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Retinoic acid enhances progesterone production via the cAMP/PKA signaling pathway in immature rat granulosa cells

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ABSTRACT

Retinoic acid (RA) is a metabolite of vitamin A and has important roles in development, differentiation, and reproduction. Activin has been shown to regulate the RA pathway and affect granulosa cell (GC) proliferation, suggesting that RA is important for early follicle development. However, little is known about the effects of RA on GC functions, particularly steroidogenesis, during the early follicle stage. The aim of this study was to investigate the effects of all-*trans*-RA (atRA) on progesterone production in immature rat GCs cultured without gonadotropin. Our results demonstrated that atRA enhanced progesterone production by upregulating the levels of steroidogenic acute regulatory protein (*StAR*) and cytochrome P450scc (*Cyp11a1*) mRNAs, but not 3β-hydroxysteroid dehydrogenase mRNA in immature rat GCs. Additionally, analysis of the mechanisms through which atRA upregulated *StAR* and *Cyp11a1* mRNAs revealed that atRA enhanced intracellular cAMP accumulation and phosphorylation of cAMP response-element binding protein (CREB). In addition, H-89, an inhibitor of protein kinase A (PKA), abolished the stimulatory effects of atRA, indicating that atRA enhanced progesterone synthesis through cAMP/PKA signaling. In conclusion, our data demonstrated that atRA has a crucial role in progesterone synthesis in rat GCs during the early follicle stage.

1. Introduction

Ovarian functions are regulated by many factors, including gonadotropin, transforming growth factor β (TGFβ), various cytokines, and retinoic acid (RA). RA is the active metabolite of vitamin A and is synthesized from retinol by retinol dehydrogenase (RDH) and retinaldehyde dehydrogenase (RALDH) [1]. RA exerts its activity by acting as a ligand for RA receptors (RARs) and retinoid X receptors (RXRs). RA mediates many physiological functions, including embryogenesis [2] and reproduction. In female rats, severe vitamin A deficiency prior to mating leads to reproductive failure prior to implantation [3]. Maternal vitamin A also plays a role in placental development and maintenance [4]. In addition, several studies have indicated that retinoids have important effects on oocyte maturation and function [5,6].

Previous studies have reported that RA enhances steroid production in several different cell types. For example, in MA-10 mouse Leydig cells, RA increases progesterone production by upregulation of steroidogenic acute regulatory protein (StAR) expression [7], and in rat hippocampal slice cultures, RA increases 17β-estradiol and testosterone levels through upregulation of cytochrome $P450_{17\alpha}$ expression [8]. In the ovary, Bagavandoss et al. reported that retinoids increase luteinized granulosa cell (GC) progesterone accumulation in gonadotropin-primed rat ovaries [9]. In a recent study, Kipp et al. reported that activin regulates the RA pathway to modulate GC proliferation and ovarian functions [10]. Because activin plays a key role in early follicle development [11], it has been hypothesized that RA may affect GC function, including steroidogenesis, during the early follicle stage. However, the effects of RA on steroidogenesis in GCs during the early follicle stage are still unclear.

In the present study, we investigated the effects of all-*trans*-RA (atRA) on progesterone production in immature rat GCs cultured without gonadotropin. Our results indicated that atRA enhanced progesterone synthesis through cAMP/protein kinase A (PKA)/cAMP response element-binding protein (CREB) signaling in GCs during the early follicle stage. Thus, these results provide important insights into the mechanisms of RA signaling in GCs.

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2. Materials and methods

2.1. Hormones and reagents

DMEM/Ham's nutrient mixture F-12, diethylstilbestrol (DES), atRA, and H-89 dihydrochloride hydrate were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Gentamicin sulfate and fungizone were purchased from Invitrogen Corp. (Carlsbad, CA, USA). The RNA labeling kit and nucleic acid detection kit were purchased from Roche Diagnostics (Indianapolis, IN, USA).

2.2. Animals

Immature female Wistar rats (Japan SLC, Inc.) were maintained at all times according to the NIH *Guide for the Care and Use of Laboratory Animals* and the policies of the Gunma University Animal Care and Use Committee. Animals were housed in a temperature- and light-controlled room (12-h light, 12-h dark cycle; lights on at 6:00 AM) with food and water provided ad libitum. All experiments were approved by the Gunma University Animal Care and Use Committee.

2.3. GC culture

GCs were obtained from immature female Wistar rats injected daily for 4 days with 2 mg DES in 0.2 mL of sesame oil. The ovaries were then excised, and the GCs were released by puncturing the follicles with 26-gauge needles. GCs were washed and collected by brief centrifugation, and cell viability was determined by trypan blue exclusion. The GCs were then cultured in DMEM/Ham's nutrient mixture F-12 supplemented with 20 mg/L gentamicin sulfate, 500 μg/L fungizone, and 1 g/L BSA on collagen-coated plates in a humidified atmosphere under 5% CO_2 at 37 °C.

