Implication of cortisol and 11 β -hydroxysteroid dehydrogenase enzymes in the development of porcine (*Sus scrofa domestica*) ovarian follicles and cysts

Neera Sunak¹, Daphne F Green², Lalantha R Abeydeera³, Lisa M Thurston⁴ and Anthony E Michael^{1,5}

¹Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, UK, ²Institute of Zoology, Zoological Society of London, Regent's Park, London NW1 4RY, UK, ³Genus Plc, 3033 Nashville Road, Franklin, Kentucky 42135, USA, ⁴Reproduction and Development Group, Department of Veterinary Basic Sciences, Royal Veterinary College, Royal College Street, London NW1 0TU, UK and ⁵Division of Clinical Developmental Sciences, Academic Section of Obstetrics and Gynaecology, St George's, University of London, Cranmer Terrace, Tooting SW17 0RE, UK

Correspondence should be addressed to A E Michael, Division of Clinical Developmental Sciences, Centre for Developmental and Endocrine Signalling, Academic Section of Obstetrics and Gynaecology, 3rd Floor, Lanesborough Wing, St George's, University of London, Cranmer Terrace, London SW17 0RE, UK; Email: tony.michael@sgul.ac.uk

Abstract

This study investigated cortisol inactivation by 11 β -hydroxysteroid dehydrogenase (11 β HSD) enzymes in porcine granulosa cells from antral follicles at different developmental stages and in ovarian cysts. In granulosa cells, cortisol oxidation increased threefold with antral follicle diameter ($P < 0.001$). This trend was paralleled by a threefold increase in NADP⁺-dependent 11 β -dehydrogenase activity in granulosa cell homogenates with follicle diameter. Intact granulosa cells from ovarian cysts exhibited significantly lower enzyme activities than cells from large antral follicles. Neither intact cells nor cell homogenates displayed net 11-ketosteroid reductase activities. Since porcine follicular fluid (FF) from large antral follicles and ovarian cysts contains hydrophobic inhibitors of glucocorticoid metabolism by type 1 11 β HSD, this study also investigated whether levels of 11 β HSD inhibitors changed during follicle growth and could affect cortisol metabolism in granulosa cells. The extent of inhibition of 11 β HSD1 activity in rat kidney homogenates decreased progressively from 50 $\pm$ 8% inhibition by FF from small antral follicles ($P < 0.001$) to 23 $\pm$ 6% by large antral FF ($P < 0.05$). Cyst fluid inhibited 11 β HSD1 activity by 59 $\pm$ 4% ($P < 0.001$). Likewise, net cortisol oxidation in granulosa cells was significantly decreased by large antral FF (35–48% inhibition, $P < 0.05$) and cyst fluid (45–75% inhibition, $P < 0.01$). We conclude that inactivation of cortisol by 11 β HSD enzymes in porcine granulosa cells increases with follicle development but is significantly decreased in ovarian cysts. Moreover, changes in ovarian cortisol metabolism are accompanied by corresponding changes in the levels of paracrine inhibitors of 11 β HSD1 within growing ovarian follicles and cysts, implicating cortisol in follicle growth and cyst development.

Reproduction (2007) 133 1149–1158

Introduction

Inter-conversion of the physiological glucocorticoid, cortisol and its inert 11-ketosteroid metabolite, cortisone, is catalysed by 11 β -hydroxysteroid dehydrogenase (11 β HSD) enzymes. To date, two biochemically distinct 11 β HSD enzymes have been cloned. 11 β HSD1, first isolated from the liver (Lakshmi & Monder 1988), can catalyse both cortisol oxidation and cortisone reduction, albeit with a relatively low substrate affinity (Agarwal *et al.* 1989, Monder & Lakshmi 1990, Tannin *et al.* 1991). Although this NADP(H)-dependent enzyme is bidirectional, the current view is that in most tissues

(e.g. liver), the regeneration of NADPH in the lumen of the smooth endoplasmic reticulum by hexose-6-phosphate dehydrogenase drives the reductase activity of 11 β HSD1 (Low *et al.* 1994, Jamieson *et al.* 1995, Seckl & Walker 2001, Draper *et al.* 2003, Atanasov *et al.* 2004, Banhegyi *et al.* 2004, McCormick *et al.* 2006). In the steroidogenic cells of the ovary and testis, 11 β HSD1 appears to act predominantly as an 11 β -dehydrogenase to inactivate glucocorticoids (Gao *et al.* 1997, Michael *et al.* 1997, Ge & Hardy 2000, Yong *et al.* 2000, Tetsuka *et al.* 2003). This has been attributed to preferential usage of NADPH for gonadal steroid synthesis (Michael *et al.* 2003, Ge *et al.* 2005).

In contrast to 11 β HSD1, 11 β HSD2 exhibits only 11 β -dehydrogenase activity and so exclusively catalyses the inactivation of glucocorticoids using NAD⁺ as its oxidant cofactor (Mercer & Krozowski 1992, Brown *et al.* 1993, Agarwal *et al.* 1994, Albiston *et al.* 1994). 11 β HSD2 is expressed primarily in aldosterone target tissues, where it restricts glucocorticoid access to mineralocorticoid receptors (Naray-Fejes-Toth *et al.* 1991, Mercer & Krozowski 1992, Agarwal *et al.* 1994, Albiston *et al.* 1994). However, 11 β HSD2 is also expressed in the placenta (Brown *et al.* 1993), prostate, testis and ovary (Albiston *et al.* 1994, Ricketts *et al.* 1998).

Previously, we have reported that follicular fluid (FF) from porcine, bovine and human ovarian follicles contains endogenous, hydrophobic compounds that can acutely inhibit the NADP(H)-dependent activities of 11 β HSD1 in homogenates of rat kidney, without altering the oxidation of cortisol by 11 β HSD2 (Thurston *et al.* 2002, 2003a). Furthermore, the levels of the intra-follicular 11 β HSD1 inhibitors in spontaneous ovarian cysts appeared to be greater than levels in large antral follicles (Thurston *et al.* 2003a), suggesting that compounds which regulate cortisol metabolism by 11 β HSD may play a role in folliculogenesis and/or cyst development in pigs.

To date, no studies have measured 11 β HSD activities in porcine granulosa cells and there are no reports in any species as to whether 11 β HSD enzyme activities in granulosa cells change during folliculogenesis. Therefore, this study aimed to assess and characterise cortisol-cortisone metabolism by 11 β HSD enzymes in porcine granulosa cells from antral follicles during folliculogenesis and in granulosa cells from ovarian cysts. In light of our previous findings, we also wanted to determine how the modulators of 11 β HSD1 activity may change in porcine FF during antral follicle growth and to assess whether these compounds could act in a paracrine manner to inhibit cortisol metabolism in porcine granulosa cells.

