Regulation of uridine diphosphate-glucuronosyltransferase 2B15 expression during ovulation in the rat

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Abstract. Uridine diphosphate-glucuronosyltransferase 2B15 (UGT2B15) conjugates 5α-androstane-3α, 17β-diol (3α-diol) to 3α-diol glucuronide (3α-diol G) in steroid target tissues. The present study investigated the regulation of UGT2B15 expression during the ovulatory process in the rat. Real-time PCR analysis revealed that treatment of immature rats with equine chorionic gonadotropin followed by human chorionic gonadotropin transiently stimulated UGT2B15 gene expression in granulosa cells of preovulatory follicles within 6 h. The progesterone receptor antagonist RU486 suppressed the gonadotropin-induced UGT2B15 expression. The expression of UGT2B15 and the levels of 3α-diol G were transiently increased by luteinizing hormone (LH) treatment in cultured preovulatory follicles. The LH-stimulated UGT2B15 mRNA level in cultured preovulatory follicles was inhibited by inhibitors of adenylyl cyclase, phosphoinositide 3-kinase and mitogen-activated protein kinase. Furthermore, a vitamin D receptor agonist (calcitriol) suppressed the LH-stimulated UGT2B15 expression in a dose-dependent manner. Taken together, these results indicate that gonadotropins transiently stimulate UGT2B15 expression and activity in preovulatory follicles, and UGT2B15 mRNA levels are regulated by the progesterone receptor and vitamin D receptor.

Key words: Uridine diphosphate-glucuronosyltransferase 2B15, 3α-diol glucuronide, Ovulation, Vitamin D receptor
Ovulation, which is triggered by surge in luteinizing hormone (LH), leads to a shift of the steroid profile from production of estrogens to that of progestins, mainly through the stimulation of P450 side-chain cleavage (P450scc; Cyp11a) and the inhibition of aromatase (Cyp19a1) expression in rodents [7], resulting in the gradual increase of androgen production during the ovulatory process [8]. Serum levels of androgens reach a peak soon (1–4 h) after LH/human chorionic gonadotropin (hCG) administration in female mice [8]. Since an excess in androgen levels could exert deleterious effects [9], a physiological system for the elimination of excess androgen during ovulation should be operated. In this present study, we investigated the regulation of UGT2B15 expression during the ovulatory process in the rat.

Materials and Methods

Animals
Immature female Sprague-Dawley rats were purchased from Samtako BioKorea (Seoul, Korea). The animals were housed in a temperature- and light-controlled room (14L:10D schedule) with food and water available ad libitum. The rats (26 days old; body weight 60–65 g) were injected subcutaneously with 15 IU equine chorionic gonadotropin (eCG/PMSG; Sigma, St. Louis, MO, USA) to stimulate multiple follicular growth. Two days later, the eCG-primed rats were injected intraperitoneally with 10 IU human chorionic gonadotropin (hCG; Sigma) to induce superovulation. At 30 min prior to the hCG treatment, six eCG-primed rats in each treatment group had received an intraperitoneal injection of vehicle (10% ethanol in sesame oil), indomethacin (10 mg/kg BW), or RU486 (10 mg/kg BW). The indomethacin and RU486 were purchased from Sigma. All animals were maintained and treated in accordance with the National Institutes of Health Guide for the Care and Use of Experimental Animals, as approved by the Institutional Animal Care and Use Committee of Chonnam National University.

Collection of preovulatory samples after hCG administration in vivo
At various time points after the hCG injection, fine forceps were used to isolate preovulatory follicles from the ovaries in culture medium under a stereomicroscope. The preovulatory follicles were then stored at –80°C for RNA extraction or used for the isolation of granulosa and theca cells.

Using a 21-gauge needle, preovulatory follicles were punctured to release granulosa cells into the medium, and the theca cells were isolated from the remaining follicle residues. The purity of the different cell populations was validated using granulosa- or theca-specific marker genes (Supplemental Fig. 1).

Culture of preovulatory follicles in vitro
Preovulatory follicles, isolated from ovaries primed with eCG for 48 h, were cultured in a glass vial containing 300–500 µL of Minimal Essential Medium Alpha (Life Technologies, Inc., Grand Island, NY, USA) supplemented with 1 mg/mL bovine serum albumin, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C under 95% O₂ and 5% CO₂. The follicles were then cultured in the presence of LH (200 ng/mL) for up to 24 h. At the end of each designated time point, the follicles were stored at –80°C for RNA extraction and the media were kept at –20°C for 3α-diol G assay.

