Inverse Relationship Between the Expression of Messenger Ribonucleic Acid for Peroxisome Proliferator-Activated Receptor γ and P450 Side Chain Cleavage in the Rat Ovary

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ABSTRACT

Messenger RNA for peroxisome proliferator-activated receptor γ (PPARγ) has been found in granulosa cells, and its expression decreases after the LH surge. We determined which developmental stage of ovarian follicles expresses mRNA for PPARγ and evaluated the impact of PPARγ agonists on steroidogenesis. Ovaries were collected from immature eCG/hCG-treated rats at 0 (no eCG), 24, and 48 h post-eCG and 4 and 24 h post-hCG. Ovarian tissue was serially sectioned and processed for in situ hybridization to localize mRNA corresponding to PPARγ, aromatase, and the LH receptor, and P450 side chain cleavage (P450scC) and to determine whether apoptotic cells were present. During follicular development, there was no correlation between the expression of mRNAs for PPARγ and aromatase or the presence of apoptotic cells, but a general inverse correlation was observed between the expression of PPARγ mRNA and LH receptor mRNA. At 4 h post-hCG, follicles expressing P450scC mRNA had lost expression of PPARγ mRNA. The inverse pattern of expression between PPARγ and P450scC mRNAs was also observed 24 h post-hCG, with developing luteal tissue expressing high levels of P450scC mRNA but little or no PPARγ mRNA. To determine the impact of PPARγ on steroidogenesis, granulosa cells were collected from ovaries 24 h post-eCG and cultured alone, with FSH alone, or with FSH in combination with the PPARγ agonists ciglitazone or 15-deoxy-D12,14-prostaglandin J2 (PGJ2). Treatment of granulosa cells with PGJ2 stimulated basal progesterone secretion, whereas ciglitazone or PGJ2 had no significant effect on FSH-stimulated steroid production. These findings suggest that 1) PPARγ may regulate genes involved with follicular differentiation and 2) the decline in PPARγ expression in response to LH is important for ovulation and/or luteinization.

INTRODUCTION

The ovarian follicle is comprised of two steroidogenic cell types, granulosa cells and theca cells. These cells work cooperatively during the follicular phase to produce estradiol. The synthesis of estradiol involves androgens produced by the theca diffusing across the basement membrane into the granulosa cell compartment, where they are converted into estrogen by aromatase. In response to the LH surge, granulosa and theca cells differentiate into large and small cells of the corpus luteum, respectively. Accompanying this cellular differentiation is a shift in steroid production from estradiol to progesterone. Although there is a vast amount of information about the changes that occur during the final maturation of follicular cells and the periovulatory shift in steroid production, the mechanisms regulating these changes are not completely understood.

The peroxisome proliferator-activated receptors (PPARs) are a family of nuclear hormone receptors that play a role in cellular differentiation and function. Three distinct PPAR family members have been identified: PPARα, PPARδ, and PPARγ. These transcription factors regulate gene expression by forming heterodimers with the 9-cis-retinoic acid receptor and binding to a PPAR response element (PPRE) in the promoter region of target genes. The presence of a PPRE in the cyclooxygenase-2 gene, whose expression is regulated by PPARs [1–3], and reports that PPAR activators can influence the expression and activity of aromatase [4–8] suggest a potential role for this class of transcription factors in ovarian steroidogenesis and cellular differentiation. Support for the hypothesis that the PPARs impact ovarian function comes from experiments in which PPARs have been identified in ovarian tissue from humans [9], cattle [10, 11], pigs [12], rats [13–15], and mice [16]. Furthermore, activators of PPARγ can regulate steroid production by cultured granulosa [4, 14, 17], theca [12], and luteal [10] cells. Recently, Cui et al. [16] reported that one-third of mice lacking expression of PPARγ in granulosa cells were infertile and the remaining two-thirds had reduced fertility. These authors speculated that the reduction in fertility was the result of inadequate ovarian function [16]. These data provide strong support for the fundamental role of PPARs in ovarian physiology.

