Potential role for peroxisome proliferator-activated receptor γ in regulating luteal lifespan in the rat

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Abstract

Peroxisome proliferator-activated receptor γ (PPAR γ) has been shown to stimulate progesterone production by bovine luteal cells. We previously reported higher expression of PPAR γ in old compared with new luteal tissue in the rat. The following studies were conducted to determine the role of PPAR γ in rat corpora lutea (CL) and test the hypothesis that PPAR γ plays a role in the metabolism of progesterone and/or luteal lifespan. Ovaries were removed from naturally cycling rats throughout the estrous cycle, and pseudopregnant rats. mRNA for PPAR γ and P450 side-chain cleavage (SCC) was localized in luteal tissue by *in situ* hybridization, and protein corresponding to PPAR γ and macrophages identified by immunohistochemistry. Luteal tissue was cultured with agonists (ciglitazone, prostaglandin J₂) or an antagonist (GW-9662) of PPAR γ . Progesterone was measured in media by RIA and levels of mRNA for 20 α -hydroxysteroid dehydrogenase (HSD) and bcl-2 were measured in luteal tissue after culture by RT-PCR. An inverse relationship existed between the expression of mRNA for SCC and PPAR γ . There was no effect of PPAR γ agonists or the antagonist on luteal progesterone production *in vitro*, or levels of mRNA for 20 α -HSD. PPAR γ protein was localized to the nuclei of luteal cells and did not correspond with the presence of macrophages. In new CL, ciglitazone decreased mRNA for bcl-2 on proestrus, estrus, and metestrus. Interestingly, GW-9662 also decreased mRNA for bcl-2 on proestrus and diestrus in old and new CL, and on metestrus in new CL. These data indicate that PPAR γ is not a major player in luteal progesterone production or metabolism but may be involved in regulating luteal lifespan.

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Introduction

The peroxisome proliferator-activated receptors (PPARs) are transcription factors and members of the nuclear receptor superfamily. There are three isoforms of PPARs: PPAR α , PPAR β/δ , and PPAR γ . Ovarian tissue from several species, including humans (Lambe & Tugwood 1996), pigs (Schoppe *et al.* 2002), sheep (Froment *et al.* 2003), mice (Cui *et al.* 2002), rats (Braissant *et al.* 1996, Komar *et al.* 2001), and cattle (Sundvold *et al.* 1997, Lohrke *et al.* 1998), expresses PPAR γ . Although this transcription factor has been detected in the ovaries of several species, to date very little is known regarding the role(s) of PPAR γ in ovarian function.

Previous work from our laboratory, using the rat as a model, has shown that PPAR γ is expressed primarily in granulosa cells and is downregulated in response to the luteinizing hormone (LH) surge (Komar *et al.* 2001). PPAR γ was also identified in luteal tissue of the naturally cycling rat (Komar & Curry 2002). Interestingly, the expression of mRNA for PPAR γ was higher in luteal tissue from previous ovulations compared with luteal tissue forming from the most recent ovulation (Komar & Curry 2002).

PPAR γ is involved in processes that are critical to normal ovarian function such as angiogenesis (reviewed by Margeli *et al.* 2003), inflammation, and cell cycle control (reviewed by Berger & Moller 2002), indicating that PPAR γ may be an important player regulating ovarian gene expression. For example, cell death via apoptosis occurs in both ovarian follicles and corpora lutea (CL). In the ovary, the anti-apoptotic gene, bcl-2, has been found to play a role in cell survival. The gene encoding bcl-2 contains a peroxisome proliferator response element (Butts *et al.* 2004), and therefore could be directly regulated by PPAR γ .

