Ovarian modulators of 11 β -hydroxysteroid dehydrogenase (11 β HSD) activity in follicular fluid from gonadotrophin-stimulated assisted conception cycles

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In the ovary, cortisol–cortisone interconversion is catalysed by isoforms of 11 β -hydroxysteroid dehydrogenase (11 β HSD). The objective of this study was to establish whether human follicular fluid (hFF), obtained after controlled ovarian hyperstimulation, contains paracrine modulators of 11 β HSD activity. Of 274 hFF samples tested for effects in rat kidney homogenates, 206 hFF samples significantly inhibited NADP⁺-dependent oxidation of cortisol within 1 h (by 11–67% of control 11 β HSD activity), whereas 42 hFF samples significantly stimulated 11 β HSD activity (16–210% increase relative to control). Although charcoal-stripping of hFF prevented the inhibition and potentiated the stimulation of NADP⁺-dependent cortisol oxidation in a renal homogenate, effects of individual hFF samples on NADP⁺-dependent cortisol oxidation were independent of intrafollicular progesterone concentrations. Hydrophilic fractions of hFF samples, isolated by C18 column chromatography, stimulated both the NADP⁺-dependent oxidation of cortisol (by $55 \pm 5\%$, $n = 98$) and

the NADPH-dependent reduction of cortisone (by $86 \pm 22\%$, $n = 5$). In contrast, the hydrophobic fractions of hFF (eluted at 65–85% methanol) inhibited both NADP⁺-dependent 11 β -dehydrogenase and NADPH-dependent 11-ketosteroid reductase activities (by $63 \pm 2\%$ and $74 \pm 4\%$, respectively). None of the C18 column fractions of 50 hFF samples had any significant effect on NADP⁺-dependent 11 β -dehydrogenase activities. The hydrophobic inhibitors of NADP(H)-dependent cortisol–cortisone metabolism did not co-elute with several candidate compounds (prostaglandins E₂ and F_{2 α} , cortisol, cortisone, oestradiol, testosterone, progesterone, pregnenolone or cholesterol). Hence, hFF aspirated from women undergoing controlled ovarian hyperstimulation for assisted conception contains both hydrophilic stimuli and hydrophobic inhibitors of glucocorticoid metabolism which appear to be selective for the NADP(H)-dependent, type 1 isoform of 11 β HSD.

Introduction

In potential target tissues, local glucocorticoid concentrations are controlled by isoforms of 11 β -hydroxysteroid dehydrogenase (11 β HSD), which catalyse the interconversion of active 11 β -hydroxysteroids (cortisol and corticosterone) with their inert 11-ketosteroid metabolites (cortisone and 11-dehydrocorticosterone, respectively) (reviewed by Monder, 1991; Krozowski, 1996; White *et al.*, 1997; Kotelevstev *et al.*, 1999; Quinkler *et al.*, 2001; Seckl and Walker, 2001). To date, two isoforms of 11 β HSD with very different biochemical properties have been cloned. Type 1 11 β HSD is a ubiquitously expressed, NADP(H)-dependent enzyme that preferentially catalyses reduction of 11-ketosteroids, but can also catalyse the dehydrogenation of active glucocorticoids (Lakshmi and Monder, 1988;

Agarwal *et al.*, 1989; Tannin *et al.*, 1991). In contrast, type 2 11 β HSD is a high affinity enzyme that acts almost exclusively as an 11 β -dehydrogenase, protecting non-specific mineralocorticoid receptors from occupation by glucocorticoids in the distal nephron, colon and parotid gland (Brown *et al.*, 1993; Rusvai and Naray-Fejes-Toth, 1993; Agarwal *et al.*, 1994; Albiston *et al.*, 1994; Zhou *et al.*, 1995).

After an initial report of conversion of cortisol to cortisone in human granulosa–lutein (hGL) cells isolated from the ovarian follicular aspirates of women undergoing oocyte retrieval for assisted conception (Owen *et al.*, 1992), it was confirmed that 11 β HSD activity modulates the biological actions of cortisol in these ovarian cells. Specifically, high 11 β HSD activities in hGL cells increase the concentration of cortisol required to inhibit LH-stimulated steroidogenesis (Michael *et al.*, 1993a). Moreover, ovarian 11 β HSD activities correlated with the clinical outcome of *in vitro* fertilization and embryo transfer (IVF–ET) in patients from whom the hGL cells had been obtained. Although none of

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the patients with high ovarian 11 β HSD activities conceived subsequent to ET, undetectable 11 β HSD activities were associated with a 64% probability of achieving pregnancy in a given IVF–ET cycle (Michael *et al.*, 1993b, 1995).

In view of the apparent relationship between low ovarian 11 β HSD activities and conception by IVF–ET, it is important to investigate potential endocrine or paracrine regulators of ovarian glucocorticoid metabolism. Michael *et al.* (1996) reported indirect evidence for paracrine inhibition of 11 β HSD activities in cultured hGL cells. Hence, low ovarian 11 β HSD activities could reflect increased amounts of a paracrine 11 β HSD inhibitor within the ovary. The objectives of the present study were to establish whether human follicular fluid (hFF) contains endogenous compounds that can acutely modulate 11 β HSD-mediated cortisol–cortisone inter-conversion, and to perform a preliminary biochemical characterization of any such paracrine modulators using rat kidney as a source of the two cloned isoforms of 11 β HSD.