In a previous study, we demonstrated that the expression of mRNA for PPARγ was restricted primarily to granulosa cells of developing follicles in the rat and that expression declined in response to the LH surge [14]. Not all follicles expressed PPARγ at the same level nor did all follicles lose expression after the gonadotropin surge. These findings led us to question whether the differential pattern of expression of mRNA for PPARγ seen between follicles reflected the ability of granulosa cells to produce estradiol and whether the decline in the expression of mRNA for PPARγ after the LH surge is coupled with an increase in the production of progesterone. To begin answering these questions, the localization and expression of mRNA for PPARγ, P450 side chain cleavage (P450scC), aromatase, and the LH receptor and the presence of apoptotic cells were identified within follicles from serial sections of rat ovarian tissue.
collected during follicular development and the periovulatory period. The potential of PPARγ to influence gonadotropin-stimulated steroid production was also investigated using granulosa cells cultured in the presence of FSH with and without the PPARγ agonists 15-deoxy-Δ12,14-prostaglandin J2 (PGJ2) and ciglitazone [18, 19].

MATERIALS AND METHODS

Materials

Unless otherwise noted, all chemicals were purchased from Sigma Chemical Company (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). OCT embedding compound was obtained from VWR Scientific (Atlanta, GA). Ciglitazone was purchased from Biomol (Plymouth Meeting, PA), and PGJ2 from Cayman Chemical Co. (Ann Arbor, MI).

Animals

All animal procedures were approved by our Institutional Animal Care and Use Committee. Female Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were injected s.c. with 10 IU eCG (provided by Dr. A.F. Parlow, National Hormone & Peptide Program, NIDDK) on Day 23 of age (n = 15) to initiate follicular development. Ovaries were collected from a subset of these animals at 0 (no eCG), 24, and 48 h post-eCG (n = 3 rats/time point). The remaining animals received 10 IU of hCG 48 h post-eCG to stimulate ovulation and luteal development. Ovaries were collected from this group of animals 4 and 24 h post-hCG (n = 3 rats/time point). All ovaries were placed in OCT and stored at −70°C.

To investigate the role of PPARγ in gonadotropin-stimulated steroidogenesis, granulosa cells were collected from eCG-primed immature rats. On Day 23 of age, female rats received 10 IU eCG s.c.; 24 h later, granulosa cells were collected as described previously [20].

In Situ Hybridization

Ovarian tissues were serially sectioned at 10 μm and mounted on ProbeOn Plus slides (Fisher). Tissue sections were processed for in situ hybridization as described previously [14]. Plasmids containing cDNAs for P450SCC, aromatase (generously provided by Dr. J.S. Richards, Baylor College of Medicine, Houston, TX), the LH receptor (a gift from Dr. Dr. O.-K. Park-Sarge, University of Kentucky, Lexington, KY), and PPARγ (kindly provided by Dr. Walter Wahli, Université de Lausanne, Lausanne, Switzerland) were linearized using the appropriate restriction enzymes. Radiolabeled sense and antisense riboprobes were synthesized using a MAXIScript kit (Ambion, Austin, TX) and [α-35S]UTP (10 μCi/ml; ICN, Irvine, CA). Tissues were hybridized with radiolabeled probe (106 cpm) in 50 μl hybridization buffer at 50°C for 15–18 h. Slides were dipped in Kodak NTB2 emulsion and stored at −70°C. After 2 weeks, slides were developed, dipped in X-ray film for 1 week, dipped in enhancer, and then counterstained with hematoxylin.

Cell Culture

Granulosa cells were pooled, and 107 cells/ml were cultured in Dulbecco modified Eagle medium:Ham's F-12 medium containing 1% BSA, 0.01% pyruvic acid, 0.22% bicarbonate, and insulin/transferrin/selenite at 37°C in an atmosphere of 5% CO2/95% air. Cultured cells were treated in duplicate as follows (n = 5 independent experiments): control (dimethyl sulfoxide), FSH (50 ng/ml; provided by Dr. A.F. Parlow), ciglitazone (50 μM), PGJ2 (50 μM), FSH + ciglitazone, and FSH + PGJ2. Treatments were added to the cells at the time of plating. Twenty-four hours after the initiation of culture, media were collected to measure the concentrations of estradiol and progesterone by RIA.

