pairs of ovaries did not become separated. (This was imperative in allowing us to assess the presumptive stage of the oestrous cycle for each ovarian follicle;) Pairs of ovaries were transported on ice to the Royal Veterinary College. Granulosa cells were recovered from follicles of different sizes (diameter < 4, 4–8, 8–11 and > 12 mm). Where an ovary contained a single follicle > 12 mm in diameter (presumed to be a dominant follicle), and either the ipsilateral or contralateral ovary contained no evidence of an active CL, the ovary was assumed to have been collected during the follicular phase. Where an ovary contained an active CL, the ovary was assessed as having been collected during the luteal phase and any follicles present on either the ipsilateral or contralateral ovary were defined for the purpose of this study as luteal phase follicles. In each case, follicular fluid was aspirated from the ovarian follicles and visually assessed for opacity before being discarded. In light of Tetsuka's prior data (Tetsuka *et al.* 2003), only those follicles that were assessed as healthy by visual inspection (i.e. well-vascularised follicles showing no evidence of collapse or leakage and having clear follicular fluid) were used in this study. The follicles were then flushed with 1:1 Dulbecco's modified Eagle's medium (DMEM):Ham's F_{12} medium supplemented with penicillin (87 000 IU/l) and streptomycin (87 mg/l), and granulosa cells were extracted by lightly scraping the superficial interior surface of the follicle wall with a sterile plastic inoculation loop taking care not to rupture the basement membrane. (Culture media and supplements were purchased from Invitrogen Life Technologies.) Media containing these granulosa cells was subsequently aspirated from the follicle. CL tissue was dissected from bovine ovaries in the luteal phase. Non-pregnant CL, assessed as being in the early luteal phase by morphological assessment according to the criteria of Ireland *et al.* (1980), were hemisected and luteal cells isolated as previously reported by Sakka *et al.* (1997). Granulosa and luteal cell numbers were estimated using a haemocytometer and viabilities were assessed by the exclusion of Trypan blue dye (Merck).

11βHSD mRNA expression in the developing follicle and CL during the ovarian cycle

Total RNA was extracted from bovine granulosa and luteal cells using a guanidine thiocyanate buffer system according to the manufacturer's instructions (GenElute Mammalian total RNA kit; Sigma–Aldrich). RNA concentrations were determined spectrophotometrically. Reverse transcription PCR (RT-PCR) was carried out in a one-step RT-PCR protocol using the AccessQuick RT-PCR kit (Promega). Briefly, 1 μg RNA was reverse transcribed in a total volume of

50 μl at 48 °C for 45 min. cDNA was then amplified by PCR using exon-spanning oligonucleotide primers designed for bovine 11βHSD1 (forward: 5′-GCCAGCAAGGGAAT CGGAAG-3′ and reverse: 5′-GCATTAAATATCCCAGC AACTG-3′) and 11βHSD2 (forward: 5′-CTGTGACT CGCTTTTGACAAC-3′ and reverse: 5′-CAGGAGAGG CCCAGAGGTTCAC-3′). (Intron-spanning primers were designed based on nucleotide sequences previously confirmed, in our laboratory, to amplify 11βHSD1 and 11βHSD2 transcripts from the rat, modified in accordance with the corresponding bovine 11βHSD nucleotide sequences: Genbank accession numbers AF548027 and NM174642 respectively.) Each sample also included internal control primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward: 5′-CCATCACCAT CTTCCAGGAGCG-3′ and reverse: 5′-TCCCGTGACG ATTACTC-3′) to facilitate semiquantitative analysis of the 11βHSD transcripts. (As these were internal control primers, GAPDH was amplified in parallel to the 11βHSD transcripts through the same number of PCR cycles.) Blank samples of cDNA were also run in each experiment as negative controls. Thermocycler parameters were 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min. The number of PCR cycles was optimised to allow semiquantitative evaluation of each sample, ensuring that cDNA transcription was within the exponential phase of amplification. Amplified cDNA was visualised on a 2% (w/v) agarose gel in the presence of ethidium bromide and each sample was quantified relative to GAPDH using a Gel Doc 1000 system and Molecular Analyst software (Bio-Rad Laboratories, Inc.). Each primer and amplicon were subjected to dideoxynucleotide sequencing (MWG Biotech, London, UK).

11βHSD protein expression in the developing follicle and CL during the ovarian cycle

Following collection, lysates of granulosa and luteal cells were prepared for immunoblotting as previously described (Dewi *et al.* 2002, Thurston *et al.* 2003). In brief, cells were centrifuged at 1000 ***g*** for 15 min at 4 °C and the DMEM:-Ham's F_{12} medium removed. Cell pellets were reconstituted in ice-cold PBS (pH 7·5; Life Technologies) containing sodium orthovanadate (Na_3VO_4; 200 μM; Sigma) and centrifuged at 1000 ***g*** for 15 min at 4 °C. PBS was removed and the cell pellets were lysed in buffer (1 ml/1×10^6 cells) containing Tris–HCl (63·5 mM, pH 6·8; Sigma), 10% (v/v) glycerol (Sigma), 2% (w/v) SDS (Sigma), Na_3VO_4 (1 mM), 4-(2-aminoethyl)benzene sulphonyl fluoride (1 mM; Sigma) and leupeptin (50 μg/ml; Sigma). The cells were then incubated on ice for 10 min. The protein content of cell lysates was quantified using the bicinchoninic acid (BCA) method according to the manufacturer's instructions (Pierce Chemical Company, Chester, Cheshire, UK). Before gel electrophoresis, samples were prepared for loading by denaturing at 95 °C. Prior to loading, bromophenol blue (Life Technologies) and β-mercaptoethanol (Sigma) were added to the lysates to give final concentrations of 5% (v/v) and 0·02% (w/v) respectively. Human embryonic kidney (HEK) cells stably transfected to express either 11βHSD1 or 11βHSD2 (Bujalska *et al.* 1997) were used as positive controls for 11βHSD in all western blotting studies. HEK cell lysates were prepared as described for bovine ovarian cells.

