Inactivation of glucocorticoids by 11 β -hydroxysteroid dehydrogenase enzymes increases during the meiotic maturation of porcine oocytes

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Abstract

Recent reports have shown that glucocorticoids can modulate oocyte maturation in both teleost fish and mammals. Within potential target cells, the actions of physiological glucocorticoids are modulated by 11 β -hydroxysteroid dehydrogenase (HSD11B) isoenzymes that catalyse the interconversion of cortisol and cortisone. Hence, the objective of this study was to establish whether HSD11B enzymes mediate cortisol–cortisone metabolism in porcine oocytes and, if so, whether the rate of glucocorticoid metabolism changes during oocyte maturation. Enzyme activities were measured in cumulus–oocyte complexes (COCs) and denuded oocytes (DOs) using radiometric conversion assays. While COCs and DOs oxidised cortisol to inert cortisone, there was no detectable regeneration of cortisol from cortisone. The rate of cortisol oxidation was higher in expanded COCs than in compact COCs containing germinal vesicle (GV) stage oocytes (111 ± 6 vs 2041 ± 115 fmol cortisone/oocyte.24 h; $P < 0.001$). Likewise, HSD11B activities were 17 ± 1 fold higher in DOs from expanded COCs than in those from compact COCs ($P < 0.001$). When GV stage oocytes were subject to a 48 h *in vitro* maturation protocol, the enzyme activities were significantly increased from 146 ± 18 to 1857 ± 276 fmol cortisone/oocyte.24 h in GV versus MII stage oocytes respectively ($P < 0.001$). Cortisol metabolism was inhibited by established pharmacological inhibitors of HSD11B (glycyrrhetic acid and carbenoxolone), and by porcine follicular and ovarian cyst fluid. We conclude that an HSD11B enzyme (or enzymes) functions within porcine oocytes to oxidise cortisol, and that this enzymatic inactivation of cortisol increases during oocyte maturation.

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Introduction

The production of an oocyte competent of fertilisation and normal embryonic development is controlled by a series of highly organised signals and intracellular events, many of which are not fully understood. Recent experiments have shown physiological glucocorticoids can exert direct actions on the oocyte and affect its meiotic competence. In several different teleost fish species, cortisol and related hydroxylated metabolites of progesterone have been shown to induce oocyte maturation (Kime *et al.* 1992, Petrino *et al.* 1993, Mugnier *et al.* 1997, Pinter & Thomas 1999, Milla *et al.* 2006). In contrast, in the two species of mammals investigated to date (mouse and pig), glucocorticoids appear to exert a negative effect on oocyte maturation (Yang *et al.* 1999, Andersen 2003). Incubation of mouse oocytes with the synthetic glucocorticoid dexamethasone (DEX) did not prevent germinal vesicle breakdown (GVBD) but decreased the proportion of oocytes

progressing to the metaphase II (MII) stage of development (Andersen 2003). Exposure of porcine oocytes to both cortisol and DEX suppressed both GVBD and progression of oocytes to MII, and this effect could be prevented using the glucocorticoid receptor (GR) antagonist RU486 (Yang *et al.* 1999). A subsequent study by the same group found that DEX decreased expression of cyclin B1. Since cyclin B1, along with P34cdc2, is a key component of the 'maturation promoting factor' (MPF) complex, the adverse effect of cortisol and DEX on maturation of porcine oocytes was attributed to the suppression of cyclin B1 (and hence MPF) levels (Chen *et al.* 2000).

