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# Expression of 11β-Hydroxysteroid Dehydrogenase, Glucocorticoid Receptor, and Mineralocorticoid Receptor Genes in Rat Ovary[1]

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## ABSTRACT

**A new concept in reproductive endocrinology is that the status of the ovary as a glucocorticoid target organ alters with follicular development. Evidence for a physiological role of glucocorticoids in the regulation of ovarian folliculogenesis has been strengthened by the discovery that 11β-hydroxysteroid dehydrogenase (11βHSD) mRNA expression in human granulosa cells is developmentally regulated. In this study, we quantified the pattern of expression and investigated the cellular location of 11βHSD type 1 (11βHSD1), 11βHSD type 2 (11βHSD2), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR) mRNAs during follicular maturation in rat ovary. Immature female rats received treatment with eCG to induce preovulatory follicular development or eCG followed by hCG to induce luteinization. 11βHSD1, 11βHSD2, GR, and MR mRNAs were all detectable by ribonuclease protection assay in ovarian total RNA. Treatment with eCG alone caused an ~8-fold increase in the ovarian level of 11βHSD1 mRNA, which rose to ~30-fold after additional treatment with hCG. Equine CG alone did not measurably affect the ovarian 11βHSD2 mRNA level, but additional treatment with hCG reduced it to 34% of the control level. Expression of GR mRNA was unchanged by any gonadotropin treatment, while MR mRNA was down-regulated. A similar pattern of 11βHSD1, 11βHSD2, GR, and MR mRNA expression was observed in isolated granulosa cells. These results provide direct experimental evidence that 11βHSD genes are gonadotropically regulated in the rat ovary, including granulosa cells, and are consistent with a shift in glucocorticoid metabolism from inactivation (due to oxidation by 11βHSD2) to activation (reduction by 11βHSD1) during hCG-induced granulosa cell luteinization.**

## INTRODUCTION

In glucocorticoid (G) and mineralocorticoid (M) target organs, access of these steroids to their receptors (GR and MR) is regulated by two 11β-hydroxysteroid dehydrogenase (11βHSD) isoforms: $NADP^+$-dependent bidirectional 11βHSD type 1 (11βHSD1) with predominant reductase activity and low binding affinity for cortisol or corticosterone [1], and $NAD^+$-dependent dehydrogenase type 2 (11βHSD2) with high binding affinity for cortisol or corticosterone [2, 3]. In G target organs such as the liver, 11βHSD1 converts cortisone to cortisol (human) and 11-dehydrocorticosterone to corticosterone (rodents), and ensures that GR is appropriately stimulated by active G [4]. In M target organs such as the kidney and colon, 11βHSD2 catalyzes active G to inactive forms, thereby protecting nonselective MR from inappropriate stimulation by active G [5].

The ovary is also a G target organ. High-affinity G binding sites, presumed to be GR, are present in rat granulosa cells [6], and G acts directly to modulate gonadotropin action on these cells in vitro [7–11]. Human granulosa cells contain 11βHSD [12–14], and a local role for G has been postulated in the regulation of follicular and oocyte function in the human ovary [12, 14–17]. The status of the ovary as a G (and possibly M) target may alter with follicular development, since up-regulation of 11βHSD1 and down-regulation of 11βHSD2 mRNA occur in human granulosa cells after hCG stimulation [18]. Both total and free cortisol levels in follicular fluid are also shown to be increased after the midcycle LH surge in spontaneously ovulating women [19].

An experimental animal model is necessary for further evaluation of the contribution of 11βHSDs to corticosteroid metabolism and action in the mammalian ovary. Rat ovaries contain both 11βHSD1 and 11βHSD2 [20–22], but it is not known whether they are differentially or developmentally regulated. Nor is it known how GR and MR expression alter with folliculogenesis in any mammalian species. In this study we examined the ovarian expression of 11βHSD1 and 11βHSD2 in relation to the levels of GR and MR mRNAs during follicular development and luteinization in immature rats treated with eCG and hCG.