Proteins from granulosa cell, luteal cell and HEK cell lysates (100 μg protein/lane) were separated using 10% (v/v) SDS-PAGE and transferred to an immobilon polyvinylidene difluoride membrane using a semi-dry western blotting apparatus (Bio-Rad Laboratories, Inc.). Prestained molecular weight markers were run and transferred in parallel lanes (Sigma). Non-specific antibody binding was blocked by incubation of the membranes for 2 h at room temperature in 10% (w/v) BSA in Tris-buffered saline–Tween (TBST; 50 mM Tris, 150 mM NaCl and 0·02% (v/v) Tween 20 (Sigma); pH 7·4). The membranes were incubated overnight at 4 °C with the appropriate primary antibody to 11βHSD at a dilution of 1/100 in 10% (w/v) BSA:TBST. Polyclonal primary antibodies to 11βHSD1 and 11βHSD2 were raised commercially (The Binding Site Ltd, Birmingham, UK) in sheep against specific peptide sequences of the human enzyme isoforms (11βHSD1 amino acids 19–33; 11βHSD2 amino acids 137–160 and 334–358). After washing with TBST (6×10 min), the membranes were incubated with donkey anti-sheep peroxidase-conjugated IgG (Sigma) for identification of 11βHSD proteins at a dilution of 1/10 000 in 0·2% (w/v) BSA/TBST for 1 h, followed by further washes in TBST (8×10 min). Immunoreactive proteins were visualised using an enhanced chemiluminescent (ECL) detection system according to the manufacturer's instructions (Amersham). After removal of excess ECL reagent, membranes were exposed to X-ray Hyperfilm (Amersham) for 1–5 min. Where indicated, densitometric analysis of immunoblots was performed using a Gel Doc 1000 system and Molecular Analyst software (Bio-Rad Laboratories, Inc.).

To correct for any minor variations in protein loading or transfer, all 11βHSD protein bands were standardised relative to the housekeeper protein GAPDH. To re-probe the membrane for GAPDH expression, bound 11βHSD primary antibody was stripped from the membrane. The membrane was incubated in stripping buffer containing Tris–HCl (63·5 mM, pH 6·7; Sigma), 2% (w/v) SDS (Sigma) and 0·7% (v/v) β-mercaptoethanol (Sigma) at 50 °C for 20 min. Following incubation, the blot was transferred for washing in TBST (4×10 min). GAPDH was probed using the same protocol that was used to determine 11βHSD expression, but using a murine anti-human GAPDH primary antibody (Sigma) and a rabbit anti-mouse conjugated secondary IgG (Sigma).

11βHSD activities in the developing follicle and CL during the ovarian cycle

Granulosa and luteal cells were assessed for 11KSR and 11β-dehydrogenase (11β-DH) activities using a modification of the radiometric conversion assay for glucocorticoid oxidation

previously described in our laboratory (Michael *et al.* 1997, Thurston *et al.* 2003). For each assay, cells were centrifuged at 1000 ***g*** for 15 min at 4 °C and the DMEM:Ham's F_{12} medium discarded. The cell pellets were washed in ice-cold PBS (pH 7·5; Life Technologies) and centrifuged at 1000 ***g*** for a further 15 min at 4 °C. PBS was removed, the cell pellets were homogenised in hypotonic Tris–EDTA lysis buffer (2·25 ml/1×10^6 cells; Rusvai & Naray-Fejes-Toth 1993, Sewell *et al.* 1998, Thompson *et al.* 2000) and isotonicity was restored by the addition of 1·5 M KCl (0·25 ml/1×10^6 cells; Merck). One hundred microlitres of each homogenate were transferred to glass screw-cap culture tubes, to each of which were added 600 µl PBS (Life Technologies). Triplicate tubes were also prepared as assay blanks containing 100 µl BSA solution (1 mg/ml prepared in PBS) in place of the ovarian cell homogenates. Each triplicate set of tubes was preincubated for 30 min at 37 °C in a gyratory water bath. To initiate the 11KSR and 11β-DH assays, each tube received 100 µl pyridine nucleotide (NADPH, $NADP^+$ or NAD^+; 4 mM in PBS; Sigma) and 100 µl PBS containing either 0·1 µCi [1,2,6,7-^{3}H]cortisone (11KSR assay) or 0·5 µCi [1,2,6,7-^{3}H]cortisol (11β-DH assay; Amersham) and unlabelled cortisol or cortisone (Sigma), each to a final steroid concentration of 100 nM. The tubes were then returned to the water bath for 60 min, after which reactions were terminated by the addition of 2 ml ice-cold chloroform (Merck) to each tube. To partition the organic and aqueous phases, these tubes were centrifuged at 1000 ***g*** for 30 min at 4 °C. After aspirating the aqueous supernatant, the organic extracts were evaporated to dryness under nitrogen at 50 °C. The steroid residues were re-suspended in 20 µl ethyl acetate containing 1 mM cortisol and 1 mM cortisone (Sigma) and were resolved by thin layer chromatography (TLC) using Silica 60 TLC plates (Merck) in an atmosphere of 92:8 (v/v) chloroform:95% (v/v) ethanol (Merck). After quantifying [^{3}H]cortisol and [^{3}H]cortisone using a Bioscan 200 TLC radiochromatogram scanner (LabLogic, Sheffield, UK), 11KSR activities were calculated as pmol cortisone reduced to cortisol and 11β-DH activities as pmol cortisol oxidised to cortisone over 60 min. 11βHSD activities were standardised per milligram protein in the cell homogenates, where protein concentrations were measured using the BCA assay, as per the manufacturer's instructions (Pierce).

Statistical analyses

Each experiment was repeated using cells from three to five individual animals. Where levels of mRNA or of enzyme protein expression were quantified, histograms show mean (± S.E.M.) optical densities for the RT-PCR products or the western blot protein bands, each expressed relative to the corresponding GAPDH mRNA/protein bands. In addition, a representative PCR agarose gel or immunoblot is also shown as appropriate. Each data set was subjected to one-way ANOVA (with repeated measures across individual PCR/blots) followed by Tukey–Kramer multiple comparisons. Enzyme activities, also presented as mean (± S.E.M.) values, were similarly subjected to one-way ANOVA followed by the Tukey–Kramer multiple comparisons as a *post hoc* test.

All statistical evaluations were performed using GraphPad Prism2 software (GraphPad, Inc., San Diego, CA, USA) and $P<0{\cdot}05$ was accepted as statistically significant in each test.

