Regulation of 11 β -hydroxysteroid dehydrogenase type 1 gene expression by LH and interleukin-1 β in cultured rat granulosa cells

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Abstract

Granulosa cells from preovulatory follicles show increased expression of 11 β -hydroxysteroid dehydrogenase type 1 (11 β HSD1) at the time of ovulation. As ovulation may be an inflammatory process, this may be a mechanism of local enhancement of the activity of anti-inflammatory glucocorticoids. In this study, we examined direct effects of LH, the proinflammatory cytokine, interleukin-1 β (IL-1 β), and pharmacological activators of protein kinase A (PKA) (forskolin and dibutyryl (db) cAMP) and PKC (LH-releasing hormone and phorbol 12-myristate 13-acetate (PMA)) signalling on the expression of 11 β HSD1 mRNA *in vitro*. Granulosa cells from immature female rat ovaries were cultured (pretreatment) in serum-free medium 199 containing recombinant human (rh) FSH (1 ng/ml) for 48 h to induce responsiveness to LH. Cell monolayers were then washed and cultured (test treatment) for a further 12 h in the presence of rhLH (0–100 ng/ml), IL-1 β (0–50 ng/ml), or both. Total RNA was extracted from granulosa cell monolayers and taken for quantitative ribonuclease protection analysis of 11 β HSD1 mRNA. The low level of 11 β HSD1 mRNA detectable in un-

stimulated (control) cultures was increased approximately twofold by the 48-h pretreatment with rhFSH. Subsequent exposure to rhLH (1–100 ng/ml) for a further 12 h dose-dependently increased 11 β HSD1 mRNA expression by an additional two- to threefold. Forskolin (10 μ M), db-cAMP (2 mM), LH-releasing hormone (LHRH; 1 μ M) and PMA (200 nM) were also stimulatory. IL-1 β (0.05–50 ng/ml) stimulated 11 β HSD1 mRNA expression in a dose-related manner, both in the absence and in the presence of rhLH (3 ng/ml). The interaction between IL-1 β (5 ng/ml) and rhLH (3 ng/ml) was additive. Co-treatment with a 50-fold excess of IL-1 receptor antagonist fully reversed the action of IL-1 β . We conclude that 11 β HSD1 mRNA expression in functionally mature granulosa cells is directly stimulated by gonadotrophins and IL-1 β *in vitro*, potentially involving post-receptor signalling via PKA- and PKC-mediated pathways. Thus both LH and IL-1 β may serve physiological roles in the upregulation of 11 β HSD1 gene expression by granulosa cells in ovulatory follicles.

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Introduction

Ovulation induced by luteinising hormone (LH) or human chorionic gonadotrophin (hCG) is the consequence of a biochemical cascade involving locally produced pro-inflammatory cytokines such as interleukin-1 β (IL-1 β) (Espey 1980, 1994, Adashi 1998). At the time of ovulation, granulosa cells are highly active sites of glucocorticoid metabolism via 11 β -hydroxysteroid dehydrogenase (11 β HSD) enzyme activity (Michael *et al.* 1997). 11 β HSD enzymes are expressed in at least two isoforms that reversibly interconvert glucocorticoids (cortisol and corticosterone) and 'inactive' 11-oxosteroid precursors (cortisone and 11-dehydrocortisone) (Michael & Cooke

1994). 11 β HSD type 1 (11 β HSD1) is principally an 11-oxoreductase (converts cortisone to cortisol, and 11-dehydrocorticosterone to corticosterone), whereas 11 β HSD type 2 (11 β HSD2) is a strong 11-dehydrogenase (back-converts cortisol to cortisone, and corticosterone to 11-dehydrocorticosterone). We have previously shown that a switch occurs in 11 β HSD gene expression during granulosa cell luteinisation, the pattern of which suggests an intraovarian role for anti-inflammatory glucocorticoids in limiting the inflammatory component of ovulation (Tetsuka *et al.* 1997, 1999, Thomas *et al.* 1998). Granulosa cells in developing follicles express mainly 11 β HSD2 mRNA, but little or no 11 β HSD1 mRNA. After exposure of preovulatory follicles to an ovulation-inducing