Within a wide range of potential target tissues, including ovarian follicles, the cellular actions of glucocorticoids are modulated by 11 β -hydroxysteroid dehydrogenase (HSD11B) enzymes that mediate the conversion of cortisol to its inert 11-ketosteroid metabolite, cortisone (reviewed by White *et al.* 1997, Kotelevtsev *et al.* 1999, Seckl & Walker 2001, Tomlinson *et al.* 2004,

Draper & Stewart 2005). To date, two biochemically distinct HSD11B enzymes have been cloned. While type 1 HSD11B (HSD11B1) is a relatively low affinity, NADP(H)-dependent bidirectional enzyme that can either inactivate cortisol or regenerate this physiological glucocorticoid from inert cortisone, HSD11B2 is a high affinity NAD⁺-dependent enzyme that acts exclusively as a dehydrogenase to inactivate cortisol (White *et al.* 1997, Kotelevtsev *et al.* 1999, Seckl & Walker 2001, Tomlinson *et al.* 2004, Draper & Stewart 2005). Previously, the balance of cortisol–cortisone metabolism by HSD11B enzymes in human ovarian follicles has been linked to the ability of human oocytes to undergo fertilisation *in vitro* and to give rise to viable embryos in women undergoing assisted conception (Michael *et al.* 1993, 1995, 1999, Keay *et al.* 2002, Lewicka *et al.* 2003, Thurston *et al.* 2003a). Recently, we have reported that both cloned HSD11B isoenzymes are co-expressed in the mural granulosa cells of bovine and porcine antral follicles, and have shown that in both cows and pigs, the ability of the HSD11B enzymes to inactivate cortisol within the mural granulosa cells increases during antral follicle growth (Sunak *et al.* 2007, Thurston *et al.* 2007).

Human, bovine and porcine antral follicles and spontaneous ovarian cysts have each been found to contain hydrophobic lipids that can selectively inhibit cortisol metabolism by the NADP(H)-dependent HSD11B1 enzyme without affecting the NAD⁺-dependent oxidation of cortisol by HSD11B2 (Thurston *et al.* 2002, 2003b, Sunak *et al.* 2007). Our most recent studies have established that in porcine antral follicles, the progressive increase in glucocorticoid inactivation with follicle growth coincides with a progressive decline in the levels of the lipid inhibitors of HSD11B1 in the follicular fluid (FF), whereas the highest level of the HSD11B1 inhibitors occur in ovarian cyst fluid (CF) accompanied by very low levels of glucocorticoid metabolism in granulosa cells from these cystic follicles (Sunak *et al.* 2007).

In terms of modulating glucocorticoid actions within the oocyte itself, Benediktsson *et al.* (1992) were the first to document the expression of high levels of HSD11B1 mRNA and protein in preovulatory rat oocytes. Subsequent studies confirmed the expression of HSD11B1 in human GV stage and MII oocytes with no detectable expression of HSD11B2 mRNA or protein in preovulatory or MII oocytes (Ricketts *et al.* 1998, Smith *et al.* 2000). However, no studies to date have established whether HSD11B enzymes are functional in mammalian oocytes and, if so, whether they catalyse net inactivation or regeneration of cortisol. Given the apparent adverse effects of glucocorticoids on the maturation of porcine oocytes, the primary aim of this study was to establish whether glucocorticoids are metabolised by HSD11B enzyme in porcine cumulus–oocyte complexes (COCs) and in

denuded porcine oocytes and, if so, whether levels of glucocorticoid metabolism change between the GV and MII stages of oocyte maturation. A secondary aim was to establish whether cortisol–cortisone metabolism in COCs and/or denuded oocytes (DOs) could be modulated by the lipid inhibitors of HSD11B1 activity in porcine FF and ovarian CF.

Results

GV stage oocytes exhibit HSD11B activity

Initial experiments were performed to determine whether either compact COCs or the DOs from these complexes had any HSD11B activity. Both COCs and denuded GV stage oocytes were capable of oxidising [³H]-cortisol to [³H]-cortisone (Fig. 1a), but did not exhibit detectable reduction of [³H]-cortisone to [³H]-cortisol. Although they did not differ significantly, 11 β -dehydrogenase activities were consistently higher in COCs (111 ± 6 , $n=10$) than in the DOs (88 ± 7 , $n=8$) within each assay, suggesting that both the cumulus cells and the oocyte may be capable of inactivating cortisol. Both carbenoxolone (CBX) and glycyrrhetic acid (GA), these being established inhibitors of both cloned HSD11B enzymes (Armanini *et al.* 1982, Stewart *et al.* 1990, Kageyama *et al.* 1992, Latif *et al.* 1992, Marandici & Monder 1993, Ulick *et al.* 1993), were able to suppress the net oxidation of cortisol by up to 81% in both compact COCs and GV stage oocytes ($P<0.01$; Fig. 1a).

