Expression of 11 β -hydroxysteroid dehydrogenase (11 β HSD) proteins in luteinizing human granulosa–lutein cells

L M Thurston, E Chin, K C Jonas¹, I J Bujalska², P M Stewart²,
D R E Abayasekara and A E Michael¹

Reproduction and Development Group, Department of Veterinary Basic Sciences, Royal Veterinary College, Royal College Street, London NW1 0TU, UK

¹Department of Biochemistry and Molecular Biology, Royal Free and University College Medical School, University College London, Rowland Hill Street, London NW3 2PF, UK

²Department of Medicine and Pathology, University of Birmingham, Queen Elizabeth II Hospital, Birmingham B15 2TH, UK

(Requests for offprints should be addressed to L Thurston; Email: lthurston@rvc.ac.uk)

Abstract

In a range of tissues, cortisol is inter-converted with cortisone by 11 β -hydroxysteroid dehydrogenase (11 β HSD). To date, two isoforms of 11 β HSD have been cloned. Previous studies have shown that human granulosa cells express type 2 11 β HSD mRNA during the follicular phase of the ovarian cycle, switching to type 1 11 β HSD mRNA expression as luteinization occurs. However, it is not known whether protein expression, and 11 β HSD enzyme activities reflect this reported pattern of mRNA expression. Hence, the aims of the current study were to investigate the expression and activities of 11 β HSD proteins in luteinizing human granulosa–lutein (hGL) cells. Luteinizing hGL cells were cultured for up to 3 days with enzyme activities (11 β -dehydrogenase (11 β BDH) and 11-ketosteroid reductase (11 KSR)) and protein expression (type 1 and type 2 11 β HSD) assessed on each day of

culture. In Western blots, an immunopurified type 1 11 β HSD antibody recognized a band of 38 kDa in hGL cells and in human embryonic kidney (HEK) cells stably transfected with human type 1 11 β HSD. The type 2 11 β HSD antibody recognized a band of 48 kDa in HEK cells transfected with human type 2 11 β HSD cDNA but the type 2 protein was not expressed in hGL cells throughout the 3 days of culture. While the expression of type 1 11 β HSD protein increased progressively by 2.7-fold over 3 days as hGL cells luteinized, both 11 β BDH and reductase activities declined (by 52.9% and 34.2%; $P < 0.05$) over this same period. Changes in enzyme expression and activity were unaffected by the suppression of ovarian steroid synthesis.

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Introduction

In a wide range of tissues, cortisol is metabolized to the inactive metabolite cortisone by the enzyme 11 β -hydroxysteroid dehydrogenase (11 β HSD) (Edwards & Stewart 1991, Monder 1991, Seckl & Walker 2001). To date, two isoforms of 11 β HSD have been cloned. Type 1 11 β HSD is a ubiquitously expressed enzyme with a relatively low affinity for cortisol ($K_m = 27 \mu\text{M}$). *In vitro*, type 1 11 β HSD can both oxidize cortisol to cortisone and reduce cortisone to cortisol in the presence of NADP⁺ and NADPH respectively (Monder *et al.* 1991). *In vivo*, this isoform appears to act predominantly as an NADPH-dependent 11-ketosteroid reductase (11 KSR), catalysing the reduction of cortisone to cortisol (Lakshmi & Monder 1988, Agarwal *et al.* 1989, Tannin *et al.* 1991). In contrast, type 2 11 β HSD is an NAD⁺-dependent, high-affinity isoform (K_m for cortisol = 30–60 nM) that appears to act exclusively as an 11 β -dehydrogenase (11 β BDH) enzyme

(Rusvai & Naray-Fejes-Toth 1993, Agarwal *et al.* 1994) protecting non-specific mineralocorticoid receptors from occupation by glucocorticoids in the distal nephron, colon and parotid gland (Rusvai & Naray-Fejes-Toth 1993, Albiston *et al.* 1994, Zhou *et al.* 1995).

Recently, 11 β HSD activities have been measured in human granulosa–lutein (hGL) cells obtained from women undergoing treatment for infertility (Owen *et al.* 1992, Michael *et al.* 1993a,b, 1995, Thomas *et al.* 1998) and these ovarian cells have been shown to express mRNA encoding both cloned isoforms of 11 β HSD. Further studies have shown that the pattern of mRNA expression appears to be differentially regulated during follicular development in granulosa cells and luteinization. Specifically, granulosa cells obtained from preovulatory follicles that have not experienced the preovulatory luteinizing hormone (LH) surge express only type 2 11 β HSD mRNA with no detectable expression of type 1 11 β HSD mRNA (Tetsuka *et al.* 1997, 1999b). Conversely, hGL cells that have been

exposed *in vivo* to an ovulatory dose of human chorionic gonadotrophin (hCG) express variable levels of mRNA encoding type 1 11 β HSD with no expression of type 2 11 β HSD mRNA (Michael *et al.* 1997, Smith *et al.* 1997). While LH and interleukin-1 β (IL-1 β) have been shown to induce expression of type 1 11 β HSD mRNA in rat granulosa cells (Tetsuka *et al.* 1999a) and human ovarian surface epithelial cells (Yong *et al.* 2002), the molecular basis for the 'follicular switch' between type 2 11 β HSD in the follicle and type 1 11 β HSD in luteinized granulosa cells is not yet defined. Moreover, it has not been confirmed whether the switch in mRNA transcripts was reflected in the pattern of type 2 and type 1 11 β HSD proteins.