dose of LH or hCG, 11 β HSD2 mRNA expression is suppressed, whereas 11 β HSD1 mRNA is enhanced. This shift in potential for glucocorticoid inactivation (oxidation by 11 β HSD2) to activation (reduction by 11 β HSD1) is reflected in the predominantly reductive (cortisone to cortisol) mode of glucocorticoid metabolism undertaken by luteinising granulosa cells *in vitro* (Hillier & Tetsuka 1998, Thomas *et al.* 1998). Differential regulation of 11 β HSD gene expression before follicular rupture may therefore be the means by which the intrafollicular concentration of cortisol is increased before ovulation (Harlow *et al.* 1997). To test the ability of gonadotrophins to act directly on granulosa cells to modulate gene 11 β HSD expression, we have optimised a rat granulosa cell culture model in which follicle-stimulating hormone (FSH) treatment is first given to induce functional maturation (i.e. LH responsiveness), followed by LH to induce luteinisation. Here, we demonstrate that LH directly upregulate granulosa cell 11 β HSD1 mRNA expression *in vitro*. We also show that this newly discovered action of LH is mimicked by IL-1 β and various agonists of protein kinase A (PKA) and PKC signalling.

Materials and Methods

Hormones and reagents

Recombinant human (rh) FSH (3860 IU/mg) and rhLH (6350 IU/mg) were generously donated by Dr C Howles (Serono Laboratories UK Ltd, Welwyn Garden City, Hertfordshire, UK). rhIL-1 β and rhIL-1 receptor antagonist (rhIL-1ra) were obtained from R&D System Europe Ltd (Abingdon, Oxfordshire, UK). Forskolin, dibutyryl 3' 5'-cAMP (db-cAMP), LH-releasing hormone (LHRH), phorbol 12-myristate 13-acetate (PMA), testosterone, diethylstilboestrol (DES), L-glutamine, penicillin, streptomycin and BSA were obtained from Sigma Chemical Co. (St Louis, MO, USA). The culture medium was medium 199 supplemented with 25 mM HEPES, 2 mM L-glutamine, penicillin (50 IU/ml), streptomycin (50 mg/ml) and 0.1% (w/v) BSA. Donor calf serum (DCS), medium 199 and Dulbecco's phosphate-buffered saline (DPBS) were supplied by Gibco BRL (Paisley, Renfrewshire, UK).

Granulosa cell culture

Immature (21-day-old) female Wistar rats from Charles River UK (Margate, Kent, UK) were given daily s.c. injections of DES (2 mg/day) for 2 days, to stimulate granulosa cell proliferation. Ovaries were removed for granulosa cell isolation after the animals were killed by asphyxiation with CO₂. All handling and treatment of animals was according to guidance issued by the British Home Office.

Preantral/early antral follicles were punctured with a 25-gauge hypodermic needle and granulosa cells gently expelled into culture medium. The cells were harvested by centrifugation, resuspended in fresh medium and counted in a haemocytometer. Cell viability assessed by trypan blue staining was $\geq 30\%$. Twenty-four-well Linbro plastic culture dishes (Flow Laboratories, Rickmansworth, Hertfordshire, UK) were precoated with DCS and washed twice with DPBS before inoculation with 0.5 ml culture medium containing approximately 2×10^5 viable cells (Hillier & de Zwart 1982). All incubations were at 37 °C in a humidified incubator gassed with 5% CO₂ in air. To induce granulosa cell maturation and responsiveness to LH, the cells were pretreated for 48 h in the presence of rhFSH (1 ng/ml) and testosterone (1 μ M). Cell monolayers were then washed once with DPBS before incubation for 12 h in fresh medium containing the test substance(s) under investigation, this being the approximate time required for hCG to induce ovulation *in vivo* (Tetsuka *et al.* 1999).

RNA preparation

Granulosa cells were lysed in ice-cold 4 M guanidinium thiocyanate solution containing 25 mM sodium citrate, 0.5% (wt/vol) sarcosyl, and 0.1 M β -mercaptoethanol (all from Sigma). Total RNA was extracted with phenol-chloroform (Chomczynski & Sacchi 1987).

