Interactions among Peroxisome Proliferator Activated Receptor-γ, Insulin Signaling Pathways, and Steroidogenic Acute Regulatory Protein in Human Ovarian Cells

Donna Seto-Young, Dimiter Avtanski, Marina Strizhevsky, Grishma Parikh, Parini Patel, Julia Kaplun, Kevin Holcomb, Zev Rosenwaks, and Leonid Poretsky

G. J. Friedman Diabetes Institute and the Division of Endocrinology, Department of Medicine (D.S.-Y., D.A., M.S., G.P., P.P., J.K., L.P.), Department of Obstetrics and Gynecology (K.H.), Beth Israel Medical Center and Albert Einstein College of Medicine, New York, New York 10003; Institute of Biology and Immunology of Reproduction (D.A.), Bulgarian Academy of Sciences, Sofia 1113, Bulgaria; and Center for Reproductive Medicine and Infertility (Z.R.), Weill Medical College of Cornell University, New York, New York 10021

Context and Objective: Peroxisome proliferator activated receptor-γ (PPAR-γ) agonists thiazolidinediones (TZDs) are thought to ameliorate hyperandrogenism in polycystic ovary syndrome by reducing hyperinsulinemia. However, TZDs also exhibit direct effects in the human ovary. We examined interactions among PPAR-γ, insulin signaling pathways, and steroidogenic acute regulatory (StAR) protein in human ovarian cells.

Materials and Methods: Mixed human ovarian tissue culture that contained granulosa, theca, and stromal cells, and a culture of purified granulosa cells obtained during *in vitro* fertilization, were established as previously described. Cells were cultured in the presence or absence of insulin, with or without 25 or 50 μM rosiglitazone or pioglitazone. Expression of PPAR-γ, insulin receptor, or insulin receptor substrate (IRS)-1 in both cell systems and of the StAR protein in granulosa cells was measured using immunoprecipitation and immunoblotting.

Results: Rosiglitazone stimulated expression of PPAR-γ, insulin receptor α- and β-subunits, and IRS-1 up to 168% ($P < 0.05$), 679% ($P <$ 0.006), 290% ($P < 0.037$), and 323% ($P < 0.01$) of baseline, respectively. Pioglitazone stimulated expression of PPAR-γ, insulin receptor α- and β-subunits, and IRS-1 up to 222% ($P < 0.01$), 362% ($P < 0.001$), 402% ($P < 0.029$), and 492% ($P < 0.03$), respectively. Insulin alone stimulated expression of PPAR-γ, α-subunit and β-subunit of insulin receptor, and IRS-1 up to 174% ($P < 0.001$), 692% ($P < 0.014$), 275% ($P < 0.024$), and 431% ($P < 0.01$), respectively. In purified granulosa cell culture, rosiglitazone stimulated expression of StAR protein up to 540% ($P < 0.007$), and pioglitazone stimulated expression of StAR protein up to 670% ($P < 0.007$). Insulin alone stimulated expression of StAR protein up to 600% ($P < 0.012$).

Conclusions: Insulin and TZDs independently stimulate expression of PPAR-γ, insulin receptor, IRS-1, and StAR protein in human ovarian cells. Thus, PPAR-γ, insulin receptor with its signaling pathways, and StAR protein constitute a novel human ovarian regulatory system with complex interactions among its components. (*J Clin Endocrinol Metab* **92: 2232–2239, 2007**)

P EROXISOME PROLIFERATOR activated receptor-γ (PPAR-γ) is involved in regulating glucose and lipid metabolism, in part through regulation of insulin signal transduction. Thiazolidinediones (TZDs), acting as PPAR-γ agonists, alter gene expression, enhance the sensitivity of tissues (fat and muscle) to insulin, ameliorate glucose intolerance, and partially reverse lipid abnormalities in patients with diabetes (1). Recently, we have reported that in human ovarian cells, TZDs exhibit direct effects, both insulin-independent and insulin-sensitizing (2).

In other tissues, PPAR-γ activation leads to the activation of insulin signaling pathways (3–6). For example, TZDs improve downstream insulin transduction in muscle (4), and

stimulate phosphatidylinositol 3-kinase and MAPK in blood vessels (5) and liver epithelial cells (6). The nature of interaction, if any, between PPAR-γ and insulin signaling pathways in human ovarian cells has not been explored.

Steroidogenic acute regulatory (StAR) protein plays an essential role in cholesterol transfer from the outer to the inner mitochondrial membrane, thus providing the substrate for steroid hormone biosynthesis (7–11). Cholesterol is then converted to pregnenolone by the cytochrome P450 side-chain cleavage (CY11A1) enzyme, initiating steroid biosynthesis. In the animal models, IGF-I activates StAR protein in mouse Leydig cells and both FSH and LH, acting synergistically with insulin, enhancing StAR protein mRNA transcription in rat and porcine granulosa cells (7–11). However, the role of insulin or PPAR-γ in the regulation of StAR protein in human ovarian cells, to our knowledge, has not been reported.

Because interactions among PPAR-γ, insulin signaling cascades, and StAR protein in the human ovary are of both physiological and clinical significance, we examined these interactions in human ovarian cell culture systems.

Materials and Methods

The institutional review boards at Beth Israel Medical Center and the Weill Medical College of Cornell University approved all studies described in this report.

Human granulosa cells

Human granulosa cells were obtained during *in vitro* fertilization over the course of 6 months and were pooled from several patients at a time to ensure adequate cell number for the experiments. The diagnoses included male factor, tubal factor and uterine factor infertility, endometriosis, and anovulation. Some cell samples were obtained from normal oocyte donors.