Results

11βHSD mRNA transcripts

PCR of cDNA prepared from granulosa and luteal cells using 11βHSD1 and 11βHSD2 primers generated amplicons of 575 and 1135 bp respectively (Fig. 1A). Sequencing of these amplicons confirmed that they had been derived from mRNA encoding bovine 11βHSD1 and 11βHSD2. Expression of 11βHSD1 mRNA in granulosa cells appeared to increase with follicle diameter in both phases of the ovarian cycle (ANOVA $F=55{\cdot}08$, $P<0{\cdot}0001$) and was greatest in the CL (Fig. 1B). Expression of mRNA encoding 11βHSD2 also appeared to increase with follicle diameter with the highest levels of mRNA expression in granulosa cells aspirated from large antral follicles (ANOVA $F=72{\cdot}93$, $P<0{\cdot}0001$; Fig. 1C). There was no detectable 11βHSD2 mRNA expression in granulosa cells from small antral follicles in the luteal phase of the ovarian cycle (Fig. 1A).

11βHSD proteins

Bovine granulosa and luteal cells were each found to express both 11βHSD1 and 11βHSD2 proteins. In western blots, the 11βHSD1 antibody recognised bands of 34 and 68 kDa, while the 11βHSD2 antibody recognised bands of 48 and 50 kDa (Fig. 2A). Alignment of primary amino-acid sequences, performed using the basic local alignment search tool (BLAST) confirmed that there were no bovine proteins other than 11βHSD1 and 11βHSD2 that should have been recognised by the respective anti-human antibodies.

Irrespective of the phase of the ovarian cycle, within granulosa cells expression of both the 34 kDa 11βHSD1 protein and the 48 kDa 11βHSD2 protein increased significantly with follicular diameter (ANOVA $F=144{\cdot}0$, $P<0{\cdot}0001$ for 11βHSD1; ANOVA $F=39{\cdot}37$, $P<0{\cdot}0001$ for 11βHSD2; Fig. 2). Expression of the 34 kDa 11βHSD1 protein was higher in granulosa cells aspirated from medium-sized antral follicles (4–8 mm in diameter) and from large antral follicles (>8 mm in diameter) in the luteal phase of the ovarian cycle than in cells aspirated from size-matched follicles in the follicular phase of the cycle (Fig. 2B). Conversely, expression of the 48 kDa 11βHSD2 protein was higher in granulosa cells harvested during the follicular phase than at the corresponding stages of folliculogenesis in the luteal phase with the highest expression of 11βHSD2 protein seen in the dominant follicle (Fig. 2C). In the corpus luteum, there was higher expression of the 11βHSD1 protein than of the 11βHSD2 protein (Fig. 2).

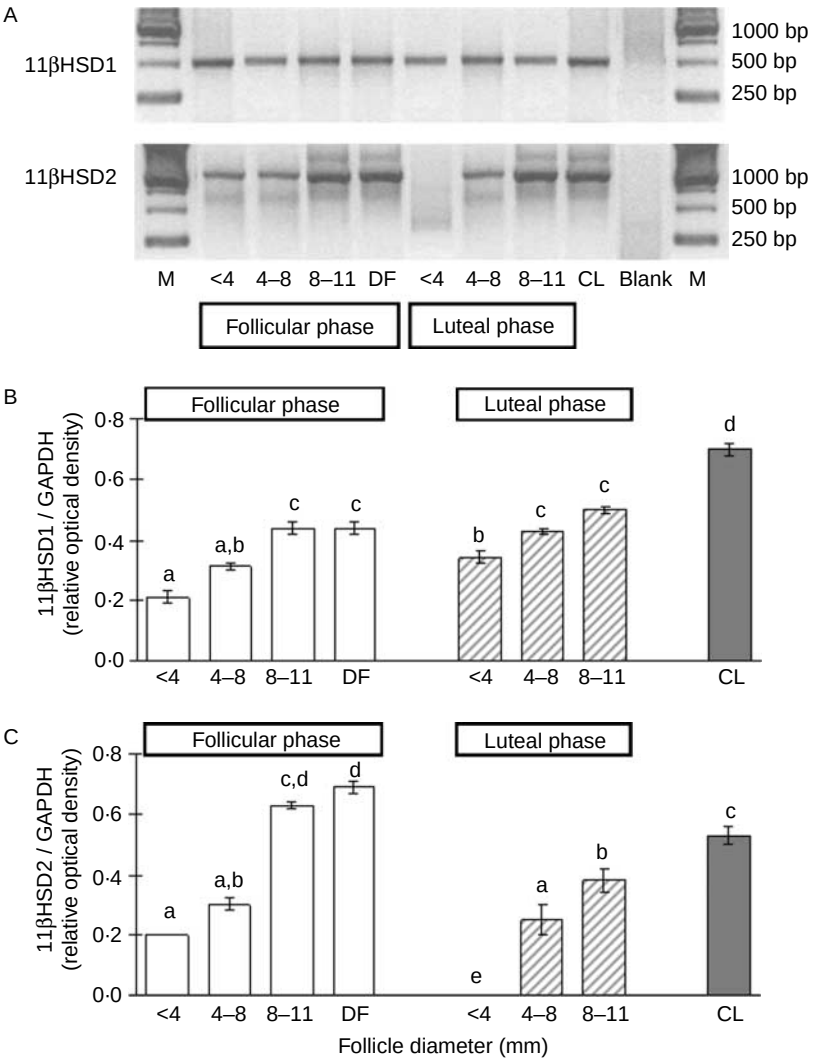

Figure 1 Expression of 11βHSD mRNA in granulosa and luteal cells during the follicular and luteal phases of the bovine ovarian cycle. Granulosa cells were extracted from follicles of different diameters (<4, 4–8 and 8–11 mm; the dominant follicle, DF) in the follicular phase (open bars) and the luteal phase (hatched bars) of the ovarian cycle. Luteal cells were prepared from the early non-pregnant corpus luteum (CL; filled bar). (A) Representative RT-PCR gels for 11βHSD1 and 11βHSD2; M, molecular weight markers. (B) 11βHSD1 mRNA expression. (C) 11βHSD2 mRNA expression. In (B) and (C), each data point represents the mean ± S.E.M. values for five independent experiments; Within each panel, mean values sharing a common superscript do not differ significantly, whereas $P<0{\cdot}05$ for means that do not share a common superscript.