³²P-Labelled probes

The 11 β HSD1 RNA probe was synthesised from cDNA generated by reverse-transcription (RT)-PCR. Oligonucleotide primer pairs were obtained from Cruachem Ltd (Glasgow, UK). The length of resultant DNA was 620 bp (nucleotides (nt) 109–728 (Agarwal *et al.* 1989), GenBank accession No. J05107).

Single-strand cDNA was reverse-transcribed from total RNA (1 μ g) obtained from immature rat liver (11 β HSD1) using Moloney's murine lymphoma virus reverse transcriptase (Stratagene Cloning Systems, La Jolla, CA, USA) at 37 °C for 60 min. The resultant cDNA was used for PCR amplification (30 cycles) using *Pyrococcus furiosus* U DNA polymerase (Stratagene Cloning Systems). Each PCR cycle consisted of: 45 s denaturing at 94 °C, 45 s annealing at 60 °C and 2 min extension at 72 °C, with the final extension for 10 min. The resultant PCR product was cloned using pCR-Script Amp SK(+) Cloning Kit (Stratagene Cloning Systems) and sequenced to verify the authenticity of the products. The 18S ribosomal RNA (18S rRNA) was synthesised from cDNA containing DNA encoding a highly conserved region of human 18S rRNA (pT7 RNA 18S; Ambion, Austin, TX, USA). RT-PCR generated 11 β HSD1 cDNA was linearised with *Eco*R V. The RNA probes were labelled with [³²P]UTP

(800 Ci/mmol; Amersham International, Aylesbury, Buckinghamshire, UK) using reagents supplied by Promega (Madison, WI, USA). The resultant RNA probes protect 257 (corresponding to nt 472–728 (Agarwal *et al.* 1989)) and 80 nt RNAs for 11 β HSD1 and 18S rRNA, respectively. The specific activities of these probes were 1.3×10^9 c.p.m./ μ g for 11 β HSD1 and 8.8×10^3 c.p.m./ μ g for 18S rRNA.

Ribonuclease protection assay

Total RNA (3–5 μ g) was hybridised with approximately 2×10^4 c.p.m. each of 11 β HSD1 and 18S rRNA probes for 16 h at 42 °C in 20 μ l hybridisation buffer containing 80% deionised formamide, 40 mM PIPES (pH 6.7), 0.4 M NaCl and 1 mM EDTA (Tetsuka & Hillier 1996). The resultant protected RNA fragments were processed as described previously and size-fractionated by electrophoresis on 5% acrylamide gels containing 8 M urea. Total RNA from liver and tRNA were used as positive and negative controls, respectively.

Data analysis

The radioactive signals given by protected RNAs were quantified by electronic autoradiography (Instant Imager, Packard, Downers Grove, IL, USA). The background value (result for tRNA) was subtracted and mRNA abundance was normalised to the 18S rRNA value. Results were expressed as percentages of the control value. Quantitative experiments were carried out at least three times. Data from replicate experiments were pooled for analysis of variance with Student's *t*-test for group comparisons. *P* values <0.05 were considered statistically significant.

Results

LH-responsive 11 β HSD1 mRNA expression

Pretreatment of immature granulosa cells with rhFSH and testosterone to induce preovulatory maturation increased the expression of 11 β HSD1 mRNA approximately two-fold (data not shown). To examine subsequent responsiveness to rhLH, mature granulosa cells were cultured for a further 12 h with increasing concentrations (0–100 ng/ml) of rhLH. In three experiments, there was a dose-related response (mean $\pm$ S.E.M.) to rhLH, producing $170.8 \pm 15.8\%$ (non-significant) stimulation by 1 ng/ml rhLH, increasing to $214.0 \pm 14.7\%$ ($P < 0.05$) at 3 ng/ml. Higher doses of rhLH (10–100 ng/ml) showed no further significant effect (Fig. 1). Time-course studies confirmed the responses to rhLH 3 and 10 ng/ml to be maximal at 12 h (data not shown).