To assess the signaling pathways, follicles were pre-treated for 30 min with the adenylyl cyclase inhibitor MDL12330A (10 µM), phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (10 µM), mitogen-activated protein kinase (MEK) inhibitor UO126 (10 µM), protein kinase C inhibitor staurosporin (100 nM), or phospholipase C inhibitor U73122 (10 µM) and then treated with LH (200 ng/mL) for 8 h. All the inhibitors were purchased from Sigma.

To determine the effect of steroids on UGT2B15 regulation, follicles were cultured in the absence or presence of LH (200 ng/mL), DHT (250 nM in 0.1% ethanol), 17β-estradiol (250 nM in 0.1% ethanol), or progesterone (250 nM in 0.1% ethanol). In parallel experiments, follicles were incubated with the androgen receptor antagonist hydroxyflutamide (10 μM in 0.1% DMSO), estrogen receptor antagonist ICI 182,780 (10 μM in 0.1% DMSO), or progesterone receptor antagonist RU486 (10 μM in 0.1% DMSO) for 30 min before the addition of LH (200 ng/mL). After 8 h, the follicles were collected and stored at –80°C for RNA extraction. All the steroids and antagonists were purchased from Sigma.

To examine the effect of the vitamin D receptor (VDR) agonist calcitriol (Sigma) on UGT2B15 regulation, follicles were cultured in the absence or presence of LH (200 ng/mL) with or without different concentrations of calcitriol. After 8 h, the follicles were collected and stored at –80°C for RNA extraction.
Real-Time PCR
Total RNA was extracted using Tri-Reagent solution (Sigma) according to the manufacturer’s protocol and reverse-transcribed with the RevertAid M-MuLV Reverse Transcriptase Kit (Fermentas, St. Leon-Rot, Germany). Real-time PCR was performed on a RotorGene Q 5plex platform (Qiagen, Hilden, Germany) located at the Korea Basic Science Institute (Gwangju, Korea), using the QuantiCect SYBR Green PCR Kit (Qiagen). The PCR program was as follows: an initial 5 min at 95°C; followed by 45 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 20 s; and a final extension at 72°C for 10 min. Primers for the PCR and real-time PCR were designed using Primer 3 software as follows: UGT2B15 (forward, 5'-CTTAGGACCCAAATACCACTGCTCAAA3'; and reverse, 5'-TTCCCTTATTTCCACAGCTGCTC3') and β-actin (forward, 5'-GAGACCTTCAACCTCCAGAGCC3'; and reverse, 5'-CCGTCAGGCCAGCTCATAAGGT3'). Transcript levels of the target genes were normalized against those of the β-actin gene. The relative gene expression levels were calculated using the comparative 2^ΔΔCt method.

Measurement of 5α-androstane-3α, 17β-diol glucuronide concentration
The concentration of 3α-diol G in culture medium was measured by enzyme immunoassay using the 3α-diol G ELISA Kit (Alpco Diagnostics, Salem, NH, USA). All samples were measured in duplicates in the same assay. The standard curve range was 0–50 ng/mL. The intra-assay and inter-assay coefficient of variation values were 7.8% and 10.4%, respectively.

Statistics
Results are presented as the mean ± SEM of at least three independent experiments. Differences among groups were analyzed by one-way analysis of variance followed by the Tukey post hoc test. Comparisons between any two points were evaluated using the Student two-tailed t test. Differences with a p value of less than 0.05 were considered statistically significant.

Results
Gonadotropin regulation of UGT2B15 expression in preovulatory follicles
To examine the regulation of UGT2B15 mRNA levels by gonadotropin during ovulation in vivo, total RNA extracted from preovulatory follicles at different time intervals after eCG/hCG administration was analyzed by real-time PCR. As shown in Fig. 1A (left panel), UGT2B15 mRNA levels were transiently stimulated: increased to reach a maximum level at 6 h, and then gradually decreased until 12 h after hCG treatment. The mRNA increase was detectable in
granulosa cells but not in theca cells of preovulatory follicles at 6 h post-hCG treatment (Fig. 1A, right panel). The expression levels of UGT2B17 and 17β-hydroxysteroid dehydrogenases were too low to be detected (data not shown).