The immunoreactive protein band at 68 kDa was identified by the anti-11βHSD1 antibody in luteal cells, granulosa cells from dominant follicles and granulosa cells from all luteal phase follicles, irrespective of their diameter (Fig. 2A). As for the 34 kDa isoform of 11βHSD1, expression of the 68 kDa 11βHSD1 protein band increased significantly with follicular diameter (ANOVA $F=137{\cdot}0$, $P<0{\cdot}0001$; Table 1). Western blotting of ovarian tissue with the 11βHSD2 antibody also revealed a second protein band of 50 kDa in granulosa cells obtained from all follicles larger than 8 mm, including the dominant follicle, although only in the follicular phase of the ovarian cycle (Fig. 2A; Table 1). This 50 kDa immunoreactive protein band was absent in all luteal phase follicles and CL (Fig. 2A; Table 1).

βHSD enzyme activities

In bovine granulosa and luteal cells, all the three assayed 11βHSD enzyme activities (i.e. the NADPH-dependent reduction of cortisone, the $NADP^+$-dependent oxidation of cortisol and the NAD^+-dependent oxidation of cortisol) were above the appropriate assay detection limits. Moreover, all three

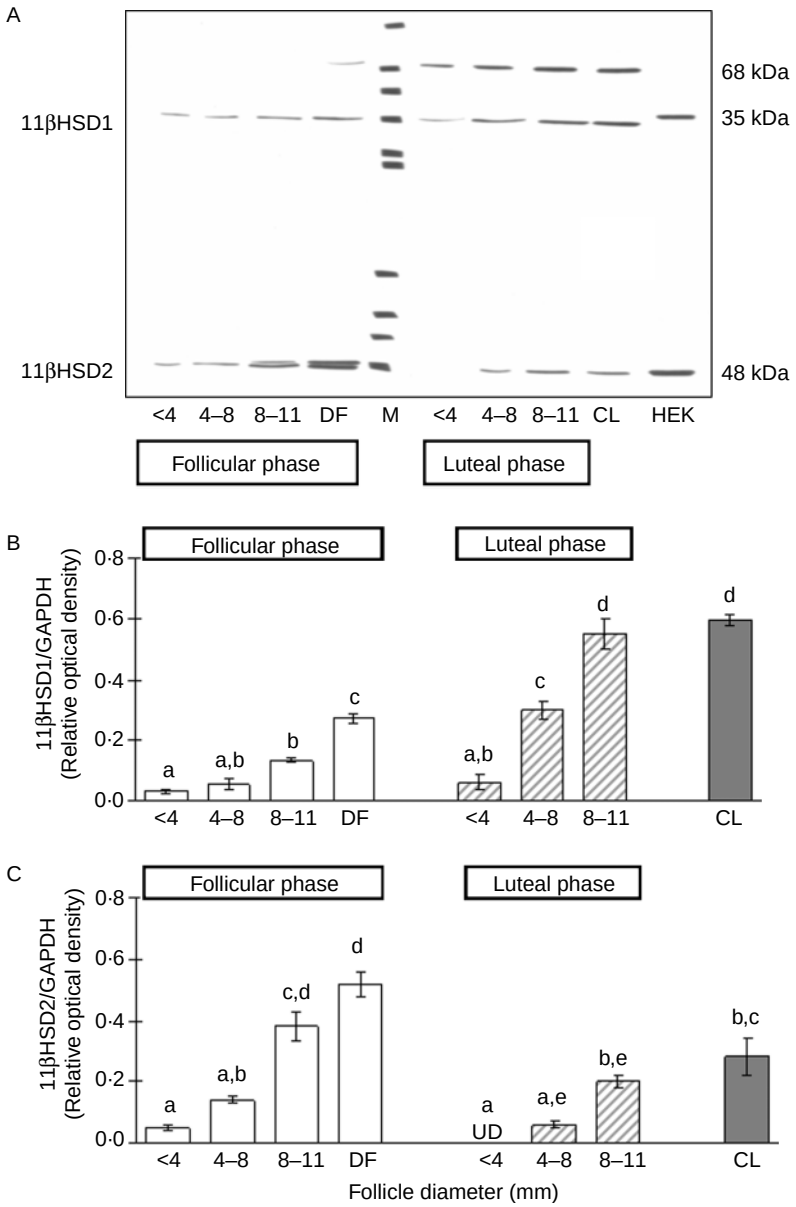

Figure 2 Expression of 11βHSD protein in granulosa and luteal cells during the follicular and luteal phases of the bovine ovarian cycle. Granulosa cells were extracted from follicles of different diameters (<4, 4–8 and 8–11 mm; the dominant follicle, DF) in the follicular phase (open bars) and the luteal phase (hatched bars) of the ovarian cycle. Luteal cells were prepared from the early non-pregnant corpus luteum (CL; filled bar). (A) Representative immunoblot for 11βHSD1 and 11βHSD2; M, molecular weight markers; HEK, protein extract from HEK cells stably transfected to express either 11βHSD1 or 11βHSD2 as positive controls. (B) 11βHSD1 protein expression. (C) 11βHSD2 protein expression. In (B) and (C), each data point represents the mean±S.E.M. values for five independent experiments; UD, undetectable levels of protein. Within each panel, mean values sharing a common superscript do not differ significantly, whereas $P<0{\cdot}05$ for means that do not share a common superscript.

enzyme activities increased by up to fourfold in granulosa cell homogenates with the diameter of the follicles from which the cells had been aspirated (ANOVA $F=288{\cdot}3$, 333·4 and 196·2 respectively, $P<0{\cdot}0001$ in each case; Fig. 3).