The objectives of the present study were to investigate whether: (1) type 1 and type 2 11 β HSD protein expression is differentially regulated as granulosa cells become progressively more luteinized; (2) 11 β DH and 11 KSR activities change as luteinization takes place; and (3) progesterone secretion by hGL cells affects the pattern of 11 β HSD protein expression and enzyme activities.

Materials and Methods

Cell isolation and culture

Samples of ovarian follicular fluid were aspirated from women undergoing oocyte retrieval for *in vitro* fertilization-embryo transfer (IVF-ET) at the Lister Private Hospital (London, UK). Controlled ovarian hyperstimulation was induced by the sequential administration of a gonadotrophin-releasing hormone (GnRH) analogue, human menopausal gonadotrophin (hMG) and hCG as previously described (Ragoobir *et al.* 2002). All samples were obtained with informed patient consent (in accordance with the Declaration of Helsinki) and with the approval of the local ethics committee. Aspirated ovarian follicles were routinely flushed with sterile Earle's Balanced Salt Solution (EBSS) to ensure oocyte retrieval. Within 4 h of collection, the follicular aspirates and flushings were transported at room temperature to the Royal Veterinary College and hGL cells were partially purified on 60% (v/v) Percoll (Sigma) as described previously (Abayasekara *et al.* 1993, Michael *et al.* 1995). Following isolation, cell numbers were estimated using a haemocytometer and viabilities were assessed by the exclusion of Trypan Blue dye (Merck, Poole, Dorset, UK). hGL cells were suspended in 1:1 Dulbecco's modified Eagle's medium (DMEM):Ham's F₁₂ medium supplemented with L-glutamine (2 mM), penicillin (87 000 IU/l), streptomycin (87 mg/l) and 10% (v/v) foetal calf serum (FCS), and were cultured for 1–3 days at 37 °C in an atmosphere of 5% (v/v) CO₂ in air. (Culture media and supplements were all purchased from Life Technologies, Strathclyde, UK.) Cells were plated at a density of 5×10^4

cells/well in 24-well plates for measurements of 11 β HSD activity and progesterone secretion. For immunoblotting studies, cells were plated at a density of 1×10^6 cells/well in six-well plates.

Transfection of human embryonic kidney cells with 11 β HSD cDNA

Human embryonic kidney (HEK) cells, stably transfected to express either type 1 or type 2 11 β HSD as described previously (Bujalska *et al.* 1997, Moore *et al.* 1999), were used as positive controls during Western blotting analyses. In brief, HEK293 cells (ECACC No. 85120602) were cultured in growth media (DMEM supplemented with 1% (v/v) non-essential amino acids and 10% (v/v) FCS) until 40% confluent. After transfer to serum-free DMEM, transfections were performed in 2 ml serum-free minimum essential medium (MEM) with human type 1 11 β HSD cDNA in pCDNA3 or human type 2 11 β HSD cDNA in pCR3 (each at 5 μ g/ml) (Invitrogen Ltd, Paisley, UK). Those clones stably transfected with type 1 or type 2 11 β HSD cDNA (HEK1 and HEK2 respectively) were selected in growth media supplemented with the selecting antibiotic, G418 (0.5 mg/ml) (Life Technologies). After 3 weeks, transfected cells formed single-cell colonies and there were no live cells in the control dishes. Twelve colonies derived from a single cell of HEK1 or HEK2 transfectants were isolated and cultured individually. HEK1 and HEK2 clones were cultured in 1:1 DMEM:Ham's F₁₂ medium supplemented with L-glutamine (2 mM), penicillin (87 000 IU/l), streptomycin (87 mg/l) and 10% (v/v) FCS at 37 °C in an atmosphere of 5% (v/v) CO₂ in air for subsequent use in Western blotting analyses.

Preparation of cell lysates

On days 1, 2 and 3 of culture, lysates of hGL cells were prepared for immunoblotting as previously described by Dewi *et al.* (2002). In brief, cells were rinsed thoroughly with warm, serum-free 1:1 DMEM:Ham's F₁₂ medium. All experimental treatments were subsequently carried out in serum-free media. After treatment, the medium was aspirated from the culture dishes and the hGL cells were rinsed with ice-cold PBS (pH 7.5, Life Technologies) containing sodium orthovanadate (Na₃VO₄; 200 μ M) (Sigma). PBS was immediately aspirated to dryness and the cells 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), Na₃VO₄ (1 mM), 4-(2-aminoethyl)benzene sulphonyl fluoride (1 mM) (Sigma) and leupeptin (50 μ g/ml) (Sigma). Cells were then incubated on ice for 10 min and monolayers were scraped into 1.5 ml centrifuge tubes. The protein content of cell lysates was quantified using the bicinchoninic acid method according to the manufacturer's instructions (Pierce

Chemical Company, Chester, 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. HEK cell lysates were prepared as described for hGL cells.