Granulosa cells were purified on Percoll gradients and cultured as previously described (12). Two milliliters of the tissue culture medium containing cells were placed in 6-well Falcon tissue culture dishes (BD Biosciences, Franklin Lakes, NJ). The cell density was approximately 2.5×10^5 cells/ml. The cells were incubated for 48 h at 37 C in 5% carbon dioxide and 90% humidity in M199 tissue culture medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen Corp., Carlsbad, CA), 10 μg/ml gentamicin, and 250 ng/ml amphotericin (Invitrogen Corp.). After a 48-h incubation, the medium supplemented with 10% FBS was replaced by medium containing 2% FBS for another 24 h. Finally, the cells were incubated for an additional 24 h in the same medium in the presence of 0, 10, 10^2, or 10^3 ng/ml insulin, with or without 25 or 50 μM rosiglitazone or pioglitazone.

Mixed ovarian cell cultures

Patients. Ovarian tissue samples were collected from 22 women at the time of indicated oophorectomy. The patients' ages ranged from 23–55 yr. None of the patients was using hormonal contraception or other hormonal therapy at oophorectomy. Pathological diagnoses included follicular cyst (3 patients), inclusion cyst (5), decidualized endometrial cyst (1), hemorrhagic corpus luteum cyst (1), benign cystic teratoma (1), tubal-ovarian adhesions (1), epithelial inclusions with focal mucinous metaplasia associated with calcification (1), and normal ovaries with abnormalities in the uterus (9).

After the pathological examination, only unaffected ovarian tissue was used for the cell culture. The ovarian cell culture system was developed as previously described, and contained a mixture of granulosa, theca, and stromal cells (13). The unaffected ovarian tissue obtained from oophorectomy specimens was placed in the M199 medium supplemented as described previously. Tissue was cut into small fragments ($\sim$5 mm^2), homogenized, and placed in tissue culture dishes. Sterile Vaseline (Unilever PLC, London, UK) was placed around the tissue, and a glass cover slide was placed on top of the tissue. Four milliliters of the culture medium were then added to the culture. Ovarian tissue was cultured without passaging until the cells were confluent. This cell culture was then trypsinized and subcultured until adequate cell numbers required for the experiments were available. In most experiments, a specimen from a single patient was used for both control and experimental cell samples. In some experiments, cells from different patients were pooled to ensure adequate cell number for the experiments.

Experimental conditions

The cells were incubated with various concentrations of insulin (0, 10, 10^2, or 10^3 ng/ml) with or without 25 or 50 μM rosiglitazone or pioglitazone. Both mixed ovarian cell culture and purified granulosa cell culture were used for the studies of TZD effects on the expression of PPAR-γ, insulin receptor, and insulin receptor substrate (IRS)-1. We used only granulosa cell culture to examine the StAR protein expression because our mixed ovarian cell culture is not capable of initiating steroidogenesis using cholesterol as substrate. This phenomenon may occur because of the loss of the cholesterol side chain cleavage enzyme or its cofactor (10, 14) during the culture process. Furthermore, the mixed ovarian cell culture expressed both inactive (37 kDa) and active (30 kDa) forms of StAR protein (15, 16) (see *Discussion* for details), while granulosa cells expressed only the active (30 kDa) form.

The cell density was approximately 5×10^4 mixed ovarian cells/ml or 2.5×10^5 purified granulosa cells/ml. For the immunoprecipitation experiments, 2 ml of cell suspension were added to each well in the 6-well tissue culture plates, and for the protein measurements, 1 ml of the cell suspension was added to each well in the 24-well tissue culture plates.

Protein measurements

After the cells were incubated with or without insulin and with or without rosiglitazone or pioglitazone, under conditions described previously, the Lowry method (17) determined cell protein concentration. The protein concentration was approximately 50 μg/ml of cell suspension (normalized for 5×10^4 cell/ml for both mixed ovarian cell culture and purified granulosa cell culture). Incubation with insulin, rosiglitazone, or pioglitazone had no effect on the cell protein concentration.

Immunoprecipitation and immunoblotting

All commercial antibodies were rabbit polyclonal antibodies that cross-reacted with human, monkey, mouse, or rat. Anti-PPAR-γ, anti-insulin receptor-β-subunit, and anti-IRS-1 antibodies were obtained from Upstate Inc. (Lake Placid, NY). Anti-insulin receptor α-subunit antibodies were from Biogenesis (Kingston, NH), and anti-StAR protein antibodies were from Affinity BioReagents, Inc. (Golden, CO). The concentrations of antibodies ranging 1.5–5 μg/ml (as described in the manufacturer's recommendations) were added to 1 ml cell lysate. The lysate buffer contained 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 1 mM Na orthovanadate, 1% NP40, 1 mM AEBSF, 0.8 μM aprotinin, 50 μM bestatin, 15 μM E-64, 20 μM leupeptin, 10 μM pepstatin A, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml trypsin inhibitor. The lysate was centrifuged at 6000 rpm for 3 min, and the supernatant was transferred into another microcentrifuge tube. Immunoprecipitation procedures were modified as described by Seto-Young *et al.* (12). The mixture was incubated for 3–4 h at 4 C. After protein A sepharose-tm-6MB was added, the mixture was incubated again overnight at 4 C. The immunoprecipitate complexes were collected by centrifugation and washed (12). The samples were then resuspended in 2X Laemmli buffer (Bio-Rad Laboratories, Hercules, CA) and 1 mM dithiothreitol (DTT) (Cleland's reagent, 1,4-dithiothreitol, threo-1,4-dimercapto-2,3-butanediol; Roche Diagnostics, Indianapolis, IN). For the experiments of total insulin receptor measurements, the samples were resuspended in 2X Laemmli buffer with or without 0.4 mM DTT. In the absence of DTT, two protein bands were observed at an apparent molecular mass of 200 kDa ($\alpha\beta$ subunits of insulin receptor) and 135 kDa (α-subunit of insulin receptor). In the presence of DTT, the β-subunit band was cleaved into a minor band at 80 kDa and a major band at 50 kDa (18, 19). Electrophoresis and immunoblotting procedures were performed as described in the manufacturer's recommendations (Bio-Rad Laboratories).