Materials and Methods

Sample collection and cell culture

Patients attending the Cardiff Assisted Reproduction Unit (University Hospital of Wales, Cardiff) underwent controlled ovarian hyperstimulation as previously described by Michael *et al.* (1995, 1996). In brief, patients were treated sequentially with a GnRH analogue (busurelin acetate), human menopausal gonadotrophin (hMG) and 10 000 iu hCG. At 36–42 h after the administration of hCG, samples of ovarian hFF were aspirated from the patients in the course of oocyte retrieval for IVF–ET. From the time of follicle aspiration until transfer to the Royal Free Campus of the Royal Free and University College Medical School (RF&UCMS), samples were stored for 1–3 days at 4°C. On arrival at RF&UCMS, hFF samples were divided into aliquots (1–5 ml) and frozen at –20°C until analysis. All samples were obtained with informed consent of the patient in accordance with the Declaration of Helsinki, and with the approval of the local ethics committee.

After aspiration of hFF, ovarian follicles were flushed with sterile Earle's balanced salt solution (EBSS; Life Technologies, Strathclyde). These follicular flushings were also transported to RF&UCMS and hGL cells were partially purified on 60% (v/v) Percoll (Sigma, Poole) as described by Michael *et al.* (1993a, 1995, 1996). Cells were suspended at a density of 50 000 cells ml^{–1} in 1:1 Dulbecco's modified Eagle's medium (DMEM):Ham's F₁₂ medium (Life Technologies) supplemented with L-glutamine (2 mmol l^{–1}; Life Technologies), penicillin (87 000 iu l^{–1}; Life Technologies), streptomycin (87 mg l^{–1}; Life Technologies) and 10% (v/v) fetal calf serum (Life Technologies). From this suspension, hGL cells were plated in 1 ml volumes into a 24-well plate and were cultured for 3 days at 37°C in an atmosphere of 5% (v/v) CO₂ in air.

Human ovarian 11 β HSD activities

Ovarian 11 β HSD activities were assayed on day 4 of cell culture using the radiometric conversion assay for

glucocorticoid oxidation previously described in our laboratory (Michael *et al.*, 1995, 1996). In brief, hGL cells were first rinsed with warm serum-free 1:1 DMEM:Ham's F₁₂ medium. One millilitre of fresh 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 nmol l^{–1}) were added to each triplicate set of wells. Cells were then incubated for 4 h at 37°C, after which samples were transferred to glass culture tubes and steroids were extracted into 2 ml ice-cold chloroform (Merck, Poole, Dorset). After centrifugation at 1000 g for 30 min at 4°C, the aqueous phases were aspirated and the organic extracts were evaporated to dryness under nitrogen at 40°C. The steroid residues were re-suspended in 20 μ l ethyl acetate containing 1 mmol cortisol l^{–1} and 1 mmol cortisone l^{–1} (Sigma) before being 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 radiochromatogram scanner (LabLogic, Sheffield), 11 β HSD activities were calculated as pmol cortisol oxidized to cortisone over 4 h. Cellular protein concentrations were determined using the Biorad protein assay (Bradford, 1976; Rosa *et al.*, 1980) and all enzyme activities were standardized per mg protein. On the basis of our previous studies (Michael *et al.*, 1993a,b, 1995), ovarian cells with net rates of cortisol oxidation < 40 pmol mg protein^{–1} (4 h)^{–1} were classified as displaying 'low' 11 β HSD activity, whereas those cells with rates > 100 pmol cortisol oxidized mg protein^{–1} (4 h)^{–1} were classified as displaying 'high' 11 β HSD activities.

Selection and preparation of hFF samples

Samples of hFF were sorted dependent upon the 11 β HSD activities in the associated granulosa–lutein cells. In the first instance, three hFF samples from follicles characterized by low enzyme activities and three fluid samples from follicles characterized by high ovarian 11 β HSD activities were assessed for subsequent effects on cortisol oxidation in homogenates of rat kidney (see below). Moreover, 5 ml was removed from each of the six hFF samples used in these initial experiments, and this sample was incubated with dextran-coated charcoal to remove steroids and other lipids. Dextran-coated charcoal was prepared by dissolving 125 mg dextran (Merck) in 50 ml PBS, followed by the addition of 1.25 g charcoal (Merck). Once thoroughly dissolved, 5 ml of this suspension was centrifuged at 1000 g for 30 min, after which the supernatant was discarded and the dextran-coated charcoal precipitate was resuspended in the 5 ml hFF. Measurements of progesterone concentrations in hFF samples before and after charcoal-stripping confirmed that this protocol decreased the progesterone concentration by > 98%.

Effects of hFF on NADP⁺-dependent 11 β HSD activities in homogenates of rat kidney

Male Sprague–Dawley rats (200–250 g) were housed in accordance with the UK Animals (Scientific Procedures) Act

1986 with access to a standard rat chow diet and drinking water *ad libitum*. Homogenates of rat kidney were prepared, using methods established in our laboratories (Sewell *et al.*, 1998; Thompson *et al.*, 2000), as a source of both NADP(H)-dependent, type 1 11 β HSD activity and NAD⁺-dependent, type 2 11 β HSD activity. For each assay, 1 g rat kidney was homogenized in 18 ml hypotonic Tris-EDTA lysis buffer (Rusvai and Naray-Fejes-Toth, 1993; Sewell *et al.*, 1998; Thompson *et al.*, 2000). After restoring isotonicity by addition of 2 ml of 1.5 mol KCl l⁻¹ (Merck), the homogenate was centrifuged at 1000 *g* for 10 min at 4°C and the supernatant was decanted into a fresh glass tube. From this supernatant, samples of 100 μ l were transferred into glass culture tubes, to each of which was added 600 μ l PBS (Life Technologies). Triplicate tubes were also prepared as assay blanks containing 100 μ l BSA solution (1 g l⁻¹ prepared in PBS) in place of renal homogenate. Subsequently, each tube received either 100 μ l PBS (controls and blanks only) or 100 μ l hFF (each added to triplicate tubes) before being pre-incubated for 30 min at 37°C in a gyratory waterbath. Assays of NADP⁺-dependent 11 β -dehydrogenase activities were initiated by adding to each tube 100 μ l NADP⁺ (4 mmol l⁻¹ in PBS; Sigma) and 100 μ l PBS containing 0.5 μ Ci [³H]cortisol plus unlabelled cortisol (to a final steroid concentration of 100 nmol l⁻¹). Tubes were then returned to the waterbath for 1 h, after which reactions were terminated by the addition to each tube of 2 ml ice-cold chloroform. 11 β HSD activities were quantified and subsequently standardized for protein concentration as described above.