Materials and Methods

Collection of ovarian samples

Porcine ovaries in the follicular phase of the ovarian cycle were obtained from a local abattoir. Ovaries were confirmed to be in the follicular phase of the ovarian cycle, determined by the absence of a corpus luteum on either ovary from an individual animal as previously described (Thurston *et al.* 2003a). Ovaries were transported to the laboratory in Medium 199 (M199; Sigma-Aldrich) supplemented with 100 IU/ml penicillin (Life Technologies), 0.1 mg/ml streptomycin (Life Technologies), 2 ml/l amphotericin B (Sigma-Aldrich), 0.1% (w/v) BSA (Sigma-Aldrich) and 200 nM L-glutamine (Life Technologies) at 25 °C within 2 h. In the laboratory, the ovaries were washed thrice in warm sterile 0.9% (w/v)

saline solution, then in 70% (v/v) ethanol (Merck, Dorset, UK) for ~30 s before being rinsed in sterile saline.

Follicles of diameter 2–3 mm (small antral follicles), 4–7 mm (medium antral follicles) and ≥ 8 mm (large antral follicles; Knox 2005) were dissected from porcine ovaries. All follicles were selected on the basis of a morphologically healthy appearance; follicles had a translucent antrum with no free-floating particles and a well-vascularised follicle wall (Maxson *et al.* 1985, Guthrie *et al.* 1995). Spontaneous ovarian cysts were dissected from cystic porcine ovaries; cysts were diagnosed as fluid-filled structures with diameters of 25–40 mm in ovaries lacking corpora lutea (Kesler & Garverick 1982, Calder *et al.* 2001). Dissected follicles and cysts were stored in Dulbecco's modified PBS (DPBS; Life Technologies) at 37 °C.

Aspiration of porcine ovarian fluids

Samples of FF from small, medium and large antral follicles and samples of cyst fluid were aspirated from dissected intact follicles and cysts respectively, then divided into 1 ml aliquots before being stored at –20 °C pending analysis. In total, five FF samples from each size category of antral follicles and five cyst fluid samples were used in this study, with each individual sample being aspirated from the ovaries of one of five different animals. In order to generate sufficient quantities of each fluid (>1 ml), samples of fluid from individual small and medium antral follicles were pooled from several follicles of the appropriate size category from the same ovary. Fluids from large antral follicles and single ovarian cysts were not pooled since single follicles/cysts each yielded more than 1 ml fluid.

Measurement of intra-follicular gonadal steroid concentrations

To confirm the visual assessment of follicles as being healthy/non-atretic, the intra-follicular oestradiol and progesterone concentrations were assayed. Oestradiol concentrations were measured by ELISA using a kit (EIA-2693) purchased from DRG Diagnostics (Marburg, Germany). The detection limit of this ELISA was 5 pM oestradiol, with intra- and inter-assay coefficients of variation of 5 and 2% respectively. Maxson *et al.* (1985) previously reported that non-atretic porcine antral follicles of medium diameter had intra-follicular oestradiol concentrations of 123 ± 50 nM, whereas FF from atretic porcine follicles contained 18 ± 5 nM oestradiol. The mean intra-follicular oestradiol concentrations for the FF samples featured in the present study ranged from 64 to 1523 nM, depending on follicle diameter (Table 1), confirming that all fluids had been aspirated from healthy follicles.

Table 1 Intra-follicular estradiol (E₂) and progesterone (P₄) concentrations in porcine FF from small, medium and large antral follicles and in fluid from spontaneous ovarian cysts.

	E ₂ (nM)	P ₄ (nM)
Small antral FF	64 ± 14*	1161 ± 244*
Medium antral FF	141 ± 32*	754 ± 110*
Large antral FF	1523 ± 321 [†]	1338 ± 121*
Ovarian cyst fluid	162 ± 133*	1099 ± 17*

Each data entry is the mean (±S.E.M.) intra-follicular hormone concentration for five individual FF or cyst fluid samples. Within each column, data that do not share a common superscript differ significantly (*[†]*P* < 0.05).

Progesterone concentrations were determined by RIA as previously described by Pallikaros *et al.* (1995). Inter- and intra-assay coefficients of variation were 9 and 14% respectively at 31% binding with a detection limit of 0.5 nM progesterone. Irrespective of follicle diameter, the concentrations of progesterone in all FF samples lay within the range of 219–1945 nM, as previously reported for healthy, pre-ovulatory porcine follicles by Conley *et al.* (1994; Table 1).

11 β HSD activities in porcine granulosa cells

Granulosa cells were isolated from small, medium and large antral follicles and spontaneous ovarian cysts. After aspiration and removal of the FF or cyst fluid, the remaining small and medium antral follicle shells were dissected and follicle shells from large antral follicles and ovarian cysts were hemisected. Granulosa cells were then gently flushed from the follicle shells using DPBS and the resultant cell preparations were washed by centrifugation in 15 ml DPBS plus 2 ml sterile water (Sigma–Aldrich; to lyse red blood cells). Viable cells were then counted by exclusion of trypan blue dye (Merck). After counting, cell pellets were resuspended in serum-free McCoy's 5A medium supplemented with 10 ng/ml bovine insulin, 10 ng/ml long R3 insulin-like growth factor-I (Gropep Limited, SA, Australia), 5 μ g/ml bovine transferrin, 0.04 ng/ml sodium selenite, 100 ng/ml androstenedione and 1 ng/ml FSH. (Unless otherwise stated, culture medium and supplements were all purchased from Sigma–Aldrich). Cells were then seeded into 24-well culture plates in 1 ml volumes at a density of 50 000 viable cells/ml and cultured in a humidified atmosphere of 5% (v/v) CO₂ in air at 37 °C.

Cells were placed into primary culture for a total period of 24 h. This allowed for an initial recovery phase of 20 h (during which cells were allowed to recover from any physical or functional injury during isolation from the ovary), followed by a 4 h assay phase. During the final 4 h, net 11 β HSD activities were assessed in intact cells using the radiometric conversion assay as previously described (Thurston *et al.* 2003b). Net 11 β -dehydrogenase activity was assessed after addition of

100 μ l fresh serum-free medium containing 0.5 μ Ci [1,2,6,7-³H]cortisol (Amersham). Prior to addition, the [1,2,6,7-³H]cortisol (specific activity 69 Ci/mmol; Amersham) was pre-diluted against a solution of non-radioactive cortisol (Sigma–Aldrich) in serum-free medium to reduce the specific activity of the cortisol to 5 Ci/mmol and to give a final steroid concentration in each well of 100 pmol/ml (i.e. 100 nM). Net 11-ketosteroid reductase activity was similarly measured with the addition of 100 μ l serum-free medium containing 0.1 μ Ci [1,2(n)-³H]cortisone (specific activity 40 Ci/mmol; Amersham) plus non-radioactive cortisone to give a final specific activity of 1 Ci/mmol and a final cortisone concentration in each well of 100 nM). Following a 4 h incubation at 37 °C, medium was decanted into glass screw-top tubes, steroids were extracted into two volumes of ice-cold chloroform, evaporated to dryness under nitrogen at 45 °C, resuspended in ethyl acetate (Merck) and resolved by thin layer chromatography (TLC) in 92:8 chloroform:95% (v/v) ethanol. To complete the radiometric assay, a Bioscan 2000 radiochromatogram scanner (LabLogic, Sheffield, UK) was used to assess the fractional conversion of [³H]cortisol to [³H]cortisone, and the resulting 11 β -dehydrogenase activity of 11 β HSD was calculated as net pmol of cortisone produced over 4 h (Thurston *et al.* 2002). Likewise, the 11-ketosteroid reductase activity of 11 β HSD in granulosa cells was determined from the fractional conversion of [³H]cortisone to [³H]cortisol, and enzyme activity calculated as net pmol of cortisol produced over 4 h.