Antibodies from a different host (mouse) were used for probing the expressed protein transferred to the nitrocellulose paper. For the experiments that examined StAR protein expression, no commercial mouse anti-StAR protein antibodies are available. Therefore, rabbit anti-StAR protein antibody was used. The proteins with a molecular mass higher than 45 kDa were cut off from the transferred nitrocellulose paper before probing with the anti-StAR protein antibodies to avoid contamination with antibodies that were added for immunoprecipitation.

For the experiments that examined IRS-1 protein expression, the rabbit anti-IRS-1 antibodies were used. After the first probing procedure, the nitrocellulose paper was washed with 0.1% sodium dodecyl sulfate buffer for 10 min, and then the second antibody probing procedure was performed as described in the manufacturer's recommendations (Bio-Rad Laboratories). The dilution factors for all antibodies were used as described in the manufacturer's recommendations [anti-PPAR-γ antibodies (Chemicon Int., Temecula, CA), anti-insulin receptor-β-subunit antibodies (AbCam, Inc., Cambridge, MA), and anti-insulin receptor-α-subunit antibodies (Biogenesis)]. The immunoblot was probed using a second antibody conjugated with horseradish peroxidase (0.5–1 μg/ml; goat antimouse or goat antirabbit IgG). Anti-α-subunit and anti-β-subunit insulin receptor antibodies used in the experiments were specific for insulin receptor (20, 21).

These immunoprecipitation and immunoblot procedures were used for analysis of protein expression for both mixed ovarian cell and purified granulosa cell cultures. The bands were detected by chemiluminescence (Pierce, Rockford, IL). For the analysis of the expression of PPAR-γ, insulin receptors, IRS-1, and StAR protein, the National Insti-

tutes of Health (Bethesda, MD) Scion Imaging program was used to evaluate integrated band intensity.

Statistical analysis

Two-way ANOVA was used to compare mean values according to insulin concentrations in the presence or absence of rosiglitazone or pioglitazone. The statistical interactions between the sets of data obtained with or without rosiglitazone or pioglitazone were examined. Pairwise Bonferroni-adjusted contrasts were used to determine statistical significance.

Results

Insulin, rosiglitazone, and pioglitazone effects on PPAR-γ

PPAR-γ was present in both human mixed ovarian cell culture and purified human granulosa cell culture (Fig. 1, A and B). Experiments demonstrating insulin, rosiglitazone (25 or 50 μM), or pioglitazone (25 or 50 μM) effects on PPAR-γ expression in the mixed ovarian cell culture and purified granulosa cell culture produced similar results. Insulin stimulated PPAR-γ expression up to 174% ($P < 0.001$) of control

(Fig. 1C). PPAR-γ expression was stimulated up to 168% ($P < 0.05$) and 222% ($P < 0.01$) by rosiglitazone or pioglitazone, respectively (Fig. 1D). When insulin was used in combination with rosiglitazone or pioglitazone (25 or 50 μM), no significant additive effect on PPAR-γ expression was observed in either cell culture system (Fig. 1, A and B). The effect on PPAR-γ expression observed with 25-μM concentration of TZDs was similar to that of 50 μM in both cell culture systems.