Immunoblotting

Proteins from HEK1, HEK2 and hGL cell lysates (100 μ g protein/lane) were separated using 10% (v/v) SDS-PAGE and transferred to an Immobilon polyvinylidene difluoride (PDF) membrane using a semi-dry Western blotting apparatus (Bio-Rad Laboratories, Inc., Hemel Hempstead, UK). Pre-stained molecular weight markers were run and transferred in parallel lanes (Sigma). Non-specific antibody binding was blocked by incubation of 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). To correct for any minor variations in protein loading or transfer, all 11 β HSD protein bands were standardized relative to the housekeeper protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Membranes were therefore incubated overnight at 4 °C with the appropriate primary antibody to either 11 β HSD or GAPDH, at a dilution of 1/100 in 10% (w/v) BSA/TBST. Polyclonal primary antibodies to type 1 and type 2 11 β HSD were raised commercially (The Binding Site Ltd, Birmingham, UK) in sheep against specific peptide sequences of the human enzyme isoforms (type 1 11 β HSD amino acids 19–33; type 2 11 β HSD amino acids 137–160 and 334–358). Monoclonal primary antibodies to GAPDH were also raised commercially (The Binding Site Ltd) in mouse against the human peptide sequence. After washing with TBST (6 $\times$ 10 min), membranes were incubated with either donkey anti-sheep peroxidase-conjugated IgG (Sigma) for identification of 11 β HSD proteins or rabbit anti-mouse conjugated IgG (Sigma) for GAPDH proteins, both at a dilution of 1/10 000 in 0.2% (w/v) BSA/TBST for 1 h, followed by further washes in TBST (8 $\times$ 10 min). Immunoreactive proteins were visualized using an enhanced chemiluminescent (ECL) detection system according to the manufacturer's instruction (Amersham, Bucks, UK). 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.).

Effects of aminoglutethimide on progesterone synthesis by luteinizing hGL cells

On each of days 1, 2 or 3 of cell culture, hGL cells were rinsed thoroughly with warm serum-free 1:1

DMEM:Ham's F₁₂ medium. To each triplicate set of wells was then added 900 μ l fresh serum-free medium and 100 μ l of either aminoglutethimide (AG; 100 μ M) (Sigma) (experimental wells) or a further 100 μ l of serum-free medium (controls). The concentration of AG was selected on the basis of previous reports demonstrating inhibition of gonadal steroidogenesis (Peluso & Pappalardo 1999, Fowkes *et al.* 2001). In order to investigate the effects of acute and chronic treatment with AG on progesterone production, cells were treated for either 4 or 24 h before addition of perchloric acid (HClO₄; 107 mM) (Merck) and transfer of culture plates to –20 °C. Control wells were treated with vehicle for 4 h only. On thawing, samples were neutralized by the addition of tripotassium phosphate (K₃PO₄; 154 mM) (Merck) before measuring progesterone concentrations in the hGL cell culture media using a standard RIA described previously with an EC₅₀ of 3.97 nM (Pallikaros *et al.* 1995). In order to ensure that samples fell on the linear region of the RIA standard curve, media samples were assayed at dilutions between 1/50 and 1/200.

Effects of AG on the expression and activities of 11 β HSD in luteinizing hGL cells

To assess effects of AG on protein expression, cells were treated in serum-free 1:1 DMEM:Ham's F₁₂ medium on day 1, 2 or 3 of culture for either 4 or 24 h with 100 μ M AG, before preparing cell lysates for Western blotting. To assess effects on 11 β HSD enzyme activities, cells were treated in parallel on each day of culture with 100 μ M AG for either 4 h (acute) during the 11 β HSD assay or, for the 4-h assay period plus the previous 20 h in culture (chronic). After a 30-min pre-incubation at 37 °C in 900 μ l serum-free medium $\pm$ AG, a further 100 μ l serum-free medium containing 0.5 μ Ci [1,2,6,7-³H]cortisol (Amersham) plus unlabelled cortisol (Sigma; to a final steroid concentration of 100 nM) was added to each well in order to assess 11 β DH activity. Assays of 11 KSR activity were performed by adding cortisone (rather than cortisol) as the assay substrate (final concentration = 100 nM containing 0.1 μ Ci [1,2,6,7-³H]cortisone per well). For these assays [³H]cortisone was prepared by overnight incubation of a rat kidney homogenate at 37 °C with 20 μ Ci [³H]cortisol per tube plus 400 μ M NADP⁺ (Sigma) (Michael *et al.* 1997).

Following the addition of the assay substrate, cells were incubated for 4 h at 37 °C, after which samples were transferred to screw-cap glass culture tubes, and steroids were extracted (Michael *et al.* 1995). The steroid residues were re-suspended in 20 μ l 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 [³H]cortisol and [³H]cortisone using a Bioscan 200 TLC

Figure 1 Type 1 and type 2 11 β HSD protein expression in hGL cells on day 3 of culture. The immunoblot shown is representative of results obtained from 4 (of 11) individual patients (P1–P4). HEK cells stably transfected with type 1 (HEK1) or type 2 11 β HSD (HEK2) were used as positive controls.

radiochromatogram scanner (LabLogic, Sheffield, UK), 11 β HSD activities were calculated as either picomoles of cortisol oxidized to cortisone, or picomoles of cortisone reduced to cortisol, over 4 h and standardized per 50 000 hGL cells present at the outset of culture (Michael *et al.* 1995, 1996).