Another possible role for PPAR γ in the ovary may be in the process of luteolysis. The expression of PPAR γ is induced during the differentiation of monocytes into macrophages, and it is expressed in activated macrophages (Ricote *et al.* 1998, Tontonoz *et al.* 1998, Cunard *et al.* 2002). Macrophages accumulate in regressing CL in the rat from late proestrus to early estrus (Gaytan *et al.* 1998). These macrophages are thought to aid in the removal of luteal tissue by phagocytosis (reviewed by Niswender *et al.* 2000). Therefore, PPAR γ may play a

role in luteolysis via its presence in activated macrophages. Alternatively, PPAR γ may be involved in luteal steroid production. In cattle, activation of PPAR γ has been reported to stimulate progesterone production by the CL (Lohrke *et al.* 1998). Luteal tissue collected during the mid-phase of the bovine estrous cycle and cultured with an agonist of PPAR γ , prostaglandin J₂ (PGJ₂), secreted more progesterone than control tissue (Lohrke *et al.* 1998).

The objective of the current study was to determine the role of PPAR γ in the rat CL. Given the ability of PPAR γ agonists to affect progesterone production by bovine luteal cells, coupled with its expression pattern in the rat CL, we hypothesized that PPAR γ plays a role in luteal progesterone metabolism. Alternatively, due to its higher expression in old CL versus new CL, and the fact that PPAR γ is expressed in macrophages, which play a role in luteolysis, we also investigated the potential involvement of PPAR γ in regulating the life cycle of the CL. Both the naturally cycling and pseudopregnant rats were used as models to assess the impact of PPAR γ on progesterone production at various physiological stages. The naturally cycling rat allowed for investigating the role of PPAR γ in both newly forming luteal tissue derived from the most recent ovulation and regressing luteal tissue present on the ovary from past ovulations. Since fully functional CL do not develop in cycling rats, the pseudopregnant rat was used to investigate the role of PPAR γ in functioning luteal tissue.

Materials and Methods

Animals

All animal procedures were approved by the Iowa State University's Animal Care and Use Committee. Adult, female Sprague–Dawley rats were obtained from Harlan (Indianapolis, IN, USA), and kept on a 14 h light:10 h darkness cycle. Vaginal smears were taken daily to monitor the estrous cycle. Animals exhibiting three normal estrous cycles were used in the following experiments.

Ovaries were collected from naturally cycling rats to investigate the relationship between PPAR γ and luteal progesterone production. In addition, the association of PPAR γ and macrophages in luteal tissue was also investigated to determine if macrophages were the cells expressing PPAR γ within the CL. Cycling rats were sacrificed on each day of the estrous cycle (proestrus, estrus, metestrus, and diestrus) between 0900 and 1000 h. Ovaries were removed, cleaned of adnexa and frozen until processed for *in situ* hybridization, or fixed in 4% paraformaldehyde for immunohistochemical analysis, as described below. Luteal tissue was also established in culture as described below, to investigate how agonists and an antagonist of PPAR γ impact progesterone production and catabolism.

The expression and function of PPAR γ were also investigated in luteal tissue from pseudopregnant rats.

Rats were mated with a vasectomized male and sacrificed 10–16 days later ($n=4$ animals). Luteal tissue was dissected from one ovary from each animal and cultured *in vitro* as described below. The second ovary from each animal was fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemical analysis.

In situ hybridization

Frozen ovarian tissue collected from rats on the days of proestrus, estrus, metestrus, and diestrus ($n=4$ animals/day) was serially sectioned at 10 μ M. Consecutive tissue sections were used for localization of mRNA for P450 side-chain cleavage (SCC) and PPAR γ . Tissue sections were fixed in 4% paraformaldehyde and dehydrated by passing through a series of increasing ethanol solutions. Slides were placed in 2 mg/ml glycine in PBS (pH 7.2), rinsed in PBS, followed by an incubation in 1.5% triethanolamine buffer with 0.25% acetic anhydride. Slides were subsequently treated with 2 $\times$ SSC (0.149 M sodium chloride, 14.9 mM sodium citrate, pH 7.0) and dehydrated by passing through a series of decreasing concentrations of ethanol.