Cofactor-dependent 11 β HSD activities in granulosa cell homogenates

Granulosa cells from small, medium and large antral follicles and ovarian cysts were suspended in DPBS, counted to assess cell density, and then precipitated by centrifugation at 1000 *g* at 4 °C for 30 min. Granulosa cell homogenates were prepared by the homogenisation of each cell pellet in hypotonic Tris–EDTA lysis buffer (2.25 ml/1 × 10⁶ cells; Thurston *et al.* 2002, 2003a). Isotonicity was restored to the cell homogenates by the addition of 1.5 M KCl (0.25 ml/1 × 10⁵ cells). One hundred microlitres of each homogenate were transferred to glass screw-cap culture tubes containing 600 μ l DPBS. Triplicate tubes were prepared as assay blanks containing 100 μ l BSA (1 mg/ml in DPBS) in place of the ovarian cell homogenates. Each triplicate set of tubes was pre-incubated for 30 min at 37 °C in a gyratory water bath. To determine net 11 β -dehydrogenase activities, each tube received 100 μ l DPBS containing either 4 mM NADP⁺ or NAD⁺ (Sigma–Aldrich) and 100 μ l DPBS containing 0.5 μ Ci [³H]cortisol substrate (prepared as above to a final specific activity of 5 Ci/mmol and a final cortisol concentration of 100 nM). To assess net 11-ketosteroid reductase

activities, each tube received 100 µl DPBS containing 4 mM NADPH (Sigma–Aldrich) $\pm$ 100 µl DPBS supplemented with 10 mM glucose-6-phosphate (Sigma–Aldrich) and 100 µl DPBS containing 0.1 µCi [3 H]cortisone (diluted specific activity = 1 Ci/mmol and final concentration = 100 nM). After topping tubes up to a final volume of 1 ml with DPBS, tubes were incubated in a gyratory water bath for 4 h at 37 °C. Reactions were terminated by the addition to each tube of 2 ml ice-cold chloroform. The radiometric conversion assay, to quantify 11 β -dehydrogenase or 11-ketosteroid reductase activities of 11 β HSD, was completed as described above.

Fractionation of porcine ovarian fluids by C18 reverse phase column chromatography

Each sample of porcine FF and cyst fluid was fractionated using reverse phase C18 column chromatography, as described by [Thurston et al. \(2002, 2003a\)](#). In brief, 1 ml aliquots of each ovarian fluid sample were applied to C18 columns (Waters Chromatography, Hertfordshire, UK) that had been conditioned with 20 ml methanol (Merck) and washed with 20 ml double-distilled water (DDW). The column was then sequentially eluted with 1 ml volumes of a stepwise gradient of 0–100% (v/v) methanol in DDW. All fractions were collected into borosilicate tubes, evaporated to dryness under nitrogen at 45 °C and resuspended in 1 ml volumes of 20% (v/v) methanol in DDW prior to assay.

Effects of porcine ovarian fluids and resolved fractions on 11 β HSD1 activity in rat kidney homogenates

The effects of porcine FF and cyst fluid samples (or resolved fractions thereof) on NADP $^+$ -dependent oxidation of cortisol by 11 β HSD1 were assessed using rat kidney homogenates as a source of both cloned 11 β HSD enzymes, as previously described ([Thurston et al. 2002, 2003a](#)). In brief, kidneys of adult male Sprague–Dawley rats, housed and fed in accordance with the UK Animals (Scientific Procedures) Act 1986, were homogenised in a hypotonic Tris–EDTA lysis buffer. Once isotonicity had been restored by the addition of 10% (v/v) 1.5 M KCl (Merck), 100 µl volumes of the homogenate were transferred to glass screw-cap culture tubes containing 600 µl DPBS. After adding 100 µl volumes of DPBS (controls), FF, cyst fluid or resolved C18 fractions of the ovarian fluids to triplicate sets of tubes, radiometric conversion assays of the 11 β -dehydrogenase activity of 11 β HSD1 were initiated by adding 100 µl DPBS containing 4 mM NADP $^+$ (Sigma–Aldrich) and 100 µl DPBS containing 0.5 µCi [3 H]cortisol (Amersham) plus non-radioactive cortisol (Sigma; as described above to give a final specific activity of 5 Ci/mmol and a final cortisol concentration in each assay tube of 100 nM). Tubes were incubated at 37 °C in a gyratory water bath for 1 h,

after which steroids were extracted into 2 ml ice-cold chloroform (Merck), then concentrated and resolved by TLC before quantifying the fractional metabolism of the [3 H]cortisol to [3 H]cortisone over 1 h.

Effects of porcine ovarian fluids and resolved fractions on 11 β HSD activity in porcine granulosa cells

Net oxidation of cortisol by 11 β HSD was reassessed in intact granulosa cells isolated from small, medium or large antral follicles, and from spontaneous ovarian cysts. For each source of granulosa cells, enzyme activities were assessed over 4 h in the presence of medium alone (control), FF aspirated from large antral follicles or ovarian cyst fluid, each tested at a final dilution of 10% by volume. In a subsequent series of experiments, the net oxidative activities of 11 β HSD were assessed in granulosa cells isolated from large antral follicles incubated in the presence of specific resolved fractions of FF from large antral follicles or of cyst fluid, each tested at 10% (v/v).

Statistical analysis

To assess whether data were normally distributed, the Kolmogorov–Smirnov test was employed and all data were then compared using one-way ANOVA followed by either the Tukey–Kramer or Dunnett's multiple comparison as the *post hoc* test (as appropriate to the data set). The correlation between the intra-follicular progesterone concentration in FF from small, medium and large antral follicles and porcine cyst fluid and the effects of those same fluid samples on 11 β HSD1 activity in rat kidney homogenates was calculated as the Pearson's correlation coefficient. For the assay involving the addition of exogenous cofactors to granulosa cell homogenates, comparisons between cofactor conditions were made within a given follicle size category by ANOVA and Dunnett's *post hoc* multiple comparison. Although selected data are presented graphically as the percentage of control enzyme activities in the absence of treatments, all statistical evaluations were performed on absolute, non-referenced data using GraphPad Prism3 software (San Diego, CA, USA). In all cases, values of $P < 0.05$ were accepted to indicate statistical significance.

