Expression of aldo-keto reductase family 1, member C14 during ovulation in the rat

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Abstract. The potent androgen 5α-dihydrotestosterone is metabolized to the weak androgen 5α-androstane-3α, 17β-diol (3α-diol) by the enzyme aldo-keto reductase family 1, member C14 (Akr1c14) in rodents. The purpose of the present study was to investigate the regulation of Akr1c14 expression during the ovulatory process in rat ovaries. Northern blot analysis revealed that treatment of immature rats with equine chorionic gonadotropin resulted in lowered Akr1c14 expression, whereas subsequent treatment with human chorionic gonadotropin (hCG) increased ovarian Akr1c14 expression within 3 h. In situ hybridization analysis showed that Akr1c14 mRNA was localized in granulosa cells of growing follicles before hCG treatment, but it was also expressed in granulosa cells of preovulatory follicles after hCG treatment. Akr1c14 protein expression increased after 6 h of hCG treatment and was sustained at high levels until 12 h. The levels of 3α-diol in preovulatory follicles isolated from ovaries in vivo were fluctuated by hCG treatment; decreased at 6 h and increased at 9 h. Human CG-induced Akr1c14 expression was suppressed by treatment with the progesterone receptor antagonist RU486, but not with the cyclooxygenase inhibitor indomethacin. Taken together, these findings demonstrate the induction of Akr1c14 by hCG in granulosa cells of rat preovulatory follicles that was regulated by progesterone receptor antagonist.

Key words: Aldo-keto reductase family 1 member C14, 5α-androstane-3α 17β-diol, Gonadotropins, Ovulation
reductase (AKR) family [10]. The enzyme Akr1c14 reduces DHT, the most potent natural androgen, to \( 5\alpha\text{-androstane-3a, 17\beta\text{-diol (3a-diol), a molecule that is unable to bind the androgen receptor} \[10] \). Akr1c14 is found in androgen target tissues, such as the liver [11] and prostate cells [12]. Akr1c14 is also abundantly expressed in progenitor and immature Leydig cells [13]. In the rodent ovary, Akr1c14 is detected during the estrous cycle [14] and is primarily localized in the granulosa and corpus luteum cells [15]. Akr1c14 is also found in porcine cumulus oocyte complexes and granulosa cells [16]. In addition, high levels of 3α-diol are detected at first proestrus in the rat [17]. However, little information is available about the regulation of Akr1c14 expression during the ovulatory process. In this study, we investigated the gonadotrophin stimulation of Akr1c14 expression and 3α-diol levels during the ovulatory process in rats.

Materials and Methods

Animal treatment and collection of ovaries

Immature female Sprague-Dawley rats were purchased from Samtako (Osan, Korea). Animals were housed in a temperature- and light-controlled room (14L:10D schedule) with food and water ad libitum. Immature female rats (26 days; body weight [BW] 60-65 g) were subcutaneously (s.c.) injected with 15 IU equine chorionic gonadotropin (eCG/PMSG; Sigma, St. Louis, MO, USA) to stimulate multiple follicular growth. Two days later, some eCG-primed rats were injected intraperitoneally (i.p.) with 10 IU human chorionic gonadotropin (hCG; Sigma) to induce superovulation. Ovaries of gonadotropin-primed rats were collected at different time intervals for Northern blot and in situ hybridization analysis. Some eCG-primed rats received i.p. injection of vehicle (10% ethanol in sesame oil), indomethacin (10 mg/kg BW; Sigma) or RU486 (10 mg/kg BW; Sigma) 30 min before hCG treatment. The ovaries of each rat 3 h after hCG were used for RNA isolation. For the examination of ovulation rate, the rats were killed, the oviduct were excised and flushed, and the oocytes were counted under a dissecting microscope 24 h after hCG. All animals were maintained and treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, as approved by the Institutional Animal Care and Use Committee of Chonnam National University.

In situ hybridization analysis

Ovaries were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS at 4°C for 6 h), followed by immersion in 0.5 M sucrose in PBS overnight. Cryostat sections (14-µm thick) were mounted on poly-L-lysine (Sigma) coated microscope slides and, fixed in 4% paraformaldehyde in PBS. The hybridization procedure was essentially the same as previously described [18]. In brief, sections were pretreated and the hybridization was carried out at 52-55°C overnight with a \( ^35\text{S-labeled} \) rat Akr1c14 cRNA probe containing the 3′-UTR. Post-hybridization washing was performed under stringent conditions, which included ribonuclease A (25 µg/mL) treatment at 37°C for 30 min and a final stringency of 0.1 × SSC. Slides were dipped into an NTB-2 emulsion (Eastman Kodak Co.) and exposed at 4°C for 4 weeks, after slides were developed. The slides were stained with hematoxylin and eosin and examined under a light microscope with bright- and dark-field illumination.