Sense and antisense riboprobes for PPAR γ and SCC (plasmids containing the cDNA for PPAR γ and SCC were generously provided by Dr Walter Walhi, Universite de Lausanne, Lausanne, Switzerland and Dr J S Richards, Baylor College of Medicine, Houston, TX, USA respectively) were synthesized using a MAXIScript kit (Ambion, Inc., Austin, TX, USA) and α -³⁵S-UTP (10 μ M Ci/ml; ICN Biomedical, Inc., Irvine, CA, USA). Tissues were hybridized with radiolabeled probe (1×10^6 c.p.m.) in 100 μ l hybridization buffer (4 M NaCl, 1 M Tris, 0.5 M EDTA, 50 $\times$ Denhardt's, 25 mg/ml yeast tRNA, 10 mg/ml polyadenylic acid). After hybridization, slides were incubated in RNase solution 1 (2 $\times$ SSC, 50% formamide, 0.1% β -mercaptoethanol), followed by incubation in RNase solution 2 (0.5 M NaCl, 10 mM Tris, 20 μ g/ml RNase A). Slides were incubated in RNase solution 1 a second time, after which they were incubated in 0.1 $\times$ SSC, 1% β -mercaptoethanol. Slides were dehydrated by rinsing with 30% ethanol containing 0.6 M NaCl, 60% ethanol containing 0.6 M NaCl, 80, 95, and 100% ethanol. The slides were air-dried, dipped in Kodak NTB2 emulsion and exposed at 4 $^{\circ}$ C for 1 week (SCC) or 4 weeks (PPAR γ). Slides were developed before being counterstained with hematoxylin. At least one slide was processed with the sense riboprobe and two slides with the antisense riboprobe (4 tissue sections/slide) per animal.

Tissue culture

Luteal tissue from ovaries collected throughout the estrous cycle was dissected from the ovary using 18-gauge needles. CL from the current cycle (new) were

differentiated from CL from previous cycles (old) by assessing the degree of vascularization (Fig. 1a). New CL were well vascularized, whereas CL present on the ovary from past cycles were less vascularized (Bassette 1943). There was no attempt to discern the differences between old CL regarding how many cycles they had been present on the ovary. Individual CL were hemisected and cultured in 500 μ l of defined media (Dulbecco's modified Eagle's medium (DMEM): F12, 0.01% sodium pyruvate, 0.22% sodium bicarbonate, 1% BSA, 0.125% gentamicin, and insulin–transferrin–sodium selenite media supplement; pH 7.2). In one experiment, CL from the day of estrus ($n=5$ animals) were cultured in the presence or absence of the PPAR γ agonists, ciglitazone (65 μ M) or PGJ $_2$ (25 μ M; Komar *et al.* 2001). Agents were added at the time tissues were established in culture and treatments were set up in duplicate. In a second experiment, luteal tissue from the days of proestrus, estrus, metestrus, and diestrus ($n=4$ animals/day of the cycle) was cultured with ciglitazone (65 μ M) or the PPAR γ antagonist GW-9662 (1 μ M). The antagonist, GW-9662, was used because it has previously been shown that there is endogenous activity of PPAR γ in the ovary (Froment *et al.* 2003, Lovekamp-Swan & Chaffin 2005). Luteal tissue from pseudopregnant rats was also cultured with PGJ $_2$ (25 μ M) or GW-9662 (1 μ M). Tissues were incubated for 24 h at 37 °C in 5% CO $_2$:95% air. At the end of culture, media were collected and stored at –20 °C until analyzed by RIA for progesterone. Tissues were frozen and stored until used for RNA and DNA extraction. Concentrations of progesterone were corrected by amount of DNA/well. RNA quality was determined by formaldehyde/agarose gel electrophoresis at the end of culture.

RIA

Concentrations of progesterone in conditioned media were determined as described previously (Komar *et al.* 2001). The intra- and inter-assay coefficients of variation were 2.3 and 13.9% respectively.

Figure 1 Image of an ovary collected on the day of metestrus from a naturally cycling rat. New luteal tissue is more vascularized than old luteal tissue (a). Arrows ($\rightarrow$) denote new luteal tissue, asterisk (*) denotes old luteal tissue. Histological image of old and new luteal tissues (b). OCL, old luteal tissue; NCL, new luteal tissue.