HSD11B activity increases during oocyte maturation

Having established the presence of an active HSD11B enzyme system in porcine oocytes at the GV stage, we investigated the balance of cortisol–cortisone metabolism in maturing porcine oocytes that had undergone GVBD. Initially, we studied oocytes obtained from abattoir tissue where the oocytes were assessed as having resumed meiosis *in vivo* as reflected by expansion of the cumulus mass. Both expanded COCs and DOs isolated from expanded COCs were able to oxidise cortisol to cortisone (Fig. 1b). As with the compact COCs/DOs, cortisol metabolism by the expanded COCs/DOs could be inhibited by both CBX and GA, by $84 \pm 1\%$ ($n=4$) and $92 \pm 1\%$ respectively ($n=4$; $P<0.01$; Fig. 1b).

Rates of cortisol oxidation in expanded COCs and DOs were consistently 20-fold higher than in the compact COCs and GV stage DOs, suggesting an increase in activity as the oocyte matures. However, to test this hypothesis directly, we performed assessments of enzyme activities in oocytes that had been stimulated to mature through to the MII stage *in vitro*. Oocytes from COC's were found to have diffuse chromatin and

Figure 1 Net oxidation of cortisol by HSD11B increases during oocyte maturation in cumulus-enclosed and denuded porcine oocytes. Enzyme activities were assessed by incubating (a) COCs and denuded oocytes from compact COCs and (b) COCs and denuded oocytes from expanded COCs with 100 nmol/l [^3H]-cortisol in the presence of medium alone (control), carbenoxolone (+CBX; 10 $\mu\text{mol/l}$) or glycyrrhetic acid (+GA; 10 $\mu\text{mol/l}$). All data are the mean $\pm$ S.E.M. values for five groups of COC/DOs. ** $P < 0.01$ versus COCs/DOs incubated in the absence of CBX or GA.

no meiotic spindle, confirming they were either in the GV stage or just commencing GVBD (Fig. 2a and b). By contrast, *in vitro* maturation (IVM) oocytes that had extruded a polar body (PB) had progressed to the MII stage as verified by the alignment of their chromosomes on a metaphase plate with a clear meiotic spindle (Fig. 2c and d). On this basis, presence of a PB was used as an indicator of progression to MII in living (non-fixed) oocytes that could then be used in the radiometric conversion assay for HSD11B enzyme activity.

In six independent experiments, net rates of cortisol oxidation were consistently and significantly higher in denuded IVM oocytes that had reached MII than in denuded GV stage oocytes ($P < 0.001$; Fig. 3). Moreover, the rates of enzymatic inactivation of cortisol in IVM MII oocytes (1857 ± 276 fmol cortisone/oocyte.24 h) were consistent with the absolute enzyme activities measured in DOs from *in vivo* expanded COCs (1485 ± 65 fmol cortisone/oocyte.24 h).

Figure 2 Confirmation of the nuclear maturation status of GV stage oocytes from compact COCs and *in vitro* matured (IVM) oocytes. (a and b) Porcine oocytes isolated from compact COCs and (c and d) oocytes generated by IVM that had extruded a polar body were confirmed to be in the GV and in MII stage by fixing and staining with PI (b and d respectively) and an FITC-labelled anti-tubulin antibody (not shown). Bar = 100 μm .

