Inhibitory effect of retinoic acid on the development of immature porcine granulosa cells to mature cells

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ABSTRACT

The present study investigated the effect of retinoic acid (RA) on the differentiation of granulosa cells prepared from porcine ovaries. The granulosa cells were precultured for 15 h, then cultured for 48 h with FSH and further treated for 24 h with LH in order to induce their transformation into luteal cells. After the cells had been exposed to 1 μ M retinoids (RA, retinal and retinol) for 87 h, analysis of the LH receptor mRNA expression, an indicator of granulosa cell differentiation, was carried out by using semiquantitative RT-PCR. The results showed that there was a decrease in LH receptor mRNA levels, and that RA had a more potent effect on these levels than the other two retinoids. When cells were exposed to RA in the immature stage (before the addition of FSH) or the early stage of development (0–24 h after the addition of FSH), expression of LH receptor mRNA was greatly

diminished. When the immature cells were cultured for 15 h with RA, then washed and cultured for 48 h with FSH and for 24 h with LH, the expression of LH receptor mRNA was not reversed. In the differentiated cells (24 h after the addition of FSH), however, RA no longer had any inhibitory effect. When the immature cells were exposed to RA, FSH-induced expression of c-fos mRNA was markedly decreased. In contrast, expression of c-jun and activating transcription factor-4 mRNAs remained constant. However, the expression of c-fos mRNA was not decreased by forskolin. The results indicate that RA is a potent inhibitor in the immature stage of porcine granulosa cell differentiation, probably through decreased expression of FSH receptor, but that RA does not inhibit differentiation in the mature stage of the cells.

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INTRODUCTION

Retinoic acid (RA) regulates cellular functions by binding to intracellular RA receptor (RAR) or retinoid X receptor (RXR). These two retinoid receptor families act via formation of either RAR–RXR heterodimer or RXR–RXR homodimer, both of which regulate the expression of RA target genes (Mangelsdorf & Evans 1995). RA has been shown to be essential for the growth and differentiation of many types of cells. Numerous studies have reported that RA induces terminal differentiation of many cell types, including P19 embryonal carcinoma (Paterno *et al.* 1997), F9 murine teratocarcinoma cells (Faria *et al.* 1998), and HL-60 human myeloblastic leukemia cells (Yen *et al.* 1999). However, the nature of the proliferation and differentiation responses elicited by RA are depen-

dent on the cell type. Thus, the differentiation of 3T3-L1 preadipocytes is inhibited in an early stage by RA (Chawla & Lazar 1994), whereas that in Ob17 and rat preadipocytes is strongly enhanced by RA (Safonova *et al.* 1994). In rat ovarian granulosa cells, retinoids have been reported to exert a biphasic effect on luteinizing hormone (LH) receptor induction, as revealed by a ligand-binding assay (Bagavandoss & Midgley 1988), suggesting that the response of granulosa cells to RA depends upon the stage of differentiation.

The expression of several proto-oncogenes has been associated with the proliferation and differentiation phases in many cell types. Transient or constitutive expression of such transcription factors as proto-oncogenes (c-fos, c-jun, c-myc) and the cAMP response element-binding protein (CREB) or activating transcription factor-4 (ATF-4) family

FIGURE 1. RT-PCR analysis of LH receptor mRNA expression in cultured granulosa cells. Cells were incubated for 15 h, cultured for 48 h with FSH (10 ng/ml), and stimulated for 24 h with LH (100 ng/ml); thereafter, RNA was extracted and reverse transcribed into cDNA, and samples were subjected to PCR amplification with primers for porcine LH receptor cDNA. The products were not detected in the RNA samples without RT reaction (no RT). M, DNA mol wt marker.

is promoted by protein kinase C and/or protein kinase A (Doucet *et al.* 1990, Sheng & Greenberg 1990). Activation of the RA nuclear receptor could act as a molecular switch for a cascade of sequential changes in gene expression involving several nuclear factors (Griep & DeLuca 1986, Thiele *et al.* 1988, Oberg & Carpenter 1989, Schule *et al.* 1991). The major actions of such transcription factors are related to the transcription of several late gene responses that are regulators of cell differentiation.