PCR

Total RNA was extracted from cultured tissues using Trizol reagent. cDNA was synthesized using SuperScript II Reverse Transcriptase and oligo (dT) random primers (Invitrogen). The cDNA obtained was analyzed by PCR to determine the expression of 20 α -hydroxysteroid dehydrogenase (20 α -HSD) and bcl-2 in luteal tissue. S16 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were also analyzed and used as internal controls (Ko *et al.* 1999, Mendoza-Rodriguez *et al.* 2003). Primers utilized for 20 α -HSD (Sugino *et al.* 1997), bcl-2, GAPDH (Mendoza-Rodriguez *et al.* 2003), and S16 (Ko *et al.* 1999) were published previously.

PCRs (25 μ l) for 20 α -HSD and S16 consisted of 2.5 mM MgCl $_2$, 1X PCR buffer (200 mM Tris–HCl, 500 mM KCl), 2 μ M of forward and reverse primers for both genes, 200 μ M dNTPs, 1.0 unit of Taq polymerase, 10 $\times$ BSA (BSA), and 50 ng of cDNA. All amplifications were carried out for 30 cycles with denaturation at 95 °C for 2 min, annealing at 65 °C for 1 min, and extension at 72 °C for 1 min/cycle.

Amplification reactions (25 μ l) for bcl-2 and GAPDH consisted of 20 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.0 mM MgCl $_2$, 0.2 mM dNTPs, 0.5 μ M forward and reverse primers for both bcl-2 and GAPDH, 2.5 units Taq DNA polymerase, 50 ng cDNA for GAPDH, and 500 ng cDNA for bcl-2. All amplifications were carried out for 35 cycles, with denaturation at 95 °C for 5 min, annealing at 60 °C for 1 min, and extension at 72 °C for 5 min per cycle.

Samples were analyzed in duplicate, and the analysis was repeated three times. PCR products were separated on a 2% agarose gel. Densitometric analysis of the resulting bands was conducted using the Spot Denso program (Fluorchem, Alpha Innotech, San Leandro, CA, USA). Relative levels of 20 α -HSD and bcl-2 were determined in relation to S16 and GAPDH respectively per sample.

Immunohistochemistry

Paraffin-embedded ovarian tissue was serially sectioned at 5 μ m. Consecutive sections were processed for immunodetection of PPAR γ and macrophages. Paraffin sections were dewaxed in xylene and rehydrated through a series of rinses in decreasing ethanol solutions. Antigen retrieval was performed by placing slides in 0.01 M Tris (pH 8.5) in a microwave on high heat for 10 min. After cooling, slides were placed in 1.5% H $_2$ O $_2$ in methanol to quench endogenous peroxidase activity. After rinsing with PBS (pH 7.2), slides were blocked with 10% normal horse (macrophage) or goat (PPAR γ) serum. Slides were then incubated with either a MAB against rat monocytes/macrophages (ED1 clone, Chemicon, Temecula, CA, USA) at a 1:200 dilution for 1 h at 37 °C or an antibody against PPAR γ (E8, Santa Cruz, CA, USA) at a

1:50 dilution overnight at 4 °C. Slides were rinsed with PBS and subsequently incubated with a species-specific biotinylated secondary antibody at a 1:200 dilution for macrophage detection (Vector Lab Inc., Burlingame, CA, USA) or 1:50 dilution for PPAR γ detection (Amersham). Slides were rinsed in PBS and treated with an avidin–peroxidase complex (ABC kit; Vector Lab Inc.). To visualize the immunocomplex, 3,3'-diaminobenzidine tetrahydrochloride was used. Slides processed for immunodetection of macrophages were counterstained with hematoxylin. All slides were mounted with Permount. For detection of both macrophages and PPAR γ , a minimum of one control slide (blocking serum used in place of primary antibody) and two treated slides were examined per rat (4 ovarian sections/slide).

Statistical analysis

Differences in levels of mRNA for 20 α -HSD and bcl-2 were analyzed by ANOVA (SAS Version 8.2, SAS Institute, Cary, NC, USA), followed by the Student–Newman–Keuls test for comparisons of multiple groups when appropriate (JMP 5.1). Concentrations of

progesterone in conditioned media from old and new CL were log transformed and analyzed independently by ANOVA. A P -value ≤ 0.05 was considered significant.