Figure 1 Dose-dependent effect of LH on 11 β HSD1 mRNA expression in FSH-pretreated granulosa cells. Granulosa cells were cultured in serum-free medium 199 supplemented with rhFSH (1 ng/ml) and testosterone (1 μ M) for 48 h (pretreatment). Cell monolayers were then washed once and incubated (test treatment) for a further 12 h in fresh medium supplemented with rhLH (0–100 ng/ml). (a) Autoradiogram of ribonuclease protection assay showing protected fragments of 11 β HSD1 mRNA (upper panel) and 18S rRNA (lower panel). tR, transfer RNA negative hybridisation control; Lv, liver total RNA positive hybridisation control. (b) Quantitative analysis of gene expression in the same experiment. Data were normalised using 18S rRNA and expressed as a percentage of the value for LH '0'. Pooled results (mean $\pm$ S.E.M.) from three separate experiments. Significant differences ($P < 0.05$): a compared with b.

Post-receptor signalling

To determine which post-receptor pathway(s) mediate upregulation of 11 β HSD1 mRNA, granulosa cells previously incubated with rhFSH and testosterone were incubated for a further 12 h in the presence of a stimulatory dose of rhLH (3 ng/ml), a 'high' dose of rhFSH (100 ng/ml) or a pharmacological activator of PKA or PKC signalling: forskolin (10 μ M), db-cAMP (2 mM), LHRH (1 μ M) or PMA (200 nM) (Fig. 2). Treatment with 3 ng/ml rhLH significantly increased the expression of 11 β HSD1 mRNA ($P < 0.01$). rhFSH and forskolin caused the greatest, but most variable, levels of stimulation ($P < 0.05$). All other pharmacological activators of PKA and PKC signalling also tested positively ($P < 0.01$).

Figure 2 Stimulation of 11 β HSD1 mRNA expression by activators of PKA and PKC signalling in cultured granulosa cells. Granulosa cells were cultured in serum-free medium 199 supplemented with rhFSH (1 ng/ml) and testosterone (1 μ M) for 48 h (FSH pretreatment). Cell monolayers were then washed once and incubated (test treatment) for a further 12 h in fresh medium supplemented with activators of PKA and PKC. (a) Autoradiogram of ribonuclease protection assay showing protected fragments of 11 β HSD1 mRNA (upper panel) and 18S rRNA (lower panel). Lane 1: negative control (no pretreatment or test treatment); lane 2: test control (no test treatment after FSH pretreatment); lane 3: rhFSH (100 ng/ml); lane 4: rhLH (3 ng/ml); lane 5: forskolin (10 μ M); lane 6: db-cAMP (2 mM); lane 7: LHRH (1 μ M); lane 8: PMA (200 nM). tR, transfer RNA negative hybridisation control; Lv, liver total RNA positive hybridisation control. Results from a single representative experiment. (b) Quantitative analysis of gene expression. Data were normalised using 18S rRNA and expressed as a percentage of the test control. Pooled results (mean $\pm$ S.E.M.) from three separate experiments. Significant differences relative to the test control (lane 2): * P <0.05, ** P <0.01.

IL-1 β responsive 11 β HSD1 mRNA expression

To determine if IL-1 β affects the expression of 11 β HSD1 mRNA, rhFSH-pretreated granulosa cells were treated for a further 12 h with increasing concentrations (0.05–50 ng/ml) of the inflammatory mediator. IL-1 β in concentrations $\geq$ 5 ng/ml significantly increased the expression of 11 β HSD1 mRNA both in the absence and in the presence of 3 ng/ml rhLH (Fig. 3). Analysis of the interaction between IL-1 β (5 ng/ml) and rhLH (3 ng/ml) in five

Figure 3 Effect of IL-1 β on 11 β HSD1 mRNA expression in cultured granulosa cells. Granulosa cells were cultured in serum-free medium 199 supplemented with rhFSH (1 ng/ml) and testosterone (1 μ M) for 48 h (FSH pretreatment). Cell monolayers were then washed once and incubated (test treatment) for a further 12 h in fresh medium supplemented with rhIL-1 β (0–50 ng/ml) with or without rhLH (3 ng/ml). (a) Autoradiogram of ribonuclease protection assay showing protected fragments of 11 β HSD1 mRNA (upper panel) and 18S rRNA (lower panel). Results from a representative experiment. tR, transfer RNA negative hybridisation control; Lv, liver total RNA positive hybridisation control. (b) Quantitative analysis of gene expression. Data were normalised using 18S rRNA and expressed as a percentage of the value obtained for test treatment without LH or IL-1 β . Pooled results from three separate experiments. Significant differences (P <0.05): a compared with b and c compared with d.

separate experiments revealed that the combined response was additive (Fig. 4).