Radioimmunoassay

Concentrations of estradiol and progesterone were determined in conditioned culture media using Coat-A-Count tubes (Diagnostic Products Co., Los Angeles, CA), which are direct solid-phase 125I RIA kits. Assay sensitivity was 50 pg/ml and 0.03 ng/ml for estradiol and progesterone, respectively. For measuring concentrations of each steroid, all samples were analyzed together in one assay.

Detection of Apoptotic Cells

Apoptotic cells were identified in serial sections of frozen ovarian tissue using ApoAlert (Clontech, Palo Alto, CA) as described previously [14].

Statistical Analysis

Concentrations of estradiol and progesterone in conditioned culture media were analyzed using a one-way ANOVA. Results were considered significant at P < 0.01. Post hoc comparisons were made using the Tukey HSD test.

RESULTS

Early in follicular development (0 h post-eCG), the majority of follicles contained granulosa cells expressing mRNA for PPARγ (Fig. 1a). As reported previously [14], some follicles contained granulosa cells that had relatively high levels of expression for PPARγ mRNA compared with other follicles of similar size. The granulosa cells in a few large antral follicles expressed mRNA for aromatase (Fig. 1c), but the expression of mRNA for the LH receptor was limited to the theca and stroma (Fig. 1b). At the early stages of follicular development, expression of mRNA for PPARγ was not correlated with localization of mRNA for aromatase. Similarly, there was no correlation observed between the expression of mRNA for PPARγ and the presence or absence of apoptotic cells (Fig. 1d).

By 24 h post-eCG, mRNA for the LH receptor was seen in the theca of most follicles and in the granulosa cells of maturing follicles (Fig. 1g). Granulosa cells in developing follicles still expressed mRNA for PPARγ (Fig. 1f). The granulosa cells in larger antral follicles that expressed high levels of mRNA for PPARγ tended to lack expression of mRNA for the LH receptor, whereas follicles containing granulosa cells with relatively low levels of PPARγ mRNA expressed high levels of LH receptor mRNA (Fig. 1, f and g, arrows). The expression of mRNA for aromatase also tended to be higher in granulosa cells expressing LH receptor mRNA relative to the expression in granulosa cells with little or no LH receptor mRNA (Fig. 1, h and g, arrows).

The same patterns of expression of mRNA for PPARγ, aromatase, and the LH receptor continued during the later phases of follicular development (48 h post-eCG, no hCG; Fig. 2, a–d). The expression of mRNA for P450SCC at this time was seen primarily in the theca and stroma (Fig. 2b). However, by 4 h post-hCG, there was a differential pattern of expression observed between P450SCC and PPARγ, with a loss of PPARγ mRNA in those follicles where granulosa cells expressed P450SCC mRNA (Figs. 2, f and g, and 3, a and c). The expression of P450SCC mRNA in granulosa cells correlated with the expression of LH receptor mRNA (Figs. 2, g and i, and 3, c and e). At this stage of the periovulatory period, the expression of mRNA for P450SCC and PPARγ appeared to be in transition. Granulosa cells in a follicle losing expression of mRNA for PPARγ began to express mRNA for P450SCC (Fig. 2, f and g, arrowhead). This transition in expression can also been seen with aromatase mRNA. In some large antral follicles that maintained expression of mRNA for aromatase 4 h post-hCG (including the follicle marked with an arrowhead in Fig. 2, f and g), expression was not uniform throughout the granulosa cell layers (Fig. 2h, arrowhead).

The differential pattern of expression between mRNA for SCC and PPARγ was also seen 24 h post-hCG. The forming luteal structures expressed high levels of mRNA for SCC whereas the expression of mRNA for PPARγ was low to undetectable (Fig. 2, k and l). One follicle with granulosa cells expressing mRNA for PPARγ only expressed mRNA for P450SCC in the theca (Fig. 2, k and l, arrows). The granulosa cells of this same follicle expressed mRNA for aromatase (Fig. 2m), indicating that it was a
healthy (Fig. 2o), growing follicle. As expected, the expression of mRNA for the LH receptor was low at this stage of luteal development (Fig. 2n).