In total, 274 individual samples of hFF (each from a different follicle) were assessed for their net effects on the NADP⁺-dependent oxidation of cortisol to cortisone.

Effects of carbenoxolone and progesterone on NADP(H)- and NAD⁺-dependent 11 β HSD activities in homogenates of rat kidney

The NAD⁺-dependent activity of type 2 11 β HSD was assessed over 1 h as for the NADP⁺-dependent activity of type 1 11 β HSD with the exception that NAD⁺ (final concentration = 400 μ mol l⁻¹; Sigma) was substituted for NADP⁺ as the pyridine nucleotide cofactor. Assays of the 11-ketosteroid reductase (11KSR) activity of type 1 11 β HSD were performed using rat kidney homogenates over 4 h as described for 11 β -dehydrogenase activities with substitution of NADPH (final concentration = 400 μ mol l⁻¹; Sigma) as the enzyme cofactor and cortisone as the assay substrate (final concentration = 100 nmol l⁻¹ containing 0.1 μ Ci [1,2,6,7-³H]cortisone per tube). For these 11KSR assays, [³H]cortisone was prepared by overnight incubation of a renal homogenate at 37°C with 20 μ Ci [³H]cortisol per tube plus 400 μ mol NADP⁺ l⁻¹ (Michael *et al.*, 1997). 11KSR activities were expressed as pmol cortisone reduced to cortisol mg protein⁻¹ h⁻¹, having confirmed this activity to be linear over 4 h.

In each assay of enzyme activity, triplicate samples of rat

kidney homogenate were treated for the duration of the assay with 1 μ mol carbenoxolone l⁻¹ (Sigma) to confirm that the measured enzyme activity could be significantly decreased by this pharmacological inhibitor of 11 β HSD (Monder *et al.*, 1989; Stewart *et al.*, 1990; Latif *et al.*, 1992; Ulick *et al.*, 1993; Gomez-Sanchez *et al.*, 1996; Sewell *et al.*, 1998). In addition, three independent homogenates of rat kidney were incubated with 0.01–10.0 μ mol progesterone l⁻¹ (Sigma) and the concentration-dependent effects of this steroid on the NADP⁺- and NAD⁺-dependent 11 β HSD activities were assessed using the methods described above.

Resolution of hFF and urine components by reverse phase C18 column chromatography

Samples of hFF were fractionated using a modification of the method described by Morris and colleagues for the partial purification of endogenous 11 β HSD inhibitors in urine (Morris *et al.*, 1992; Lo *et al.*, 1997). For 98 hFF samples (each from a different follicle), 3 ml samples were applied to separate C18 Sepak cartridges (Waters, Elstree, Hertford) that had been conditioned previously with 20 ml methanol. After collecting the loading eluent, the column was sequentially eluted with 1 ml samples of a stepwise gradient of methanol (Merck) in double-distilled water (0–40% (v/v) methanol in 10% increments followed by 40–100% (v/v) methanol in 5% increments). All 1 ml fractions were collected into borosilicate tubes and samples eluted at methanol concentrations > 20% (v/v) were evaporated to dryness under nitrogen before being re-suspended in 1 ml of 20% (v/v) methanol in water.

A further six samples of hFF were each divided into 2 $\times$ 5 ml aliquots, and one aliquot of each pair was charcoal-stripped before loading all 12 aliquots on to separate C18 cartridges and eluting the hFF fractions with increasing concentrations of methanol. Samples of urine, obtained from three different IVF patients within 2 h before collection of the hFF, were similarly fractionated, as were parallel samples of the follicular flushing medium (EBSS), double-distilled water and PBS.

Effects of hFF fractions on rat renal 11 β HSD activities

Assays of renal NADP(H)- and NAD⁺-dependent 11 β HSD activities were performed as described above with the following modification. Samples were incubated in triplicate in the presence of (i) 100 μ l of a specific hFF fraction, or (ii) 100 μ l of 20% (v/v) methanol in water (that is, final methanol concentration in 1 ml = 2%), or (iii) 100 μ l of water alone. Enzyme activities in the presence of the loading eluent, 0 and 10% methanol fractions were compared with those measured in the controls incubated with water alone, whereas enzyme activities in the presence of fractions eluted at $\geq$ 20% methanol were compared with the 20% methanol control.

Fig. 1. Effects of human follicular fluid (hFF) samples, obtained from follicles with low human ovarian 11β-hydroxysteroid dehydrogenase (11βHSD) activities (< 40 pmol cortisone mg protein⁻¹ (4 h)⁻¹) (low FF) or with high human ovarian 11βHSD activities (> 100 pmol cortisone mg protein⁻¹ (4 h)⁻¹) (high FF), before and after charcoal-stripping, on subsequent NADP⁺-dependent 11β-dehydrogenase activities in rat kidney homogenates. Each data point represents the mean ± SEM for three independent experiments. ***P* < 0.01 versus control.