Results

To investigate the relationship between PPAR γ and progesterone production, mRNAs for SCC and PPAR γ were localized in luteal tissue from naturally cycling rats by *in situ* hybridization. New and old CL were differentiated histologically, with new CL containing more luteal cells and less stromal elements than old CL (Fig. 1b; Malven & Sawyer 1966, Gaytan 1997, Simpson *et al.* 2001). An inverse relationship was observed between the expression of mRNA for SCC and PPAR γ . The expression of mRNA for SCC was higher in newly forming luteal tissue compared with that observed in old luteal tissue on all days of the cycle (Fig. 2). In contrast, the expression of mRNA for PPAR γ was higher in old luteal tissue compared with newly forming luteal tissue (Fig. 3). The expression of mRNA for PPAR γ appeared in a punctate pattern in some luteal tissue, most notably on the day of diestrus (Fig. 3j).

Figure 2 Localization of mRNA corresponding to SCC in ovarian tissue collected from rats on the day of proestrus (a, b and c), estrus (d, e and f), metestrus (g, h and i), and diestrus (j, k and l). Tissue sections (10 μ m) were hybridized with 35 S-labeled sense and antisense riboprobes as described in the Materials and Methods. The first column represents darkfield images of ovarian tissue sections labeled with an antisense riboprobe (a, d, g, j) with the corresponding brightfield image in the second column (b, e, h, k). Darkfield images of tissue hybridized with a sense riboprobe appear in the third column (c, f, i, l). GC, Granulosa cells; OCL, old luteal tissue; NCL, new luteal tissue; Original magnification, 100 $\times$.

Figure 3 Localization of mRNA corresponding to PPAR γ in ovarian tissue collected from rats on the day of proestrus (a, b and c), estrus (d, e and f), metestrus (g, h and i), and diestrus (j, k and l). Panels a and b contain an inset image of a NCL from another area of the ovary for comparison. Tissue sections (10 μ M) were hybridized with 35 S-labeled sense and antisense riboprobes as described in the Materials and Methods. The first column represents darkfield images of ovarian tissue sections labeled with an antisense riboprobe (a, d, g, j) with the corresponding brightfield image in the second column (b, e, h, k). Darkfield images of tissue hybridized with a sense riboprobe appear in the third column (c, f, i, l). GC, Granulosa cells; OCL, old luteal tissue; NCL, new luteal tissue; original magnification, 100 $\times$.

Due to the inverse relationship between mRNA for SCC and PPAR γ in luteal tissue with PPAR γ being expressed at a higher level in old CL versus new CL, we investigated the potential of PPAR γ to influence the metabolism of progesterone. Levels of mRNA for 20 α -HSD, the enzyme responsible for the breakdown of progesterone into its inactive metabolite, were measured in luteal tissue collected on the day of estrus and cultured with ciglitazone or PGJ $_2$. mRNA for 20 α -HSD was expressed in new and old CL, but expression did not change in response to treatment with either PPAR γ agonist (Fig. 4).

Luteal tissue from the days of proestrus, estrus, metestrus, and diestrus was cultured *in vitro* to determine how activation or inhibition of PPAR γ would impact progesterone production throughout the estrous cycle. Old and new CL were separated and cultured with ciglitazone (65 μ M) or GW-9662 (1 μ M). There was no effect of activating or inhibiting PPAR γ on progesterone production by new or old CL collected throughout the estrous cycle (Fig. 5a and b). However, administration of GW-9662 to old CL on proestrus tended to increase progesterone production compared with controls ($P=0.06$, Fig. 5b). The secretion of

progesterone *in vitro* by fully functional luteal tissue collected from pseudopregnant rats was also not affected by treatment with either PGJ $_2$ or GW-9662 (data not shown).

Figure 4 Relative levels of mRNA (mean $\pm$ S.E.M.) for 20 α -HSD in rat luteal tissue on the day of estrus as described in the Materials and Methods. The image depicts PCR products for 20 α -HSD and S16 with corresponding levels of mRNA for 20 α -HSD in new and old CL cultured alone (control) or with ciglitazone (65 μ M) or PGJ $_2$ (25 μ M). OCL, old luteal tissue, NCL, new luteal tissue.