2.4. RNA isolation and reverse transcription

GCs were cultured in 35-mm dishes containing 2.5×10^6 viable cells in 2.5 mL of medium, and the test substances were added to the medium after 24 h of culture. GCs were further incubated, and the cultures were stopped at selected times using Isogen (Nippon Gene, Toyama, Japan). The final RNA pellet was dissolved in diethylpyrocarbonate-treated H_2O. Total RNA was quantified by measuring the absorbance of samples at 260 nm. Isolated RNAs (2 μg of each sample) from the GC cultures were treated with deoxyribonuclease I (Invitrogen) to eliminate residual genomic DNA. These RNAs were reverse transcribed with random primers, 10 mM deoxynucleoside triphosphate mix, and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. The reactions were incubated for 5 min at 25 °C, 60 min at 50 °C, and 15 min at 70 °C in a thermal cycler. To remove cRNA, ribonuclease H was added to the cDNAs and incubated for 20 min at 37 °C.

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

To amplify rat Rar, Rxr, Raldh, and ribosomal protein L19 (Rpl19) cDNAs, RT-PCR was employed according to the manufacturer's instructions using an Advantage2 PCR Kit (TaKaRa Bio, Inc., Shiga, Japan). The PCR protocol was as follows: 95 °C for 1 min, 33 cycles of 95 °C for 30 s and 68 °C for 1 min, and a final extension at 68 °C for 1 min. The primer pairs use in this study are shown in Table 1. Amplification products were resolved on 2% agarose gels and stained with 0.25 μg/mL ethidium bromide. The gel image was photographed, and band intensities were analyzed using NIH ImageJ (version 1.60). The bands were excised from the gel, purified, and characterized by DNA sequencing.

Table 1
RT-PCR primer pairs.

Gene	Primer
Rarα	
Forward	5′-GTGTCACCGGGACAAGAACT-3′
Reverse	5′-GGGCTTGGGTGTTTCTTTCT-3′
Rarβ	
Forward	5′-GCTTCGGTCCTCTGACTGAC-3′
Reverse	5′-GGCGGTCTCCACAGATTAAG-3′
Rarγ	
Forward	5′-CAGCATCCAGAAAAACATGG-3′
Reverse	5′-TTCCGGTCATTCCTTACAGC-3′
Rxrα	
Forward	5′-GTCAAGCAGCAGACAAGCAG-3′
Reverse	5′-GAGAAGGAGGCAATCAGCAG-3′
Rxrβ	
Forward	5′-AGACTGCACAGTGGACAAGC-3′
Reverse	5′-GTTGTCGCTCCTCCTGTACC-3′
Rxrγ	
Forward	5′-TTGCTGATTGCCTCCTTCTC-3′
Reverse	5′-CTTGGACACCAGCTCTGTGA-3′
Raldh1	
Forward	5′-GGGCCATCACTGTGTCTTCT-3′
Reverse	5′-CATCTTGAATCCACCGAAGG-3′
Raldh2	
Forward	5′-TGAGTTTGGCTTACGGGAGT-3′
Reverse	5′-AAGGAGGCCTGGTGATAGGT-3′
Raldh3	
Forward	5′-ATCAACAATGACTGGCACGA-3′
Reverse	5′-CTTGTCCACATCGGGCTTAT-3′
Rpl19	
Forward	5′-AGCCTGTGACTGTCCATTCC-3′
Reverse	5′-GGCAGTACCCTTCCTCTTCC-3′

2.6. Quantitative RT-PCR

Quantitative RT-PCR was performed using the EagleTaq Universal Master Mix with ROX (Roche Diagnostics) and an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) according to manufacturers' instructions. The quantitative PCR conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The primers and probes for each gene were purchased from Applied Biosystems (steroidogenic acute regulatory protein: Rn00580695_m1; cytochrome P450, family 11, subfamily a, polypeptide 1: Rn00568733_m1; 3 beta-hydroxysteroid dehydrogenase/delta-5-delta-4 isomerase type II: Rn01789220_m1; and eukaryotic *18S* rRNA: Hs99999901_s1 as an internal control). Relative quantification of mRNA was carried out using the comparative threshold cycle (CT) method.

2.7. Intracellular cAMP assay

Intracellular accumulation of cAMP was measured using a Cyclic AMP EIA Kit (Cayman Chemical Co., Ann Arbor, MI, USA). GCs were cultured in 24-well culture plates containing 5×10^5 viable cells per well in 0.5 mL of medium. After 24 h, the cells were incubated for 30 min in fresh medium in the presence of 0.5 mM 3-isobutyl-1-methylxanthine. Various concentrations of RA were then added to the wells, and the cells were incubated for an additional 30 min. After incubation, the cells were lysed, and intracellular cAMP levels were measured according to the manufacturer's instructions.

2.8. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

GCs were cultured in 35-mm dishes containing 2.5×10^6 viable