At all time points examined during the periovulatory period, there was no correlation observed between the expression of mRNA for PPARγ and the presence or absence of apoptotic cells (Fig. 2). Additionally, there was no labeling observed at any time point investigated in tissue sections hybridized with sense riboprobes for PPARγ, aromatase, the LH receptor, or P450SCC (representative images from tissue collected 4 h post-hCG shown in Fig. 3).

To determine whether activators of PPARγ modified go-
FIG. 2. Localization of mRNAs for PPARγ (a, f, and k), P450SCC (b, g, and l), aromatase (c, h, and m), and the LH receptor (d, i, and n) and the detection of apoptotic cells (e, j, and o) in serial sections of ovarian tissue collected from rats at 0 h (48 h post-eCG, no hCG; a–e), 4 h (f–j), and 24 h (k–o) post-hCG. Ten-micrometer tissue sections were hybridized with 35S-labeled antisense riboprobes. Symbols identify a single follicle throughout the serial sections. Arrowheads indicate the transition in expression of mRNAs for PPARγ, P450SCC, and aromatase 4 h post-hCG. Arrows in f and g denote the inverse relationship between the expression of mRNA for PPARγ and P450SCC. Arrows in k, l, m, and o point to a healthy growing follicle, demonstrating the differential pattern of expression for PPARγ mRNA, P450SCC mRNA, and aromatase mRNA. ×40.

nadotropin-stimulated steroid production, granulosa cells were cultured with FSH and agonists of PPARγ, PGJ2 or ciglitazone. Cells treated with FSH secreted >2 times more estradiol (FSH: 3188 ± 593 pg/ml; control: 1417 ± 531 pg/ml; Fig. 4A) and 300 times more progesterone (FSH: 224 ± 45 ng/ml; control: 0.66 ± 0.24 ng/ml; P < 0.01; Fig. 4B) than control cultures. Treatment with PGJ2 stimulated basal progesterone production (P < 0.01; Fig. 4B) but not estradiol production. Neither PPARγ agonist significantly influenced FSH-stimulated steroidogenesis, although there was a trend toward inhibition of gonadotropin-stimulated steroid production by PGJ2 (P = 0.15 for estradiol, P = 0.14 for progesterone).

DISCUSSION

We have previously shown that mRNA for PPARγ is restricted primarily to granulosa cells of developing follicles and that expression decreases in response to the LH surge [14]. PPARγ was first identified as an adipocyte differentiation factor (reviewed in [21, 22]). It also plays a role in the cell cycle and apoptosis (reviewed in [18, 23, 24]) and in steroidogenesis [4, 10, 12, 14, 17]. Because these processes occur in granulosa cells during the course of follicular development and/or luteinization, the current studies were designed to begin investigation of the role(s) of PPARγ in the ovary by characterizing the follicles expressing mRNA for PPARγ.
The expression of mRNA for PPARγ in granulosa cells was compared with the expression of markers for follicular development, differentiation, and subsequent luteinization. During follicular development, mRNA for aromatase is restricted to granulosa cells and its expression increases as follicles differentiate into preovulatory follicles [25, 26]. Messenger RNA for the LH receptor is expressed in the theca from very early in follicular development, but its expression in granulosa cells does not occur until follicles grow from small antral to preovulatory in size [27–30]. In response to an LH surge, the expression of mRNAs for the LH receptor and aromatase is downregulated [27–30], whereas the expression of mRNA for P450SCC is stimulated as the follicle becomes luteinized [31–34]. Therefore, mRNAs for aromatase, the LH receptor, and P450SCC were used to identify developing follicles with granulosa cells that were in the later stages of development and capable of responding to an LH surge and those follicles that had responded to the LH surge and were in the early stages of luteinization.