To investigate the regulation of UGT2B15 gene expression in vivo, eCG-primed rats were injected with the progesterone receptor antagonist RU486 or cyclooxygenase inhibitor indomethacin at 30 min before hCG administration. As shown in Fig. 1B, treatment with RU486 suppressed the hCG-stimulated UGT2B15 mRNA level, whereas indomethacin had no effect, indicating the mediation of the progesterone receptor pathway.

To confirm the gonadotropin regulation in vitro, preovulatory follicles isolated from rat ovaries primed with eCG for 48 h were cultured with LH for up to 24 h. As shown in Fig. 2A, LH treatment transiently stimulated the UGT2B15 mRNA level, with a peak at 12 h. The activity of UGT2B15 was estimated by measuring the concentrations of 3α-diol G, a metabolite generated by the action of UGT2B15, in medium. In parallel with UGT2B15 expression, LH treatment significantly elevated the 3α-diol G levels, reaching a maximum at 12 h (Fig. 2B). To determine the signaling pathway involved, follicles were treated with various signal inhibitors for 8 h. As shown in Fig. 2C, the LH-stimulated UGT2B15 mRNA levels

![Fig. 2](image-url)

**Fig. 2** Regulation of UGT2B15 expression in cultured preovulatory follicles

Preovulatory follicles isolated from ovaries treated with equine chorionic gonadotropin for 48 h were cultured in the presence of luteinizing hormone (LH; 200 ng/mL) for up to 24 h. Follicular RNA was analyzed for UGT2B15 expression using real-time PCR (A) and media were used to measure the 3α-diol glucuronide (3α-diol G) levels (B) at indicated time points. Results show the mean ± SEM of five independent experiments. * p < 0.05 vs. 0 h. (C) Preovulatory follicles were cultured in the absence (C, control) or presence of LH (200 ng/mL), with or without signal inhibitors, including the different doses of the adenylyl cyclase inhibitor MDL12330A (MDL), phosphonoisitide 3-kinase inhibitor LY294002 (LY) or mitogen-activated protein kinase inhibitor UO126 (UO), and a single dose of the protein kinase C inhibitor staurosporin (STS; 100 nM) or phospholipase C inhibitor U73122 (U73; 10 μM) for 8 h. Treatment with maximal dose of inhibitor alone or negative control (NC; 50 μM LY30351 or UO124) was also included. The expression of UGT2B15 was analyzed using real-time PCR. Results show the mean ± SEM of four independent experiments. * p < 0.05 vs. LH treatment.
were dose-dependently inhibited by the adenylyl cyclase inhibitor MDL12330A (36% inhibition at 50 µM), by the PI3K inhibitor LY294002 (84% inhibition at 50 µM) and by the MEK inhibitor UO126 (74% inhibition at 50 µM). Treatment with the inhibitor alone or negative control (NC) did not affect UGT2B15 expression. The protein kinase C inhibitor staurosporin (STS; 100 nM) or phospholipase C inhibitor U73122 (U73; 10 µM) did not significantly affect the LH-stimulated UGT2B15 expression.

**Regulation of UGT2B15 by steroids**

Since steroid hormones regulate UGT2B15 expression in breast cancer cells [10] and prostate cells [2], the effect of steroids on UGT2B15 expression was examined in preovulatory follicles cultured for 8 h. LH alone significantly increased the UGT2B15 mRNA levels, as expected (Fig. 3). Co-treatment with RU486 suppressed the LH-induced UGT2B15 expression, similar to the in vivo result, whereas addition of the androgen receptor antagonist hydroxyflutamide or estrogen receptor antagonist ICI 182,780 had no effect. Treatment with DHT, estrogen, or progesterone alone did not stimulate the UGT2B15 mRNA levels.

**Suppression of UGT2B15 expression by vitamin D**

Since vitamin D inhibits UGT2B15 expression in prostate cancer cells [4, 11], preovulatory follicles were treated with LH and the vitamin D receptor (VDR) agonist calcitriol for 8 h. The addition of calcitriol suppressed the stimulatory action of LH on UGT2B15 expression in a dose-dependent manner, resulting in a final 34% suppression of the hormone action (Fig. 4). Calcitriol alone did not affect the UGT2B15 expression.

**Discussion**

The present study indicates that the ovary, like other steroid target tissues, possesses the androgen-eliminating system of UGT2B15 enzyme during ovulation. UGT2B15 expression and 3α-diol G production were transiently stimulated by LH/hCG in preovulatory follicles. The role of UGT2B15 on conjugating 3α-diol G is well demonstrated in steroid target tissues [5, 6]. The LH/hCG-stimulated UGT2B15 expression is regulated by the extracellular signal-regulated kinases (ERK) signaling pathway and progesterone receptor
pathway, both of which play an important role in ovulation [12, 13]. Furthermore, activation of the VDR suppressed the LH/hCG-stimulated UGT2B15 expression, supporting the fact that VDR-knockout mice have impaired follicle maturation [14].