Resolution of standard components of hFF by reverse phase C18 column chromatography

After testing each C18 column fraction for effects on renal 11βHSD activities, the protein concentration of each fraction was assessed using the Biorad assay. The elution profiles for reference hydrophobic compounds known to be present in hFF were established by equilibrating each sample of hFF (for 18 h at 4°C) with 1 μCi of the following compounds (each purchased from Amersham International, Amersham or from NEN-DuPont Ltd, Stevenage): [5,6,8,11,12,14,15(n)-³H]prostaglandin (PGE₂) (130 Ci mmol⁻¹), [5,6,8,9,11,12,14(n)-³H]PGF_{2α} (130 Ci mmol⁻¹), [1,2,6,7-³H]cortisol (50 Ci mmol⁻¹), [1,2,6,7-³H]cortisone (50 Ci mmol⁻¹), [2,4,6,7-³H]oestradiol (70 Ci mmol⁻¹), [1,2,6,7-³H]testosterone (70 Ci mmol⁻¹), [1,2,6,7-³H]progesterone (60 Ci mmol⁻¹), [7-³H]-pregnenolone (21.1 Ci mmol⁻¹) or [7-³H]cholesterol (2 Ci mmol⁻¹). Each reference compound was added to at least four independent samples of hFF, each of which was loaded on to a separate C18 Sepak cartridge. Fractions were eluted from each cartridge as described above, and duplicate 100 μl aliquots of each column fraction were transferred to scintillation vials. After adding Ultima-gold scintillant (Packard BioScience Ltd, Pangbourne; 2 ml per vial), the radioactivity present in each eluted C18 fraction was quantified using a LS500E liquid scintillation counter (Beckman, High Wycombe).

Radioimmunoassays

Progesterone concentrations in hFF samples and in fractions thereof were measured using a standard radio-

immunoassay described previously with an EC₅₀ value of 3.97 nmol l⁻¹ (Pallikaros *et al.*, 1995). hFF samples were assayed at sequential dilutions of 1:100 to 1:100 000 to ensure that samples fell on the linear region of the radioimmunoassay standard curve (0.6–14.0 nmol l⁻¹). Charcoal-stripped samples were assayed neat and at 1:10. Concentrations of PGE₂ and PGF_{2α} were also assessed in C18 column fractions of hFF (to which no ³H-lipid had been added) using previously published radioimmunoassay protocols (Kelly *et al.*, 1986; Poyser *et al.*, 1987).

Statistical analysis

The effects of particular hFF samples or hFF fractions on renal 11βHSD activities were assessed by comparison of enzyme activities in the presence of a treatment and in the matched untreated control samples using one-way ANOVA followed by Dunnett's or Bonferroni's multiple comparisons as appropriate. In selected experiments, the Tukey *post hoc* test was applied to compare the effects of multiple hFF samples on 11βHSD activities. Before performing correlation tests, each data set was tested for conformation to a normal (Gaussian) distribution by application of Kolmogorov–Smirnov tests. As several data sets proved to deviate from the normal data distribution, correlations were assessed by the application of Spearman's rank correlation tests. Specifically, the effects of hFF samples on NADP⁺-dependent 11β-dehydrogenase activities were correlated with their progesterone concentrations and with the effects of the corresponding hydrophilic and hydrophobic fractions of those hFF samples on the NADP⁺-dependent oxidation of cortisol.

All statistical evaluations were performed using the GraphPad Prism2 program (San Diego, CA). In all cases, *P* < 0.05 was accepted to indicate statistical significance. Although all data are presented graphically as percentage values (mean ± SEM) (where the appropriate control enzyme activity for each experimental design was standardized to 100%), all statistical analyses were performed on non-referenced data. Each experiment was repeated at least three times using a different renal homogenate on each occasion.

Results

Effects of hFF on rat renal 11βHSD activities

Samples of hFF (10%, v/v) obtained from follicles with low ovarian 11βHSD activities inhibited NADP⁺-dependent oxidation of cortisol in rat kidney homogenates by 34 ± 5% (*P* < 0.01), whereas hFF samples associated with high ovarian 11βHSD activities had no significant effect on renal NADP⁺-dependent 11β-dehydrogenase activity (Fig. 1). After charcoal-stripping, the same samples of hFF increased NADP⁺-dependent 11βHSD activities by 59 ± 5% and 45 ± 9%, respectively (*P* < 0.01; Fig. 1).

Of the 274 individual hFF samples tested for effects on NADP⁺-dependent 11β-dehydrogenase activity, 206

Fig. 2. Effects of human follicular fluid (hFF) samples from different individual follicles in a given cohort from (a) patient 1 and (b) patient 2 on NADP⁺-dependent 11 β -dehydrogenase activity in homogenates of rat kidney. For each hFF sample, the uppercase letters (L (left) and R (right)) indicate from which ovary the sample was obtained and the number indicates the sequential order in which follicles were aspirated. Each data point represents the mean $\pm$ SEM for triplicate measures in a single assay. Enzyme activities differ significantly ($P < 0.05$) between bars that do not share the same letter within an enzyme assay; bars sharing the same letter do not differ significantly. *** $P < 0.001$ versus control.

significantly decreased this enzyme activity over 1 h (by 11–67% relative to the control enzyme activity), whereas approximately one in six of the tested samples (42 of 274) significantly stimulated NADP⁺-dependent oxidation of cortisol (16–210% increase over control enzyme activity). For a given patient, the effects on NADP⁺-dependent 11 β -dehydrogenase activity differed significantly between hFF samples aspirated from different follicles (Fig. 2).

Effects of carbenoxolone and progesterone on rat renal 11 β HSD activities; relationship to the actions of hFF samples

In the 11 β -dehydrogenase assays, carbenoxolone consistently inhibited the NADP⁺- and NAD⁺-dependent oxidation of cortisol by $93 \pm 3\%$ ($n = 16$) and $94 \pm 2\%$ ($n = 5$), respectively ($P < 0.001$ in both cases). In comparison,

Fig. 3. Concentration-dependent effects of progesterone on NADP⁺-dependent (●) and NAD⁺-dependent (○) oxidation of cortisol in renal homogenates. Each data point represents the mean $\pm$ SEM for three independent experiments. ** $P < 0.01$, *** $P < 0.01$ versus respective enzyme activity in the presence of 0 $\mu\text{mol l}^{-1}$ progesterone (l^{-1}) (dashed horizontal line).