## MATERIALS AND METHODS

### *Animals and Treatments*

Immature female Wistar rats (21–25 days old; Charles River UK, Margate, UK) were kept in a temperature-controlled room on a 12L:12D cycle and fed rat chow and water ad libitum. Animals were assigned to three groups and treated (subcutaneous injection in PBS vehicle) as follows before being killed: 1) eCG (Sigma Chemical Co., Poole, UK; 10 IU for 48 h: group P); 2) eCG (10 IU for 48 h) and hCG (Sigma; 10 IU for 12 h after eCG treatment: group H); 3) controls (no treatment: group C). To examine expression of 11βHSD mRNAs during luteinization, animals were treated in the same manner as group H and killed at 0, 3, 6, 9, 12, 24, and 120 h after the hCG injection. Ovaries were removed and kept on ice in PBS. They were cleaned under a dissection microscope then deep frozen in liquid nitrogen. All animal handling and treatments were performed according to the guidance issued by the Home Office, UK.

### *Granulosa Cell Isolation*

Follicles were punctured with a 25-gauge hypodermic needle, and granulosa cells were gently expelled into medium. Granulosa cell viability as assessed by trypan blue staining was 33–62%. Residual ovaries were compressed

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with forceps and washed vigorously to remove as many granulosa cells as possible. Using this method, residual ovaries comprised less than 15% granulosa cells as estimated by expression of 17βHSD1 mRNA, a gene that is expressed in granulosa cells but not in thecal-interstitial cells [23] (data not shown). Corpora lutea (CL) were dissected from ovaries 120 h after hCG treatment.

### *RNA Preparation*

Ovaries were homogenized in ice-cold 4 M guanidium thiocyanate solution containing 25 mM sodium citrate, 0.5% (w:v) sarcosyl, and 0.1 M β-mercaptoethanol (all from Sigma). Total RNA was extracted with phenol-chloroform [24].

### *$^{32}$P-Labeled Probes*

The 11βHSD1, 11βHSD2, GR, and MR RNA probes were synthesized from DNAs generated by reverse transcription-polymerase chain reaction (RT-PCR). Oligonucleotide primer pairs of 23–26 nucleotides were obtained from Cruachem Ltd. (Glasgow, UK). The lengths of resultant DNAs were as follows: 11βHSD1, 620 base pairs (bp) (nucleotides [nt] 109–728 [25], GenBank accession no. J05107); 11βHSD2, 412 bp (nt 534–945 [26], U22424); GR, 772 bp (nt 1383–2154 [27], M14053); MR, 331 bp (nt 3206–3536 [28], M36074).

Single-strand cDNA was reverse-transcribed from total RNA (1 μg) obtained from immature rat liver (11βHSD1 and GR) and kidney (11βHSD2 and MR) using Moloney murine leukemia virus reverse transcriptase (Stratagene Cloning Systems, La Jolla, CA) at 37°C for 60 min. The resultant cDNA was used for PCR amplification (30 cycles) using Pfu-DNA polymerase (Stratagene Cloning Systems). Each PCR cycle consisted of 45-sec denaturing at 94°C, 45-sec annealing at 60°C, and 2-min extension at 72°C with the final extension for 10 min. Resultant PCR products were cloned using pCR-Script Amp SK(+) Cloning Kit (Stratagene Cloning Systems) and sequenced to verify the authenticity of the products. The 18S rRNA was synthesized from cDNA containing the 80-bp fragment of a highly conserved region of human 18S rRNA (pT7 RNA 18S; Ambion, Austin, TX). RT-PCR-generated cDNAs were linearized with either *Eco*RI or *Not*I. The RNA probes were labeled with [$^{32}$P]UTP (400 Ci/mmol; Amersham International, Aylesbury, UK) using either T3 or T7 RNA polymerase and reagents supplied by Promega (Madison, WI). Resultant RNA probes protect 620-, 412-, 772-, and 254-nt mRNAs for 11βHSD1, 11βHSD2, GR, and MR, respectively. The specific activities of these probes were $6.7 \times 10^8$ cpm/μg for 11βHSD1, 11βHSD2, GR, and MR and $4.4 \times 10^3$ cpm/μg for 18S rRNA.

### *Ribonuclease Protection Assay*

Total RNA (20–40 μg) was hybridized with approximately $5 \times 10^3$ cpm of each of the 11βHSD1, 11βHSD2, GR, and MR probes together with $1 \times 10^4$ cpm 18S rRNA probe for 16 h at 42°C in 20 μl of hybridization buffer containing 80% deionized formamide, 40 mM Pipes (pH 6.7), 0.4 M NaCl, and 1 mM EDTA [29]. The resultant protected RNA fragments were processed according to the procedure outlined previously [29] and size-fractionated by electrophoresis on 5% acrylamide gels containing 8 M urea. Total RNA from kidney and liver was used as a positive control, and tRNA was used as a negative control.