Figure 5 Progesterone production (mean $\pm$ S.E.M.) by new (a) and old (b) luteal tissues collected from naturally cycling rats on all days of the cycle and cultured alone (control) or with ciglitazone (65 μ M) or GW-9662 (1 μ M).

PPAR γ and macrophages were immunolocalized in serial tissue sections of ovaries collected throughout the cycle and during pseudopregnancy to determine if the expression of PPAR γ in CL corresponded to macrophages within the tissue. Positive staining for ED1 (macrophages) was observed in luteal tissue from cycling (Fig. 6a–d) and pseudopregnant (Fig. 6e) rats. Luteal tissue on the day of diestrus contained low levels of labeling for ED1 (Fig. 6d) relative to the other days of the cycle. Protein corresponding to PPAR γ was detected in the nuclei of luteal cells on all days of the cycle, albeit at a lower level than in granulosa cells of developing follicles (Fig. 7a). There was little if any co-localization of protein for PPAR γ and macrophages (Fig. 7a–d). Protein corresponding to PPAR γ was also observed in nuclei of luteal cells during pseudopregnancy at a relatively low level (data not shown).

The role of PPAR γ in luteal lifespan was investigated by measuring levels of mRNA for bcl-2 in tissue collected throughout the cycle and cultured with ciglitazone or GW-9662. In newly forming luteal tissue collected on all days of the cycle except diestrus, treatment with ciglitazone reduced levels of mRNA for bcl-2 compared with controls (Fig. 8a, $P < 0.05$, within day of the cycle). However, the administration of ciglitazone to old CL *in vitro* resulted in a decrease in levels of mRNA for bcl-2 only when the tissue was collected on the day of estrus (Fig. 8b, $P < 0.05$, within

Figure 6 Immunodetection of macrophages (brown color) in sections of ovarian tissue collected from rats on proestrus (a), estrus (b), metestrus (c), diestrus (d), and during pseudopregnancy (e). Tissue section from an ovary collected on the day of proestrus with normal horse serum used in place of the primary antibody as a control (f). Original magnification, 50 $\times$.

the day of the cycle). Interestingly, treating luteal tissue *in vitro* with the PPAR γ antagonist, GW-9662, also reduced levels of mRNA for bcl-2 in both new and old CL collected on the days of proestrus and diestrus, as well as new CL collected on metestrus (Fig. 8a and b, $P < 0.05$, within day of the cycle).

Figure 7 Immunodetection of PPAR γ (a and c) and macrophages (b and d) in consecutive tissue sections of new (a and b) and old (c and d) CL in ovaries collected from rats on proestrus. Original magnification, 100 $\times$. Arrows indicate labeling for PPAR γ in the nuclei of luteal cells. GC, Granulosa cells.

Figure 8 Relative levels of mRNA (mean $\pm$ S.E.M.) for bcl-2 in rat luteal tissue collected throughout the cycle as described in the Materials and Methods. (a) PCR products for bcl-2 and GAPDH with corresponding levels of mRNA for bcl-2 in new CL cultured alone (control) or with ciglitazone (65 μ M) or GW-9662 (1 μ M). (b) PCR products for bcl-2 and GAPDH with corresponding levels of mRNA for bcl-2 in old CL cultured alone (control) or with ciglitazone (65 μ M) or GW-9662 (1 μ M). Bars with different superscripts denote significant differences within the day of the cycle ($P < 0.05$).

Discussion

PPAR γ has been detected in ovarian tissue from several species. However, at this time, it is unknown how this transcription factor impacts ovarian function. Research conducted by Cui *et al.* (2002) demonstrated that in transgenic mice with the expression of PPAR γ disrupted in the ovary, the females were sub- or infertile. Since the expression of PPAR γ was not disrupted in the uterus of these transgenic animals, the authors concluded that the lesion causing the sub- and infertility resided in the ovary. These authors reported that on the day of estrus, circulating concentrations of progesterone were 50% lower in animals with PPAR γ disrupted in the ovary compared with controls, although the difference was not significant most likely due to the small sample size ($n=4$). In cattle, agonists of PPAR γ were found to stimulate progesterone production from mid-phase luteal cells over a 24-h culture period (Lohrke *et al.*

1998). Treatment of human granulosa-lutein cells with PGJ₂ had no effect on basal progesterone production, but inhibited LH-stimulated progesterone production (Willis *et al.* 1999). Taken together, these data suggest that PPAR γ may influence the ability of luteal tissue to produce progesterone, which is necessary for the establishment and maintenance of pregnancy.