Fig. 4. Effects of C18 fractions of individual human follicular fluid (hFF) samples on NADP⁺-dependent 11 β -dehydrogenase activities in renal homogenates. Each data point represents the mean $\pm$ SEM for 98 individual hFF samples. ** $P < 0.01$ versus respective control enzyme activity (dashed horizontal line).

and consistent with previous reports, the effects of carbenoxolone were relatively modest in the 11KSR assays; at a concentration of 1 $\mu\text{mol l}^{-1}$, the mean inhibition achieved by carbenoxolone was $52 \pm 15\%$ ($n = 9$; $P < 0.05$).

In three independent assays, progesterone inhibited both the NADP⁺- and NAD⁺-dependent oxidation of cortisol in a concentration-dependent manner. The IC_{50} values for these actions were 1.2 $\mu\text{mol l}^{-1}$ and 7.0 $\mu\text{mol l}^{-1}$, respectively, and at a concentration of 10 $\mu\text{mol l}^{-1}$, progesterone inhibited the NADP⁺- and NAD⁺-dependent 11 β -dehydrogenase activities in rat kidney homogenates by $83 \pm 3\%$ and $62 \pm 10\%$, respectively ($P < 0.001$; Fig. 3).

Progesterone concentrations measured in individual samples of hFF (before charcoal-stripping) ranged between 0.021 and 0.368 $\mu\text{mol l}^{-1}$. Hence, the inhibitory effects of hFF samples on NADP⁺-dependent 11 β HSD activities were greater than would have been anticipated solely on the basis of the intrafollicular progesterone concentrations. In addition, intrafollicular progesterone concentrations did not

Table 1. Effects of duration of storage of human follicular fluid (hFF) samples at 4°C before freezing on the modulation of NADP⁺-dependent 11 β -dehydrogenase activities in rat kidney homogenates

	Number of days stored at 4°C before freezing		
	1 (n = 35)	2 (n = 46)	3 (n = 60)
Inhibition by whole hFF (% decrease)	27 $\pm$ 2	27 $\pm$ 3	19 $\pm$ 3
Stimulation by hydrophilic hFF fraction (% increase)	56 $\pm$ 7	53 $\pm$ 6	55 $\pm$ 6
Inhibition by hydrophobic hFF fraction (% decrease)	56 $\pm$ 4	62 $\pm$ 3	62 $\pm$ 2

There were no significant differences in values within each row ($P > 0.05$). The hFF samples tested were before fractionation (whole hFF), the hydrophilic hFF fractions eluted at 0% (v/v) methanol, and the hydrophobic hFF fractions eluted at 80% (v/v) methanol.

correlate with the effects of individual hFF samples on the NADP⁺-dependent oxidation of cortisol ($r = 0.221$; $n = 35$; $P = 0.201$).

Resolution of 11 β HSD modulators by C18 column chromatography

When 98 individual hFF samples (each aspirated from a different follicle) were applied to C18 cartridges, the 0% (v/v) methanol fractions of each increased the NADP⁺-dependent oxidation of cortisol in rat kidney homogenates by up to 171%, irrespective of the net effects of the same hFF samples on enzyme activities before fractionation. Although the hFF fractions eluted at 20–50% (v/v) methanol exerted no significant effects, fractions eluted at methanol concentrations of 65–85% (v/v) inhibited NADP⁺-dependent inactivation of cortisol by up to 78%, with peak inhibitory activity eluted at 80% methanol (Fig. 4). In contrast, when C18 cartridges were loaded with follicular flushing medium, double-distilled water or PBS in place of hFF, none of the eluted fractions had any significant effect on the NADP⁺-dependent oxidation of cortisol (data not shown).

Since samples of hFF had been stored at 4°C for up to 3 days before freezing, the effects of hFF and fractions thereof on NADP⁺-dependent 11 β -dehydrogenase activities were compared among hFF samples stored for 1, 2 or 3 days before receipt. Duration of storage had no impact on the extent to which the NADP⁺-dependent oxidation of cortisol was modulated by the hFF samples before fractionation, or by the hydrophilic and hydrophobic fractions of these samples, eluted at 0% and 80% (v/v) methanol, respectively (Table 1).

For 145 hFF samples, the inhibition of NADP⁺-dependent 11 β -dehydrogenase by the hFF samples before fractionation was inversely proportional to the stimulation exerted by the hydrophilic fractions of these samples ($r = -0.552$; $P < 0.0001$; Fig. 5a) but directly proportional to

the degree of enzyme inhibition by the hydrophobic fractions ($r = 0.471$; $P < 0.0001$; Fig. 5b). Moreover, the stimulation of NADP⁺-dependent cortisol oxidation by the hydrophilic fractions of these 145 hFF samples was also inversely correlated with the inhibition of this enzyme activity by the corresponding hydrophobic hFF fractions ($r = -0.356$; $P < 0.0001$; Fig. 5c).

In six randomly selected hFF samples, the elution profile for endogenous ovarian modulators of NADP⁺-dependent 11 β -dehydrogenase activity was comparable to that for the initial series of 98 individual hFF samples. However, charcoal-stripping of each of these six samples before loading on to the C18 cartridges depleted the hFF samples of the hydrophobic enzyme inhibitors without affecting the content of the hydrophilic stimuli to NADP⁺-dependent 11 β -dehydrogenase activities (Fig. 6).