Differentiation of ovarian granulosa cells involves follicle-stimulating hormone (FSH)-stimulated transformation of immature into mature cells. A prominent biochemical event in this differentiation is the expression of LH receptor by the cAMP signaling pathway (Hsueh *et al.* 1989). In a study using rat granulosa cells, RA inhibited LH receptor induction in a dose-dependent manner, and this inhibition was associated with a decrease in cAMP and progesterone levels (Bagavandoss & Midgley 1988). Despite these studies, however, the effects of RA on granulosa cell differentiation have not been adequately characterized. In the present study, we used semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) to determine whether RA exposure would induce any changes in

FIGURE 2. Inhibition of LH receptor mRNA levels by retinoid derivatives. Cells were incubated for 15 h in the presence or absence of 1 μ M retinoids, cultured for 48 h with FSH (10 ng/ml) in the presence or absence of retinoids, and stimulated for 24 h with LH (100 ng/ml). Then, RNA was extracted and reverse transcribed into cDNA, and samples were subjected to PCR amplification with primers for porcine LH receptor cDNA. Similar results were obtained in two different mRNA preparations. M, DNA mol wt marker.

the expression of LH receptor mRNA and the transcription factors c-fos, c-jun and ATF-4 in association with the differentiation of porcine granulosa cells.

MATERIALS AND METHODS

Materials

Ovine FSH (NIDDK-oFSH-20) and LH (NIDDK-oLH-26) were supplied by Dr A F Parlow (Harbor-UCLA Medical Center, Torrance, CA, USA). All trans-retinoids (RA, retinal and retinol) were from Sigma Chemicals (St Louis, MO, USA); Ham's F-10 and Dulbecco's minimum essential medium (DMEM) were from Gibco Laboratories (Grand Island, NY, USA); ISOGEN was from Wako (Osaka, Japan); the T-primed first-strand kit was from Amersham Pharmacia Biotech (Tokyo, Japan); and AmpliTaq Gold polymerase was from Perkin-Elmer (Norwalk, CT, USA). All the other chemicals used were of reagent grade and obtained from commercial sources.

Cell culture

Porcine ovaries were obtained at a local slaughterhouse. Granulosa cells were prepared from medium-sized (1–4 mm diameter) follicles by aspirating with a 20 gauge needle and then filtering through a fine stainless steel mesh. They were treated with 50 µg DNase I/ml at 37 °C for 5 min, and washed with Ham's F-10 and DMEM (1:1) supplemented with 10 mM HEPES, 50 µg gentamycin/ml, and 20 U nystatin/ml (Mondschein *et al.* 1990). Cell viability was determined to be >95% by trypan blue exclusion. Cells were seeded at 2×10^5 /well in 48-well plates coated with fibronectin (Falcon; Becton-Dickinson, Oxnard, CA, USA) with 100 nM androstenedione, 110 nM hydrocortisone, 1 µg insulin/ml, 5 µg transferrin/ml and 0.1% (w/v) bovine serum albumin at 39 °C in a humidified atmosphere of 95% air and 5% CO₂.

Hormonal treatment of cultured granulosa cells

After culturing the granulosa cells for 15 h with various concentrations (10, 100 and 1000 nM) of retinoids, 10 ng FSH/ml was added to the cultures in the presence of retinoids (culture 0 h). The cells were matured by culturing for an additional 48 h, and then they were washed with culture medium

FIGURE 3. Dose-dependent inhibition of LH receptor (LH-R) mRNA levels by RA. Cells were incubated for 15 h in the presence or absence of RA (0.01, 0.1 and 1 µM), cultured for 48 h with FSH (10 ng/ml) in the presence or absence of RA, and stimulated for 24 h with LH (100 ng/ml). Then, RNA was extracted and reverse transcribed into cDNA, and samples were subjected to PCR amplification with primers for porcine LH receptor cDNA. Similar results were obtained in two different mRNA preparations. The data are normalized for GAPDH RT-PCR product levels in each sample and presented as the means $\pm$ s.e. M, DNA mol wt marker.

and exposed to 100 ng LH/ml. In some experiments, RA was added to the culture at the indicated times.

RNA isolation and cDNA preparation

After removal of the media, 0.5 ml ISOGEN was added to each well to dissolve the cells. Each resulting solution was transferred to a microtube, extracted with 0.1 ml chloroform and centrifuged at 12 000 g for 15 min. The aqueous phase was collected and precipitated with 0.2 ml isopropyl-alcohol at room temperature, and the precipitate was washed with 75% (v/v) ethanol. After drying, the recovered RNA was dissolved in 20 µl diethylpyrocarbonate-treated water. Reverse transcription was performed using a T-primed first-strand kit as follows. Samples of 10 µl RNA mixed with the reagent were incubated at 45 °C for 1 h, diethylpyrocarbonate-treated water was added to each tube (final volume, 50 µl) and the mixture was heated at 70 °C for 5 min.