RNA extraction, reverse transcription (RT) and real-time polymerase chain reaction (PCR)

Total RNA was extracted from preovulatory follicles of eCG/hCG-treated ovaries using TRIzol (Life Technologies). These RNAs were reverse transcribed with a RevertAid M-MuLV Reverse Transcriptase Kit (Fermentas, St. Leon-Rot, Germany) to evaluate gene expression.
Akr1c14 expression during ovulation

expression. Real-time PCR was performed on a Rotor-Gene Q 5plex (Qiagen, Hilden, Germany) located at Korea Basic Science Institute (Gwangju, Korea), using a QuantiTect SYBR Green PCR kit (Qiagen). The PCR program was as follows: 95°C at 5 min, followed by 45 cycles at 95°C for 15 s, 60°C for 20 s, and 72°C for 20 s; with a final extension at 72°C for 10 min. All primers were designed using Primer 3 software (Table 1). Transcript levels of target genes were normalized against those of β-actin. Relative gene expression levels were calculated using the comparative 2^ΔΔCt method.

Western blot analysis

Lysates of preovulatory follicles (20 µg protein per lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 12% gels and transferred to nitrocellulose membranes (Amersham Bioscience, Arlington Heights, IL, USA), as previously described [18]. After blocking in 5% skim milk, the membranes were incubated with anti-Akr1c14 goat polyclonal antibodies (1:1,000 final dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by horseradish peroxidase-conjugated secondary IgG (1:1,000 final dilution). After washing with 1× TBST, reactive bands were visualized with enhanced chemiluminescence (Amersham). The band intensities were quantified using UN-SCAN-IT Gel 6.1 software (Silk, Scientific, Orem, UT, USA) after subtraction of background signals, and normalized using GAPDH (Santa Cruz Biotechnology).

Assay for 3α-diol levels

Pre-ovulatory follicles were dissected from the ovaries of gonadotropin-primed rats at different time intervals. Follicular 3α-diol was extracted, and assays were performed according to previously described methods [19]. Briefly, preovulatory follicles were homogenized in Tris buffer (pH 7.0), and extracted with 10 volumes of ethyl acetate. The ethyl acetate fraction was transferred into clean tubes and evaporated to dryness under nitrogen. The dried extracts were then resuspended in 0.5 ml PBS (pH 7.0). The efficiency of extraction was at least 90%. 3α-Diol levels were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (MyBioSource, San Diego, CA, USA). The intra-assay and inter-assay coefficients of variation (CVs) were 7.8% and 10%, respectively. 3α-Diol contents were presented as ng/mg follicle protein.

Statistics

All data are presented as the mean ± standard error of the mean (SEM) from at least three independent experiments. Differences among groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test (Prism software version 5 [GraphPad]). 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 the Akr1c14 expression in the ovary in vivo

To examine regulation of Akr1c14 mRNA levels in response to gonadotropin treatment, immature rats were treated with eCG followed by hCG. As shown in Fig. 1A, Northern blot analysis revealed that eCG treatment markedly decreased levels of the 2.7 kb Akr1c14 transcript in the ovaries (right panel, 4-fold decrease at 24 h). However, treatment with hCG resulted in a transient increase in Akr1c14 mRNA levels, reaching a peak at 3 h (right panel, 4.3-fold increase). In situ hybridization was performed to determine the cell types expressing Akr1c14 mRNA. Akr1c14 mRNA was detected in granulosa cells of growing follicles in the ovaries of untreated 26-day-old rats (Fig. 1B).