At each stage of folliculogenesis, the level of $NADP^+$-dependent 11β-DH activity in granulosa cell homogenates was consistently higher than the NADPH-dependent 11-KSR activity, irrespective of the phase of the ovarian

Table 1 Expression of 11β-hydroxysteroid dehydrogenase (11βHSD) protein bands of defined molecular weights (relative optical densities with respect to glyceraldehyde-3-phosphate dehydrogenase) in granulosa and luteal cells during the follicular and luteal phases of the bovine ovarian cycle. Each value represents the mean±S.E.M. for five independent experiments

	34 kDa	68 kDa	Total 11βHSD1	Ratio of total 11βHSD1:11βHSD2
11βHSD1				
Follicular phase				
<4 mm	0·03±0·01^{a}	UD^{a}	0·03±0·01^{a}	0·56±0·06^{a}
4–8 mm	0·05±0·02^{a,b}	UD^{a}	0·05±0·02^{a}	0·37±0·12^{a}
8–11 mm	0·13±0·01^{b}	UD^{a}	0·13±0·01^{a}	0·27±0·04^{a}
DF	0·27±0·02^{c}	0·13±0·01^{b}	0·40±0·03^{b,c}	0·45±0·04^{a}
Luteal phase				
<4 mm	0·06±0·02^{a,b}	0·16±0·02^{b}	0·22±0·04^{a,b}	∞
4–8 mm	0·30±0·03^{c}	0·31±0·04^{c}	0·61±0·06^{c}	9·76±0·97^{b}
8–11 mm	0·55±0·05^{d}	0·52±0·05^{d}	1·07±0·10^{d}	5·53±0·96^{c}
CL	0·60±0·02^{d}	0·61±0·01^{d}	1·20±0·03^{d}	4·62±0·96^{c}
	48 kDa	**50 kDa**	**Total 11βHSD2**	
11βHSD2				
Follicular phase				
<4 mm	0·05±0·01^{a}	UD^{a}	0·05±0·01^{a,b}	
4–8 mm	0·14±0·01^{a,b}	UD^{a}	0·14±0·01^{a,b}	
8–11 mm	0·38±0·05^{c,d}	0·14±0·03^{b}	0·53±0·08^{c}	
DF	0·52±0·04^{c}	0·37±0·07^{c}	0·90±0·10^{d}	
Luteal phase				
<4 mm	UD^{a}	UD^{a}	UD^{a}	
4–8 mm	0·06±0·01^{a,e}	UD^{a}	0·06±0·01^{a,b}	
8–11 mm	0·20±0·02^{b,e}	UD^{a}	0·20±0·02^{a,b}	
CL	0·28±0·02^{b,d}	UD^{a}	0·28±0·06^{b}	

Granulosa cells were extracted from follicles of different diameters (<4, 4–8 and 8–11 mm; the dominant follicle, DF) in the follicular and luteal phases of the ovarian cycle. Luteal cells were prepared from the corpus luteum (CL). Within each data set (protein band of a defined size, total expression of a given 11βHSD enzyme or the total 11βHSD1:11βHSD2 ratios), means sharing a common superscript do not differ significantly, whereas $P<0·05$ for means that do not share a common superscript. UD, undetectable levels of protein expression. Ratios of 11βHSD1:11βHSD2 expression were calculated using the total relative optical density summed for the 34+68 kDa immunoreactive proteins and the 48+50 kDa immunoreactive proteins recognised by the 11βHSD1 and 11βHSD2 antibodies respectively.

cycle (Fig. 3A and B; Table 2). This same bias in favour of $NADP^+$-dependent cortisol oxidation was also observed in luteal cell homogenates.

In granulosa cell homogenates prepared from follicles during the follicular phase of the ovarian cycle, the level of 11β-DH activity was consistently higher in the presence of NAD^+ than with $NADP^+$ irrespective of follicle diameter, reaching a maximum value in granulosa cells aspirated from dominant follicles (Fig. 3C). In contrast, in granulosa cell homogenates prepared from each size of follicle during the luteal phase of the ovarian cycle and in the CL, the level of 11β-DH activity was higher in the presence of $NADP^+$ than with NAD^+ (Fig. 3). In addition, the NAD^+-dependent 11β-DH activities were higher in granulosa cells harvested during the follicular phase than in granulosa cells from follicles of equivalent diameters aspirated in the luteal phase (Fig. 3C).

Irrespective of the preferred cofactor, the summed $NADP^+$- and NAD^+-dependent 11β-DH activities were five- to tenfold higher than the corresponding NADPH-dependent 11KSR activities in granulosa cells from follicular phase follicles (including the dominant follicle), but this predominance fell to less than a fourfold excess of dehydrogenase activity in cells from luteal phase follicles and the early CL (Table 2).

Discussion

Previous studies of 11βHSD in the bovine ovary have been confined to studies of mRNA expression (Tetsuka *et al*. 2003). This study is the first to document changes in enzyme protein expression and cofactor-dependent enzyme activities across the bovine ovarian cycle in granulosa cells and the early CL. Trends in NADP(H)- and NAD^+-dependent 11βHSD activities paralleled changes in the expression of the 11βHSD1 and 11βHSD2 proteins, which in turn reflected the expression of mRNA encoding these enzyme proteins. This suggests that the balance of 11βHSD enzyme activities may be set, to a large extent, at the level of enzyme translation, if not at the level of enzyme transcription.

In women and rats, follicular granulosa cells express exclusively 11βHSD2 with a switch at ovulation to expression of 11βHSD1 in the luteinised granulosa cells that comprise the CL (Michael *et al*. 1997, Tetsuka *et al*. 1997, 1999, Ricketts *et al*. 1998). We can now extend the findings of Tetsuka *et al*. (2003) in reporting that both cloned isoforms of 11βHSD are co-expressed in bovine granulosa cells and CL at the mRNA, protein and functional levels. In granulosa cells aspirated during the follicular phase of the ovarian cycle, the