As regards effects of the C18 column fractions on NADPH-dependent 11KSR activities, the 0% (v/v) methanol fractions of each of five individual hFF samples increased renal cortisone reduction by $86 \pm 22\%$ ($P < 0.01$), whereas the 80% (v/v) methanol fractions decreased 11KSR activity by $74 \pm 4\%$ ($P < 0.01$; Fig. 7). (In assays of 11KSR activities, control enzyme activities ranged from 24 to 33 pmol cortisone reduced mg protein⁻¹ h⁻¹ with an interassay coefficient of variation of 8%). When 50 different hFF samples were applied to C18 columns, none of the eluted fractions had any significant effect on NAD⁺-dependent 11 β HSD activity (Fig. 8).

In excess of 97% of the total protein content of hFF eluted in either the C18 column loading eluent or in the 0% (v/v) methanol fraction. In contrast, when samples of hFF were spiked with [³H]lipids, these were found to elute from the C18 cartridges across the range of 20–100% (v/v) methanol (Fig. 9). In general, the peak elution of [³H]prostaglandins and of [³H]steroids was at methanol concentrations of 30% (v/v) and 55–70% (v/v) methanol, respectively. (The elution profiles of [³H]progesterone, [³H]PGE₂ and [³H]PGF_{2 α} showed complete agreement with the profiles as determined by radioimmunoassays for intra-follicular lipid hormones; data not shown.) The peak elution of [³H]pregnenolone and of [³H]cholesterol occurred at 75% and 100% methanol, respectively (Fig. 9a).

The profile of 11 β HSD modulators as resolved from hFF samples was different from the profile derived from urine samples obtained from patients undergoing preparation for IVF–ET < 2 h before collection of the matched hFF samples. Urine contained no evidence of water-soluble 11 β HSD stimuli, and although the 20–50% methanol eluents elicited relatively small increases in NADP⁺-dependent cortisol oxidation ($P < 0.01$), urinary fractions eluted at 60–90% methanol were able to inhibit 11 β HSD activities by only 22–30% ($P < 0.01$; Fig. 10).

Discussion

This study demonstrates that human follicular fluid, recovered from patients undergoing fertility treatment,

Fig. 5. Correlations between: (a) the effects of human follicular fluid (hFF) samples before fractionation versus the effects of the hydrophilic fractions of the same hFF samples, eluted at 0% (v/v) methanol, on NADP⁺-dependent 11 β -dehydrogenase activities; (b) the effects of hFF samples before fractionation versus the effects of the hydrophobic fractions of the same hFF samples, eluted at 80% (v/v) methanol, on NADP⁺-dependent 11 β -dehydrogenase activities; and (c) the stimulation of NADP⁺-dependent 11 β -dehydrogenase activities by the hydrophilic fractions of hFF versus the effects of the hydrophobic fractions of the same hFF samples. Each correlation plots data for the same 145 hFF samples.

Fig. 6. Effects of C18 fractions of six individual human follicular fluid (hFF) samples on NADP⁺-dependent 11 β -dehydrogenase activities in renal homogenates both before (●) and after (○) charcoal-stripping. Each data point represents the mean $\pm$ SEM for six individual hFF samples. * P < 0.05, ** P < 0.01 versus respective control enzyme activity (dashed horizontal line).

Fig. 7. Effects of C18 fractions of individual human follicular fluid (hFF) samples on NADPH-dependent 11-ketosteroid reductase (11KSR) activities in renal homogenates. Each data point represents the mean $\pm$ SEM for five individual hFF samples. * P < 0.05, ** P < 0.01 versus control enzyme activity (dashed horizontal line).

contains both hydrophilic stimuli and hydrophobic inhibitors of 11 β HSD-mediated cortisol–cortisone interconversion. In the rat kidney, the NAD(P)(H)-dependent 11 β -dehydrogenase and 11KSR activities have been attributed to the expression of type 1 11 β HSD. Hence, the ability of hFF samples, and the hydrophilic and hydrophobic constituents thereof, to modulate acutely the NAD(P)(H)-dependent metabolism of cortisol and cortisone without significantly affecting the NAD⁺-dependent oxidation of cortisol indicates that these components of hFF might act as selective inhibitors of type 1 11 β HSD.

At present, it is not possible to state whether the modulators of 11 β HSD activity resolved in this study arise from within the ovary or are derived from the circulation. However, we have previously reported data indicating that human granulosa–lutein cells, which line pre-ovulatory ovarian follicles, may synthesize and secrete a paracrine inhibitor(s) of ovarian 11 β HSD activity (Michael *et al.*, 1996). As type 1 11 β HSD appears to be the major 11 β HSD

Fig. 8. Effects of C18 fractions of individual human follicular fluid (hFF) samples on subsequent NAD⁺-dependent 11β-dehydrogenase activities in renal homogenates. Each data point represents the mean ± SEM for 50 individual hFF samples.

isoform expressed in human granulosa-lutein cells (Michael *et al.*, 1997; Smith *et al.*, 1997a; Tetsuka *et al.*, 1997), it is possible that the ovarian enzyme modulators resolved in the present study act in a paracrine manner to influence net cortisol–cortisone interconversion by NADP(H)-dependent isoforms of 11βHSD in the human ovary. This speculation is supported by two lines of evidence. First, samples of hFF obtained from three follicles characterized by low ovarian 11βHSD activities inhibited renal 11βHSD activities to a greater extent than did three hFF samples obtained from follicles with high ovarian 11βHSD activities. Second, in an ongoing study, we have observed that the ratio of cortisol:cortisone in 80 hFF samples increases with the degree of inhibition of NADP⁺-dependent cortisol oxidation by the hydrophobic fractions of those hFF samples, consistent with local inhibition of ovarian 11β-dehydrogenase activities *in vivo* (L. M. Thurston, L. Gregory and A. E. Michael, unpublished).