To assess the involvement of IL-1 receptors in mediating the action of IL-1 β , rhFSH-pretreated granulosa cells were treated for 12 h with IL-1 β (5 ng/ml) in the presence and absence of IL-1ra (250 ng/ml). IL-1ra completely blocked the stimulatory effect of IL-1 β in all three of three experiments (Fig. 5).

Discussion

These findings pinpoint a cellular level at which gonadotrophins and cytokines act to increase follicular expression

Figure 4 Interaction between LH and IL-1 β on granulosa cell 11 β HSD1 mRNA expression in cultured granulosa cells. Granulosa cells were cultured in serum-free medium 199 supplemented with rhFSH (1 ng/ml) and testosterone (1 μ M) for 48 h (FSH pretreatment). Cell monolayers were then washed once and incubated (test treatment) for a further 12 h in fresh medium supplemented with or without rhIL-1 β (50 ng/ml), with or without rhLH (3 ng/ml). 11 β HSD1 mRNA was quantified by ribonuclease protection assay, normalised using 18S rRNA and expressed as a percentage of the value obtained for test treatment without LH or IL-1 β . Bars represent mean ($\pm$ S.E.M.) values from five separate experiments. Significant differences ($P < 0.05$): a compared with b and b compared with c.

of 11 β HSD1 expression. Previously, we reported that, in human beings (Tetsuka *et al.* 1997) and rats (Tetsuka *et al.* 1999), administration of hCG (surrogate LH) to induce ovulation induces upregulation of 11 β HSD1 mRNA expression in granulosa cells of ovulatory follicles. Other studies confirmed the presence of a high level of 11 β HSD1 mRNA in human granulosa-lutein cells (Michael *et al.* 1997, Smith *et al.* 1997). However, it had not been determined if the action of hCG was direct on granulosa cells or mediated by paracrine interactions with thecal cells. Here, we have proved that LH is able to stimulate granulosa cell 11 β HSD1 mRNA expression directly in the absence of any other LH-responsive cell type. This leaves open the possibility of other cell types contributing to the overall response to LH/hCG *in vivo*.

Upregulation of granulosa cell 11 β HSD1 by LH/hCG is accompanied by downregulation of 11 β HSD2 *in vivo* (Tetsuka *et al.* 1997, 1999). As, in contrast to 11 β HSD1, 11 β HSD2 is principally oxidative (Stewart & Mason 1995, Edwards *et al.* 1996, Seckl & Chapman 1997, White *et al.*

Figure 5 Effect of IL-1ra on IL-1 β -stimulated 11 β HSD1 mRNA expression in cultured granulosa cells. Granulosa cells were cultured in serum-free medium 199 supplemented with rhFSH (1 ng/ml) and testosterone (1 μ M) for 48 h (FSH pretreatment). Cell monolayers were then washed once and incubated (test treatment) for a further 12 h in fresh medium supplemented with or without rhIL-1 β (5 ng/ml), rhIL-1ra (250 ng/ml), or both. (a) Autoradiogram of ribonuclease protection assay showing protected fragments of 11 β HSD1 mRNA (upper panel) and 18S rRNA (lower panel). Results from a representative experiment. tR, transfer RNA negative hybridisation control. (b) Quantitative analysis of data from three separate experiments. Data were normalised using 18S rRNA and expressed as a percentage of the value obtained for test treatment without IL-1ra or IL-1 β . Bars represent mean ($\pm$ S.E.M.) values from three separate experiments. Significant differences ($P < 0.01$): a compared with b.