Statistical analyses

The initial experiment, to identify which isoforms of 11 β HSD were expressed on day 3 of culture, was repeated using cells from 11 individual patients. All subsequent experiments were repeated four times using cells from a different patient on each occasion. Where levels of protein expression were quantified, the histograms show the mean ($\pm$ SE) densities of each 11 β HSD protein band relative to the corresponding GAPDH band from four independent experiments and a representative immunoblot for a single patient is also shown. For studies measuring progesterone secretion, each treatment was performed in triplicate for cells from each patient. The effects of days in culture or treatment with AG on progesterone secretion by hGL cells, 11 β HSD activities and 11 β HSD protein expression were each assessed using one-way ANOVA followed by Dunnett's multiple comparison. All statistical evaluations were performed using GraphPad Prism2 software (GraphPad Inc., San Diego, CA, USA). $P < 0.05$ was accepted as statistically significant.

Results

Type 1 and type 2 11 β HSD protein expression in luteinizing human granulosa cells

The type 1 11 β HSD antibody recognized a single band of approximately 38 kDa in lysates of HEK1 cells (stably expressing type 1 11 β HSD) and of cultured hGL from each of the 11 different patients studied (Fig. 1). (The

Figure 2 Type 1 11 β HSD protein expression in hGL cells on days 1–3 of culture (D1–D3). (a) The immunoblot shown is representative of data from 4 (of 11) individual patients (P1–P4). HEK cells stably transfected with type 1 11 β HSD (HEK1) were used as a positive control. (b) Densitometric analysis of the type 1 11 β HSD protein band relative to the corresponding GAPDH protein band; mean $\pm$ S.E. for 11 different patients. * $P < 0.01$ versus day 1 of culture.

apparent molecular weight of this band was in keeping with previously identified type 1 11 β HSD protein bands in human liver and decidua (Ricketts *et al.* 1998).) In marked contrast, the type 2 11 β HSD antibody did not detect any proteins in the 11 different hGL lysates (Fig. 1). However, the same antibody detected a protein of approximately 48 kDa in lysates prepared from type 2 11 β HSD-transfected HEK2 cells, consistent with previous studies using human kidney tissue lysates (Shimojo *et al.* 1997).

To determine if the pattern of 11 β HSD protein expression changed as granulosa cells became progressively more luteinized, Western blots were performed using hGL lysates prepared on days 1, 2 and 3 of culture. Expression of type 1 11 β HSD protein increased by up to 2.7-fold from day 1 to day 3 of culture ($P < 0.01$; Fig. 2). There was no type 2 11 β HSD protein expression in hGL cells at any stage of culture up to and including day 3.

Figure 3 Progesterone production in hGL cells on successive days in culture in the presence or absence of AG; mean $\pm$ S.E. for 11 different patients. Cells were treated with vehicle for 4 h (open bar), or treated with AG for either 4 h (grey bar) or 24 h (black bar). * $P < 0.05$ versus the respective control value on each day of culture.

Progesterone production in hGL cells on days 1–3 of culture

Progesterone production in hGL cells significantly increased by up to 3.4-fold between day 1 and day 3 of culture ($P < 0.0001$) (Fig. 3). Both acute (4 h) and chronic (24 h) treatment of hGL cells with AG decreased progesterone production by up to 75% when compared with untreated cells on each day in culture ($P < 0.001$) (Fig. 3).

Effect of AG on type 1 11 β HSD protein expression in luteinizing hGL cells

In control hGL cells, incubated in the absence of AG, type 1 11 β HSD protein expression increased by up to 4.7-fold between day 1 and day 3 of culture ($P < 0.001$; Fig. 4), consistent with the results of our initial experiments. However, there were no significant differences in type 1 11 β HSD protein expression between the control hGL cells and those treated acutely or chronically with an effective concentration of AG on each day of culture ($P > 0.05$; Fig. 4). Moreover, the acute and chronic exposure to AG did not prevent the progressive increase in expression of type 1 11 β HSD protein between days 1 and 3 of culture ($P < 0.01$ for each treatment condition).

11 β DH and 11 KSR activities in luteinizing human granulosa cells

In the absence of AG, 11 β DH activity in hGL cells decreased by up to 56% between day 1 and day 3 of culture ($P < 0.0001$; Fig. 5). Acute and chronic treatment of hGL cells with AG significantly increased 11 β DH activities when compared with untreated cells on each day in culture (up to 2.1-fold and 1.3-fold respectively; $P < 0.05$; Fig. 5). Enzyme activities were consistently

Figure 4 Type 1 11 β HSD protein expression in hGL cells on days 1–3 of culture (D1–D3) in the presence or absence of AG. (a) A representative immunoblot for cells from one patient is shown. HEK cells stably transfected with type 1 11 β HSD (HEK1) were used as a positive control. Cells were treated with vehicle only for 4 h (–), or treated with AG for either 4 h (acute, A) or 24 h (chronic, C). (b) Densitometric analysis of the type 1 11 β HSD protein band relative to the corresponding GAPDH protein band; mean $\pm$ S.E. for 11 different patients. Cells were treated with vehicle for 4 h (open bar), or treated with AG for either 4 h (grey bar) or 24 h (black bar).

higher in hGL cells exposed to AG for only 4 h as compared with cells exposed for 24 h ($P < 0.05$). However, acute and chronic exposure of hGL cells to AG had no impact on the progressive decline in 11 β DH activities over days 1–3 of culture (Fig. 5).