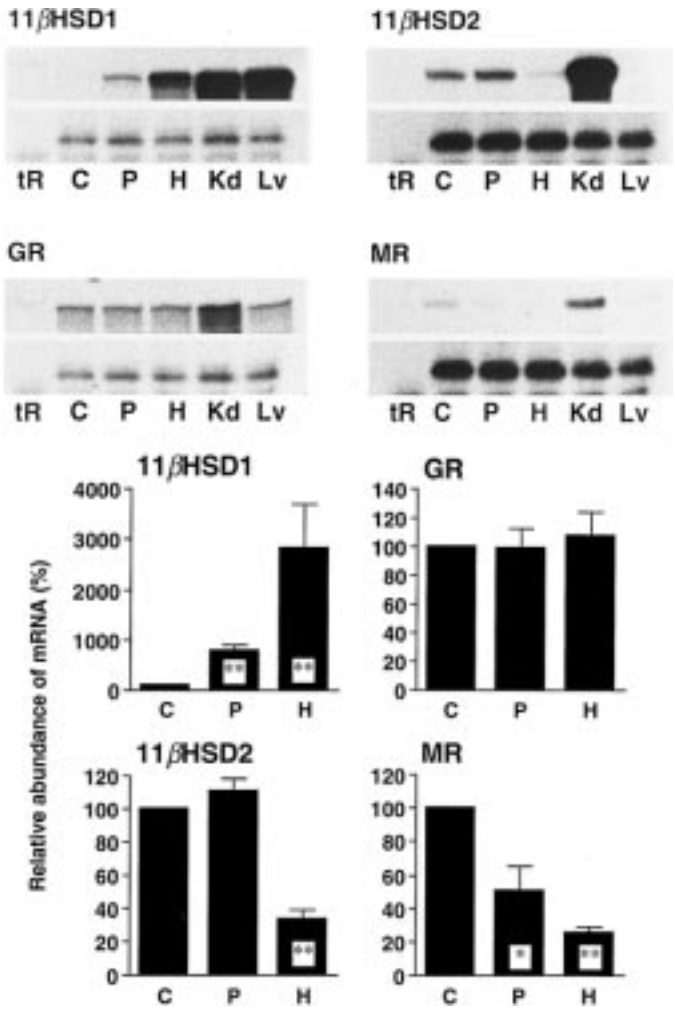


FIG. 1. Effect of gonadotropins on ovarian 11βHSD1, 11βHSD2, GR, and MR mRNA expression in immature rats. **Top**) Autoradiogram of ribonuclease protection assay showing protected fragments of 11βHSD1, 11βHSD2, GR, and MR mRNAs and 18S rRNA (lower panels). Approximately 20 μg of total RNA was analyzed for 11βHSD1, GR, tRNA, kidney (Kd), and liver (Lv) and 40 μg for 11βHSD2 and MR. Films were exposed for 4 days (11βHSD1 and GR), 9 days (11βHSD2 and MR), or 1 day (18S rRNA). **Bottom**) Quantitative analysis of gene expression. Data were normalized using 18S rRNA and expressed as a percentage of the control values. Results were expressed as mean ± SEM of three separate trials. * and **, Significantly different from the control values; $p < 0.05$ and $p < 0.01$, respectively (ANOVA with Student's *t*-test).

### *Data Analysis*

The radioactive signal of the protected RNAs was quantified by electronic autoradiography (Instant Imager; Packard, Downers Grove, IL). The background value (tRNA) was subtracted, and the abundance of mRNAs was normalized to the 18S rRNA. The results were expressed as a percentage of the control values and analyzed using one-way ANOVA with Student's *t*-test.