Results

11 β HSD activities in intact porcine granulosa cells and granulosa cell homogenates

In primary cultures of granulosa cells from antral follicles, net oxidation of cortisol increased by threefold with increasing antral follicle diameter ($P < 0.001$; [Table 2](#)). Net 11 β -dehydrogenase activities in granulosa cells from ovarian cysts were significantly lower than in

Table 2 11 β -Hydroxysteroid dehydrogenase (11 β HSD) activities (net oxidation of cortisol to cortisone) in porcine granulosa cells isolated from small, medium and large antral follicles and from spontaneous ovarian cysts.

Follicle category from which granulosa cells were isolated	11 β HSD activity (pmol cortisone/50 000 cells for 4 h)
Small antral follicle	0.5 $\pm$ 0.1 ^a
Medium antral follicle	0.7 $\pm$ 0.1 ^a
Large antral follicle	1.4 $\pm$ 0.2 ^b
Ovarian cyst	0.6 $\pm$ 0.0 ^a

Each data entry is the mean ($\pm$ S.E.M.) enzyme activity for five assays, each performed in triplicate, on separate primary cultures of granulosa cells prepared from independent follicles and cysts. Mean values that do not share a common superscript differ significantly, $P < 0.05$.

cells from large antral follicles ($P < 0.001$) and comparable to those in granulosa cells from small antral follicles (Table 2). There was no detectable reduction of cortisone to cortisol in granulosa cells from ovarian cysts or antral follicles, irrespective of their diameter.

In homogenates of granulosa cells isolated from antral follicles or ovarian cysts, there was no significant difference between the net oxidation of cortisol in the presence of NADP⁺ versus NAD⁺, irrespective of follicle category (Fig. 1). The net NADP⁺-dependent oxidation of cortisol by granulosa cell homogenates increased with follicle diameter from a minimum of 0.5 $\pm$ 0.1 pmol/4 h in small antral follicles to a maximum of 1.2 $\pm$ 0.3 pmol/4 h in large antral follicles. Net NAD⁺-dependent activities of 11 β HSD increased from 0.5 $\pm$ 0.1 pmol/4 h in cell homogenates from small antral follicles to 1.0 $\pm$ 0.2 pmol/4 h. In granulosa cells isolated from spontaneous ovarian cysts, the level of NADP⁺-dependent cortisol inactivation was the same as that observed in cell homogenates from large antral follicles, whereas the NAD⁺-dependent inactivation of cortisol was $\sim$ 50% of that measured in granulosa

Figure 1 Effects of exogenous nucleotide cofactors on the net oxidation of cortisol by 11 β HSD in homogenates of porcine granulosa cells isolated from small, medium and large antral follicles and from spontaneous ovarian cysts. Each data point represents the mean ($\pm$ S.E.M.) enzyme activity (pmol cortisone/50 000 cells for 4 h) for five assays, each performed in triplicate, on individual homogenates of granulosa cells isolated from independent follicles and cysts, in the absence of cofactors (open bars), and in the presence of 4 mM NADP⁺ (dotted bar) or NAD⁺ (diagonal hatched bar). Within a given follicle size category, * $P < 0.05$ and ** $P < 0.01$ versus enzyme activity measured in the absence of cofactors.

cell homogenates from large antral follicles, but comparable to the levels of NAD⁺-dependent cortisol oxidation in small and medium antral follicles ($P < 0.05$; Fig. 1). There was no detectable reduction of cortisone to cortisol in granulosa cell homogenates from any antral follicles or cysts, despite the addition of NADPH and 10 mM glucose-6-phosphate.

Effects of ovarian fluids and NADP⁺-dependent cortisol oxidation by 11 β HSD1 in rat kidney homogenates

All samples of porcine FF and ovarian cyst fluid had a significant net inhibitory effect on NADP⁺-dependent cortisol oxidation over 1 h in homogenates of rat kidney (Table 3). The extent of inhibition of 11 β HSD1 activity by FF aspirated from antral follicles decreased as follicle diameter increased. Hence, the extent of enzyme inhibition decreased progressively from a maximum of 50 $\pm$ 5% by FF from small antral follicles ($P < 0.001$) to only 23 $\pm$ 3% inhibition by FF from large antral follicles ($P < 0.05$; Table 3). Fluid aspirated from spontaneous ovarian cysts exerted the greatest inhibition of NADP⁺-dependent cortisol inactivation, suppressing 11 β HSD1 activity by 59 $\pm$ 3% of control enzyme activity ($P < 0.001$).

The majority of eluted fractions of porcine FF and cyst fluid significantly inhibited NADP⁺-dependent 11 β HSD activity in rat kidney homogenates (Fig. 2). The most inhibitory fraction for all fluids, irrespective of whether they were aspirated from small, medium or large antral follicles, or even from ovarian cysts, eluted between 70 and 80% (v/v) methanol and inhibited 11 β HSD-mediated inactivation of cortisol by around 50% (Fig. 2).

The hydrophilic fraction of FF aspirated from large antral follicles and eluted at 0% (v/v) methanol increased NADP⁺-dependent cortisol oxidation by 26 $\pm$ 5% ($P < 0.001$; Fig. 2c). However, none of the tested fractions eluted from FF aspirated from small (Fig. 2a) and medium (Fig. 2b) antral follicles or from

Table 3 Effects of porcine follicular fluid (FF) from small, medium and large antral follicles and of porcine cyst fluid from spontaneous ovarian cysts on NADP⁺-dependent cortisol oxidation by type 1 11 β -hydroxysteroid dehydrogenase (11 β HSD1) in rat kidney homogenates.

	NADP ⁺ -dependent 11 β HSD1 activity (% of control)
Small antral FF	50 $\pm$ 5***
Medium antral FF	68 $\pm$ 3***
Large antral FF	77 $\pm$ 3*
Ovarian cyst fluid	41 $\pm$ 3***

Each data entry represents the mean ($\pm$ S.E.M.) 11 β HSD1 activity (expressed as a percentage of the control enzyme activity measured in the absence of FF or cyst fluid) for five independent assays, where each assay was performed over 1 h in triplicate, using separate rat kidney homogenates with individual FF or cyst fluid samples. The control 11 β HSD1 activity, standardised to 100%, equated to 11.2 $\pm$ 0.1 pmol cortisone/h. Within the column, * $P < 0.05$ and *** $P < 0.001$ versus control enzyme activity measured in the absence of FF or cyst fluid.