11 KSR activities followed the same pattern as 11 β DH activities, in so far as enzyme activities decreased by up to 43% over the first 3 days of culture irrespective of the presence or absence of AG ($P < 0.001$; Fig. 6). However, while acute exposure of hGL cells to AG increased 11 KSR activities by up to 1.9-fold when compared with untreated cells on each day in culture ($P < 0.001$), chronic

Figure 5 Net 11 β DH activity in hGL cells on days 1–3 of culture in the presence or absence of AG; mean $\pm$ S.E. for 11 different patients. Cells were treated with vehicle for 4 h (open bar), or treated with AG for either 4 h (grey bar) or 24 h (black bar). * P < 0.05 versus the respective control value on each day of culture.

Figure 6 Net 11 KSR activity in hGL cells on days 1–3 of culture in the presence or absence of AG; mean $\pm$ S.E. for 11 different patients. Cells were treated with vehicle for 4 h (open bar), or treated with AG for either 4 h (grey bar) or 24 h (black bar). * P < 0.05 versus the respective control value on each day of culture.

treatment of hGL cells with AG for 24 h produced relatively modest increases in 11 KSR activities (Fig. 6).

Discussion

Human granulosa cells, which exhibit both oxidative and reductive 11 β HSD activities (Michael *et al.* 1997, Thomas *et al.* 1998), express both type 1 and type 2 11 β HSD mRNA (Tetsuka *et al.* 1997). The pattern of mRNA expression appears to be developmentally regulated during

the ovarian cycle with follicular granulosa cells exclusively expressing type 2 11 β HSD mRNA and luteinized granulosa cells switching to expression of mRNA encoding the type 1 11 β HSD isoform (Tetsuka *et al.* 1997). In this paper, we report evidence that after administration of hCG *in vivo*, hGL cells undergoing functional luteinization *in vitro* express type 1 11 β HSD protein but not type 2 11 β HSD protein, consistent with the mRNA expression pattern documented previously (Michael *et al.* 1997, Tetsuka *et al.* 1997). Furthermore, type 1 11 β HSD protein expression increases over days 1–3 of culture as hGL cells become progressively more luteinized, reflected by a progressive increase in the production of progesterone (Fowkes *et al.* 2001).

The molecular basis of the ovarian switch in the expression of the 11 β HSD isoforms remains unclear. It has been suggested that the switch from expression of type 2 to type 1 11 β HSD may reflect a change in the cortisol requirements of the ovarian follicle in the periovulatory phase of the cycle, moving from inactivation of cortisol by type 2 11 β HSD to net generation of cortisol by the 11 KSR activity of type 1 11 β HSD (Tetsuka *et al.* 1997). Consistent with this hypothesis, cortisol concentrations do indeed increase in follicular fluid with follicular maturation (Fateh *et al.* 1989, Harlow *et al.* 1997). While this increase in the intra-follicular free cortisol concentration with follicular maturation could reflect an increase in the 11 KSR activity of ovarian 11 β HSD enzymes, it may also reflect a decrease in the rate of cortisol inactivation by 11 β DH activities. In addition, it is possible that the observed increase in free cortisol within the preovulatory follicle reflects increasing displacement of cortisol from its corticosteroid-binding-globulin (CBG) by progesterone (Andersen 1990, Hammond *et al.* 1991).

Since hGL cells exhibit both 11 β DH and 11 KSR activities, the progressive decrease in the net oxidation of cortisol by luteinizing cells *in vitro* may have reflected increasing reduction of cortisone to cortisol. However, this was found not to be the case since both the 11 β DH and 11 KSR activities of luteinizing hGL cells decreased in parallel. Moreover, this decline in both enzyme activities occurred in the face of increasing expression of type 1 11 β HSD protein. There would appear to be three possible explanations for this discord between 11 β HSD enzyme activities and the levels of expression of the type 1 11 β HSD protein: (1) hGL cell membranes have become disrupted during cell culture, allowing pyridine nucleotides to 'leak' out of the cells, which may result in a loss in enzyme activities, (2) the measured enzyme activities may be attributable to an isoform of 11 β HSD other than type 1 or type 2 11 β HSD, or (3) there may be post-translational inhibition of the enzymatic activities of type 1 11 β HSD as hGL luteinize *in vitro*.