Previous studies have reported changes in the expression of mRNA encoding type 1 and type 2 11βHSD across the course of the ovarian cycle and in pregnancy (Benediktsson *et al.*, 1992; Burton and Waddell, 1994; Albiston *et al.*, 1995; Burton *et al.*, 1996; Pepe *et al.*, 1996; Waddell *et al.*, 1996; Yang *et al.*, 1996; Smith *et al.*, 1997b; Tetsuka *et al.*, 1997, 1998; Sampath-Kumar *et al.*, 1998; Yong *et al.*, 2000), indicating regulation of 11βHSD expression by ovarian steroid hormones. Indeed, oestradiol inhibits hepatic type 1 11βHSD expression while increasing type 2 11βHSD activity in the kidney and placenta (Baggia *et al.*, 1990; Low *et al.*, 1993; Sun *et al.*, 1998). Moreover, progesterone can repress type 2 11βHSD expression in the placenta and inhibits this enzyme activity at the post-transcriptional level (Lopez-Bernal *et al.*, 1980). Insulin and activators of protein kinase A also stimulate expression of the cloned 11βHSD isoforms (Hammami and Siiteri, 1990; Alfaidy *et al.*, 1997; Sun *et al.*, 1998). However, the ability of paracrine ovarian modulators to regulate 11βHSD activities within 1 h in homogenized renal tissue indicates that these compounds probably act at the post-transcriptional or post-translational level.

In correlation analyses, it was found that the degree of inhibition of NADP⁺-dependent 11β-dehydrogenase by the hFF samples before fractionation was inversely correlated with the stimulation by the hydrophilic fractions of these same samples, but directly proportional to the inhibition of enzyme activity by the hydrophobic fractions. Furthermore, the extent to which the hydrophilic fractions of hFF stimulated NADP⁺-dependent cortisol oxidation was also inversely correlated with the inhibition of type 1 11βHSD activities by the hydrophobic fractions of the same hFF samples. Hence, it would appear that the net effect of a given hFF sample on NADP(H)-dependent glucocorticoid metabolism by type 1 11βHSD reflects the balance between hydrophilic components of hFF that tend to stimulate cortisol–cortisone interconversion and the hydrophobic enzyme inhibitors. Thus, hFF samples that are rich in the hydrophilic enzyme stimuli with relatively low content of the hydrophobic enzyme inhibitors tend to exert a net stimulation of NADP⁺-dependent cortisol oxidation. At the other extreme, the majority of hFF samples, which contain relatively low amounts of the hydrophilic stimuli to 11βHSD and high concentrations of the hydrophobic enzyme inhibitors exert a significant net inhibition of type 1 11βHSD activities. Presumably, the 26 hFF samples that exerted no significant effect on the NADP⁺-dependent oxidation of cortisol had no net effect because they contained roughly equal proportions of the antagonistic enzyme stimuli and inhibitors.

Within a given cohort of follicles from a single patient, the amounts of ovarian enzyme modulators appear to differ between individual follicles, as did the balance between the stimulatory and inhibitory components of the hFF samples.

Treatment of hFF samples with dextran-coated charcoal negated the inhibition of NADP(H)-dependent 11βHSD activities and facilitated a significant stimulation of NADP⁺-dependent cortisol oxidation by the same hFF samples. This finding indicated that the inhibitors of type 1 11βHSD in hFF are likely to be hydrophobic lipids, whereas the enzyme stimuli that remain after charcoal-stripping are probably hydrophilic compounds. This conclusion was supported by the fractionation of hFF samples using C18 reverse phase column chromatography: the major stimulatory component of hFF eluted at methanol concentrations ≤ 20% (v/v), whereas the peak inhibitory activity of hFF was eluted from the C18 column by 80% (v/v) methanol and was depleted by prior charcoal-stripping.

The contention that the human ovary contains lipid inhibitors of type 1 11βHSD activity is not surprising given that cholesterol, progesterone and bile pigments, each a major constituent of hFF, have been shown to inhibit 11βHSD activities (Perschel *et al.*, 1991; Latif *et al.*, 1994; Souness *et al.*, 1995; Gomez-Sanchez *et al.*, 1996; Morita *et al.*, 1996; Souness and Morris, 1996; Quinkler *et al.*, 1999; Diederich *et al.*, 2000). As progesterone is a major component of ovarian hFF and a physiological inhibitor of type 1 11βHSD, this C₂₁ steroid was an obvious candidate for the dominant ovarian 11βHSD inhibitor in hFF. However, the

Fig. 9. Elution profile for candidate hydrophobic modulators of 11 β -hydroxysteroid dehydrogenase (11 β HSD) in human follicular fluid (hFF), eluted from a C18 Sepak cartridge with increasing concentrations of methanol. (a) Candidate modulators are cortisol (closed square, red; F), cortisone (open square, yellow; E), oestradiol (closed circle, green; E2), testosterone (open circle, light blue; T), progesterone (closed triangle, indigo; P4), pregnenolone (open triangle, violet; P5) and cholesterol (closed diamond, black). (b) Candidate modulators are PGF_{2 α} (■, red line) and PGE₂ (□, light blue line). Each data point is the mean $\pm$ SEM for four independent profiles.

intrafollicular progesterone concentrations did not correlate with the net effects of a given hFF sample on the NADP⁺-dependent oxidation of cortisol. Moreover, on the basis solely of the intrafollicular concentrations of progesterone (0.021–0.368 μ mol l⁻¹), the results obtained would have predicted less inhibition of NADP⁺-dependent 11 β -dehydrogenase activities than was actually observed, supporting the existence of selective type 1 11 β HSD inhibitors other than progesterone in hFF (Fig. 3).