Figure 2 Effects of C18 fractions of porcine FF from (a) small, (b) medium and (c) large antral follicles and (d) porcine cyst fluid from spontaneous ovarian cysts on NADP⁺-dependent cortisol oxidation by 11 β HSD1 in rat kidney homogenates. Each data point represents the mean ($\pm$ S.E.M.) 11 β HSD1 activity (percentage of control) for five assays, each of which was performed in triplicate, on individual rat kidney homogenates using sequential fractions of separate FF or cyst fluid samples. The horizontal line indicates a control enzyme activity of 100%, measured in the absence of fractions of FF or cyst fluid. The control 11 β HSD1 activities in rat kidney homogenates to which enzyme activities measured in the presence of FF or cyst fluid fractions were compared equated to 7.0 ± 0.3 , 8.5 ± 0.8 , 6.2 ± 0.3 and 12.3 ± 1.3 pmol cortisone/h respectively. Within each panel, * $P < 0.05$ and *** $P < 0.001$ versus respective control enzyme activity measured in the absence of FF or cyst fluid.

spontaneous ovarian cysts (Fig. 2d) significantly stimulated NADP⁺-dependent cortisol oxidation.

There was no significant correlation between the percentage inhibition of 11 β HSD1 activity in rat kidney homogenates by FF from antral follicles or porcine cyst

fluid and the progesterone concentration in those fluid samples ($R^2 = 0.052$; $P = 0.395$).

Effects of ovarian fluids and resolved fractions on 11 β HSD activities in porcine granulosa cells

Irrespective of the follicle type (small, medium and large antral follicles and ovarian cysts), co-incubation of granulosa cells with fluids derived from large antral follicles or ovarian cysts suppressed cortisol oxidation. The extent of inhibition of 11 β HSD activity by cyst fluid was consistently greater than the inhibition achieved with FF in each set of cells (Table 4). For example, in cells from large antral follicles, large antral FF inhibited cortisol oxidation by $40 \pm 5\%$ ($P < 0.01$) when compared with the $73 \pm 4\%$ inhibition ($P < 0.001$) achieved by co-incubation with cyst fluid. Likewise, in granulosa cells isolated from ovarian cysts, large antral FF and cyst fluid inhibited cortisol inactivation via 11 β HSD by $44 \pm 16\%$ ($P < 0.05$) vs $74 \pm 9\%$ ($P < 0.001$) respectively (Table 4).

After C18 column chromatography, most of the resolved fractions of porcine FF (from large antral follicles) and of porcine cyst fluid were able to inhibit net oxidation of cortisol by 11 β HSD in granulosa cells from large antral follicles (Fig. 3). The profiles of enzyme inhibition by the resolved fractions of FF and cyst fluid differed slightly from those observed when rat kidney homogenate was used as the source of enzyme activity. Cortisol oxidation in granulosa cells was significantly inhibited by those fractions of FF eluted at 20% and 60–100% (v/v) methanol (Fig. 3a) and by

Table 4 Effects of porcine follicular fluid (FF) from large antral follicles and of fluid from spontaneous ovarian cysts on cortisol oxidation by 11 β -hydroxysteroid dehydrogenase (11 β HSD) in porcine mural granulosa cells from small, medium and large antral follicles and from spontaneous ovarian cysts.

Follicle category from which granulosa cells were isolated	11 β HSD activity (% of control) in the presence of	
	FF	Cyst fluid
Small antral follicle	$65 \pm 12^*$	$55 \pm 2^*$
Medium antral follicle	$54 \pm 3^{***}$	$36 \pm 4^{***}$
Large antral follicle	$60 \pm 5^{**}$	$27 \pm 4^{***}$
Ovarian cyst	$56 \pm 16^*$	$26 \pm 9^{***}$

Each data entry represents the mean ($\pm$ S.E.M.) 11 β HSD activity (expressed as a percentage of the control enzyme activity for granulosa cells from the appropriate follicle category measured in the absence of FF or cyst fluid) for five independent assays, where each assay was performed over 4 h in triplicate, using granulosa cells from independent follicles/cysts. The control 11 β HSD activities for granulosa cells from each follicle category, standardised to 100%, were as defined in Table 2. Each individual granulosa cell culture was incubated in medium alone (control), with 10% (v/v) porcine large antral follicular fluid (FF) or with 10% (v/v) porcine cyst fluid. Within each column, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus the corresponding control enzyme activity measured in the absence of FF or cyst fluid.

Figure 3 Effects of C18 fractions of (a) porcine FF from large antral follicles and of (b) porcine cyst fluid from spontaneous ovarian cysts on the net oxidation of cortisol by 11 β HSD in porcine granulosa cells isolated from large antral follicles. Each data point represents the mean ($\pm$ S.E.M.) enzyme activity (percentage of control) for five assays, each performed in triplicate, on individual granulosa cell cultures with sequential fractions of separate FF or cyst fluid samples. The horizontal line indicates a control net 11 β HSD activity of 100%, measured in the absence of fractions of FF or cyst fluid. The control 11 β HSD activities in granulosa cells to which enzyme activities measured in the presence of FF and cyst fluid fractions were compared equated to 1.3 ± 0.3 and 3.5 ± 1.3 pmol cortisone/h respectively. Within each panel, * $P < 0.05$ and ** $P < 0.01$ versus respective control cortisol oxidation measured in the absence of FF or cyst fluid.

fractions of cyst fluid eluted at 70, 80 and 100% (v/v) methanol (Fig. 3b).

Discussion

This study is the first to document changes in the enzyme-catalysed inactivation of cortisol during ovarian follicle growth. Net 11 β -dehydrogenase activities increased in granulosa cells during antral follicle growth but low levels of cortisol oxidation were observed in granulosa cells from ovarian cysts. This indicates that within small antral follicles, and indeed, ovarian cysts, mural granulosa cells may experience higher intracellular glucocorticoid concentrations than in cells from large antral follicles. The inhibitory effects of FF and cyst fluid on cortisol oxidation in porcine granulosa cells could be attributed to hydrophobic components eluted at high concentrations of methanol, consistent with our previous findings regarding suppression of NADP(H)-dependent cortisol metabolism in rat kidney homogenates by hydrophobic components of porcine follicular fluid (FF; [Thurston et al. 2003a](#)). This study has also shown that levels of the enzyme inhibitors in FF progressively decrease during porcine antral follicle growth, but are increased in cyst

fluid. Therefore, the increasing levels of cortisol oxidation within mural granulosa cells during follicle growth are associated with decreasing levels of intra-follicular inhibitors of 11 β HSD1 activity in the antral follicles. Furthermore, the low 11 β -dehydrogenase activity in granulosa cells from ovarian cysts is associated with the highest levels of the intra-follicular 11 β HSD1 inhibitors in cyst fluid. These trends would be consistent with the compounds in FF and in cyst fluid exerting a local modulation of cortisol oxidation in granulosa cells. Indeed, this study shows that both FF and cyst fluid prior to fractionation, and the resolved hydrophobic constituents of these fluids, could significantly suppress cortisol oxidation by 11 β HSD enzymes in granulosa cells from ovarian follicles and cysts.