Previously, Ferguson *et al.* (1999) showed that in rat Leydig cells, supplementation of cultured medium with pyridine nucleotides resulted in increased 11 β HSD

activities. It has been suggested that the decline in 11 β HSD activities observed in this study may therefore have been due to the loss of enzyme cofactors as cultured cell membranes became 'leaky'. However, we have previously established that over the first 4 days of cell culture there is no significant increase in the admission of vital dyes (trypan blue, crystal violet or propidium iodide) into hGL cells. Furthermore, in the current study we confirmed that the output of progesterone from luteinizing granulosa cells increased progressively over the first 3 days in culture. Hence, we assume that the cells must retain sufficient NADPH to support P450 cholesterol side-chain cleavage (CSCC) activity and sufficient NAD⁺ to support increasing 3 β HSD activity. Since NADP⁺ is even less likely to leak out of cells than either the 'smaller' nucleotide, NAD⁺, or the uncharged nucleotide, NADPH, we would argue that leakage of NADP⁺ would not be sufficient to explain the progressive decline in 11 β HSD activities as human granulosa cells undergo functional luteinization *in vitro*.

As regards the possibility that the declining 11 β DH and 11 KSR activities of hGL cells may be attributable to a novel isoform of 11 β HSD, our previous biochemical studies found the major enzyme activity in these ovarian cells to be an NADP⁺-dependent 11 β DH activity with a K_m for cortisol more than an order of magnitude lower than that for type 1 11 β HSD (Michael *et al.* 1997). Although a similar enzyme activity has also been measured in rat Leydig cells (Gao *et al.* 1997), in the LLC-PK1 porcine renal cell line (Mobus *et al.* 1999) and in the JEG-3 human choriocarcinoma cell line (Gomez-Sanchez *et al.* 1996), a type 3 11 β HSD isoform has yet to be cloned.

The third hypothesis, that type 1 11 β HSD activities may be subject to post-translational inhibition in hGL cells, would be consistent with our previously published study which indicated production of a paracrine/autocrine 11 β HSD inhibitor by hGL cells *in vitro* (Michael *et al.* 1996). Recently, we have reported data that human follicular fluid samples, from which the hGL cells are isolated, contain both a hydrophilic compound (or compounds) that can acutely increase 11 β HSD activities and a hydrophobic compound(s) that can inhibit NADP(H)-dependent 11 β HSD activities by up to 62% within 1 h in rat kidney homogenates. The inhibitory compound in human follicular fluid is known to be a lipid that is neither a prostaglandin nor one of several candidate steroids (cortisol, cortisone, oestradiol, testosterone, pregnenolone or progesterone) (Thurston *et al.* 2002). It is possible that the increase in expression of type 1 11 β HSD protein observed in this study as human granulosa cells luteinize *in vitro* may simply be a compensatory rise in enzyme expression due to the increasing inhibition of type 1 11 β HSD activities by a paracrine HSD inhibitor.

As discussed previously, the level of expression of mRNA encoding type 1 and type 2 11 β HSD changes in a variety of tissues over the course of the ovarian cycle

and during pregnancy, suggesting regulation of 11 β HSD expression and activity by ovarian steroid hormones (Benediktsson *et al.* 1992, Waddell *et al.* 1996, Yong *et al.* 2000). As a specific example, progesterone, and its hydroxylated metabolites, can inhibit the activities of both cloned isoforms of 11 β HSD in a range of cells and tissues (Lopez-Bernal *et al.* 1980, Souness & Morris 1996, Bujalska *et al.* 1997). In our studies of human follicular fluid, we have excluded progesterone as the major endogenous ovarian inhibitor of type 1 11 β HSD activities based on: (1) its relative potencies for type 2 versus type 1 11 β HSD; (2) the lack of correlation between degree of enzyme inhibition and progesterone concentrations in individual follicular fluid samples; and (3) the fact that the major inhibitor of type 1 11 β HSD elutes from a C18 cartridge at higher methanol concentrations than does progesterone (Thurston *et al.* 2002). To establish whether the decline in 11 β HSD activities and/or the increase in the expression of type 1 11 β HSD protein in luteinizing hGL cells could simply reflect increasing progesterone production, experiments were repeated in the presence of AG: an inhibitor of cytochrome P450 cholesterol side-chain cleavage. Although neither acute nor chronic exposure to AG affected the level of expression of the type 1 11 β HSD protein, these treatments did significantly increase both the 11 β DH and 11 KSR activities on each day of culture, consistent with post-translational inhibition of type 1 11 β HSD by progesterone or a metabolite thereof. The fact that chronic exposure to AG did not prevent the decrease in enzyme activities over successive days in culture suggests that progesterone cannot be the only hydrophobic inhibitor of type 1 11 β HSD responsible for the decline in 11 β DH and 11 KSR activities as cells luteinize *in vitro*.

In summary, this study supports the postulated switch from expression of type 2 to type 1 11 β HSD as ovarian granulosa cells differentiate from the preovulatory, follicular phenotype to the functionally luteinized phenotype. Moreover, we speculate that a paracrine/autocrine factor, other than progesterone or pregnenolone, produced by luteinizing human granulosa cells might be responsible for the decline in 11 β HSD activities and for the concomitant rise in type 1 11 β HSD protein expression over successive days in culture.