The data presented here illustrate that PPAR γ is not a major player in progesterone production in the rat CL. In luteal tissue collected throughout the estrous cycle, as well as from pseudopregnant rats, there was no significant effect of PPAR γ agonists or the antagonist on progesterone production. Our data also show that there is an inverse relationship between the expression of PPAR γ in luteal tissue and the ability of that tissue to produce progesterone – as determined by levels of mRNA for SCC. These data agree with a previously published report on the gonadotropin-treated immature rat where an inverse relationship between the expression of mRNA for PPAR γ and SCC in granulosa cells was detected during the periovulatory period (Komar & Curry 2003). These findings indicate that downregulation of PPAR γ may be important for the changes in gene expression involved in luteinization and the switch in steroid production from estrogen to progesterone in the rat. The finding in the current study that an antagonist of PPAR γ tended to increase progesterone production by cultured luteal tissue further supports the hypothesis that downregulation of PPAR γ may be important for periovulatory progesterone production in the rat. These findings differ from those reported by Lohrke *et al.* (1998) and could be the result of differences in culture methods and/or species differences in luteal development and function. The current study utilized hemisected luteal tissue *in vitro*, whereas Lohrke *et al.* (1998) cultured dispersed luteal cells. Separation of large and small luteal cells alters steroidogenesis. Dispersed luteal cells produced lower amounts of hormones *in vitro* compared to intact tissue from the same CL (Pate & Nephew 1988, Jaroszewski *et al.* 2003). Therefore, the use of intact luteal tissue provides a more accurate model of *in vivo* luteal function. In cycling rodents, mating or cervical stimulation is necessary for the development of fully functional corpora lutea. In contrast, after ovulation in cattle, luteal tissue develops regardless of whether the female is mated. The hormonal environment of the luteal phase also differs between the rat and cow. In the rat, the hormone prolactin is needed for luteal development and demise (reviewed by Freeman *et al.* 2000), while prolactin does not play a role in luteal function in cattle (reviewed by Niswender *et al.* 2000).

Another difference between the bovine and rat is that the rat ovary contains CL from past ovulations (old CL) as well as CL from the most recent ovulation (new CL). Multiple cycles are needed for the complete regression of old CL (reviewed by Freeman *et al.* 2000). This is in

contrast to the bovine, whose luteal tissue develops from the most recent ovulation and regresses in the open animal before the next estrous cycle. In the rat, the secretion of progesterone from newly forming luteal tissue increases on the evening of proestrus. By diestrus, there is an increase in the activity of 20 α -HSD, the enzyme responsible for converting progesterone into its inactive metabolite, 20 α -hydroxyprogesterone (Diaz *et al.* 2002). A decrease in progesterone may trigger the increase in expression of 20 α -HSD, leading to progesterone catabolism (Stocco *et al.* 2001). In a study by Yoshida *et al.* (1999), the expression of 20 α -HSD was higher in new luteal tissue collected at the end of pseudopregnancy compared with that at the beginning. This expression pattern of 20 α -HSD most likely reflects the fact that at the beginning of pseudopregnancy, luteal tissue would secrete progesterone, which is needed for the establishment and maintenance of pregnancy, whereas at the end of pseudopregnancy a decline in progesterone would correspond with parturition at the end of gestation. In the current study, agonists of PPAR γ had no effect on the expression of mRNA for 20 α -HSD in either new or old luteal tissue. These findings indicate that just as PPAR γ is not a major player in progesterone production, it also does not affect the breakdown of progesterone via regulating the expression of mRNA for 20 α -HSD.