In granulosa cell homogenates, addition of NADP⁺ and NAD⁺ both increased cortisol oxidation. Enzyme activities were consistently higher in the presence of NADP⁺ than NAD⁺, particularly in granulosa cell homogenates from large antral follicles and ovarian cysts. While NADP⁺, being the larger pyridine nucleotide, binds selectively to the cofactor-binding site for 11 β HSD1, NAD⁺ can be utilised by both 11 β HSD1 and 11 β HSD2. Hence, inactivation of cortisol in the presence of exogenous NAD⁺ may reflect oxidation by either cloned 11 β HSD enzyme, whereas the ability of exogenous NADP⁺ to increase cortisol oxidation specifically indicates the presence of functional 11 β HSD1 in the granulosa cells.

In all granulosa cell preparations, there was no detectable reduction of cortisone to cortisol despite incubation with exogenous NADPH and glucose-6-phosphate. The results of this study complement recent evidence from human granulosa-lutein cells, bovine granulosa cells and rat testis Leydig cells, where 11 β HSD1 acts predominantly as an 11 β -dehydrogenase ([Gao et al. 1997](#), [Michael et al. 1997](#), [Ge & Hardy 2000](#), [Yong et al. 2000](#), [Tetsuka et al. 2003](#)). This has been attributed to the preferential usage of NADPH by the steroidogenic cytochrome P450 enzymes, resulting in the greater availability of NADP⁺ for the dehydrogenase activity of 11 β HSD1 ([Michael et al. 2003](#), [Ge et al. 2005](#)).

The progressive decline in the inhibition of NADP⁺-dependent 11 β -dehydrogenase activities in rat kidney homogenates by FF from antral follicles of increasing diameter could simply reflect dilution of locally synthesised enzyme inhibitors given that as antral follicles increase in diameter, FF volume increases at a faster rate than cell division in the follicle wall. However, this explanation seems unlikely given that the greatest inhibition of 11 β HSD1 activity was exerted by fluid from ovarian cysts, which had an antral volume $\sim$ 100-fold greater than large antral follicles. Hence, it seems more likely that the local synthesis of enzyme inhibitors changes during antral follicle and cyst growth. Furthermore, a single hydrophilic fraction eluted from FF of

large antral follicles could acutely increase NADP⁺-dependent cortisol oxidation by around 25% such that the opposing actions of a hydrophilic compound (or compounds) that stimulates cortisol metabolism might explain the lower inhibition of 11 β HSD1 activity by FF from large antral follicles.

The progressive increase in net cortisol oxidation in granulosa cells from follicles of increasing diameter, associated with progressively decreasing levels of 11 β HSD1 inhibitors in FF, indicates that intracellular glucocorticoids may be favourable for the development of small antral follicles but less so for large follicles. In noting that the lowest levels of cortisol inactivation occurred in granulosa cells from small antral follicles and spontaneous ovarian cysts, it may be relevant that these structures share the highest potential for growth. To attain ovulatory status, small antral follicles must increase in volume by ~60-fold, which is comparable to the size differential between large antral follicles and spontaneous ovarian cysts. Hence, low rates of glucocorticoid metabolism in small antral follicles and ovarian cysts may be functionally linked to follicle/cyst growth and/or fluid accretion in the follicle/cyst antrum. Glucocorticoids have been shown to stimulate granulosa cell differentiation (Schoonmaker & Erickson 1983) and so may participate in the differentiation of granulosa cell types in the antral follicle wall during early folliculogenesis. Since atresia appears to occur via an apoptotic mechanism (Hughes & Gorospe 1991, Tilly *et al.* 1992), the ability of glucocorticoids to inhibit granulosa cell apoptosis (Sasson *et al.* 2001) may also be important in limiting atresia in small antral follicles (and may even prevent apoptotic degeneration of ovarian cysts). With regards to the low levels of net cortisol oxidation in granulosa cells from porcine large antral follicles, glucocorticoids have been reported to inhibit porcine oocyte maturation (Yang *et al.* 1999), such that increased metabolism of cortisol by 11 β HSD in mural granulosa cells from large follicles may limit exposure of the preovulatory oocyte to glucocorticoids during oocyte maturation.

Irrespective of follicle diameter, fractions of FF eluted at methanol concentrations of >40% (v/v), and fractions eluted from cyst fluid above 50% (v/v) methanol, inhibited NADP⁺-dependent oxidation of cortisol in rat kidney homogenates. Thus, the 11 β HSD1 inhibitors in porcine FF eluted across a wider range of methanol concentrations than those published for human and bovine large antral follicles (Thurston *et al.* 2002, 2003a). While these inhibitors have not yet been identified, it appears that these are predominantly hydrophobic compounds and therefore most likely to be steroids or sterols, either produced locally or derived from the circulation. Furthermore, as the inhibitors elute across several methanol concentrations, they might also be various metabolites of steroids or sterols, with varying degrees of

hydrophobicity. Recent literature has documented hydrophobic substrates for renal or hepatic 11 β HSD1 other than the glucocorticoids, such as DHEA and its metabolites, 7 α - and 7 β -hydroxy-DHEA (Robinson *et al.* 2003, Robinson & Prough 2005), as well as 7 β -hydroxy- and 7-ketocholesterol (Hult *et al.* 2004, Schweizer *et al.* 2004).

Since progesterone and its 11-hydroxy-metabolites are potent inhibitors of cortisol metabolism (Souness *et al.* 1995, Souness & Morris 1996, Sun *et al.* 1998, Thurston *et al.* 2002, Latif *et al.* 2005, Robinson & Prough 2005), progesterone would be a strong candidate for an intra-follicular inhibitor of 11 β HSD1 activity. We had provisionally excluded progesterone as the major 11 β HSD1 inhibitor in FF on the basis that progesterone inhibits both 11 β HSD1 and 11 β HSD2 and elutes from a C18 column at lower methanol concentrations than are required to resolve the predominant 11 β HSD1 inhibitor from human and bovine FF (Thurston *et al.* 2002, 2003a). We now present new evidence to show that the progesterone concentration in individual FF or cyst fluid samples did not correlate to the extent to which those fluid samples inhibited 11 β HSD1-mediated cortisol metabolism.

In summary, we have demonstrated that porcine FF and cyst fluid contain hydrophobic compounds that inhibit the NADP⁺-dependent activity of 11 β HSD1 in rat kidney homogenates and can also inhibit enzymatic inactivation of cortisol in porcine granulosa cells. The levels of the paracrine enzyme inhibitors progressively decreased during growth of the antral follicle, coincident with an increase in the rate of cortisol metabolism by mural granulosa cells, but levels of the intra-follicular inhibitors of 11 β HSD1 were increased in spontaneous ovarian cysts, wherein granulosa cells exhibited low 11 β -dehydrogenase activities. These findings indicate that small antral follicles and ovarian cysts may be exposed to relatively high intracellular concentrations of active glucocorticoids and suggest a local role for cortisol in follicle development and/or cystic ovarian disease.