Table 1 Prime sequences used for real-time PCR analysis

| Gene name | Primer sequence (5’→3’) | Product size (bp) | GenBank index |
|-----------|--------------------------|------------------|---------------|
| Akr1c14   | F: CTGACACCAAGCTGATTTGCTA R: ACAGGTGCCCTACCTCAAAACT | 219 | NM_138547 |
| β-actin   | F: GAGACCTTCAAACCCCCAGCC R: CCGTCAGGCAGCTCATAGCTC | 362 | NM_031144 |

F, Forward; R, Reverse.
Fig. 1  Expression of Akr1c14 gene in the ovary

(A) Twenty micrograms of ovarian total RNA were assayed for mRNA levels by Northern blotting using a rat Akr1c14 cDNA probe. The size of Akr1c14 transcript (2.7 kb) is indicated. The transcript was quantified using a phospho-imager and normalized for 28S RNA levels (n = 3; mean ± SEM).  * p < 0.05 versus the 0 h eCG or 48 h eCG (= 0 h hCG).  (B) Ovarian sections were hybridized with 35S-labeled rat cRNA probes for in situ localization. Asterisk, Growing follicles; PoF, pre-ovulatory follicles.
High levels of Akr1c14 mRNA were localized in granulosa cells of growing follicles but not of preovulatory follicles in ovaries treated with eCG for 2 days. Interestingly, Akr1c14 mRNA was induced in granulosa cells of preovulatory follicles by hCG treatment for 3 h. Ovarian sections hybridized with the sense cRNA probe did not show a hybridization signal (data not shown).

Akr1c14 gene and protein expression were then determined in preovulatory follicles isolated from eCG/hCG-treated ovaries at each time point by real-time PCR and Western analysis. Akr1c14 mRNA levels were reached a maximum level at 6 and then gradually decreased until 12 h after hCG treatment (Fig. 2A). Akr1c14 protein levels were increased at 3 h, reached a maximum level at 6 h and were sustained at high levels until 12 h (Fig. 2B).

Concentration of 3α-diol in preovulatory follicles

Because Akr1c14 was stimulated by hCG, concentrations of 3α-diol, a steroid metabolite generated by the activity of Akr1c14, were measured in preovulatory follicles isolated from eCG/hCG-treated ovaries at each time point. In response to hCG treatment, 3α-diol levels were decreased at 6 h, transiently increased at 9 h, and returned to control levels at 12 h (Fig. 3).

Regulation of the Akr1c14 expression by progesterone receptor in the ovary in vivo

To evaluate the regulation of Akr1c14 gene expression in vivo, ovulation-inhibiting doses of the progesterone receptor antagonist RU486 or indomethacin, a cyclooxygenase inhibitor were administered 30 min before hCG stimulation in eCG-primed immature rats. As expected, the ovulation rates were significantly inhibited by RU486 (72.8% inhibition) or indomethacin (87.5% inhibition) (Fig. 4A). There was substantial expression of Akr1c14 mRNA at 3 h post-hCG (Fig. 4B). In animals treated with RU486, the hCG-stimulated Akr1c14 mRNA levels were significantly decreased (62.4% inhibition). Treatment with indomethacin suppressed slightly, but not significantly, hCG-induced Akr1c14 mRNA levels (17.6% inhibition).

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**Fig. 2** Expression of Akr1c14 in preovulatory follicles isolated from eCG/hCG-treated rat ovaries

(A) Total RNA (5 μg) of preovulatory follicles was assayed for Akr1c14 gene expression using real-time RT-PCR. All values are expressed as a relative amount to β-actin. Data points represent the mean ± SEM of three independently performed experiments. *p < 0.05 vs. 0 h hCG. (B) Western blot analysis was performed to detect the expression of Akr1c14 protein in preovulatory follicles. Data for each time point are means ± SEs from five different assays. S, Spleen (−, negative control); L, liver (+, positive control). *p < 0.05 versus the 0 h hCG.
Discussion

The present study demonstrates that eCG/hCG induces Akr1c14 expression in granulosa cells of pre-ovulatory follicles in the rat ovary. Northern analysis revealed the presence of the 2.7-kb transcript of Akr1c14, which encodes for a long 3′-UTR that may be an important determinant in mRNA stability [10]. The present findings, which demonstrated the involvement of the progesterone receptor pathway in hCG-induced Akr1c14 expression supported the observation that Akr1c14 expression is regulated by steroid hormones [20]. Stimulation of ovarian 3α-HSD/Akr1c14 expression by an ovulatory dose of gonadotropin has been
reported in the rat ovary [21]; however, this gene has been identified as a pseudogene product. Furthermore, follicular levels of 3α-diol were increased by hCG, indicating that Akr1c14 is active during ovulation.