FIGURE 4. Effect of RA administration at different times on LH receptor mRNA levels. Cells were incubated for 15 h, cultured for 48 h with FSH (10 ng/ml), and stimulated for 24 h with LH (100 ng/ml). RA (final concentration: 1 μ M) was added to cultures at different times (–15, 0, 24 and 48 h after the addition of FSH). RNA was then extracted and reverse transcribed into cDNA, and samples were subjected to PCR amplification with primers for porcine LH receptor cDNA. The results were obtained from two independent RT-PCR experiments performed for two different mRNA preparations. M, DNA mol wt marker.

Analysis of granulosa cells for mRNA of LH receptor and transcription factors using RT-PCR

After an initial denaturation step (95 °C for 10 min), amplification of each cDNA (1–3 μ l RT template) was performed in 10 μ l 1 $\times$ PCR buffer, 0.2 mM dNTPs, 0.25 U AmpliTaq Gold polymerase, and 0.2 μ M each of the synthetic primers. A pair of primers were selected for detecting the mRNAs of porcine LH receptor isoforms (Loosfelt *et al.* 1989). The sense primer was a 25 mer oligonucleotide corresponding to positions 861–885 (5'-CCAATCTCCTAGATGCCACATTGAC-3') on the porcine LH receptor mRNA. The antisense primer was a 23 mer oligonucleotide corresponding to positions 1981–1959 (5'-GCTCAGCAACAGAAAGAAATCCC-3'). The predicted sizes of LH receptor RT-PCR products were 1114 bp, 855 bp, 411 bp and 185 bp. However, two large sizes (1114 bp, 855 bp) were not detected. The amplification step for porcine LH receptor consisted of 30–34 cycles under a thermal profile of 95 °C for 30 s (denaturation), 54 °C for 30 s (annealing), and 72 °C for 2 min (synthesis). The RT-PCR

products of 411 bp and 185 bp linearly increased from 30 to 35 cycles under this condition (data not shown).

Amplification of porcine c-fos, c-jun and ATF-4 consisted of 34 cycles under the same thermal schedule using the specific primer sets of c-fos (sense, 5'-CCGAAGGGAAAGGAATAAGATGG-3'; antisense, 5'-TCGGTGTCTGTAGAGAA GACC-3') (Chung *et al.* 1996), c-jun (sense, 5'-CCGAAGGGAAAGGAATAAGATGG-3'; antisense, 5'-ACGTTGACGACGCAATCGTAC-3') (Chung *et al.* 1996), and ATF-4 (sense, 5'-GCAGAGCAGGAGGCCCTCACTG-3'; antisense, 5'-ACGTTGACGACGCAATCGTAC-3') (Kato *et al.* 1999). The predicted sizes of c-fos, c-jun and ATF-4 PCR products were 232 bp, 179 bp and 216 bp respectively. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal amplification control (sense, 5'-TCCTGT CCTGCACCACCAACTGCTTAG-3'; antisense, 5'-GTAGGTGTCAGAAGACTCACCGTC-3') to generate a PCR product of 118 bp. The amplified products were resolved by 2% agarose gel electrophoresis, stained with ethidium bromide, and photographed under u.v. light. The intensities of

FIGURE 5. Maximum induction of RA inhibition in the immature granulosa cells. Cells were incubated for 15 h in the presence or absence of 1 μ M RA, then cells were washed twice and cultured for 48 h with FSH (10 ng/ml) in the presence or absence of 1 μ M RA, then stimulated for 24 h with LH (100 ng/ml). RNA was extracted and reverse transcribed into cDNA, and samples were subjected to PCR amplification with primers for porcine LH receptor cDNA. The results were obtained from three independent RT-PCR experiments performed for three different mRNA preparations.

the bands were quantified as a reference gene of GAPDH using a densitometry program (NIH Image Version 1.58).