Insulin, rosiglitazone, and pioglitazone effects on the insulin receptor

Experiments demonstrating insulin, rosiglitazone (25 or 50 μM), or pioglitazone (25 or 50 μM) effects on insulin receptor expression (total, membrane and cytoplasmic fractions) in the mixed ovarian cell and granulosa cell cultures produced similar results (Fig. 2, A and B). In the absence of insulin, rosiglitazone stimulated insulin receptor α-subunit expression up to 679% ($P < 0.006$) and pioglitazone up to 368% ($P < 0.001$) of baseline (Fig. 2C). Similarly, rosiglitazone stimulated insulin receptor β-subunit up to 290% ($P < 0.037$)
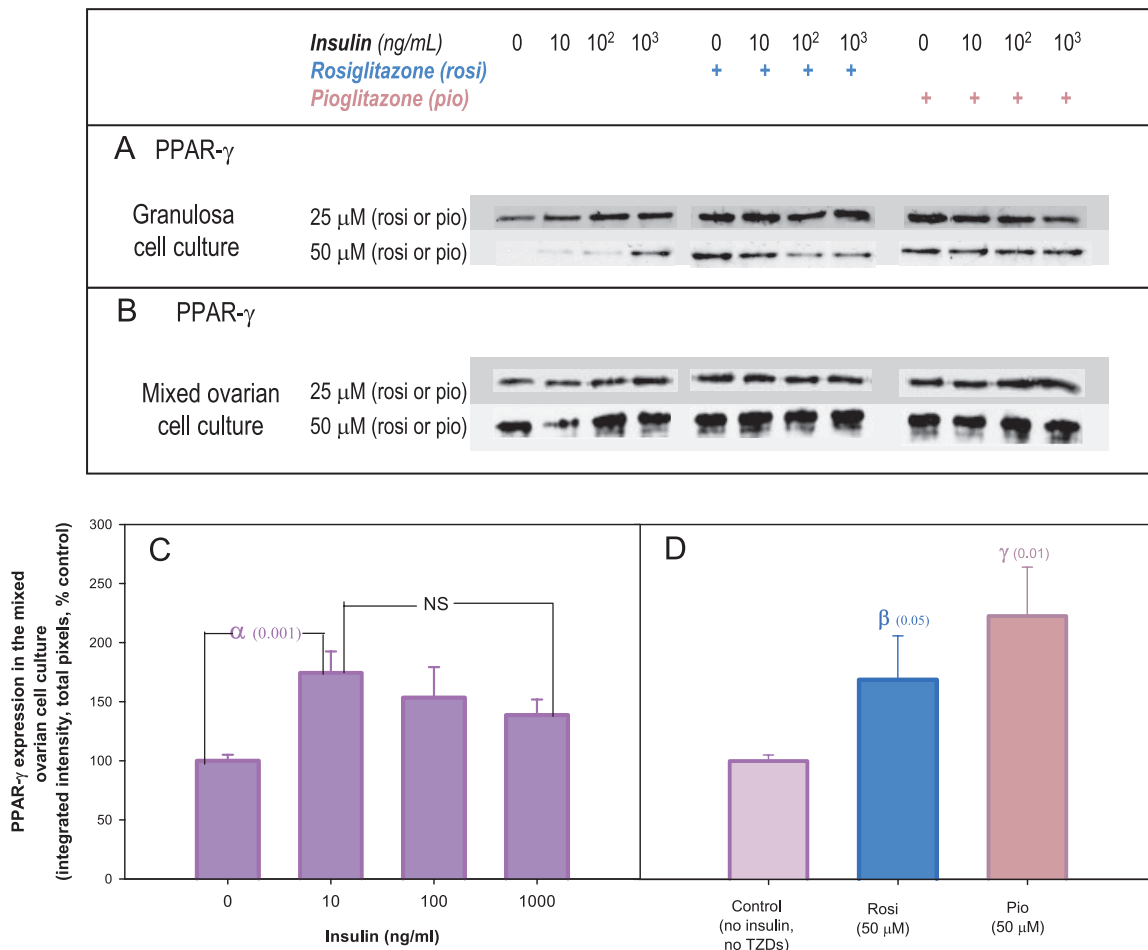

FIG. 1. The effects of TZDs on PPAR-γ expression. A and B, Representative immunoblot analysis of PPAR-γ expression in mixed ovarian cell culture or purified granulosa cell culture in the presence of insulin, with or without rosiglitazone (rosi) or pioglitazone (pio) (25 or 50 μM). A, Granulosa cell culture. B, Mixed ovarian cell culture. C and D, Integrated intensity (total pixels) of the bands of PPAR-γ expression. C, The effects of insulin alone; α [$P < 0.001$ (n = 9)] compared with the control (in the absence of insulin). D, The effects of 50 μM rosiglitazone or 50 μM pioglitazone; β [$P < 0.05$ (n = 9)] and γ [$P < 0.01$ (n = 9)] compared with the control (in the absence of rosiglitazone or pioglitazone). NS, Not significant.

FIG. 2. The effects of TZDs on insulin receptor expression. A and B, Representative immunoblot analysis of insulin receptor α-subunit or β-subunit expression in mixed ovarian cell culture or purified granulosa cell culture in the presence or absence of insulin, with or without rosiglitazone (rosi) or pioglitazone (pio) (25 or 50 μM). A, Granulosa cell culture. B, Mixed ovarian cell culture. C–E, Integrated intensity (total pixels) of the bands of α-subunit and β-subunit of the insulin receptor. C, α-Subunit of insulin receptor, in the presence of 50 μM rosiglitazone or 50 μM pioglitazone; γ [$P < 0.006$ (n = 5)] and δ [$P < 0.001$ (n = 5)] compared with the control (in the absence of rosiglitazone or pioglitazone). D, β-Subunit of insulin receptor, in the presence of 50 μM rosiglitazone or 50 μM pioglitazone; ε [$P < 0.037$ (n = 4)] and κ [$P < 0.029$ (n = 4)] compared with the control (in the absence of rosiglitazone or pioglitazone). E, α-Subunit and β-subunit of the insulin receptor, in the presence or absence of insulin; τ [$P < 0.014$ (n = 5)] and ρ [$P < 0.024$ (n = 4)] compared with the control (in the absence of insulin).
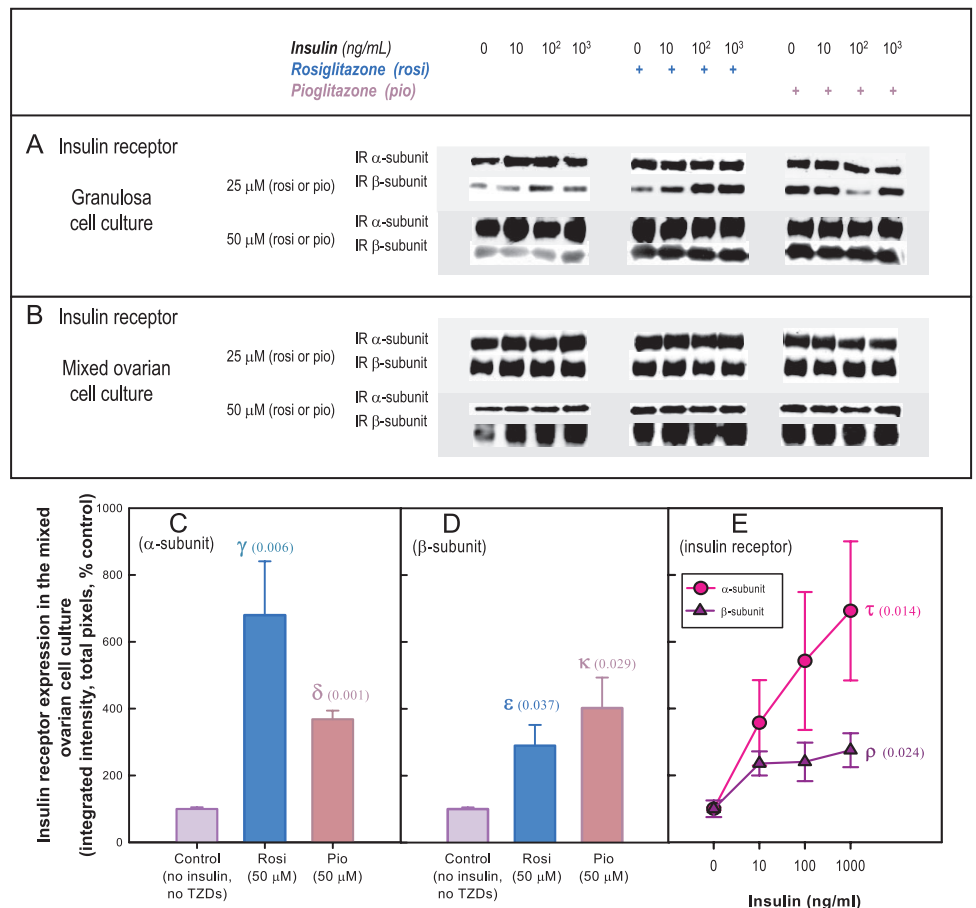
and pioglitazone up to 402% ($P < 0.029$) of baseline (Fig. 2D). When insulin was used with rosiglitazone (25 or 50 μM) or pioglitazone (25 or 50 μM), no significant additive effect on either α- or β-subunit insulin receptor expression was observed. Insulin stimulated expression of the insulin receptor α-subunit up to 692% ($P < 0.014$) (Fig. 2E) and of the insulin receptor β-subunit, up to 275% ($P < 0.024$) of baseline (Fig. 2E). The effect on insulin receptor expression observed with 25-μM concentration of TZDs was similar to that of 50 μM in both cell culture systems.