The expression of Akr1c14 showed a transient increase in granulosa cells of preovulatory follicles after hCG administration. This transient activation of Akr1c14 may be necessary for the removal of excess androgens produced by LH/hCG. Serum levels of androgens reach a peak soon (1–4 h) after hCG administration in female mice [2]. If Akr1c14 is not expressed, excessive androgens would accumulate in preovulatory follicles and thus affect the ovulatory process. Indeed, in vivo administration of DHT to immature female rats at the time of hCG injection reduces the ovulation rate [7]. The importance of 3α-HSD/Akr1c14 in the removal of excessive androgens is well documented in the prostate [12] and liver [11]. Inadequate metabolism of DHT by 3α-HSD/Akr1c14 can lead to the development of benign prostatic hyperplasia and prostate cancer [22].

The Akr1c14 is also involved in dihydroprogesterone (DHP) catabolism into allopregnanolone in different tissues such as testis [23]. However, progesterone is mainly catabolized into its biologically inactive form, 20α-hydroxyprogesterone, by 20α-hydroxysteroid dehydrogenase (20-HSD) in the ovary [24]. The 20-HSD is expressed in corpus luteum and ovarian theca cells which produce progesterone [25]. Although Akr1c14 has 20-HSD activity [25], Akr1c14 may not be involved in progesterone catabolism because it was exclusively expressed in ovarian granulosa cells.

Akr1c14 was not expressed in granulosa cells of preovulatory follicles in vivo unless they receive an acute LH/hCG stimulus. One of the possible explanations for gonadotropin-induced transactivation of this gene is the recruitment of transcriptional activators such as progesterone receptor. The Akr1c14 gene promoter contains multiple steroid response elements (SREs), which may comprise a steroid response unit (Supplemental Fig. 1) [26]. Our studies in vivo suggest that progesterone receptor may interact via the PREs to mediate the regulation of Akr1c14 expression. Akr1c14 gene can be induced by estrogens in rat liver, indicating that this gene transcription may protect against steroid hormone excess [27]. However, Akr1c14 gene and protein were spontaneously induced in preovulatory follicles cultured in vitro under the serum-free condition without LH/hCG stimulation (Supplemental Fig. 2), providing the technical limitation for the study of Akr1c14 regulation. The difference in Akr1c14 expression in vitro compared with that in vivo may depend on the absence of serum in culture. The Akr1c14 gene promoter contains a powerful silencer that binds Oct-1 [26], and a strong distal enhancer with GAGA repeats that contribute to high constitutive expression of this gene [28]. Circulating transcriptional repressors in serum may bind to the site of a powerful silencer in the Akr1c14 gene promoter to suppress its expression. The absence of repressors in serum-free culture may result in a relief of pausing that leads to a constitutive expression of the Akr1c14 gene.

Fluctuations in 3α-diol concentrations in preovulatory follicles after hCG treatment indicate the active role of Akr1c14 during ovulation. The increase in 3α-diol levels between 6 and 9 h post-hCG reflected that Akr1c14 was most active during this period. In contrast, the decrease in 3α-diol levels after 9 h suggested that 3α-diol may be metabolized to 3α-diol-glucuronide by the enzyme uridine diphosphate-glucuronosyltransferases (UGT) [29]. Thus, investigation of UGT isoforms expressed in the ovary during ovulation would be interesting.

In summary, LH/hCG stimulates the transient expression of Akr1c14 gene and protein in preovulatory follicles probably to eliminate excess androgens during ovulation. Levels of 3α-diol, an androgen metabolite generated by the activity of Akr1c14, were also increased after gonadotropin treatment, indicating an active role of Akr1c14. Based on the present study, Akr1c14 may contribute the normal ovulatory process by maintaining physiological levels of androgens.

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Disclosure

No financial or other conflict of interest to declare.
Supplemental Fig. 1  The cis-acting elements on the 5′-flanking region of the rat Akr1c14 gene

The direction of the arrow indicates whether the consensus sequence is on the positive or complementary strand. The diagram was adopted from Lin and Penning, 1995 [26] with some modification.

Supplemental Fig. 2  Expression of Akr1c14 in cultured preovulatory follicles of rat ovary

The preovulatory follicles isolated from eCG-primed ovary for 48 h were cultured for 6 h in serum-free conditions under 95% O2-5% CO2 at 37°C in the absence (control; C) or presence of LH (200 ng/mL), forskolin (Fsk; 10 µM), or TPA (200 nM). Total RNA (20 µg) was assayed for Akr1c14 levels by Northern blotting using a rat Akr1c14 cDNA probe. The size of Akr1c14 transcript (2.7 kb) is indicated.

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