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References

- Abayasekara DR, Michael AE, Webley GE & Flint AP 1993 Mode of action of prostaglandin F2 alpha in human luteinized granulosa

- cells: role of protein kinase C. *Molecular and Cellular Endocrinology* **97** 81–91.
- 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 of 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.
- Andersen CY 1990 Levels of steroid-binding proteins and steroids in human preovulatory follicle fluid and serum as predictors of success in *in vitro* fertilization–embryo transfer treatment. *Journal of Clinical Endocrinology and Metabolism* **71** 1375–1381.
- Benediktsson R, Yau JLW, Low SC, Brett LP, Cooke BE, Edwards CRW & Seckl JR 1992 11 β -hydroxysteroid dehydrogenase in the rat ovary: high expression in the oocyte. *Journal of Endocrinology* **135** 53–58.
- Bujalska I, Shimojo M, Howie A & Stewart PM 1997 Human 11 β -hydroxysteroid dehydrogenase: studies on the stably transfected isoforms and localization of the type 2 isozyme within renal tissue. *Steroids* **62** 77–82.
- Dewi DA, Abayasekara DRE & Wheeler-Jones CPD 2002 Requirement for ERK1/2 activation in the regulation of progesterone production in human granulosa–lutein cells is stimulus specific. *Endocrinology* **143** 877–888.
- Edwards CRW & Stewart PM 1991 The cortisol–cortisone shuttle and the apparent specificity of glucocorticoid and mineralocorticoid receptors. *Journal of Steroid Biochemistry and Molecular Biology* **39** 859–865.
- Fateh M, BenRafael Z, Benadiva CA, Mastroianni L Jr & Flickinger GL 1989 Cortisol levels in human follicular fluid. *Fertility and Sterility* **51** 538–541.
- Ferguson SE, Pallikaros Z, Michael AE & Cooke BA 1999 The effects of different culture media, glucose, pyridine nucleotides and adenosine on the activity of 11 β -hydroxysteroid dehydrogenase in rat Leydig cells. *Molecular and Cellular Endocrinology* **158** 37–44.
- Fowkes RC, Chandras C, Chin EC, Okolo S, Abayasekara DR & Michael AE 2001 Relationship between the production of prostaglandins and progesterone by luteinizing human granulosa cells. *Journal of Endocrinology* **171** 455–462.
- Gao H-B, Ge R-S, 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.
- Gomez-Sanchez EP, Cox D, Foecking M, Ganjam V & Gomez-Sanchez CE 1996 11 β -hydroxysteroid dehydrogenase of the choriocarcinoma cell line JEG-3 and their inhibition by glycyrrhetic acid and other natural substances. *Steroids* **61** 110–115.
- Hammond GL, Smith CL & Underhill DA 1991 Molecular studies of corticosteroid binding globulin, biosynthesis and function. *Journal of Biological Chemistry and Molecular Biology* **40** 755–762.
- Harlow CR, Jenkins JM & Winston RM 1997 Increased follicular fluid total and free cortisol levels during the luteinizing hormone surge. *Fertility and Sterility* **68** 48–53.
- 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.
- Lopez-Bernal A, Flint APF, Anderson, ABP & Turnbull AC 1980 11 β -hydroxysteroid dehydrogenase activity in human placenta and decidua. *Journal of Steroid Biochemistry* **13** 1081–1087.
- Michael AE, Gregory L, Walker SM, Antoniow JW, Shaw RW, Edwards CRW & Cooke BA 1993a Ovarian 11 β -hydroxysteroid dehydrogenase: potential predictor of conception by *in-vitro* fertilization and embryo transfer. *Lancet* **342** 711–712.
- Michael AE, Pester LA, Curtis P, Shaw RW, Edwards CRW & Cooke BA 1993b Direct inhibition of ovarian steroidogenesis by cortisol and the modulatory role of 11 β -hydroxysteroid dehydrogenase. *Clinical Endocrinology* **38** 641–644.
- Michael AE, Gregory L, Piercy EC, Walker SM, Shaw RW & Cooke BA 1995 Ovarian 11 β -hydroxysteroid dehydrogenase activity is inversely related to the outcome of *in vitro* fertilisation–embryo transfer treatment cycles. *Fertility and Sterility* **64** 590–598.
- Michael AE, Gregory L, Thaventhiran L, Antoniow JW & Cooke BA 1996 Follicular variation in ovarian 11 β -hydroxysteroid dehydrogenase (11 β HSD) activities: evidence for the paracrine inhibition of 11 β HSD in human granulosa–lutein cells. *Journal of Endocrinology* **148** 419–425.
- Michael AE, Evagelatou M, Norgate DP, Clarke RJ, Antoniow JW, Stedman B, Brennan A, Welsby R, Bujalska I, Stewart PM *et al.* 1997 Isoforms of 11 β -hydroxysteroid dehydrogenase in human granulosa–lutein cells. *Molecular and Cellular Endocrinology* **132** 43–52.
- Mobus E, Hermans JJ & Maser E 1999 Characterisation of 11 β -hydroxysteroid dehydrogenase activities in the renal cell line LLC-PK1: evidence for a third isoform? *Endocrine* **11** 301–307.
- Monder C 1991 Corticosteroids, receptors, and the organ-specific functions of 11 β -hydroxysteroid dehydrogenase. *FASEB Journal* **5** 3047–3054.
- Monder C, Lakshmi V & Miroff Y 1991 Kinetic studies on rat liver 11 beta-hydroxysteroid dehydrogenase. *Biochimica et Biophysica Acta* **1115** 23–29.
- Moore JS, Monson JP, Kaltsas G, Putignano P, Wood PJ, Sheppard MC, Besser GM, Taylor NF & Stewart PM 1999 Modulation of 11 β -hydroxysteroid dehydrogenase isozymes by growth hormone and insulin-like growth factor: *in vivo* and *in vitro* studies. *Journal of Clinical Endocrinology and Metabolism* **84** 4172–4177.
- Owen EJ, Holownia P, Conway GS, Jacobs HS & Honour JW 1992 11 β -hydroxyandrostenedione in plasma, follicular fluid, and granulosa cells of women with normal and polycystic ovaries. *Fertility and Sterility* **58** 713–718.
- Pallikaros Z, Schulster D, Baldwin SA, Helliwell RJA, 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.
- Peluso JJ & Papalardo A 1999 Progesterone maintains large rat granulosa cell viability indirectly by stimulating small granulosa cells to synthesise basic fibroblast growth factor. *Biology of Reproduction* **60** 290–296.
- Ragoobir J, Abayasekara DR, Bruckdorfer KR & Michael AE 2002 Stimulation of progesterone production in human granulosa–lutein cells by lipoproteins: evidence for cholesterol-independent actions of high-density lipoproteins. *Journal of Endocrinology* **173** 103–111.
- Ricketts ML, Verhaeg JM, Bujalska I, Howie AJ, Rainey WE & Stewart PM 1998 Immunohistochemical localisation of type 1 11 β hydroxysteroid dehydrogenase in human tissues. *Journal of Clinical Endocrinology and Metabolism* **83** 1325–1335.
- Rusvai E & Naray-Fejes-Toth A 1993 A new isoform of 11 β -hydroxysteroid dehydrogenase in aldosterone target cells. *Journal of Biological Chemistry* **268** 10717–10720.
- Seckl JR & Walker BR 2001 11 β -hydroxysteroid dehydrogenase type 1 – a tissue-specific amplifier of glucocorticoid action. *Endocrinology* **142** 1371–1376.
- Shimojo M, Ricketts ML, Petrelli MD, Moradi P, Johnson GD, Bradwell AR, Hewison M, Howie AJ & Stewart PM 1997