Insulin, rosiglitazone, and pioglitazone effects on IRS-1

Experiments demonstrating insulin, rosiglitazone (25 or 50 μM), or pioglitazone (25 or 50 μM) effects on IRS-1 expression produced similar results in both cell culture systems (Fig. 3, A and B). In the absence of insulin, rosiglitazone stimulated IRS-1 expression up to 323% ($P < 0.01$) and pioglitazone up to 492% ($P < 0.03$) of baseline (Fig. 3C). Insulin alone stimulated IRS-1 expression in a dose-dependent manner up to 431% ($P < 0.01$) (Fig. 3D). When a combination of insulin and rosiglitazone (25 or 50 μM) or insulin and pioglitazone (25 or 50 μM) was used, no significant additive effect on IRS-1 expression was observed. The effect of 25-μM concentration of TZDs on IRS-1 expression was similar to that of 50 μM in both cell culture systems.

Insulin, rosiglitazone, and pioglitazone effects on StAR protein expression

Figure 4A shows experiments that examined the effects of insulin, rosiglitazone, or pioglitazone (25 or 50 μM) on the StAR protein expression in human granulosa cell culture. In the absence of insulin, rosiglitazone stimulated StAR protein expression up to 540% ($P < 0.007$) and pioglitazone up to 670% ($P < 0.007$) of baseline (Fig. 4B). Insulin stimulated StAR protein expression in a dose-dependent manner up to 600% ($P < 0.012$) (Fig. 4C). When insulin was used with rosiglitazone (25 or 50 μM) or pioglitazone (25 or 50 μM), no significant additive effect on StAR protein expression was observed (Fig. 4A). The effect of 25-μM concentration of TZDs on StAR protein expression was similar to that of 50 μM.