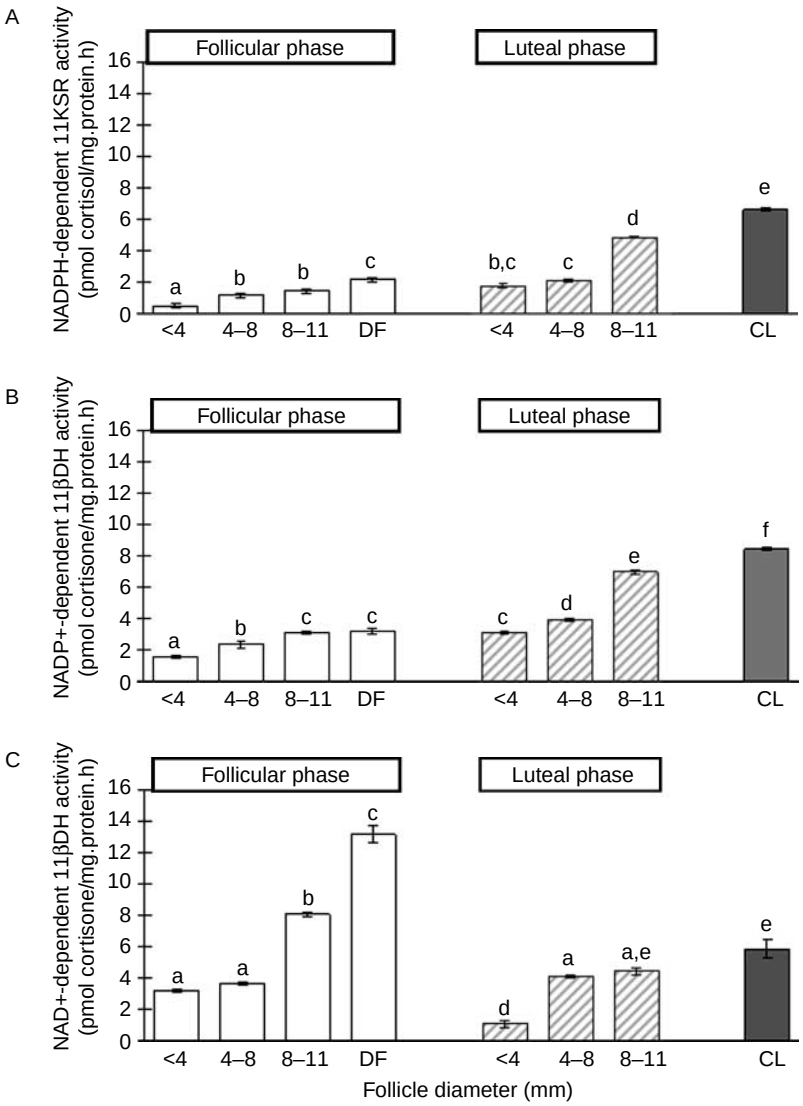

Figure 3 11βHSD activities in homogenised granulosa and luteal cells during the follicular and luteal phases of the bovine ovarian cycle. Granulosa cells were obtained from follicles of different diameters (<4, 4–8 and 8–11 mm; the dominant follicle, DF) in the follicular phase (open bars) and the luteal phase (hatched bars) of the ovarian cycle. Luteal cells were prepared from the corpus luteum (CL; filled bar). (A) NADPH-dependent 11-ketosteroid reductase activities. (B) $NADP^+$-dependent 11β-dehydrogenase activities. (C) NAD^+-dependent 11β-dehydrogenase activities. Each value represents the mean±S.E.M. for three independent experiments. Within each panel, means sharing a common superscript do not differ significantly, whereas $P<0{\cdot}05$ for means that do not share a common superscript.

expression and NAD^+-dependent oxidase activity of 11βHSD2 appears to predominate, whereas in luteal phase granulosa cells and CL, the expression and NADP(H)-dependent activities of 11βHSD1 predominate. Hence, while cows do not appear to show the discrete switch from 11βHSD2 to 11βHSD1 at ovulation, comparing levels of 11βHSD protein expression and $NADP^+/NAD^+$-dependent enzyme activities between dominant follicles and early CL indicates that there may be a more subtle transition between the predominance of these two cloned enzyme isoforms around ovulation.

In interpreting the enzyme activity data, it must be conceded that the use of different pyridine dinucleotide cofactors does not allow for absolute discrimination between the activities of 11βHSD1 versus 11βHSD2. Although the pioneering studies that characterised 11βHSD1 found the enzyme to show a preference for $NADP^+$ as its oxidant cofactor (Lakshmi & Monder 1988, Agarwal *et al.* 1989),

Table 2 Balance of 11β-hydroxysteroid dehydrogenase (11βHSD) enzyme activities (11β-DH, net 11β-dehydrogenase; 11KSR, net 11-ketosteroid reductase; both expressed in pmol product/mg protein per h) in granulosa and luteal cell homogenates during the follicular and luteal phases of the bovine ovarian cycle. Each value represents the mean±S.E.M. for three independent experiments

	$NADP^+$	NAD^+	Total 11β-DH	Ratio of total 11β-DH:11KSR
11β-DH				
Follicular phase				
<4 mm	$1{\cdot}6\pm0{\cdot}1^{a}$	$3{\cdot}2\pm0{\cdot}1^{a}$	$4{\cdot}8\pm0{\cdot}2^{a,b}$	$9{\cdot}6\pm1{\cdot}8^{a}$
4–8 mm	$2{\cdot}3\pm0{\cdot}2^{b}$	$3{\cdot}7\pm0{\cdot}1^{a}$	$6{\cdot}0\pm0{\cdot}2^{b}$	$5{\cdot}1\pm0{\cdot}7^{b,c}$
8–11 mm	$3{\cdot}1\pm0{\cdot}1^{c}$	$8{\cdot}0\pm0{\cdot}1^{b}$	$11{\cdot}1\pm0{\cdot}1^{c}$	$7{\cdot}8\pm0{\cdot}9^{a,b}$
DF	$3{\cdot}2\pm0{\cdot}2^{c}$	$13{\cdot}2\pm0{\cdot}6^{c}$	$16{\cdot}4\pm0{\cdot}7^{d}$	$7{\cdot}6\pm0{\cdot}3^{a,b,d}$
Luteal phase				
<4 mm	$3{\cdot}1\pm0{\cdot}1^{c}$	$1{\cdot}1\pm0{\cdot}2^{d}$	$4{\cdot}1\pm0{\cdot}3^{a}$	$2{\cdot}3\pm0{\cdot}2^{c}$
4–8 mm	$3{\cdot}9\pm0{\cdot}1^{d}$	$4{\cdot}1\pm0{\cdot}1^{a}$	$8{\cdot}1\pm0{\cdot}2^{e}$	$3{\cdot}8\pm0{\cdot}1^{c,d}$
8–11 mm	$6{\cdot}9\pm0{\cdot}1^{e}$	$4{\cdot}5\pm0{\cdot}2^{a,e}$	$11{\cdot}4\pm0{\cdot}4^{c}$	$2{\cdot}3\pm0{\cdot}1^{c}$
CL	$8{\cdot}4\pm0{\cdot}1^{f}$	$5{\cdot}8\pm0{\cdot}5^{e}$	$14{\cdot}3\pm0{\cdot}5^{f}$	$2{\cdot}2\pm0{\cdot}1^{c}$
	NADPH			
11KSR				
Follicular phase				
<4 mm	$0{\cdot}5\pm0{\cdot}2^{a}$			
4–8 mm	$1{\cdot}2\pm0{\cdot}1^{b}$			
8–11 mm	$1{\cdot}4\pm0{\cdot}1^{b}$			
DF	$2{\cdot}2\pm0{\cdot}1^{c}$			
Luteal phase				
<4 mm	$1{\cdot}8\pm0{\cdot}1^{b,c}$			
4–8 mm	$2{\cdot}1\pm0{\cdot}1^{c}$			
8–11 mm	$4{\cdot}9\pm0{\cdot}0^{d}$			
CL	$6{\cdot}6\pm0{\cdot}1^{e}$			

