Direct Thiazolidinedione Action in the Human Ovary: Insulin-Independent and Insulin-Sensitizing Effects on Steroidogenesis and Insulin-Like Growth Factor Binding Protein-1 Production

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Context and Objective: Hyperinsulinemia contributes to the pathogenesis of ovarian dysfunction in insulin-resistant states, including polycystic ovary syndrome (PCOS). Peroxisome proliferator activated receptor-γ (PPAR-γ) agonists [thiazolidinediones (TZDs)] ameliorate hyperandrogenism in polycystic ovary syndrome presumably because they reduce systemic hyperinsulinemia. Direct effects of TZDs in the ovary, however, cannot be excluded. We explored direct effects of TZDs in cultured human ovarian cells.

Methods: Human ovarian cells, obtained from oophorectomy specimens, were cultured in the presence or absence of rosiglitazone or pioglitazone. Steroid hormone and IGF-binding protein-1 (IGFBP-1) concentrations were measured in conditioned tissue culture medium.

Results: Rosiglitazone or pioglitazone stimulated progesterone production up to 156% (P < 0.001) and 131% (P < 0.001) of baseline, respectively. Pioglitazone but not rosiglitazone, inhibited baseline and FSH-stimulated estradiol production by 20% (P < 0.001) and 50% (P < 0.001), respectively. Both rosiglitazone and pioglitazone abolished insulin-dependent stimulation of estradiol production in the presence of FSH. Rosiglitazone and pioglitazone inhibited testosterone production by 10% (P < 0.012) and 15% (P < 0.023), respectively, and abolished insulin-induced stimulation of testosterone production. In the absence of insulin, pioglitazone or rosiglitazone stimulated IGFBP-1 production up to 160% (P < 0.001) and 125% (P < 0.036) of baseline, respectively. Pioglitazone and rosiglitazone enhanced insulin-induced inhibition of IGFBP-1 production by 13% and 20%, respectively (P < 0.001).

Conclusions: PPAR-γ agonists directly stimulate progesterone and IGFBP-1 production, inhibit estradiol and testosterone production, abolish insulin-induced stimulation of testosterone production and insulin-dependent stimulation of estradiol production in the presence of FSH, and enhance insulin-induced inhibition of IGFBP-1 production in human ovarian cells. PPAR-γ represents a novel system of ovarian regulation. (J Clin Endocrinol Metab 90: 6099–6105, 2005)

In insulin participates in the regulation of ovarian steroidogenesis and folliculogenesis and plays an important role in the pathogenesis of ovarian dysfunction in patients with insulin-resistant states (1–5). It is thought that in these conditions hyperinsulinemia excessively stimulates ovarian androgen production, contributing to hyperandrogenism, anovulation, and infertility (1, 4, 5). On the other hand, insulin inhibits IGF-binding protein-1 (IGFBP-1) production, resulting in increased bioavailability of IGFs and enhancement of folliculogenesis (1).

Ovarian hyperandrogenism, commonly observed in insulin-resistant states, can be ameliorated by insulin-sensitizing agents, including thiazolidinediones (TZDs) (6–12). TZDs act as peroxisome proliferator activated receptor-γ (PPAR-γ) agonists and include, among other agents, troglitazone, rosiglitazone, and pioglitazone. Recent studies suggested that, in addition to reducing circulating androgen levels (9–12), TZDs can normalize (increase) circulating progesterone levels, which are commonly reduced in patients with polycystic ovary syndrome (PCOS) (13) and in a primate model of this syndrome (14).

Although it is thought that the inhibitory effects of TZDs on ovarian androgen production are a result of the reduction of circulating insulin levels induced by TZDs, in several in vitro and in vivo studies in animal and human ovarian cells, TZDs have been reported to directly inhibit or increase activity of ovarian steroidogenic enzymes (15–20), progesterone (18), and estradiol (14, 19) production as well as androgen biosynthesis induced by LH and insulin (20).

In this report, we explored whether PPAR-γ agonists, rosiglitazone and pioglitazone, have direct effects on ovarian steroidogenesis and IGFBP-1 production.