Discussion

Classically, human ovarian function is thought to be regulated by gonadotropins (LH and FSH). In the last two decades, it became apparent that insulin also participates in the regulation of human ovarian function (22). Multiple effects of insulin that are relevant to the human ovary are either direct (*e.g.* stimulation of ovarian steroidogenesis) or indirect (*e.g.* effects on IGF binding protein-1 and SHBG production in the liver). The ovarian effects of insulin are both of physiological and pathophysiological significance. For example, insulin participates in the regulation of normal ovulation and
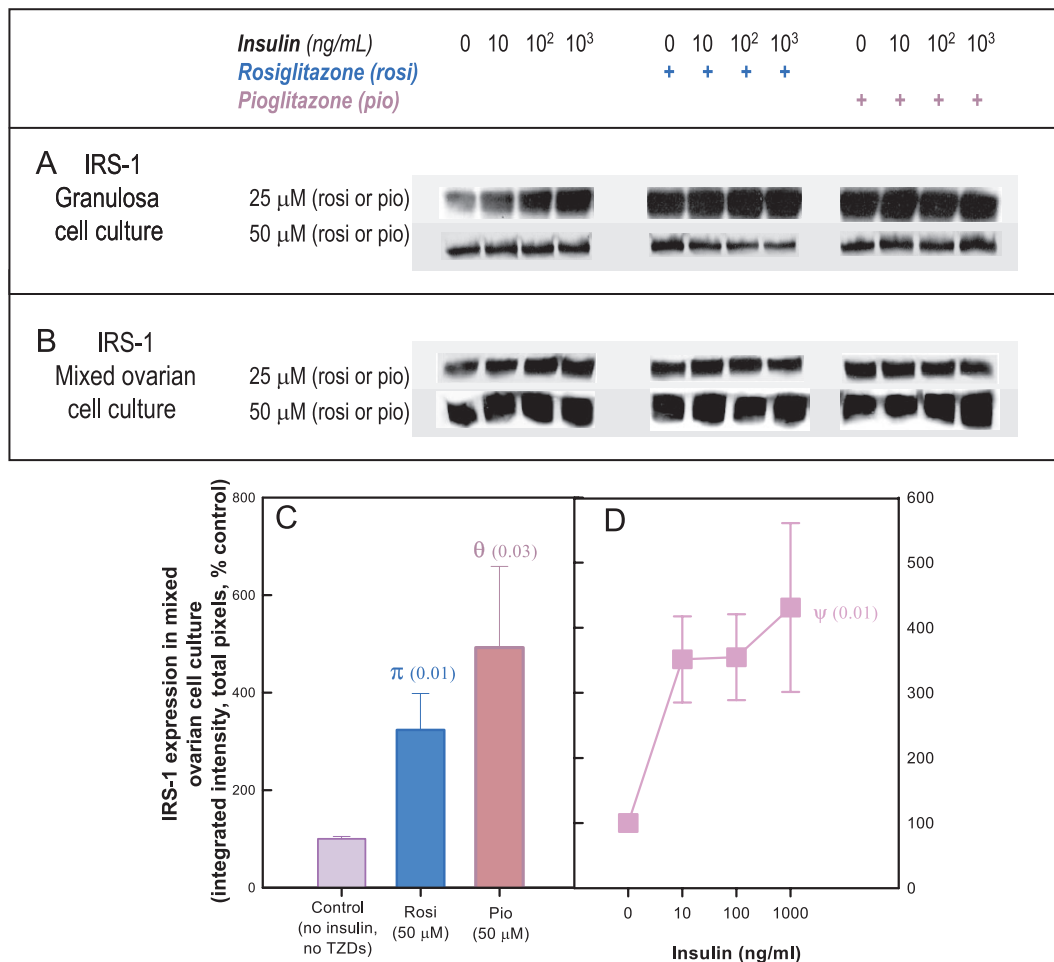
FIG. 3. The effects of TZDs on IRS-1 expression. A and B, Representative immunoblot analysis of IRS-1 expression in mixed ovarian cell culture or purified granulosa cell culture in the presence or absence of insulin, with or without rosiglitazone (rosi) or pioglitazone (pio) (25 or 50 μM). A, Granulosa cell culture. B, Mixed ovarian cell culture. C and D, Integrated intensity (total pixels) of the bands of IRS-1 expression. C, In the presence of 50 μM rosiglitazone or 50 μM pioglitazone, π [$P < 0.01$ (n = 12)] and θ [$P < 0.03$ (n = 12)] compared with the control (in the absence of rosiglitazone or pioglitazone). D, In the presence of insulin, ψ [$P < 0.01$ (n = 12)] compared with the control (in the absence of insulin).

can excessively stimulate ovarian androgen production in insulin-resistant states (22).

The concentrations of insulin, which were used in this study, ranged from physiological (1–10 ng/ml), to moderately supraphysiological (10–10^2 ng/ml), to significantly supraphysiological (up to 10^3 ng/ml). In physiological concentrations, insulin participates in the regulation of normal ovarian function (22). Moderately supraphysiological circulating concentrations of insulin are observed in patients with polycystic ovary syndrome (PCOS), obesity, or type 2 diabetes (22), whereas significantly supraphysiological concentrations are present in patients with syndromes of extreme insulin resistance and acanthosis nigricans (23–25). Thus, the range of insulin concentrations that we used in this study is relevant for both physiological and pathological conditions in the ovary.

Recently, it became apparent that the effects of insulin in its classical target tissues (*e.g.* fat and muscle) can be modulated by PPAR-γ agonists, TZDs, which improve insulin sensitivity in these organs. The resultant reduction of systemic hyperinsulinemia served as a rationale for therapeutic

trials of TZDs in patients with PCOS (26, 27). These trials indeed demonstrated a reduction of hyperandrogenism in PCOS patients. This effect of TZDs was attributed to their systemic insulin-sensitizing action, which leads to a reduction of systemic circulating insulin concentration and, consequently, a reduced ovarian androgen production.

However, very recently we demonstrated that the direct TZD action in the human ovary can be potentially of both physiological and pathophysiological significance (2). TZDs exhibited both insulin-independent and insulin-sensitizing direct effects on steroidogenesis and IGF binding protein-1 production in human ovarian cells (2). These data implied the presence of PPAR-γ in the human ovary.

Although transcription of PPAR-γ has been demonstrated in rat granulosa cells (28), to our knowledge, there are no published data on PPAR-γ protein expression in human ovarian cells. In this study we conclusively demonstrated that PPAR-γ is expressed in human ovarian cell cultures and that its expression in these cultures is enhanced by insulin and TZDs.

