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Rosiglitazone stimulates peroxisome proliferator-activated receptor gamma expression and directly affects *in vitro* steroidogenesis in porcine ovarian follicles

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ABSTRACT

Rosiglitazone is a peroxisome proliferator-activated receptor gamma (PPARγ) synthetic activator from the group of thiazolidinediones often used in the treatment of chronic diseases such as type 2 diabetes and other forms of insulin resistance. The present *in vitro* study assessed the direct effects of rosiglitazone at 25 and 50 μM doses on PPARγ gene expression, steroid secretion (progesterone [P4], androstenedione [A4], testosterone [T], and estradiol), and protein expression of PPARγ, 3βHSD, CYP17, 17βHSD, CYP19 by porcine ovarian follicles from prepubertal and cycling animals. We analyzed also steroid enzymatic activity by conversion of pregnen-3β-ol-20-one to P4, P4 to A4, and A4 to T. Our results indicated that rosiglitazone increased significantly PPARγ expression, P4 secretion, 3βHSD activity, and protein expression. Rosiglitazone decreased A4 and T secretion by reducing the expression and activity of CYP17 and 17βHSD and did not change estradiol secretion and CYP19. Similarly results was observed both in prepubertal and cycling pigs. Our results indicate that these direct effects of rosiglitazone on ovarian steroidogenesis provide a framework for testing several potential new mechanisms of PPAR-γ actions on porcine ovarian function.

1. Introduction

Thiazolidinediones (TZDs) are synthetic ligands also known as glitazones (troglitazone, rosiglitazone, or pioglitazone) [1], which can bind and activate the nuclear receptor, peroxisome proliferator-activated receptor (PPAR) [2]. By binding to PPARγ, TZDs are implicated in the transcription of several factors involved in the regulation of glucose and lipid metabolism, mainly in the adipose and muscle tissues [3,4]. Recent studies showed PPARγ expression in the ovary and indicated an important role for this receptor in ovarian functions such as steroid synthesis, angiogenesis, tissue remodeling, cell-cycle regulation, and apoptosis [5,6]. Thiazolidinediones improve peripheral insulin resistance and decrease hyperinsulinemia, and as such, they may also be used for the treatment of polycystic ovarian syndrome (PCOS) in women. Administration of troglitazone, rosiglitazone, or pioglitazone is able to induce ovulation and to increase the ovulation rate and pregnancy in women with PCOS [7]. Moreover, several studies showed that TZDs improve insulin sensitivity, decrease the insulin concentration, and reduce androgenic activity in women with PCOS [8].

Rosiglitazone, which has a binding affinity for PPARγ 100-fold greater than that of troglitazone [9], can directly influence ovarian function and ultimately exert positive effects on the developmental competence of oocytes in mice [10]. Additionally, Minge, et al. [11] demonstrated that rosiglitazone improved blastocyst quality in obese female mice, suggesting that PPARγ was a key target for metabolic regulation of ovarian function and oocyte quality. In human granulosa lutein cells, rosiglitazone stimulated steroidogenic

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acute regulatory protein (StAR) gene expression but did not significantly affect progesterone (P4) and estradiol (E2) secretion [12]. In contrast, in *in vitro* ovine granulosa cell culture, rosiglitazone increased P4 secretion but had no effect on CYP11A and 3βHSD protein expression in small and large follicles [13].

Although there is evidence that rosiglitazone modulates ovarian function, direct effects of rosiglitazone on steroid synthesis and its mechanism of action have not been reported in porcine ovaries. Pigs and mini pigs are becoming a valid alternative to traditional nonrodent species in pharmacologic, physiological, and toxicologic studies because many of their physiological characteristics resemble those of humans [14]. The present investigation examines the hypothesis that rosiglitazone directly affects porcine ovarian steroid production; thus, in this study, we used ovarian follicles from prepubertal and cycling animals to investigate the direct *in vitro* effects of rosiglitazone on mRNA and protein expression of PPARγ and steroidogenesis by measurement of P4, androstenedione (A4), testosterone (T), and E2 secretion and 3βHSD, CYP17, 17βHSD, CYP19 activity, and protein expression.