Patients and Methods

All studies described in this report were approved by the Institutional Review Board at Beth Israel Medical Center. Patient’s consent, allowing the use of ovarian tissue in research studies, was obtained before surgery.
**Patients**

Ovarian tissue samples were collected from 25 women at the time of indicated oophorectomy. All patients were premenopausal or perimenopausal. The patient age ranged from 25–54 yr. No patient was using hormonal contraception or other hormonal therapy at the time of oophorectomy. Reasons for oophorectomy included follicular or dermoid cysts and benign uterine or ovarian neoplasms. Only unaffected ovarian tissue was used for the cell culture. For each experiment, the ovarian tissue from a single patient was used. The experiments were repeated with ovarian tissue obtained from four to 10 patients.

**Cell culture system**

The cell culture used in these studies was developed and previously described by us (21). Briefly, the unaffected ovarian tissue was obtained from oophorectomy specimens after dissection, which separated cysts or benign tumors from the tissue used to establish the culture. Tissue fragments were placed in the McCoy’s 5A tissue culture medium supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, CA), 100 μg/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin. The capsule was removed, and the tissue was cut into small pieces (~0.5 cm² × 0.1 cm) and then homogenized in a glass homogenizer. The tissue was then plated in 60 × 15-mm tissue culture dishes. Sterile Vaseline was placed around the tissue fragments, and the fragments were covered with a 24 × 30-mm glass cover slide. Four milliliters of the culture medium were added to the culture dishes. The ovarian tissue was cultured without passing (usually for 2–5 wk) until the cells were confluent. The cells were then trypsinized and subcultured until adequate cell numbers required for the experiments were available. Enzymatic and immunocytochemistry studies demonstrated the presence of stroma, theca, and granulosa cells in this tissue culture system (21).

**IGFBP-1 production**

The cells were incubated in tissue culture medium as described in the experiments assessing progesterone production, except that, during the final 24-h incubation, the medium was supplemented with 0.1% (rather than 1%) FBS. A lower concentration of FBS was used because FBS, while increasing cell survival, inhibits IGFBP-1 synthesis (23, 24). The medium was supplemented with 0, 10, 10², 10³, or 10⁴ ng/ml insulin in the presence of 50 μM rosiglitazone or pioglitazone. IGFBP-1 concentration in the conditioned medium was determined by immunoradiometric assay (Diagnostic Systems Laboratories).

**Materials**

Dehydroepiandrosterone, pregnenolone, progesterone, and testosterone were obtained from Sigma Chemical Co. (St. Louis, MO). LH and FSH were obtained from Calbiochem (San Diego, CA). Insulin was obtained from Roche Applied Science (Indianapolis, IN). Rosiglitazone was obtained from Cayman Chemical (Ann Arbor, MI), and pioglitazone was obtained from Takeda Pharmaceuticals America, Inc. (Lincolnshire, IL).

**Statistical analysis**

All experiments were carried out in quadruplicate and repeated four to 10 times. 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. Because absolute concentration of steroid hormones and IGFBP-1 in the tissue culture medium at baseline differed among the samples from different patients, the results were normalized to the each sample’s baseline level, which was considered 100%.

**Results**

The concentration of progesterone in the conditioned tissue culture medium at baseline ranged from 300–600 nmol/liter, of estradiol from 1.1–4.8 nmol/liter, of testosterone from 70–420 nmol/liter, and of IGFBP-1 from 0.05–0.31 nmol/liter.

**Progesterone production**

Insulin alone stimulated progesterone production up to 125% of baseline (P < 0.004) (Fig. 1). Rosiglitazone (30 μM) alone stimulated progesterone production up to 156% (P < 0.001), and pioglitazone (30 μM) alone stimulated progesterone production up to 131% of baseline (P < 0.001). Adding insulin to either rosiglitazone or pioglitazone produced no significant additional effect on progesterone production. There was no statistically significant difference between rosiglitazone or pioglitazone effects on progesterone production.