The results reported here illustrate that the expression of mRNA for PPARγ is inversely related to the expression of mRNA for P450SCC. As follicles matured, those follicles containing granulosa cells expressing high levels of mRNA for the LH receptor tended to express lower relative levels of mRNA for PPARγ. These observations suggest that those follicles capable of responding to the LH surge have a reduction in expression of mRNA for PPARγ and that a decline in this transcription factor may be important for the processes of ovulation and luteinization. This conclusion is supported by the lack of expression of mRNA for PPARγ during early luteal development. However, in the naturally
cycling animal, mRNA for PPARγ is expressed in luteal tissue and at relatively high levels in luteal tissue remaining from previous cycles [35]. These data and the findings reported here indicate that PPARγ plays a role in luteal function, but it may be more involved in luteal maintenance and/or regression than in development.

The lack of correlation of mRNA expression for aromatase and PPARγ either before or after the LH surge suggests that PPARγ does not play a critical role in the expression of this P450 enzyme in the rat ovary. However, this finding does not rule out the ability of PPARγ to influence aromatase expression or subsequent activity. It has been shown that activators of PPARγ inhibited the expression and activity of aromatase in human granulosa-lutein cells [4], malignant granulosa cells [6], and breast adipose tissue [5, 7]. One mechanism by which activators of PPARγ inhibit aromatase in human breast adipose is via an indirect effect on promoter II of aromatase [7]. Ovarian expression of aromatase is under the regulation of promoter II [36]. Thus, the possibility exists that PPARγ may regulate the activity of aromatase by interactions with the promoter during the latter stages of follicular development.

In previous studies, activators of PPARγ influenced steroid production by cultured granulosa, luteal, and theca cells [10, 12, 14]. Because the expression of mRNA for PPARγ is high in granulosa cells during folliculogenesis, we investigated the ability of PPARγ activators to modify FSH-stimulated steroid production during the early stages of follicular development. As expected, cultured granulosa cells responded to FSH with increased steroid production. The addition of PGJ2 or ciglitazone to granulosa cells in culture had no effect on FSH-stimulated estradiol or progesterone secretion. However, treatment with PGJ2 significantly increased basal secretion of progesterone. Because ciglitazone did not stimulate basal progesterone production, the increase in progesterone secretion may be a direct effect of PGJ2 on granulosa cells independent of PPARγ activation. This conclusion is supported by the fact that progestogens can stimulate follicular steroidogenesis [37, 38]. Previously, we reported that activators of PPARγ stimulated the secretion of both progesterone and estradiol from cultured rat granulosa cells collected during the latter stages of follicular development [14]. Although the changes reported in the current study were not significant, the trends in estradiol and progesterone production paralleled the findings in the previous study. The difference in magnitude of response between the previous study and the current study is most likely the result of using granulosa cells at different stages of development. In the current study, granulosa cells were collected from developing follicles 24 h post-eCG, whereas in the previous study [14] cultured granulosa cells were collected from maturing follicles 48 h post-eCG. More follicles expressed mRNA for aromatase and the LH receptor at 48 h post-eCG than at 24 h post-eCG (Figs. 1 and 2). These changes are associated with a more advanced stage of follicular maturation. Because levels of mRNA for PPARγ were high at both time points, the influence of PPARγ activation on steroid production most likely reflects the cellular environment and how PPARγ interacts with cellular events particular to those stages of development. Thus, the response of rat granulosa cells to PPARγ agonists may be dependent upon the stage of follicular differentiation.

Ovarian follicles undergo atresia by a process of programmed cell death called apoptosis [39]. In the current study, exogenous gonadotropins were administered to immature rats to increase the number of developing follicles. In this model, the presence or absence of apoptotic cells in follicles was not correlated with high, low, or loss of expression of mRNA for PPARγ. Hence, although PPARγ is involved in apoptosis in other cell systems, its expression pattern observed in the current study indicates that it may not play a major role in follicular atresia in this animal model.

The findings reported here show that the expression of mRNA for PPARγ is inversely correlated with the expression of mRNA for P450SCC. Because the expression of PPARγ mRNA is high in the granulosa cells of developing follicles, PPARγ may be responsible for regulating the expression of genes involved with growth, development, and/or differentiation of the follicle. Studies are underway to further investigate the role of PPARγ in the ovary, which will allow us to better understand how this transcription factor influences ovarian function.

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REFERENCES