Porcine FF and CF inhibit HSD11B activity in COCs and DOs

To determine whether compounds present in FF from large antral follicles and CF could affect the HSD11B enzyme activities in porcine oocytes, cortisol metabolism was assayed in compact and expanded COCs and DOs, each incubated in the presence of medium alone (control), with 10% (v/v) large antral FF or with 10% (v/v) CF from spontaneous ovarian cysts. FF had no significant effect on cortisol metabolism in compact COCs and their enclosed immature oocytes, but inhibited HSD11B activity by $42 \pm 6\%$ and $36 \pm 8\%$ in expanded COCs and DOs from those expanded COCs respectively ($P < 0.001$; Fig. 4). Although the inhibitory effect of CF on cortisol metabolism in compact COCs ($41 \pm 18\%$ inhibition) did not achieve statistical significance (Fig. 4a), CF significantly inhibited 11β -dehydrogenase activities by between $47 \pm 5\%$ and $62 \pm 9\%$ in expanded COCs and in DOs from both compact and expanded COCs ($P < 0.001$ in each case; Fig. 4).

Discussion

The physiological glucocorticoid cortisol is present throughout the development of porcine antral follicles at concentrations ranging from 115 to 144 nmol/l (Sunak 2007), which are sufficient to bind and activate intracellular GRs. Although several studies have reported the expression of HSD11B1 mRNA and protein in the mammalian oocyte and surrounding cumulus granulosa cells (Benediktsson *et al.* 1992, Ricketts *et al.* 1998,

Figure 3 The net oxidation of cortisol by HSD11B differs between GV and MII stage porcine oocytes. GV stage denuded oocytes were incubated in M199 medium containing FSH (500 ng/ml) for 48 h and confirmed to have reached MII by extrusion of a polar body. These denuded MII oocytes were assayed for cortisol metabolism in groups of three to five per group alongside groups of five freshly collected denuded GV stage oocytes. For each of the six independent batches of MII oocytes, the mean enzyme activities are represented by different symbols, connected to the corresponding mean value for GV stage oocytes in the same enzyme assay by a diagonal line.

Smith *et al.* 2000), this study is the first to demonstrate that at least one HSD11B enzyme is functional in the mammalian (porcine) oocyte/COC, controlling the exposure of oocytes to physiological glucocorticoids by catalysing the conversion of cortisol to inactive cortisone. Furthermore, during the nuclear maturation of porcine oocytes, the oxidative activity of HSD11B is dramatically increased. Since glucocorticoids have been reported to inhibit the nuclear maturation of porcine oocytes, apparently by repressing the expression of the cyclin D1 component of the MPF complex (Yang *et al.* 1999, Chen *et al.* 2000), this raises the possibility that progression of porcine oocytes to the ovulatory MII stage may require an increase in the enzymatic capacity of those oocytes to inactivate cortisol.

In the present study, cortisol oxidation could be measured in completely denuded porcine oocytes, confirming that there must be an operational HSD11B enzyme (or enzymes) expressed within the oocyte itself. The finding that rates of cortisol metabolism were consistently higher in COCs than in DOs suggests that either HSD11B enzymes may also act to metabolise cortisol within the associated cumulus granulosa cells or that the presence of cumulus granulosa cells serves to stimulate HSD11B activity within the oocyte. In both COCs and DOs, the oxidation of cortisol was inhibited by both GA and CBX and while these compounds are established inhibitors of both cloned HSD11B enzymes (Monder *et al.* 1989, Stewart *et al.* 1990,

Figure 4 Follicular fluid from large antral follicles (FF) and from spontaneous ovarian cysts (CF) inhibit the on the net oxidation of cortisol by HSD11B in porcine COCs and DOs. Enzyme activities were assessed by incubating (a) COCs and denuded oocytes from compact COCs and (b) COCs and denuded oocytes from expanded COCs with 100 nmol/l [3 H]-cortisol in the presence of medium alone (control), 10% (v/v) FF or 10% (v/v) CF. The bars represent the mean $\pm$ S.E.M. enzyme activity in the presence of FF or CF as a percentage of that control value ($n=5$ in each case). *** $P<0.001$ versus the corresponding COCs/DOs incubated in the absence of FF or CF.