## RESULTS

### *Ovarian Expression of 11βHSD1, 11βHSD2, GR, and MR mRNAs in Immature Gonadotropin-Treated Rats (Fig. 1)*

All mRNAs were detected in the ovary, although signal intensity varied between mRNA species and stage of follicular development. The expression of 11βHSD1 was very low in group C but dramatically up-regulated in groups P

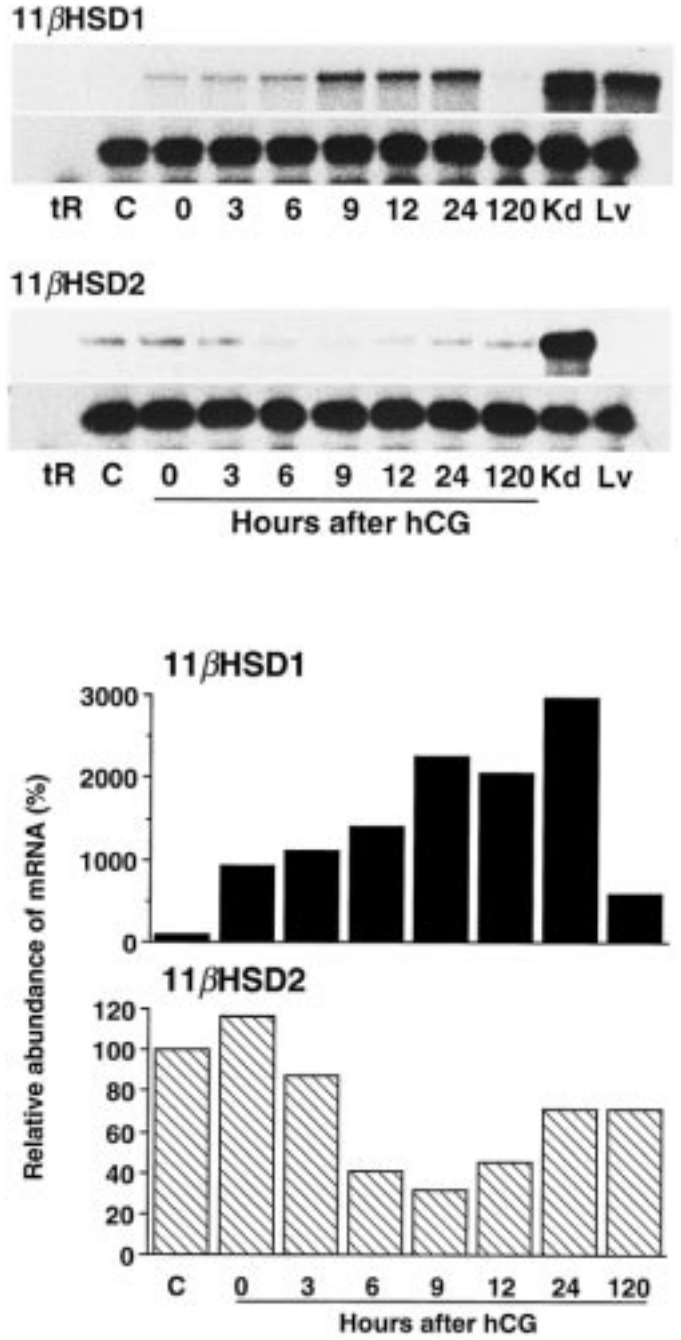


FIG. 2. Ovarian expression of 11βHSD1 and 11βHSD2 mRNAs during gonadotropin-stimulated luteinization in immature rats. **Top**) Autoradiogram of ribonuclease protection assay showing protected fragments of 11βHSD1, 11βHSD2, and 18S rRNA (lower panels). Approximately 20 μg of total RNA was analyzed for 11βHSD1 and 40 μg for 11βHSD2. Films were exposed for 4 days (11βHSD1), 7 days (11βHSD2), or 1 day (18S rRNA). Kd, kidney; Lv, liver. **Bottom**) Quantitative analysis of gene expression. Data were normalized using 18S rRNA and expressed as a percentage of the control values.