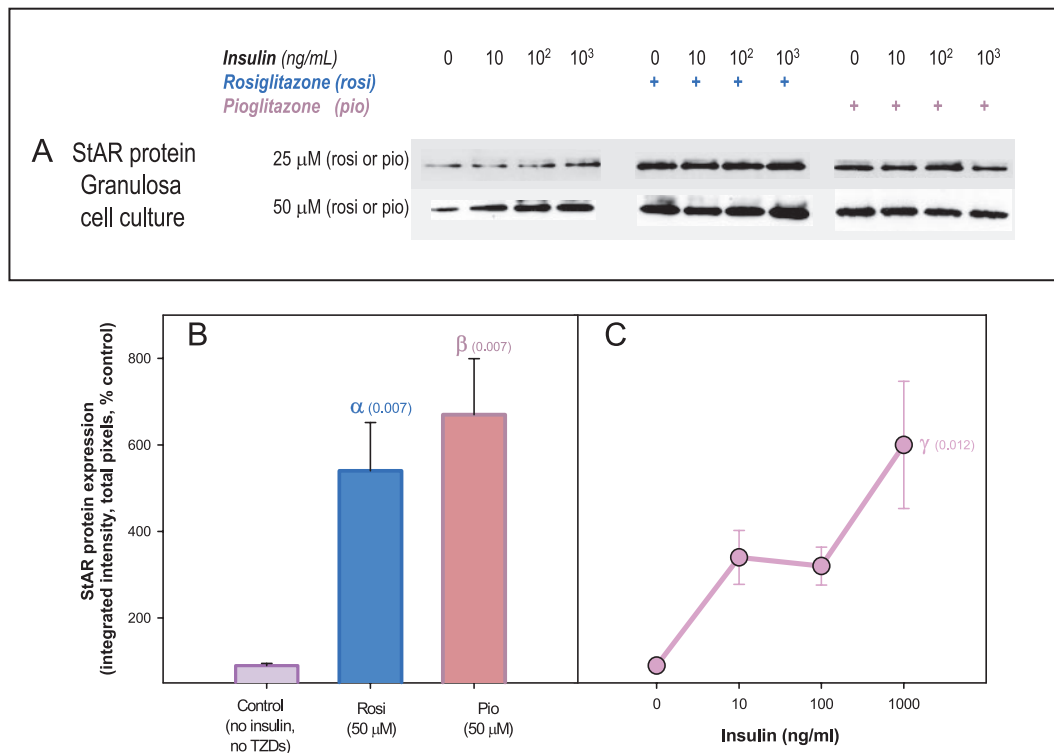The circulating concentration of TZDs achieved in patients

FIG. 4. The effects of TZDs on StAR protein expression in human granulosa cells. A, Representative immunoblot analysis of StAR protein expression in purified granulosa cell culture in the presence or absence of insulin, rosiglitazone (rosi) (25 or 50 μM), or pioglitazone (pio) (25 or 50 μM). B and C, Integrated intensity (total pixels) of the bands of StAR protein. B, In the presence of 50 μM rosiglitazone or 50 μM pioglitazone, α [$P < 0.007$ (n = 9)] and β [$P < 0.007$ (n = 9)] compared with the control (in the absence of rosiglitazone or pioglitazone). C, In the presence of insulin, γ [$P < 0.012$ (n = 9)] compared with the control (in the absence of insulin).

receiving these medications is in the range of 0.5–5 μM (29, 30). However, target tissue concentrations may be quite different [10–20 times higher; Law, R. (Takeda Pharmaceuticals North America, Inc., Deerfield, IL), personal communication]. Therefore, higher concentrations of these substances are usually used when tissue cultures are used to examine the effects of these agents. In our previous studies, we found the effective concentrations of rosiglitazone and pioglitazone for steroid hormone production to be between 20 and 30 μM (2). In the current study, we used 25 and 50-μM TZD concentrations and found that these concentrations produced similar effects.

A combination of rosiglitazone or pioglitazone (25 or 50 μM) and insulin (10–10^3 ng/ml) did not produce significant synergistic effects on the expression of PPAR-γ, insulin receptor, IRS-1, or StAR protein. These findings may appear to be in contrast with our earlier studies in which TZDs inhibited insulin-induced testosterone production and insulin/FSH-induced estradiol production (2). This discrepancy may be due to the fact that protein expression in the current study was examined at a single point after 18-h incubation. In the previously reported steroid hormone experiments (2), final steroid hormone concentration was a result of accumulated steroid hormone production over the time of the experiment. Further investigation of the initial rates of mRNA expression is needed to clarify whether it is affected by a combination of insulin with rosiglitazone or pioglitazone. The lack of synergism between insulin and TZDs in the current experiments may be also due

to the fact that maximal stimulation of protein expression by each of these stimulants was achieved, so that adding another stimulant had no additional effect. Finally, steroid hormone production is a multiple-step process, requiring activation of multiple enzymes, and is, therefore, quite different from activation of a single-protein synthesis measured in the current study. Therefore, the responses of these two distinct processes to TZDs may be quite different.

As mentioned previously, TZDs have been shown to increase insulin sensitivity, and affect metabolic regulation in adipose tissue, muscle, and liver. The mechanisms of these effects include PPAR-γ interaction with retinoid X receptors and activation of the gene transcription for a variety of proteins that participate in insulin signaling cascades. These proteins include IRS-1, phosphatidylinositol 3-kinase, Glut 4, Erk-MAPK, p38-MAPK, and Akt-MAPK (3–6, 31–33). We now report that in cultured human ovarian cells, like in classical insulin target tissues, TZDs interact with insulin receptor and its signaling pathway molecules.

To our knowledge, direct effects of TZDs on the insulin receptor in the human ovary have not been previously reported. In our experiments, both rosiglitazone and pioglitazone activated insulin receptor expression, the phenomenon that may explain, at least in part, insulin-sensitizing action of TZDs in the ovary.

It may appear surprising that insulin increased insulin receptor expression (total insulin receptors from membrane and cytoplasmic fractions) in both mixed ovarian cell culture

and purified granulosa cell culture, while it down-regulates insulin receptors in the muscle, fat, and liver cells (34). However, in human lymphocytes, hydrocortisone and insulin, acting synergistically, produced an increase in insulin receptor mRNA transcription (35). Furthermore, progestin has increased insulin receptor expression in human breast carcinoma cells (36), and androgens have promoted IGF-I receptor gene expression in the primate ovary (37). Because insulin enhances steroidogenesis in our ovarian cell culture (2, 13), the secondary effects of steroid hormones may explain the effect of insulin on its receptor expression (36, 37). An increased expression of insulin receptor α - and β-subunits under the influence of insulin also could be due to the formation of hybrid insulin/IGF-I receptors, which are up-regulated by insulin in other tissues (38–40). Finally, the methods that we used to measure the total insulin receptor protein included both cell membrane and the cytoplasmic compartments. Further examination of insulin receptor expression in different cellular compartments is needed to clarify the effect of insulin on the membrane receptor in this system.