Buhler *et al.* 1991, Kageyama *et al.* 1992, Latif *et al.* 1992, Marandici & Monder 1993, Ulick *et al.* 1993), they are also known to block gap-junctional communication (Davidson *et al.* 1986, Davidson & Baumgarten 1988, Goldberg *et al.* 1996). Their potential effects on gap junctions in the COCs are unlikely to account for the suppression of cortisol metabolism since both GA and CBX were used a concentration of 10 μ mol/l, which is an order of magnitude lower than the concentration of CBX (100 μ mol/l) required to block the passage of small molecules and ions between the oocyte and cumulus cells (Webb *et al.* 2002a, 2002b). Moreover, GA and CBX significantly inhibited cortisol metabolism in DOs where the blockade of gap junctions would be irrelevant.

In addition to testing the effects of GA and CBX, we also assessed the impact of porcine FF from large antral follicles and of porcine CF on cortisol metabolism in oocytes. Previously, we have reported that antral fluid contains compounds that can selectively inhibit the

NADP(H)-dependent activities of HSD11B1 without affecting the NAD^+ -dependent oxidation of cortisol by HSD11B2 in homogenates of rat kidney (Thurston *et al.* 2003b, Sunak *et al.* 2007). Levels of these endogenous ovarian inhibitors are lowest in FF from large antral follicles, but highest in CF from spontaneous ovarian cysts (Sunak *et al.* 2007). In the present studies, enzymatic inactivation of cortisol within porcine COCs and DOs could be inhibited by both CF (tested on the basis of its maximal content of ovarian HSD11B inhibitors) and, more importantly, by large antral FF. These findings indicate that compounds present within antral fluid of porcine ovarian follicles are capable of modulating cortisol–cortisone metabolism in porcine oocytes.

Irrespective of their nuclear maturation status, both COCs and DOs only exhibited net oxidation of cortisol to cortisone with no detectable reduction of cortisone back to cortisol. Since the 11-ketosteroid reductase activity of HSD11B1 has been reported to be labile (Lakshmi & Monder 1985, Agarwal *et al.* 1995, Blum *et al.* 2000, Blum & Maser 2003), we cannot categorically exclude the possibility that there is reduction of cortisone to cortisol by porcine oocytes *in vivo* and that, this reductase activity is lost during the isolation of the oocytes and the subsequent enzyme assays. However, the absence of any detectable 11-ketosteroid reductase activity in porcine COCs and DOs is in good agreement with our previous studies of cortisol–cortisone metabolism in mural granulosa cells recovered from porcine antral follicles at different stages of development (Sunak *et al.* 2007). Hence, it would appear that in the porcine ovary, the HSD11B enzyme(s) functions predominantly as an 11 β -dehydrogenase enzyme to inactivate cortisol with no capacity to regenerate cortisol from circulating cortisone.

Although HSD11B1 is intrinsically bidirectional, it usually acts predominantly (if not exclusively) as a reductase to regenerate cortisol in tissues such as the liver (Seckl & Walker 2001, Tomlinson *et al.* 2004, Draper & Stewart 2005). It had been assumed that this was true of all tissues, but recent studies have established that the predominant direction of activity for HSD11B1 is sensitive to the ratio of $\text{NADPH}/\text{NADP}^+$ in the lumen of the smooth endoplasmic reticulum, which in turn depends on carbohydrate metabolism by hexose-6-phosphate dehydrogenase (Draper *et al.* 2003, Atanasov *et al.* 2004, Banhegyi *et al.* 2004, Bujalska *et al.* 2005, Czegle *et al.* 2006, McCormick *et al.* 2006, Odermatt *et al.* 2006, White *et al.* 2007). While it is conventionally accepted that pyruvate and glutamine (and to a lesser extent acetyl-CoA derived from non-esterified fatty acids) are the major respiratory substrates for mammalian oocytes (Downs *et al.* 1997, 2002, Downs & Hudson 2000, Johnson *et al.* 2007, Songsasen *et al.* 2007), there are emerging roles for glycolytic oxidation of glucose and the pentose phosphate pathway in oocyte maturation (Downs *et al.* 1998, Harris *et al.* 2007). This could

set the balance of $\text{NADPH}/\text{NADP}^+$ in the mammalian oocyte such that HSD11B1 acts as a predominant 11 β -dehydrogenase enzyme to inactivate glucocorticoids.