RESULTS

Expression of LH receptor mRNA during luteinization

The induction of LH receptor mRNA was determined as an indicator of differentiation and luteinization. After culturing the granulosa cells for 15 h, FSH was added (0 h) and the cells were cultured for an additional 48 h. LH was then added and, after culturing for a final 24 h, total RNA was isolated. Although four LH receptor isoforms are expressed in the porcine ovary (Loosfelt *et al.* 1989), only two receptor isoforms were detected in the primer sets, one with a product size of 411 bp and one of 185 bp (Fig. 1). These isoforms were not amplified in the RNA sample without the RT reaction. Thus, LH receptor mRNA was expressed during luteinization induced by LH.

Retinoid suppression of LH receptor mRNA expression

We next examined the effect of retinoids on the expression of porcine LH receptor mRNA during luteinization of the granulosa cells. The granulosa cells were cultured for 15 h in the presence of 1 μ M RA, retinal or retinol, and then FSH was added to the cultures. After treatment with FSH for 48 h, the cells were washed and exposed to LH in the presence or absence of retinoids. A maximal level of LH receptor PCR products was observed 24 h after LH stimulation, and the level decreased thereafter. RA decreased the expression of LH receptor mRNA (Fig. 2). The precursors of RA, retinal and retinol also inhibited receptor expression. RA decreased receptor mRNA expression in a dose-dependent manner, showing a maximum effect at 1 μ M (Fig. 3).

Maximum inhibitory effect of RA on the immature cells

In order to further examine the inhibitory action of RA, it was added to cultures at various times

(–15, 0, 24 or 48 h), and FSH was added at 0 h. After washing the cells at 48 h, the cells were treated for 24 h with LH in the presence or absence of RA. The inhibitory action of RA was clearly observed at –15 h and 0 h, whereas it was not observed at 24 h and 48 h (Fig. 4). Thus, the inhibitory effect of RA may be dependent upon the progress of differentiation.

Effect of RA on immature cells

To test whether the inhibitory action of RA is more effective in immature than in mature cells, granulosa cells were exposed to RA only before FSH stimulation. The cells were cultured for 15 h in the presence of RA, then washed twice and cultured for 48 h with FSH and for 24 h with LH. As shown in Fig. 5, expression of LH receptor mRNA was suppressed by the initial 15-h exposure to RA, and was not further suppressed thereafter. Thus, the immature granulosa cells were much more sensitive to the inhibitory action of RA than were the mature cells.

Suppression of FSH-stimulated expression of c-fos mRNA in RA-treated cells

After treatment of the granulosa cells with RA, FSH-induced expression of the c-fos, c-jun and ATF-4 mRNAs was tested. A maximal expression of c-fos was observed 30 min after exposure to FSH, whereas c-jun and ATF-4 were constitutively expressed (data not shown). Figure 6 shows the effect of RA on expression of the c-fos, c-jun and ATF-4 mRNAs. When the cells were exposed to RA for 15 h, FSH-induced expression of c-fos mRNA was markedly decreased. In contrast, expression of the c-jun and ATF-4 mRNAs remained constant.

Forskolin-induced expression of c-fos mRNA in RA-treated cells

After treatment of the granulosa cells with RA for 15 h, expression of c-fos mRNA was tested by 10 μ M forskolin. In contrast to FSH stimulation, forskolin induced the expression of c-fos mRNA (Fig. 7).

DISCUSSION

In the present study, RA has been shown to inhibit the differentiation of granulosa cells prepared from porcine ovaries. RA inhibition has previously been reported to occur in granulosa cells in association

FIGURE 6. Effect of RA pretreatment on FSH-induced levels of transcription factor c-fos, c-jun and ATF-4 mRNAs. Cells were incubated for 15 h in the presence or absence of 1 μ M RA, then cells were washed twice and treated for 30 min with FSH (10 ng/ml); thereafter, RNA was extracted and reverse transcribed into cDNA, and samples were subjected to PCR amplification with primers for porcine c-fos, c-jun and ATF-4. The results were obtained from three independent RT-PCR experiments performed for three different mRNA preparations. M, DNA mol wt marker.

with FSH-induced LH/human chorionic gonadotropin receptor induction as well as cAMP and progesterone accumulation (Bagavandoss & Midgley 1988). In this work, RA clearly suppressed the expression of LH receptor mRNA in granulosa cells matured by FSH, as revealed by RT-PCR analysis. To ascertain the granulosa cell differentiation stage at which the inhibition of RA was maximal, RA was added at different time-points during culturing. The inhibitory effect of RA on receptor mRNA expression was time-dependent and much more pronounced in immature than in mature cells. After immature cells were exposed to RA for an initial

FIGURE 7. Effect of RA pretreatment on forskolin-induced levels of transcription factor c-fos, c-jun and ATF-4 mRNAs. Cells were incubated for 15 h in the presence or absence of 1 μ M RA, then cells were washed twice and treated for 30 min with 5 μ M forskolin; thereafter, RNA was extracted and reverse transcribed into cDNA, and samples were subjected to PCR amplification with primers for porcine c-fos, c-jun and ATF-4. The results were obtained from three independent RT-PCR experiments performed for three different mRNA preparations. M, DNA mol wt marker.