1997), its negative regulation would be expected to promote glucocorticoid activation, thereby supplementing the reductase function of 11 β HSD1. During the present study, we found that the relatively high level of 11 β HSD2 mRNA initially present in immature granulosa cells rapidly declined *in vitro*, regardless of hormone treatment (data not shown). This is in accord with previous evidence that the two 11 β HSD isoforms are differentially regulated

during follicular maturation *in vivo*. However, the regulatory mechanism giving rise to downregulation of 11 β HSD2 in the face of upregulation of 11 β HSD1 remains to be determined.

The ability of IL-1 β to mimic LH-induced upregulation of 11 β HSD1 mRNA expression by granulosa cells is consistent with other evidence for the involvement of proinflammatory factors in ovulation (Espey 1980, 1994, Terranova & Rice 1997, Adashi 1998). Work on the control of 11 β HSD1 expression in glomerular mesangial cells has shown that exposure to proinflammatory cytokine increases the expression and reductase activity of 11 β HSD1, serving to augment the anti-inflammatory effect of glucocorticoids (Escher *et al.* 1997). Increased production of tumour necrosis factor α and IL-1 β (Terranova & Rice 1997, Adashi 1998) with upregulation of phospholipase A2 (Ben-Shlomo *et al.* 1997), cyclo-oxygenase 2 (Morris & Richards 1995, Hellberg *et al.* 1996, Ando *et al.* 1998) and 11 β HSD1 (Tetsuka *et al.* 1997) are common features of ovulation. Glucocorticoid receptor mRNA expression in rat granulosa cells is not measurably influenced by LH/hCG treatment *in vivo* (Tetsuka *et al.* 1999). Thus local activation of glucocorticoids by 11 β HSD could be a major determinant of glucocorticoid action in the ovulatory follicle.

The finding that IL-1 β directly stimulates granulosa cell 11 β HSD1 mRNA *in vitro* does not take account of any cell-cell interactions that might influence cytokine responsiveness *in vivo*. Others have shown that association with thecal-interstitial cells strongly influences IL-1 β -responsive prostaglandin synthesis by granulosa cells *in vitro* (Kokia *et al.* 1992). It will be of interest to determine if a similar synergy occurs between these cell types with respect to IL-1 β -responsive 11 β HSD1 mRNA expression.

Whereas LH and IL-1 β can each stimulate granulosa 11 β HSD1 expression directly, the principal post-receptor signalling pathways involved are likely to differ. Actions of LH/hCG on granulosa cells classically involve PKA-mediated signalling leading to increased expression of cAMP-responsive genes (Morris & Richards 1995), including IL-1 β (Adashi 1998). However, granulosa cell luteinisation also involves PKC- and tyrosine kinase(s)-mediated signalling pathways (Morris & Richards 1995), which are more likely to be activated by IL-1 β . As shown here, pharmacological agonists of both the PKA (forskolin, db-cAMP) and PKC (PMA, LHRH) signalling pathways upregulate 11 β HSD1 expression *in vitro*. These pharmacological data complement the finding that combined treatment with LH and IL-1 β causes additive stimulation of 11 β HSD1 mRNA expression *in vitro* (Figs 3 and 4), suggesting that more than one post-receptor signalling mechanism is likely to regulate granulosa cell 11 β HSD1 expression *in vivo*.

Regardless of the signalling pathways involved, it is of interest that an LH-inducible proinflammatory component

(IL-1 β) in the periovulatory cascade has the capacity to set in train a means by which inflammation might be quelled – that is, through 11 β HSD1-mediated formation of anti-inflammatory glucocorticoid. Further work is required to determine if the spatio-temporal expression of genes encoding inflammatory mediators in the wall of the periovulatory follicle is consistent with this postulated sequence of events *in vivo*.

In conclusion, our results suggest a mechanism by which the potentially deleterious inflammatory component of follicular rupture is ultimately self-limiting. LH can act directly to upregulate 11 β HSD1 gene expression in granulosa cells *in vitro* and its action is mimicked by the proinflammatory mediator, IL-1 β . This supports a working hypothesis that upregulation of 11 β HSD1 expression during granulosa cell luteinisation (with reciprocal downregulation of 11 β HSD2 gene expression) is a mechanism to augment local production of anti-inflammatory glucocorticoids, serving to minimise inflammatory tissue damage at the time of rupture and encourage rapid healing of the ovarian surface in anticipation of the next ovulation.

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