Acknowledgements

We would like to thank Dr Kim Jonas (Royal Veterinary College, London, UK) for her assistance in measuring concentrations of progesterone in porcine follicular fluid and cyst fluid samples. We also thank Mrs Sarah Winyard (St George's, University of London, UK) for her assistance in the final production of this manuscript. This work was financed by a BBSRC-CASE PhD studentship BBS/S/L/2003/10221, awarded in support of Neera Sunak and co-funded by the Biotechnology and Biological Sciences Research Council of the UK in partnership with Genus Plc. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Agarwal AK, Monder C, Eckstein B & White PC 1989 Cloning and expression of rat cDNA encoding corticosteroid 11 β -dehydrogenase. *Journal of Biological Chemistry* **264** 18939–18943.
- Agarwal AK, Mune T, Monder C & White PC 1994 NAD⁺-dependent isoform of 11 β -hydroxysteroid dehydrogenase. Cloning and characterization cDNA from sheep kidney. *Journal of Biological Chemistry* **269** 25959–25962.
- Albiston AL, Obeyesekere VR, Smith RE & Krozowski ZS 1994 Cloning and tissue distribution of the human 11 β -hydroxysteroid dehydrogenase type 2 enzyme. *Molecular and Cellular Endocrinology* **105** R11–R17.
- Atanasov AG, Nashev LG, Schweizer RA, Frick C & Odermatt A 2004 Hexose-6-phosphate dehydrogenase determines the reaction direction of 11 β -hydroxysteroid dehydrogenase type 1 as an oxoreductase. *FEBS Letters* **571** 129–133.
- Banhegyi G, Benedetti A, Fulceri R & Senesi S 2004 Cooperativity between 11 β -hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate dehydrogenase in the lumen of the endoplasmic reticulum. *Journal of Biological Chemistry* **279** 27017–27021.
- Brown RW, Chapman KE, Edwards CR & Seckl JR 1993 Human placental 11 β -hydroxysteroid dehydrogenase: evidence for and partial purification of a distinct NAD-dependent isoform. *Endocrinology* **132** 2614–2621.
- Calder MD, Manikkam M, Salfen BE, Youngquist RS, Lubahn BD, Lamberson WR & Garverick HA 2001 Dominant bovine ovarian follicular cysts express increased levels of messenger RNAs for luteinizing hormone receptor and 3 β -hydroxysteroid dehydrogenase delta(4),delta(5) isomerase compared to normal dominant follicles. *Biology of Reproduction* **65** 471–476.
- Conley AJ, Howard HJ, Slanger WD & Ford JJ 1994 Steroidogenesis in the preovulatory porcine follicles. *Biology of Reproduction* **51** 655–661.
- Draper N, Walker EA, Bujalska IJ, Tomlinson JW, Chalder SM, Arlt W, Lavery GG, Bedendo O, Ray DW, Laing I, Malunowicz E, White PC, Hewison M, Mason PJ, Connell JM, Shackleton CH & Stewart PM 2003 Mutations in the genes encoding 11 β -hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate dehydrogenase interact to cause cortisone reductase deficiency. *Nature Genetics* **34** 434–439.
- Gao HB, Ge RS, Lakshmi V, Marandici A & Hardy MP 1997 Hormonal regulation of oxidative and reductive activities of 11 β -hydroxysteroid dehydrogenase in rat Leydig cells. *Endocrinology* **138** 156–161.
- Ge RS & Hardy MP 2000 Initial predominance of the oxidative activity of type I 11 β -hydroxysteroid dehydrogenase in primary rat Leydig cells and transfected cell lines. *Journal of Andrology* **21** 303–310.
- Ge RS, Dong Q, Niu EM, Sottas CM, Hardy DO, Catterall JF, Latif SA, Morris DJ & Hardy MP 2005 11 β -hydroxysteroid dehydrogenase 2 in rat Leydig cells: its role in blunting glucocorticoid action at physiological levels of substrate. *Endocrinology* **146** 2657–2664.
- Guthrie HD, Grimes RW, Cooper BS & Hammond JM 1995 Follicular atresia in pigs: measurement and physiology. *Journal of Animal Science* **73** 2834–2844.
- Hughes FM Jr & Gorospe WC 1991 Biochemical identification of apoptosis (programmed cell death) in granulosa cells: evidence for a potential mechanism underlying follicular atresia. *Endocrinology* **129** 2415–2422.
- Hult M, Elleby B, Shafqat N, Svensson S, Rane A, Jornvall H, Abrahmsen L & Oppermann U 2004 Human and rodent type 1 11 β -hydroxysteroid dehydrogenases are 7 β -hydroxycholesterol dehydrogenases involved in oxysterol metabolism. *Cellular and Molecular Life Sciences* **61** 992–999.
- Jamieson PM, Chapman KE, Edwards CR & Seckl JR 1995 11 β -hydroxysteroid dehydrogenase is an exclusive 11 β -reductase in primary cultures of rat hepatocytes: effect of physicochemical and hormonal manipulations. *Endocrinology* **136** 4754–4761.
- Kesler DJ & Garverick HA 1982 Ovarian cysts in dairy cattle: a review. *Journal of Animal Science* **55** 1147–1159.
- Knox RV 2005 Recruitment and selection of ovarian follicles for determination of ovulation rate in the pig. *Domestic Animal Endocrinology* **29** 385–397.
- Lakshmi V & Monder C 1988 Purification and characterization of the corticosteroid 11 β -dehydrogenase component of the rat liver 11 β -hydroxysteroid dehydrogenase complex. *Endocrinology* **123** 2390–2398.
- Latif SA, Pardo HA, Hardy MP & Morris DJ 2005 Endogenous selective inhibitors of 11 β -hydroxysteroid dehydrogenase isoforms 1 and 2 of adrenal origin. *Molecular and Cellular Endocrinology* **243** 43–50.
- Low SC, Chapman KE, Edwards CR & Seckl JR 1994 Liver-type 11 β -hydroxysteroid dehydrogenase cDNA encodes reductase but not dehydrogenase activity in intact mammalian COS-7 cells. *Journal of Molecular Endocrinology* **13** 167–174.
- Maxson WS, Haney AF & Schomberg DW 1985 Steroidogenesis in porcine atretic follicles: loss of aromatase activity in isolated granulosa and theca. *Biology of Reproduction* **33** 495–501.
- McCormick KL, Wang X & Mick GJ 2006 Evidence that the 11 β -hydroxysteroid dehydrogenase (11 β -HSD1) is regulated by pentose pathway flux. Studies in rat adipocytes and microsomes. *Journal of Biological Chemistry* **281** 341–347.
- Mercer WR & Krozowski ZS 1992 Localization of an 11 β -hydroxysteroid dehydrogenase activity to the distal nephron. Evidence for the existence of two species of dehydrogenase in the rat kidney. *Endocrinology* **130** 540–543.
- Michael AE, Evagelatou M, Norgate DP, Clarke RJ, Antoniw JW, Stedman BA, Brennan A, Welsby R, Bujalska I, Stewart PM & Cooke BA 1997 Isoforms of 11 β -hydroxysteroid dehydrogenase in human granulosa-lutein cells. *Molecular and Cellular Endocrinology* **132** 43–52.
- Michael AE, Thurston LM & Rae MT 2003 Glucocorticoid metabolism and reproduction: a tale of two enzymes. *Reproduction* **126** 425–441.
- Monder C & Lakshmi V 1990 Corticosteroid 11 β -dehydrogenase of rat tissues: immunological studies. *Endocrinology* **126** 2435–2443.
- Naray-Fejes-Toth A, Watlington CO & Fejes-Toth G 1991 11 β -hydroxysteroid dehydrogenase activity in the renal target cells of aldosterone. *Endocrinology* **129** 17–21.
- Pallikaros Z, Schulster D, Baldwin SA, Helliwell RJ, Michael AE & Cooke BA 1995 Characterization of site-directed antibodies to the LH receptor in functionally active gonadal cells and their differential effects on LH-stimulated signal transduction in Leydig tumour (MA10) cells. *Molecular and Cellular Endocrinology* **114** 57–68.
- Ricketts ML, Verhaeg JM, Bujalska I, Howie AJ, Rainey WE & Stewart PM 1998 Immunohistochemical localization of type 1 11 β -hydroxysteroid dehydrogenase in human tissues. *Journal of Clinical Endocrinology and Metabolism* **83** 1325–1335.
- Robinson B & Prough RA 2005 Interactions between dehydroepiandrosterone and glucocorticoid metabolism in pig kidney: nuclear and microsomal 11 β -hydroxysteroid dehydrogenases. *Archives of Biochemistry and Biophysics* **442** 33–40.
- Robinson B, Michael KK, Ripp SL, Winters SJ & Prough RA 2003 Glucocorticoids inhibit interconversion of 7-hydroxy and 7-oxo metabolites of dehydroepiandrosterone: a role for 11 β -hydroxysteroid dehydrogenases? *Archives of Biochemistry and Biophysics* **412** 251–258.
- Sasson R, Tajima K & Amsterdam A 2001 Glucocorticoids protect against apoptosis induced by serum deprivation, cyclic adenosine 30,50-monophosphate and p53 activation in immortalized human granulosa cells: involvement of Bcl-2. *Endocrinology* **142** 802–811.
- Schoonmaker JN & Erickson GF 1983 Glucocorticoid modulation of follicle-stimulating hormone-mediated granulosa cell differentiation. *Endocrinology* **113** 1356–1363.
- Schweizer RA, Zurcher M, Balazs Z, Dick B & Odermatt A 2004 Rapid hepatic metabolism of 7-ketocholesterol by 11 β -hydroxysteroid