The transient stimulation of UGT2B15 is in conformance with the steroid profile during ovulation in rodents. The LH surge causes an increase in progesterone, mainly by stimulating P450scc, and at the same time causes a decrease in estrogens, mainly by inhibiting aromatase, resulting in a transient increase of testosterone levels [8]. Since excess androgens are deleterious for ovulation [9, 15], UGT2B15 plays a part in their removal. The transient increase in 3α-diol G levels in preovulatory follicles observed in the present study support this idea. The physiological importance of UGT2B15 has been demonstrated in the prostate, wherein the reduction of glucuronidation by inhibition of UGT2B15 decreases the 3α-diol G levels, leading to an accumulation of androgens that causes hyperplasia [16].

In humans, high levels of 3α-diol G in plasma [17] and in follicular fluid [18] reflect the active role of UGT2B enzymes in ovarian androgen metabolism. The cell type-specific expression of UGT2B enzymes during follicular development in monkey ovary provides evidence for a potential role of glucuronidation as a modulator of the androgen response [19]. Since human P450c17 (CYP17) acts on 17-hydroxyprogrenolone but not 17-hydroxyprogesterone almost exclusively [20], the future study on the role of P450c17 in androgen metabolism in human ovary would be of interest. Indeed, P450c17 plays an active role in causing an excess of ovarian androgens that leads to polycistic ovary syndrome [21]. Further experiments will be needed to assess the role of UGT2B enzymes in ovarian diseases and to verify the potential of 3α-diol G as a biomarker for fertility in females.

UGT2B15 mRNA remained elevated for up to 12 h in preovulatory follicles cultured in vitro with LH, compared with ovaries treated with hCG in vivo, indicating that the mechanism mediating the decline in UGT2B15 mRNA after hCG treatment in vivo is reduced in vitro after LH treatment. One possible explanation for the difference is that UGT2B15 mRNA expressed in preovulatory follicles may be diluted with other ovarian tissues as the size of ovary increases after hCG treatment in vivo. Another possible explanation for the difference is that the production of signal molecules including cAMP seems to be sustained longer in vitro after LH treatment [22].

Since the progesterone receptor is indispensable for ovulation [23], the role of the progesterone receptor pathway in the stimulation of UGT2B15 provides evidence for the physiological relevance of this enzyme, which is further supported by the suppression of UGT2B15 expression via blocking of the ERK signaling pathway. The progesterone receptor is activated by the ERK pathway [12], and blocking ERK signals during ovulation elicits defects in follicle rupture, cumulus expansion, and corpus luteum formation in mice [12, 24]. The inhibition of UGT2B15 expression by the cAMP inhibitor confirms the fact that ERK is activated by cAMP-PKA pathway in preovulatory follicles [12]. The inhibition of UGT2B15 expression by blocking of the PI3K signal suggests the possible role of UGT2B15 in the formation of the corpus luteum. PI3K signal activation induces CCAAT-enhancer-binding proteins, which play an important role in the formation of the corpus luteum [25-27].

Suppression of the LH-stimulated UGT2B15 expression by the VDR agonist indicates that the VDR modulates androgen metabolism during ovulation. The VDR is expressed in rat ovarian follicles [28]. VDR-knockout female mice show reproductive defect due to impaired follicle maturation [14], which is possibly due to the accumulation of androgens via UGT2B15 defectiveness, as found in the present study. The VDR also plays a role in regulating testicular androgen production [29]. VDR ligands suppress UGT2B15 expression in the prostate [4, 11].

In summary, to the best of our knowledge, our study is the first to demonstrate the presence of an androgen-eliminating system comprising UGT2B15 in the ovary. LH/hCG transiently stimulated both the expression of UGT2B15 and the glucuronidation of 3α-diol in preovulatory follicles. The regulation of UGT2B15 expression by the progesterone receptor, ERK signals, and VDR, which play a role in ovulation, suggests the physiological relevance of UGT2B15 in ovulation. Further studies are needed to clarify the correlation between 3α-diol G and female infertility.

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Disclosure

No financial or other conflict of interest to declare.

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