- Immunodetection of 11 β -hydroxysteroid dehydrogenase type 2 in human mineralocorticoid target tissues: evidence for nuclear localisation. *Endocrinology* **138** 1305–1311.
- Smith MP, Keay SD, Hall L, Harlow CR & Jenkins JM 1997 The detection and confirmation of 11 β -hydroxysteroid dehydrogenase type 1 transcripts in human luteinized granulosa cells using RT-PCR and plasmid pUC18. *Molecular Human Reproduction* **3** 651–654.
- 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.
- 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, Thomas FJ, Thomas MJ, Anderson RA, Mason JI & Hillier SG 1997 Differential expression of messenger ribonucleic acids encoding 11 β -hydroxysteroid dehydrogenase types 1 and 2 in human granulosa cells. *Journal of Clinical Endocrinology and Metabolism* **82** 2006–2009.
- Tetsuka M, Haines LC, Milne M, Simpson GE & Hillier SG 1999a Regulation of 11 β -hydroxysteroid dehydrogenase type 1 gene expression by LH and interleukin-1 β in cultured rat granulosa cells. *Journal of Endocrinology* **163** 417–423.
- Tetsuka M, Milne M, Simpson GE & Hillier SG 1999b Expression of 11 β -hydroxysteroid dehydrogenase, glucocorticoid receptor, and mineralocorticoid receptor genes in rat ovary. *Biology of Reproduction* **60** 330–335.
- Thomas FJ, Thomas MJ, Tetsuka M, Mason JI & Hillier SG 1998 Corticosteroid metabolism in human granulosa–lutein cells. *Clinical Endocrinology* **48** 509–513.
- Thurston LM, Norgate DP, Jonas KC, Chandras C, Kloosterboer HJ, Cooke BA & Michael AE 2002 Paracrine modulators of 11 β -hydroxysteroid dehydrogenase (11 β HSD) activities in follicular fluid from gonadotrophin-stimulated assisted conception cycles. *Reproduction* **124** 801–812.
- Waddell BJ, Benediktsson R & Seckl JR 1996 11 β -hydroxysteroid dehydrogenase type 2 in the rat corpus luteum: induction of messenger ribonucleic acid expression and bioactivity coincident with luteal regression. *Endocrinology* **137** 5386–5391.
- 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.
- Yong PYK, Harlow C, Thong KJ & Hillier SG 2002 Regulation of 11 β -hydroxysteroid dehydrogenase type 1 gene expression in human ovarian surface epithelial cells by interleukin-1. *Human Reproduction* **17** 2300–2306.
- Zhou M-Y, Gomez-Sanchez EP, Cox DL, Cosby D & Gomez-Sanchez CE 1995 Cloning, expression and tissue distribution of the rat nicotinamide adenine dinucleotide-dependent isoform of 11 β -hydroxysteroid dehydrogenase. *Endocrinology* **136** 3729–3734.

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