- dehydrogenase type 1: species-specific differences between the rat, human, and hamster enzyme. *Journal of Biological Chemistry* **279** 18415–18424.
- Seckl JR & Walker BR** 2001 11 β -hydroxysteroid dehydrogenase type 1 – a tissue-specific amplifier of glucocorticoid action. *Endocrinology* **142** 1371–1376.
- Souness GW & Morris DJ** 1996 11 α - and 11 β -hydroxyprogesterone, potent inhibitors of 11 β -hydroxysteroid dehydrogenase, possess hypertensinogenic activity in the rat. *Hypertension* **27** 421–425.
- Souness GW, Latif SA, Laurenzo JL & Morris DJ** 1995 11 α - and 11 β -hydroxyprogesterone, potent inhibitors of 11 β -hydroxysteroid dehydrogenase (isoforms 1 and 2), confer marked mineralocorticoid activity on corticosterone in the ADX rat. *Endocrinology* **136** 1809–1812.
- Sun K, Yang K & Challis JR** 1998 Regulation of 11 β -hydroxysteroid dehydrogenase type 2 by progesterone, estrogen, and the cyclic adenosine 5'-monophosphate pathway in cultured human placental and chorionic trophoblasts. *Biology of Reproduction* **58** 1379–1384.
- Tannin GM, Agarwal AK, Monder C, New MI & White PC** 1991 The human gene for 11 β -hydroxysteroid dehydrogenase. Structure, tissue distribution and chromosomal localization. *Journal of Biological Chemistry* **266** 16653–16658.
- Tetsuka M, Yamamoto S, Hayashida N, Hayashi KG, Hayashi M, Acosta TJ & Miyamoto A** 2003 Expression of 11 β -hydroxysteroid dehydrogenase in bovine follicle and corpus luteum. *Journal of Endocrinology* **177** 445–452.
- Thurston LM, Norgate DP, Jonas KC, Chandras C, Kloosterboer HJ, Cooke BA & Michael AE** 2002 Ovarian modulators of 11 β -hydroxysteroid dehydrogenase (11 β HSD) in follicular fluid from gonadotrophin-stimulated assisted conception cycles. *Reproduction* **124** 801–812.
- Thurston LM, Jonas KC, Abayasekara DR & Michael AE** 2003a Ovarian modulators of 11 β -hydroxysteroid dehydrogenase (11 β HSD) activity in follicular fluid from bovine and porcine large antral follicles and spontaneous ovarian cysts. *Biology of Reproduction* **68** 2157–2163.
- Thurston LM, Chin E, Jonas KC, Bujalska IJ, Stewart PM, Abayasekara - DR & Michael AE** 2003b Expression of 11 β -hydroxysteroid dehydrogenase (11 β HSD) proteins in luteinizing human granulosa-lutein cells. *Journal of Endocrinology* **178** 127–135.
- Tilly JL, Kowalski KI, Schomberg DW & Hsueh AJ** 1992 Apoptosis in atretic ovarian follicles is associated with selective decreases in messenger ribonucleic acid transcripts for gonadotropin receptors - and cytochrome P450 aromatase. *Endocrinology* **131** 1670–1676.
- Yang JG, Chen WY & Li PS** 1999 Effects of glucocorticoids on maturation of pig oocytes and their subsequent fertilizing capacity *in vitro*. *Biology of Reproduction* **60** 929–936.
- Yong PY, Thong KJ, Andrew R, Walker BR & Hillier SG** 2000 Development-related increase in cortisol biosynthesis by human granulosa cells. *Journal of Clinical Endocrinology and Metabolism* **85** 4728–4733.

Received 3 January 2007

First decision 26 January 2007

Accepted 27 February 2007