15 h, washed twice, cultured for 48 h with FSH, and stimulated with LH for 24 h, their expression of LH receptor mRNA was markedly decreased. These data indicate that RA acts as an inhibitory factor in the immature stage of granulosa cells, and that FSH cannot initiate the differentiation of RA-treated cells. After the initiation of the differentiation signal by FSH, however, RA was less effective. These results indicate that RA blocks the development of immature, but not mature, granulosa cells.

RA is known to cause G0 arrest in S91 melanoma cells (Spanjaard *et al.* 1997), F9 murine teratocarcinoma cells (Faria *et al.* 1998), and HL-60 human myeloblastic leukemia cells (Yen *et al.* 1999). In contrast, RA causes cell differentiation in PCC7-Mz1 embryonic carcinoma cells (Herget *et al.* 1998), P19 mouse embryonic carcinoma cells (Jho *et al.* 1997) and porcine preadipocytes (Suryawan & Hu 1997). Thus, the differentiation response elicited by RA may depend on cell type. Further, RA alone cannot induce the differentiation of rat granulosa cells (Bagavandoss & Midgley 1988). Retinoids have been shown to inhibit FSH-stimulated cAMP formation and aromatase activation in cultured rat Sertoli cells (Galdieri &

Nistico 1994) and to inhibit expression of FSH receptor mRNA in cultured rat granulosa cells (Minegishi *et al.* 1996). The action of FSH is exerted through the FSH receptor, resulting in development of both Sertoli and granulosa cells. Expression of FSH receptor mRNA is maximally induced after 24-h incubation with FSH (Minegishi *et al.* 1996). In the present study, simultaneous addition of RA and FSH caused a decrease in the LH receptor mRNA level, although this decreased level was still higher than the level of LH receptor mRNA after RA pretreatment for 15 h. After 24-h incubation with FSH, the addition of RA did not decrease LH receptor mRNA level, showing that RA is effective until 24 h after FSH stimulation. Taken together, these results indicate that the response of granulosa cells to RA may depend on the stage of differentiation. Thus, cells may be RA-sensitive over a period beginning with the immature stage or onset of differentiation and ending 24 h later.

Protein kinase A is involved in the regulation of c-fos transcription through a well-characterized mechanism of phosphorylation of the dimeric transcription factor, CREB or ATF-4 (Meyer & Habener 1993). RA has been reported to act as a

negative regulator of activator protein (AP)-1-responsive genes (Schule *et al.* 1991). In addition, the primary response gene products encode proteins that form hetero- or homodimeric AP-1 factors, i.e. jun/fos or jun/jun, which are involved in activation or repression of several late response genes, depending on their composition. Therefore, expression of these transcription factor mRNAs was determined by using RT-PCR. RA significantly decreased the expression of c-fos mRNA induced by FSH stimulation, whereas c-jun and ATF-4 were not influenced by RA. Alteration of c-fos mRNA expression may result in the decreased level of LH receptor mRNA, because the major actions of these transcription factor gene products are related to the transcription of several late gene responses that are associated with cell proliferation or differentiation. However, there has been no report of the presence of an AP-1 site in the promoter domain of the LH receptor gene, suggesting that AP-1 factors do not bind directly to the LH receptor gene. The finding that c-fos mRNA expression was inhibited by RA may indicate that the FSH receptor was not activated. Actually, forskolin could increase c-fos mRNA level in the RA-treated cells. This result suggests that the signaling pathway from the action of cAMP to expression of c-fos mRNA is at least normally maintained in the RA-treated cells.

In conclusion, RA had a profound effect on the differentiation of granulosa cells in the present study, provided that cells were exposed to RA in the immature rather than in the mature stage, through inhibition of FSH-induced expression of c-fos and/or expression of FSH receptor. It seems likely that RA does not directly inhibit the expression of c-fos mRNA, at least in granulosa cells.

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