Regression of the CL involves functional (decrease in progesterone production) and structural changes (regression of luteal tissue, apoptosis, and macrophage infiltration) to the tissue. Accumulation of macrophages in CL has been found to occur from the evening of proestrus to the morning of estrus in the rat (Gaytan *et al.* 1998). The current study examined the expression of macrophages in both the naturally cycling and pseudopregnant rat models. In the cycling rat, prolactin is necessary for the establishment of the macrophage population in new luteal tissue as well as for the influx of monocytes and macrophages into regressing CL (Gaytan *et al.* 1997). Immunodetection of macrophages and PPAR γ in serial sections of ovarian tissue demonstrated that they are localized primarily to different areas within luteal tissue. Macrophages were situated mostly around luteal cells, while PPAR γ was detected in the nuclei of luteal cells. These findings are similar to those reported by Minge *et al.* (2006). These authors detected PPAR γ in the nuclei of luteal cells in the mouse, and also reported co-localization of PPAR γ with select macrophages present in the theca and stroma, as well as luteal tissue. The antibody employed in the study by Minge *et al.* (2006) identified recently recruited and mature macrophages, whereas the antibody utilized in the current study recognized both monocytes and macrophages. Since PPAR γ is expressed in activated macrophages (Ricote *et al.* 1998, Tontonoz *et al.* 1998, Cunard *et al.* 2002), it would not co-localize with monocytes. Therefore, the detection of PPAR γ in nuclei of luteal cells (current study; Minge *et al.* 2006), and limited

co-localization with macrophages in luteal tissue (Minge *et al.* 2006), indicates that the higher expression of PPAR γ in old compared with newly forming luteal tissue is not due solely to the presence of macrophages within the tissue.

Apoptosis, or programmed cell death, plays a role in luteal regression. Apoptosis is modulated by a number of regulatory genes, such as bcl-2. Bcl-2 is a member of the B-cell leukemia/lymphoma family, and plays a role in cell survival in the ovary. In the human and rodent, protein corresponding to bcl-2 has been detected in the corpus luteum and granulosa cells (reviewed by Richards 1994, Rodger *et al.* 1998, Leng *et al.* 2001, Kim & Tilly 2004). As mentioned previously, the human gene encoding bcl-2 contains a peroxisome proliferator response element (Butts *et al.* 2004). Since bcl-2 and PPAR γ are located in the same tissues within the ovary, PPAR γ may affect ovarian cell survival via regulating the expression of bcl-2. In support of that hypothesis, a study using cultured granulosa cells showed that treatment with the PPAR γ agonist, troglitazone, decreased protein levels for bcl-2 (Lovekamp-Swan & Chaffin 2005). In the current study, levels of mRNA for bcl-2 in old CL were reduced by treatment with a PPAR γ agonist when the tissue was collected on the day of estrus, and by treatment with a PPAR γ antagonist when tissue was collected on proestrus and diestrus. This reduction in mRNA for bcl-2 in response to both an agonist and antagonist of PPAR γ was also seen in new CL collected on proestrus and metestrus. These apparent incongruous findings of both an agonist and antagonist of PPAR γ inhibiting expression of mRNA for bcl-2 may be due to PPAR γ -dependent and -independent effects of these agents. Ciglitazone is a member of the thiazolidinedione (TZD) family of drugs. Troglitazone, another TZD, has been shown to have cellular effects that are not mediated by activation of PPAR γ (Chawla *et al.* 2001), and reportedly can have direct effects on ovarian cells (Gasic *et al.* 2001). GW-9662 has also been shown to act as a PPAR γ agonist in a normal human mammary epithelial cell line (Allred & Kilgore 2005). Further study is warranted to determine how activation/inhibition of PPAR γ impacts luteal cell survival.

In conclusion, our data suggest that PPAR γ does not play a significant role in progesterone production in the naturally cycling or pseudopregnant rat. The expression pattern of PPAR γ is inversely related to the ability of luteal tissue to produce progesterone, and agonists of PPAR γ had no effect on the ability of luteal tissue to secrete or breakdown progesterone. PPAR γ is located in the nucleus of luteal cells where it may influence luteal cell survival via regulating the expression of bcl-2. Future studies are needed to further investigate the role of PPAR γ in luteal cell apoptosis and how its varying expression is regulated during the lifespan of the CL.

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