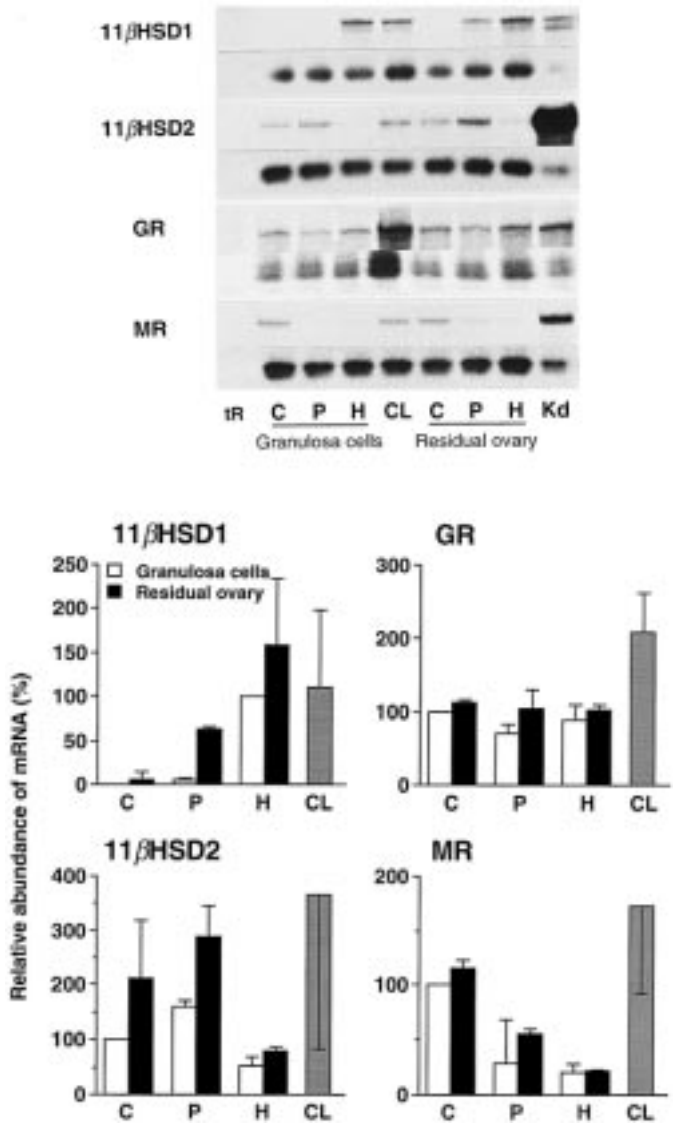


FIG. 3. Expression of 11βHSD1, 11βHSD2, GR, and MR mRNA in isolated granulosa cells, residual ovary, and CL during gonadotropin-stimulated follicular maturation and luteinization in immature rats. **Top**) Autoradiogram of ribonuclease protection assay showing protected fragments of 11βHSD1, 11βHSD2, GR, and MR mRNAs and 18S rRNA (lower panels). Approximately 20 μg of total RNA was analyzed for 11βHSD1, 11βHSD2, and GR; 40 μg for MR. Films were exposed for 6 days (11βHSD1 and GR), 14 days (11βHSD2 and MR), or 1 day (18S rRNA). **Bottom**) Quantitative analysis of 11βHSD1, 11βHSD2, GR, and MR mRNA gene expression. Data were normalized using 18S rRNA and expressed as a percentage of the control values. Results were expressed as mean ± SD of two separate trials.

(783 ± 133% of the control value, mean ± SEM; $p < 0.01$) and H (2835 ± 878%; $p < 0.01$). The expression of 11βHSD2 remained unchanged in group P (111 ± 7%) and was decreased in group H (34 ± 6%; $p < 0.01$). Expression of GR was unchanged in both groups P (99 ± 14%) and H (107 ± 18%), while expression of MR was down-regulated in groups P (51 ± 15%; $p < 0.05$) and H (25 ± 4%; $p < 0.01$).

### *Ovarian Expression of 11βHSD1 and 11βHSD2 mRNAs during Luteinization (Fig. 2)*

Expression of 11βHSD1 increased steadily after hCG treatment, reaching a maximum of 2000–3000% of the control value at 9–24 h and falling to 600% at 120 h. Conversely, expression of 11βHSD2 decreased after hCG, reaching a nadir of 40–30% of the control value at 6–12 h and recovering to 70% at 24–120 h.

### *Expression of 11βHSD1, 11βHSD2, GR, and MR mRNAs in Isolated Granulosa Cells, Residual Ovary, and CL (Fig. 3)*

All four mRNAs were detected in isolated granulosa cells, residual ovary, and CL. Levels of both 11βHSD

mRNAs were 1.5–2 times higher in the residual ovary than in the granulosa cells, while equivalent levels of GR and MR mRNAs were detected in both tissues. Relatively high but variable levels of these mRNAs were also detected in CL.