2. Materials and methods

2.1. Reagents and antibodies

M199 medium and PBS were purchased from CytoGen (Łódź, Poland). Antibiotic/antimycotic solution (10,000 units/mL penicillin, 10 mg/mL streptomycin, and 25 μg/mL amphotericin B), TRIS, Na-deoxycholate, Nonidet NP-40, SDS, protease inhibitor (EDTA-free), dithiothreitol, Tween-20, bromophenol blue, DMSO, rosiglitazone, pregnen-3β-ol-20-one (P5), 17α-hydroxyprogesterone (P4), 4-androstene-3, 17-dione (A4), and β-actin (cat. #A5316) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against PPARγ (cat. #sc-271392), 3βHSD (cat. #sc-30820), CYP17A1 (cat. #sc-46084), 17βHSD (cat. #sc-26963), and CYP19 (cat. #sc-14244), horseradish peroxidase-conjugated secondary antibody and Western blotting luminol reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A Bradford protein assay kit and Image Lab 2.0 software were obtained from Bio-Rad laboratories (Hercules, CA, USA). Polyvinylidene difluoride membrane was purchased from Millipore (Darmstadt, Germany).

2.2. Sample collection

Porcine ovaries were collected from prepubertal (4–6 months of age) or cycling (7–8 months of age) crossbred gilts (Large White and Polish Landrace) at a local abattoir. Average weight of these animals is a prepubertal: 100 to 110 kg and mature (cycling): 120 to 140 kg. Veterinarian determined the age, weight, and gender of all animals before slaughter. Approximately 15 minutes elapsed between slaughter and ovary collection. Before ovarian incubation, we thoroughly analyzed ovarian morphology and to the culture, we used follicles of the same size. Medium-sized ovarian follicles (4 mm) were obtained from prepubertal or cycling pigs on Days 10 to 12, as described previously [15]. Individually, each ovary derived from cycling pigs is checked for their size, morphology, and of the corpus luteum (CL) phase. Estrus was designated as Day 0. For each experiment described below, eight to 10 ovaries from four to five different animals were selected.

2.3. Ovarian follicles incubation

After isolation, follicles were cut using small scissors to facilitate penetration of the compounds into the tissue. Follicular walls, including theca and granulosa cells and excluding oocytes and follicular fluids, were individually placed in 24-well plates in 250 μL per well M199 medium without phenol red. To choose doses and time, in the first experiment, we used a wide range of rosiglitazone doses (5–100 μM) and incubation time (12, 24, and 48 hours) on P4 secretion. The doses of rosiglitazone based on the literature [16]. Stock solutions of rosiglitazone were dissolved in DMSO. The final concentration of DMSO in the medium was 0.1%. DMSO had no effect on steroid secretion (data not shown). Additionally, in our lab, we demonstrated that DMSO had no effect also on cell viability and apoptosis in porcine ovarian cells [17]. The follicles were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. After incubation, the medium was collected from the follicles and stored at −20 °C for steroid hormone determination. Cultured follicular tissue was homogenized twice in ice-cold lysis buffer (50 mM Tris-HCl [pH 7.5] containing 100 mM NaCl, 0.5% sodium deoxycholate [wt/vol], 0.5% NP-40 [vol/vol], 0.5% SDS [wt/vol], and protease inhibitor). Pool samples, from four follicles were used. Lysates were cleared by centrifugation at 15,000× *g* at 4 °C for 30 minutes, and protein content was determined by a protein assay using BSA as a standard. All samples were stored at −20 °C for 3βHSD, CYP17, 17βHSD, and CYP19 protein expression analysis.

To examine effects of rosiglitazone on PPARγ mRNA expression, we performed similar experiments, with the exception that ovarian follicles were immediately frozen in liquid nitrogen and then stored at −70 °C for RNA isolation.

Steroid enzymatic activity was studied by adding substrates (10^{-7} M) for 3βHSD activity (measured by conversion of P5 to P4), CYP17 (measured by conversion of P4 to A4), and 17βHSD activity (measured by conversion of A4 to T) and measuring the amount of the resulting products in the culture medium after a 24-hour incubation. It should be noted that this *in vitro* model was previously used to study the effects of leptin [18] or resistin [19] on porcine ovarian follicle steroid synthesis.

2.4. Analysis of steroid levels

Steroid hormone (i.e., P4, A4, T, and E2) levels were determined in conditioned culture media using commercially available ELISA kits (cat. #s EIA-1561, EIA-3265, EIA-1559, and EIA-2693, DRG Diagnostic, Marburg, Germany). All samples were run in duplicate in the same assay. 25-μL culture medium was added to each well, according to the manufacturer's protocol. The sensitivity of each assay was 0.045 ng/mL for P4, 0.019 to 10 ng/mL for A4, 0.083 to 16 ng/mL for T, and 9.7 to 2000 pg/mL for E2. The intra- and