Previous studies have focused on the activities of the more conventional hydroxysteroid dehydrogenase enzymes, namely HSD11B, HSD17B, 20 α HSD and 20 β HSD, during oocyte maturation. Immunohistochemical studies of mouse oocytes suggest that the activities of HSD3B1, HSD17B and 20 β HSD remain constant during oocyte maturation, and while the activity of 20 α HSD increases following hCG administration (either *in vivo* or *in vitro*), this occurs independently of meiotic maturation (Niimura & Kawakami 2003). Hence, this is the first report of a quantitative association between changes in the activity of any specific HSD enzyme as oocytes mature from the GV to the MII stage. At this time, we are unable to state whether the marked increase in cortisol metabolism by HSD11B is a cause or the consequence of the progression of porcine oocytes to MII: we have yet to determine whether meiotic progression can only occur in those oocytes in which the ability of the HSD11B enzymes to inactivate cortisol has already been increased.

In the mammalian ovary, the expression and activity of HSD11B1 are sensitive to a variety of endocrine/paracrine regulators, including LH/hCG (Tetsuka *et al.* 1997, 1999, Thurston *et al.* 2003c) and prostaglandin E_2 (Jonas *et al.* 2006, Chandras *et al.* 2007). In expanded COCs, it seems likely that the enclosed oocytes would have been subjected to the start of an LH surge *in vivo* prior to isolation which could account for their relatively high levels of cortisol oxidation. Similarly, in the IVM experiments reported herein, all COCs were exposed for 48 h to both FSH and LH, which may have increased the expression of HSD11B1 in those oocytes that progressed to MII.

In conclusion, these studies have established that the porcine oocyte expresses at least one functional HSD11B enzyme that acts to decrease the local glucocorticoid concentration by catalysing the oxidative inactivation of cortisol to cortisone. Moreover, the rate of steroid metabolism increases markedly coincident with oocyte maturation from the GV to the MII stage, suggesting that the increase in HSD11B enzyme activity may limit the potentially adverse effects of cortisol on the nuclear maturation of porcine oocytes.

Materials and Methods

Collection of oocytes, follicular and CFs

Sow ovaries were obtained from ANG Barbers abattoir (Chelmsford, Essex, UK) and transported in a Dewar flask at room temperature. On arrival in the lab, ovaries were washed twice in 0.9% (w/v) saline, after which antral follicles were classified as small (2–3 mm), medium (4–7 mm), large

(8–12 mm) or cystic follicles (20–40 mm in diameter). Within 5 h of collecting the ovaries, COCs were aspirated from non-cystic antral follicles in FF using a 21.1 gauge needle. The COCs were transferred from the FF into warm M199 medium (Invitrogen) supplemented with 10% (v/v) foetal bovine serum (FBS; Invitrogen), penicillin (100 IU/ml) and streptomycin (0.1 mg/ml; Invitrogen) and held thereafter at 39 °C. The remaining FF from large antral follicles and CF aspirated from spontaneous ovarian cysts during oocyte retrieval were each centrifuged at 250 *g* for 10 min at 4 °C to precipitate any contaminating granulosa cells. The acellular samples of FF and CF were then transferred into prelabelled polypropylene tubes and stored at –20 °C for up to 3 months (over which time the levels of lipid inhibitors of HSD11B1 activity did not change on storage).

COCs were examined under a Leica MZ 12.5 dissecting microscope and classified as either compact COCs or expanded COCs, dependent on the appearance of the cumulus cells. Compact COCs contained GV stage oocytes with three or more concentric layers of tightly attached cumulus cells, whereas expanded COCs contained oocytes that had undergone GVBD and were surrounded by a diffuse arrangement of cumulus mass embedded in matrix of hyaluronic acid. In selected experiments, oocytes were denuded from either compact COCs or expanded COCs by incubating these with 1% (w/v) hyaluronidase (Sigma) in M199 medium and vortexing gently for 2–5 min. The cumulus free DOs were then collected and washed three times in fresh pre-warmed M199 medium.