Granulosa cells were extracted from follicles of different diameters (<4, 4–8 and 8–11 mm; the dominant follicle, DF) in the follicular and luteal phases of the ovarian cycle. Luteal cells were prepared from the corpus luteum (CL). Within each data set (a given 11βHSD enzyme activity, the summed 11β-DH activities and the ratio of 11β-DH to 11KSR activities), means sharing a common superscript do not differ significantly, whereas $P<0{\cdot}05$ for means that do not share a common superscript. Ratios of 11β-DH:11KSR activities were calculated using the total oxidation of cortisol, irrespective of pyridine nucleotide cofactor.

11βHSD1 was also able to bind and use NAD^+ as a cofactor such that the NAD^+-dependent oxidation of cortisol reflects the oxidative activities of both 11βHSD2 and, to a lesser extent, 11βHSD1. In contrast, 11βHSD2 is unable to utilise $NADP^+$ and shows an absolute requirement for NAD^+ as its co-substrate. Consequently, any $NADP^+$-dependent oxidation of cortisol can be attributed, at present, to 11βHSD1. In this study, the fact that the trends in NAD^+-dependent oxidation of cortisol paralleled the changes in expression of 11βHSD2 protein but did not mirror the changes in expression of 11βHSD1 and $NADP^+$-dependent cortisol metabolism supports the assumption that cortisol oxidation in the presence of NAD^+ can be ascribed to 11βHSD2 activity.

Initial biochemical studies of 11βHSD1 as isolated from the liver determined that while this enzyme isoform is intrinsically bi-directional, its predominant activity, at least *in vivo*, is as an 11KSR that regenerates cortisol from cortisone (Lakshmi & Monder 1988, Agarwal *et al.* 1989, Tannin *et al.* 1991). However, we have contested that in human granulosa-lutein cells, 11βHSD1 acts principally as an oxidative enzyme (Michael *et al.* 1997), and Tetsuka *et al.* (2003) have also interpreted their recent findings to indicate that 11βHSD1 may act predominantly as an oxidative enzyme to inactivate glucocorticoids in bovine ovarian cells. In the steroidogenic Leydig cells of the testis, it is established that in mature Leydig cells obtained from post-pubertal male rats and incubated with physiological concentrations of respiratory substrates, 11βHSD1 acts primarily as an oxidase, protecting steroidogenesis from the inhibitory actions of glucocorticoids (Ge *et al.* 1997). That said, the predominant direction of reaction for 11βHSD1 is highly dependent on the precise assay conditions *in vitro*. For example, while Ge *et al.* (1997) found that 11βHSD1 acted predominantly to oxidise cortisol in cultured rat Leydig cells, Leckie *et al.* (1998) found that reductase activity predominated in the same cells under remarkably similar conditions. We subsequently confirmed that in the MA10 mouse tumour Leydig cell line, the direction of the reaction for 11βHSD1 was determined by the concentrations of respiratory substrates (e.g. glucose) in the medium (Ferguson *et al.* 1999).

The data reported in the present study suggest that in bovine granulosa cells and in early non-pregnant CL, the sum of the two 11β-DH activities and, specifically, the $NADP^+$-dependent oxidation of cortisol exceeds the NADPH-dependent reduction of cortisone. This bias towards 11β-DH versus 11KSR activity was greatest in granulosa cells aspirated during the follicular phase of the cycle, but was decreased in luteal phase granulosa cells and early CL,

suggesting two things. First, these data imply that a balance in favour of the oxidative inactivation of cortisol is more important in developing follicles than in accessory follicles or the early CL. Secondly, these data indicate that the predominant activity of 11βHSD1 may depend upon the prevalent endocrine and/or paracrine environment at a specific stage of the ovarian cycle.

Recent studies have established that in tissues such as liver and adipose tissue, the predominant direction of activity for 11βHSD1 depends on the redox state of NADPH, determined by the pentose phosphate pathway and/or the oxidation of glucose-6-phosphate by hexose-6-phosphate dehydrogenase (H6PDH). The current accepted view is that the oxidation of glucose-6-phosphate to 6-phosphogluconate by H6PDH maintains a high concentration of NADPH in the lumen of the smooth endoplasmic reticulum, which favours the 11KSR activity of 11βHSD1 (Draper *et al.* 2003, Atanasov *et al.* 2004, Banhegyi *et al.* 2004, Bujalska *et al.* 2005, McCormick *et al.* 2006). In steroidogenic gonadal cells, specifically in testis Leydig cells and ovarian granulosa-lutein cells, it has been suggested that the preferential usage of NADPH by steroidogenic cytochrome P450 enzymes alters the balance of $NADPH/NADP^+$ such that 11βHSD1 can act as an effective 11β-DH enzyme to inactivate glucocorticoids (Michael *et al.* 2003, Ge *et al.* 2005). Our latest findings support the view that 11βHSD1 can act predominantly as an oxidative enzyme in ovarian cells. However, the fact that the rate of cortisol oxidation exceeded the rate of cortisone reduction in bovine granulosa and luteal cell homogenates supplied with excess cofactor suggests that the redox state of NADP(H) may not be the only determinant of the balance of 11βHSD1 enzyme activity in these gonadal cells.