Expression of 11βHSD1 was very low in granulosa cells from group P but was clearly detectable in residual ovary. After hCG treatment, the expression of 11βHSD1 increased in both granulosa cells and residual ovary. The expression of 11βHSD2 and MR in granulosa cells and residual ovary responded to gonadotropin treatment in the same way and to a similar extent. The expression of GR was not measurably altered by any treatment, and similar levels of GR mRNA were detected in isolated granulosa cells and residual ovary.

## DISCUSSION

This study provides direct evidence that 11βHSD genes are gonadotropically regulated in the mammalian ovary. Previously we showed that luteinized granulosa cells from the ovaries of in vitro fertilization patients, collected 36 h after ovulation induction with hCG, express increased levels of 11βHSD1 mRNA but reduced levels of 11βHSD2 mRNA as compared with granulosa cells from the resected ovaries of cyclic women removed before onset of the midcycle LH surge [18]. The clinical context precluded experimental observation of 11βHSD isoform expression in relation to stage of gonadotropin-induced follicular development. Here, we used the eCG-primed immature rat ovary to demonstrate unequivocally that hCG induces up-regulation of 11βHSD1 and down-regulation of 11β-HSD2 expression in the preovulatory follicle. In addition we found that the rat ovary expresses MR mRNA, which unlike GR mRNA is also down-regulated by gonadotropins. This confirms the mammalian ovary as a corticosteroid target organ, the status of which alters in relation to follicular activity.

Our results extend previous reports of 11βHSD expression in the rat ovary, demonstrating that both 11βHSD type 1 and 2 are transcribed, albeit subject to differential control. 11βHSD1 has previously been localized by immunocytochemistry and in situ hybridization to oocytes and CL, but not granulosa cells or thecal-interstitial cells on the morning of proestrus, i.e., shortly before onset of the LH surge [20]. It was also demonstrated in that study that NAD-dependent 11βHSD activity (i.e., 11βHSD2) is present in the ovary of the proestrous rat [20]. This is consistent with the low level of 11βHSD1 and high level of 11βHSD2 mRNAs detected at an equivalent stage of follicular maturity in eCG-treated ovaries, particularly in isolated granulosa cells. 11βHSD2 mRNA was also localized to the rat CL during late pregnancy [22], when no signal for 11βHSD1 mRNA was detected [22]. The present data, based on a sensitive ribonuclease protection assay, show that mRNAs encoding both 11βHSD isoforms, as well as GR and MR, can be detected in CL, indicating the CL as a potential site of both of corticosteroid metabolism and action in the rat.

Our results unequivocally locate all four mRNAs to isolated granulosa cells, where both 11βHSD isoforms and MR, but not GR, are developmentally regulated. Since the isolated granulosa cells would have contained some oocytes, we do not rule out the possibility that 11βHSD expression by the oocyte also contributes to these results. Residual ovary (which inevitably contained residual granulosa cells and oocytes) displayed development-related changes in mRNA expression that were qualitatively and quantitatively similar to those shown by isolated granulosa cells, except for 11βHSD1 in group P, where expression of the mRNA was much higher in residual ovary than in granulosa cells. This is presumably due to the difference in LH responsiveness of thecal-interstitial cells and granulosa cells, since the latter only express LH receptor after adequate exposure to FSH [30]. The inability to remove all granulosa cells from the ovary using the puncture technique also clouds the interpretation of the ''residual ovary'' MR mRNA data. It is possible that contaminating granulosa cells, especially those from unpunctured small follicles, were responsible for MR signals detected in residual ovary. However, it seems reasonable to conclude from the present data that the dramatic up-regulation of 11βHSD1 mRNA by hCG was mainly at the level of granulosa cells in large follicles, since granulosa cells in small follicles do not possess LH/hCG receptors [30]. We have preliminary evidence to support this conclusion, based on measurements of 11βHSD1 mRNA expression and enzymatic activity in isolated granulosa cells after gonadotropin treatment in vitro (unpublished results). Whether any other ovarian cell type contributes directly to this pattern of mRNA expression remains to be determined. To date, the few attempts that have been made to localize 11βHSD mRNAs in ovarian follicles by in situ hybridization [20–22] have been inconclusive, presumably due to the relatively low abundance of the transcripts. We are presently optimizing highly sensitive nonisotopic in situ hybridization methodology in order to resolve this issue.