IVM of oocytes

Compact COCs, aspirated from medium diameter antral follicles, were collected and placed into an IVM medium comprising M199 medium supplemented with penicillin (100 mg/ml), streptomycin (0.1 mg/ml), epidermal growth factor (10 ng/ml), LH (500 ng/ml), FSH (500 ng/ml), cysteine (0.57 mmol/l), glutamine (3 mmol/l) and BSA (4 mg/ml). The COCs were then incubated at 39 °C under an atmosphere of 5% (v/v) CO₂ in air for 48 h, during which time the cumulus masses underwent expansion. Following the 48 h IVM protocol, the expanded cumulus cells were removed by vortexing in the presence of 1% (w/v) hyaluronidase, and the oocytes were visually assessed using a Leica MZ 12.5 dissection microscope for the presence of a PB as a marker of the nuclear maturation of the oocytes. Oocytes were then segregated according to the presence or absence of an extruded PB. Although the majority of these DOs were used directly in the radiometric conversion assay of HSD11B enzyme activities (described below), 12 oocytes that had extruded a PB within 48 h of IVM and ten oocytes denuded from compact COCs were each fixed in 4% (w/v) paraformaldehyde and 0.1% (w/v) TritonX-100. After blocking with FBS for 1 h at room temperature, these fixed oocytes were stained with a primary goat anti-tubulin antibody (1/200; 1 h) washed and incubated with a FITC-labelled anti-goat secondary antibody (1/400; 1 h, 37 °C) and with propidium iodide (10 µg/ml) to confirm the meiotic status of the oocytes. Fluorescence was recorded using a Olympus AX81 microscope.

HSD11B activity

Enzyme activities were measured using modifications of the radiometric conversion assays, which we have reported previously (Michael *et al.* 1997, Thurston *et al.* 2003c). In brief, COCs and DOs were incubated in 100 µl volumes of serum-free M199 medium either individually (expanded COCs) or in groups of five oocytes per well (compact COCs and all DOs). Assays of enzyme activity, conducted over a 24-h incubation phase at 39 °C, were initiated by the addition of either 100 nmol/l [1,2,6,7-³H]-cortisol (specific activity = 69 Ci/mmol; GE Healthcare, Buckinghamshire, UK) or 100 nmol/l [1,2-³H]-cortisone (specific activity = 40 Ci/mmol; GE Healthcare). Enzyme activities were also assessed in the presence of either CBX (10 µmol/l; Sigma) or GA (10 µmol/l; Sigma), or in the presence of porcine FF from large antral follicles or porcine CF from spontaneous ovarian cysts, each tested at a final dilution of 10% by volume. In all assays, incubations were terminated after 24 h, at which point the oocytes were visually examined to confirm no obvious degeneration had occurred, followed by extraction of the [³H]-steroids into two volumes of chloroform (Merck). After resolving [³H]-cortisol from [³H]-cortisone by thin layer chromatography in an atmosphere of 92: 8 chloroform: 95% (v/v) ethanol, the net fractional oxidation of [³H]-cortisol to [³H]-cortisone, or reduction of [³H]-cortisone to [³H]-cortisol, was quantified using an AR200 radiochromatogramme scanner with inline Laura Lite 3.0 software (LabLogic, Sheffield, UK).

Statistical analysis

In the first instance, all data were subjected to Kolmogorov–Smirnov testing to assess whether data conformed to Gaussian (Normal) distributions. Having confirmed all data to be normally distributed, mean values were then compared between groups using either unpaired *t*-tests or one-way ANOVA followed by the Bonferroni multiple comparison, as appropriate to the experimental design. All statistical tests were performed using GraphPad Prism 3.0 software (San Diego, CA, USA) and *P* values less than 0.05 were accepted as indicating statistical significance.

Declaration of interest

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

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