As mentioned previously, TZDs increase insulin sensitivity and promote glucose transport in the classical target tissues, at least in part by activating IRS-1 (3, 4). In this study we demonstrated that TZDs activate both insulin receptor and IRS-1 expression in human ovarian cells. It remains unclear whether IRS-1 activation by TZDs is entirely dependent on activation of the insulin receptor. Regardless of the mechanism of IRS-1 activation, TZDs activated PPAR-γ, insulin receptor, IRS-1, and StAR protein maximally. Therefore, when TZDs were added to insulin, whether in moderate or high concentration, no additional effect was observed.

StAR protein plays a major role in the regulation of steroid hormone synthesis. It mediates the delivery of sterol substrate to cholesterol side-chain cleavage enzyme and initiates the enzymatic reactions of steroidogenesis (10, 14–16). IGF-I and LH or FSH regulates the StAR protein (8–11). In this report we demonstrate that insulin, rosiglitazone, or pioglitazone increased StAR protein expression in human granulosa cells. These results suggest that activation of steroid hormone synthesis in human ovarian cells by insulin or TZDs may be mediated via activation of StAR protein.

Granulosa cells were used for StAR protein expression experiments because steroidogenesis could not be initiated on our mixed ovarian cell culture system when cholesterol was used as a substrate, indicating a possible loss of the cholesterol cleavage enzyme or its cofactors (10, 14). Furthermore, the mixed ovarian cell culture expressed both active (30 kDa) and inactive (37 kDa) forms of StAR protein (15, 16), while granulosa cell cultures expressed only the active (30 kDa) form. Therefore, only granulosa cell culture was used to examine the active StAR protein expression.

Our previous report (2) demonstrated that, while TZDs stimulated progesterone production, they inhibited estradiol and testosterone production in human ovarian cells. However, in these previously published experiments, the StAR protein involvement in steroidogenesis was unlikely because the substrates that we provided for synthesis of the three latter hormones (pregnenolone for progesterone synthesis, testosterone for estradiol synthesis, and dehydroepiandrosterone for testosterone synthesis) were distal to the StAR

protein. Further studies of the TZD role in the regulation of the synthesis of specific steroid hormones are needed.

We propose hypothetical interactions among PPAR-γ, insulin signaling pathways, and StAR protein in human ovarian cells, as shown in Fig. 5. Both insulin (by activating insulin receptor) and TZDs (by activating PPAR-γ) may lead to stimulation of StAR protein expression. In addition, TZDs activate insulin receptor expression while insulin activates expression of PPAR-γ, further enhancing StAR protein. Furthermore, both insulin and PPAR-γ stimulate the expression of a downstream component of insulin signaling pathway, IRS-1, the role of which in mediating the ovarian effects of insulin needs to be clarified. These interactions may be important under both physiological and pathological conditions. The former include regulation of ovulation and normal steroidogenesis; the latter include PCOS and syndromes of extreme insulin resistance and acanthosis nigricans. Finally, the effects of PPAR-γ in this system, as discussed earlier, appear to have therapeutic implications. For example, stimulation of insulin receptor or hybrid insulin/IGF-I receptor by TZDs may increase ovarian sensitivity to insulin and/or IGFs that, when combined with the direct inhibitory effect of TZDs on androgen synthesis (2), may lead to an improved ovulatory function in patients with PCOS.

We conclude that PPAR-γ is present in human ovarian cells. Activation of PPAR-γ enhances steroidogenesis via activation of StAR protein and leads to the activation of insulin-signaling pathways. Insulin or TZDs up-regulate PPAR-γ, insulin receptor, IRS-1. and StAR protein expression. Thus, insulin receptor, PPAR-γ, and StAR protein constitute a novel system of ovarian regulation with complex interactions among its components.
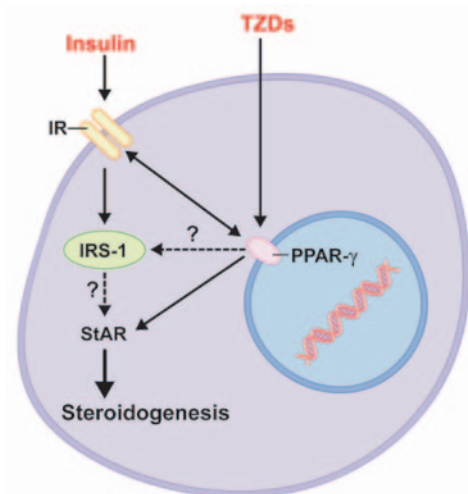

FIG. 5. Proposed interactions among PPAR-γ, insulin receptor (IR), IRS-1, and StAR protein in human ovarian cells. Both insulin (by activating primarily insulin receptor) and TZDs (by activating primarily PPAR-γ) lead to stimulation of StAR protein expression. In addition, TZDs activate insulin receptor expression while insulin activates expression of PPAR-γ, thus further enhancing StAR protein expression and stimulating steroidogenesis. Both insulin and TZDs activate a downstream component of insulin signaling pathway, IRS-1. This effect of TZDs may be mediated with or without activation of the insulin receptor.

Acknowledgments

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