The presence of GR mRNA confirms isolated granulosa cells and CL as G target tissues [6–11, 22, 31, 32]. Glucocorticoids or synthetic G, dexamethasone, has been shown to increase FSH-stimulated progesterone (P) production in cultured immature granulosa cells [7, 8, 32]. This is at least partially due to a selective increase in 3βHSD activity and a decrease in 20αHSD activity [32]. Glucocorticoids also synergistically enhance FSH-, GnRH-, and epidermal growth factor-stimulated tissue-type plasminogen activator (tPA) activity and gene expression [10, 11]. These results are different from those of an early study in which an inhibitory effect of G on basal and FSH-stimulated tPA activity was shown in immature granulosa cells [9]. The reason for the discrepancy between these studies is not clear. An inhibitory effect of G was reported on FSH-stimulated aromatase activity and LH receptor levels [7, 8]. G appears to inhibit induction of aromatase, since it did not inhibit aromatase activity in mature granulosa cells [7]. Overall, these results indicate that G enhances FSH-stimulated P production and tPA activity, without inhibiting already existing aromatase activity, while it inhibits induction of aromatase and LH receptor in rat granulosa cells. The G receptor may also act as a P receptor. It has been reported that P inhibits 20αHSD expression in rat CL through GR [31]. However, it has not yet been investigated whether P exerts any physiological effect through GR in granulosa or thecal-interstitial cells. Since GR is expressed constitutively throughout follicular development and luteinization, the differential expression of 11βHSD1 reductase and 11βHSD2 dehydrogenase observed in the present study may act as a key mechanism to control G action during follicular development.

We find that in addition to GR, the rat ovary expresses MR mRNA. Coexpression of MR and 11βHSD2 is a feature of M target organs such as kidney and colon [21, 33]. Coordinate regulation of both genes in the ovary suggests that it may also be an M target organ. However, there is no known physiological role for M in the regulation of ovarian

function. Aldosterone has been shown to modulate FSH-induced differentiation of rat granulosa cells in vitro [8, 10], but this effect is likely to be due to action via GR [8, 10]. Mineralocorticoids serve physiological roles via association with MR to regulate fluid dynamics in classic M targets, where 11βHSD2 is expressed to ensure inactivation of glucocorticoids that would otherwise inappropriately activate MR [33]. Whether MR plays a similar role in the developing follicle remains to be determined.

The physiological importance of these results lies in the fact that the ovarian follicle is likely to operate a cortisol/cortisone (corticosterone/11-dehydrocorticosterone in the rat) shuttle based on the differential expression of 11βHSD isoforms, similar to that shown to operate in other G and M target tissues [34, 35]. Although ovaries do not undertake de novo corticosteroid synthesis due to lack of expression of the cytochrome P450s that catalyze C21 (P450C21) and C11 (P450C11β) hydroxylation, they clearly undertake corticosteroid metabolism. The finding that 11βHSD genes are gonadotropically regulated in rat ovarian granulosa cells is consistent with a shift in G metabolism from inactivation (due to oxidation by 11βHSD2) to activation (reduction by 11βHSD1) during hCG-induced granulosa cell luteinization. Together with the elevated circulating level of adrenal-derived corticosterone that occurs around the time of the LH surge in the afternoon of proestrus [36], up-regulation of 11βHSD1 and down-regulation of 11βHSD2 would ensure maximum availability of G for action via GR within periovulatory follicles. In human ovaries, the simultaneous up-regulation of 11βHSD1 and down-regulation of 11βHSD2 [18] cause luteinized granulosa cells to be rich in 11β-reductase activity [37], which presumably explains why both total and free cortisol levels in human follicular fluid become elevated after the ovulation-inducing LH surge [19].

In conclusion, a developmental shift in the potential for G metabolism occurs within the rat ovarian follicle during hCG-induced luteinization: from inactivation (via 11βHSD2-catalyzed oxidation) to activation (via 11βHSD1-catalyzed reduction). This suggests a local mechanism for governing the intrafollicular corticosteroid milieu whereby immature follicles receive ''protection'' from inappropriate stimulation by G while ovulatory follicles gain exposure to G at levels required for involvement in oogenesis or the process of follicular rupture, possibly as an anti-inflammatory modulator [38, 39].

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