In the present study, the relative decrease in the 11β-DH activity and/or increase in the reductase activity of 11βHSD1 coincided with the appearance of the 68 kDa immunoreactive protein recognised by the antibody directed to the N-terminus of 11βHSD1 in the western blots. This 68 kDa band has been identified in previous reports and has been attributed to the fact that the 11βHSD1 enzyme as isolated from liver can form homodimers (Monder & Lakshmi 1990, Ricketts *et al.* 1998, Walker *et al.* 2001, Maser *et al.* 2002, Blum & Maser 2003, Blum *et al.* 2003). Recently, it has been established that 11βHSD1 is only active in its dimeric state with the individual enzyme proteins acting cooperatively to catalyse the 11-oxoreductase reaction (Maser *et al.* 2002, Elleby *et al.* 2004). Since, in the present study, all immunoblots were performed under reducing conditions, we would have expected the 11βHSD1 dimers to have been fully dissociated into 34 kDa monomeric subunits. However, we note that the 11βHSD1 dimer was not fully dissociated by incubation of protein samples with a reducing agent (β-mercaptoethanol or dithiothreitol) in previous reports (Ricketts *et al.* 1998, Maser *et al.* 2002, Blum & Maser 2003, Blum *et al.* 2003, Elleby *et al.* 2004). In the case of human 11βHSD1, the enzyme homodimers are stabilised by interchain disulphide bonds between Cys_{272} residues on the individual protein molecules (Walker *et al.* 2001). While Cys_{272} is not conserved in the bovine 11βHSD1 enzyme, we cannot yet exclude the possibility that bovine 11βHSD1 dimers are stabilised by alternative structural features.

Although the 68 kDa dimeric form of 11βHSD1 was not detected in proteins prepared from bovine granulosa cells aspirated from antral follicles smaller than 12 mm in diameter during the follicular phase of the cycle, this higher molecular weight form was consistently observed in the early CL and granulosa cells aspirated from dominant and all luteal phase follicles. There are two obvious explanations for this finding. First, the formation of an 11βHSD1 homodimer may be favoured by the paracrine/autocrine actions of progesterone synthesised within the granulosa cells of the dominant follicle or by a neighbouring CL. Secondly, the persistence of 11βHSD1 homodimers may depend on the absolute level of expression of the 11βHSD1 protein affecting the probability of dimer formation. In support of the latter explanation, 11βHSD1 only migrated as a 68 kDa dimer in those ovarian samples expressing 11βHSD1 above a threshold level (equal, in the present study, to 0·22 relative to GAPDH). Hence, while a high molecular weight form of 11βHSD1 was consistently observed in proteins prepared from the CL, dominant follicle and luteal phase follicles, the level of expression of 11βHSD1 may have simply been too low to form or detect 11βHSD1 protein dimers in granulosa cells from follicular phase follicles.

Turning to the 50 kDa protein recognised by the 11βHSD2 antibody, the fact that this protein was only 2 kDa larger than the 48 kDa protein observed in all cell types (including the HEK cells stably transfected with 11βHSD2 cDNA) would be consistent with the postulate that this 50 kDa protein is simply a form of 11βHSD2 that has been post-translationally modified (e.g. by phosphorylation and/or glycosylation). We also note that the appearance of this higher molecular weight form of 11βHSD2 is only observed in large follicles during the follicular phase of the cycle and in dominant ovarian follicles, suggesting that the presence of this form of 11βHSD2 either depends on paracrine agents produced at increased concentrations in the final stages of follicle maturation (oestradiol or inhibin) or is suppressed in luteal phase granulosa cells and CL by a luteal secretory product (progesterone or oxytocin). For most mammalian species studied to date, 11βHSD2 migrates with a molecular mass of 40–42 kDa. While the primary sequence of bovine 11βHSD2 would predict a larger protein, we did not find any published reports to confirm the size of the bovine 11βHSD2 as 48 or 50 kDa. However, the antibodies used in the present study recognise only those proteins migrating at these positions on the gel (in both bovine protein preparations and HEK-positive control lanes).

In terms of the physiological implications of these findings, this study has shown that the rate of glucocorticoid inactivation in mural granulosa cells from bovine follicles increases during follicle growth. We have recently observed the same trends in mural granulosa cells from healthy porcine

follicles of increasing diameter (Sunak *et al.* 2007), and so would infer that growing antral follicles, at least from cows and pigs, need to increase their capacity to inactivate glucocorticoids as follicle growth progresses. In the case of porcine follicles, this may reflect the ability of glucocorticoid steroids to inhibit oocyte maturation (by repression of cyclin B1; Yang *et al.* 1999, Chen *et al.* 2000), such that there is a need to limit the actions of glucocorticoids in the later stages of porcine follicle development (Sunak *et al.* 2007). In bovine follicles, mineralocorticoids have been implicated in bovine oocyte maturation by the finding that mineralocorticoid receptor (MR) transcripts are upregulated during oocyte maturation (Robert *et al.* 2000). Given the intrinsic lack of specificity of the MR for mineralocorticoid ligands and the associated physiological role for 11βHSD2 in conferring specificity on this promiscuous receptor, we would suggest that the increase in NAD^+-dependent 11βHSD activity during the growth of bovine follicles is important to coordinate the access of corticosteroids to the MR during maturation of bovine oocytes.

In conclusion, the data reported herein suggest that the expression and activity of 11βHSD2 predominate in bovine granulosa cells at all stages of folliculogenesis with a transition to dominance by 11βHSD1 in the CL, possibly due to factors secreted by the CL that can also act on follicles in the ipsilateral ovary containing that gland during the luteal phase. From a functional perspective, there appears to be increasing capacity of the granulosa cells to inactivate glucocorticoids as the follicle develops (principally via the high affinity, NAD^+-dependent 11βHSD2 enzyme), whereas in luteal phase follicles and CL, there is increased capacity to regenerate active glucocorticoids through the NADPH-dependent reductase activity of 11βHSD1.

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