Most candidate lipids equilibrated in hFF eluted from the C18 columns at different concentrations of methanol from those required maximally to elute the ovarian inhibitors of

NADP(H)-dependent 11 β HSD activities. Specifically, PGE₂, PGF_{2 α} , cortisol, cortisone, oestradiol, testosterone and progesterone each eluted at < 75% (v/v) methanol, whereas cholesterol did not elute until 100% (v/v) methanol. It is also highly relevant that steroids, such as cortisol, cortisone and progesterone, would be expected to inhibit the activities of both type 1 and type 2 11 β HSD, whereas the ovarian enzyme inhibitors eluted from hFF at 65–85% (v/v) methanol selectively inhibited NADP(H)-dependent cortisol–cortisone interconversion without affecting the NAD⁺-dependent oxidation of cortisol. Taking both the elution profiles and the apparent selectivity for type 1

Fig. 10. Effects of C18 fractions of urine, obtained from patients 2 h before follicular aspiration, on subsequent NADP⁺-dependent 11β-dehydrogenase activities in renal homogenates. Each data point represents the mean ± SEM for three individual urine samples, each from a different patient. ***P* < 0.01 versus control enzyme activity (dashed horizontal line).

11βHSD into account, it seems unlikely that the predominant hydrophobic enzyme inhibitor in hFF, eluted at 75–85% methanol, is one of the aforementioned steroids or prostaglandins. It is not possible to exclude pregnenolone as a candidate ovarian enzyme inhibitor since this C₂₁ steroid eluted over a broad range of methanol concentrations with peak elution at 75% (v/v) methanol. Additional lipids present in hFF, such as meiosis-activating sterol (4,4-dimethyl-5α-cholestane-8,14,24-triene,3β-ol) (Bykov *et al.*, 1995), are also likely to inhibit 11βHSD, although their effects on the enzyme isoforms have yet to be evaluated, as have their C18 elution profiles.

Although hFF samples were fractionated using a method originally described for the partial purification of 'glycyrhethinic acid-like factor' from urine (Morris *et al.*, 1992; Lo *et al.*, 1997), the profile of 11βHSD modulators resolved from hFF differed from the profile for urine obtained from the same IVF patients < 2 h before the hFF samples. This finding indicates that the paracrine 11βHSD modulators resolved from hFF differ from the endogenous modulators of 11βHSD activity excreted in urine.

Other studies have reported an association between low ovarian 11βHSD activities and conception by IVF–ET (Michael *et al.*, 1993b, 1995), although this association was not borne out by subsequent studies (O'Shaughnessy *et al.*, 1997; Thomas *et al.*, 1998). In addition, other studies have reported an association between pregnancy and high intrafollicular cortisol:cortisone ratios (consistent with low ovarian 11βHSD activities) (Michael *et al.*, 1999), a relationship recently observed in non-stimulated IVF cycles by Keay *et al.* (2002). In view of the present data, high ovarian 11βHSD activities may reflect increased production of the paracrine 11βHSD stimuli or absence of the ovarian 11βHSD inhibitors, whereas low ovarian 11βHSD activities could reflect high intrafollicular concentrations of 11βHSD inhibitors or absence of the endogenous 11βHSD stimuli.

The potential to correlate ovarian 11βHSD activities with the clinical outcome of fertility treatment may, therefore, rely on the degree to which a particular study reflects concentrations of the paracrine enzyme modulators present in hFF before measurement of 11βHSD activities. A salient feature of our previous studies has been that cells were stored at 4°C for up to 3 days in hFF before isolation and then cultured for 3 days before assessing net oxidation of cortisol. In contrast, Thomas *et al.* (1998) measured 11βHSD activities in freshly isolated cells within 3–6 h of aspiration, and O'Shaughnessy *et al.* (1997) stored cells at –20°C for several days before enzyme assay. Hence, measurements made in previous studies of ovarian 11βHSD activities may have been influenced either by the presence of endogenous modulators of enzyme expression or activity in the hFF before cell culture, or by the production of such compounds by the cultured cells *in vitro*.

In reporting the present findings, two caveats must be added. First, all studies were performed using follicular aspirates of women who had undergone controlled ovarian hyperstimulation for the purpose of assisted conception. Hence, the concentrations of enzyme modulators in these hFF samples may have been influenced by prior exposure to pharmacological doses of gonadotrophins. Second, the rat kidney is known to be unusual with regard to glucocorticoid metabolism in that it co-expresses both type 1 and type 2 11βHSD activities. Although the use of NADP⁺ and NAD⁺ allows for some distinction to be made between the oxidative activities of these two enzyme isoforms, it is not possible to discriminate absolutely between changes in type 1 and type 2 11βHSD activities. Hence, further characterization of the enzyme modulators is ongoing using mammalian cell lines and stably transfected cells that express individual isoforms of 11βHSD.

Although 11βHSD appears to play a key role in determining the concentrations of active glucocorticoids within the ovary, corticosteroid-binding globulin is also important in the modulating the bioavailability of cortisol. Within hFF from preovulatory follicles, progesterone increases the free cortisol concentration after competitive displacement of the glucocorticoid from corticosteroid-binding globulin (Yding Andersen, 1990). Thus, as the preovulatory follicle develops, corticosteroid-binding globulin, 11βHSD and local modulators of 11βHSD activity may all act in concert to regulate the availability of free cortisol to interact with corticosteroid receptors.

In conclusion, the present study demonstrates the presence of hydrophilic 11βHSD stimuli and hydrophobic 11βHSD inhibitors in the human ovary. These compounds, which appear to modulate selectively the activities of type 1 11βHSD, may act locally to determine the balance of cortisol–cortisone interconversion within ovarian follicles.

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