**Estradiol production**

Insulin alone stimulated estradiol production up to 122% of baseline (P < 0.049) (Fig. 2A). In the absence of insulin, pioglitazone (30 μM) inhibited estradiol production by 15% (P < 0.003), whereas rosiglitazone had no effect. The stimulatory effect of insulin on estradiol production was reduced by 20% (P < 0.001) in the presence of pioglitazone, whereas rosiglitazone, once again, had no effect. Pioglitazone inhibited FSH-dependent estradiol production by 50% (P < 0.001) (Fig. 2B), whereas rosiglitazone had no effect. In the presence of FSH (100 ng/ml), insulin-induced estradiol production was stimulated up to 160% of baseline (P < 0.001). Rosigli-
tazone and pioglitazone completely abolished insulin-dependent stimulation of estradiol production in the presence of FSH. The inhibitory effect of pioglitazone (30 μM) on FSH/insulin-stimulated estradiol production was more potent than that of rosiglitazone (P < 0.001).

**Testosterone production**

Insulin alone stimulated testosterone production up to 131% of baseline (P < 0.001) (Fig. 3). Rosiglitazone (20 μM) alone inhibited testosterone production by up to 10% (P < 0.012), and pioglitazone (20 μM) alone inhibited testosterone production by up to 15% (P < 0.023). Rosiglitazone and pioglitazone completely abolished insulin-induced stimulation of testosterone production (Fig. 3). The inhibitory effect of pioglitazone on basal and insulin-induced testosterone production was more potent than that of rosiglitazone (P < 0.05).

**IGFBP-1 production**

In the absence of insulin, rosiglitazone (50 μM) stimulated IGFBP-1 production up to 160% of baseline (P < 0.001) and pioglitazone (50 μM) up to 125% of baseline (P < 0.036) (Fig. 4A). The stimulatory effect of rosiglitazone on IGFBP-1 production in the absence of insulin was more potent than that of pioglitazone (P < 0.024). Insulin alone inhibited IGFBP-1 production.
duction by 42% (P < 0.001) (Fig. 4B). In the presence of insulin, pioglitazone enhanced insulin-induced inhibition of IGFBP-1 production by 13% (P < 0.001, compared to insulin alone) and rosiglitazone by 20% (P < 0.001, compared to insulin alone) (Fig. 4B). There was no statistically significant difference between the effects of pioglitazone or rosiglitazone on insulin-induced inhibition of IGFBP-1 production.

Discussion

Traditionally, gonadotropins have been viewed as exclusive regulators of ovarian function. More recently, it has been demonstrated that insulin and IGFs, their receptors, IGFBPs, and IGFBP-proteases participate in the regulation of ovarian function (1–5, 25–27). We examined...
whether PPAR-γ may represent yet another system of ovarian regulation.

In this report, we demonstrated that PPAR-γ agonists rosiglitazone and pioglitazone directly regulate ovarian steroidogenesis. Both rosiglitazone and pioglitazone enhanced progesterone production and inhibited testosterone synthesis. Pioglitazone inhibited basal and FSH-stimulated estradiol production whereas rosiglitazone had no significant effect. However, both PPAR-γ agonists completely abolished stimulation of estradiol production induced synergistically by FSH and insulin as well as insulin-induced stimulation of testosterone production. In the absence of insulin, both pioglitazone and rosiglitazone stimulated IGFBP-1 production. Both TZDs, however, enhanced insulin-induced inhibition of IGFBP-1 production.

The cell system, used in this study, has been described by us in detail and is derived from oophorectomy specimens (21). Our cell culture system contains cells from three types of ovarian steroidogenic compartments: granulosa, theca, and stroma. In this kind of system, it is difficult to be certain whether an observed effect is a result of a primary action on the cells representing one compartment, because primary effects on one cellular compartment may produce secondary effects in another compartment. For example, if theca cells are stimulated by gonadotropins or insulin to increase production of androgens (primary effect), the increased amount of androgens may be used as a substrate for aromatization by granulosa cells, resulting in increased synthesis of estrogens (secondary effect). Experiments with isolated cell cultures derived from a single steroidogenic cell compartment (i.e. isolated granulosa, stroma, or theca cultures) are necessary to clarify these mechanisms. However, our cell culture system, which contains all steroidogenic ovarian compartments, reflects normal ovarian physiology in vivo, where all cellular components are present simultaneously and interact with each other.

Oophorectomy specimens were used as a source for our cell culture system. Although we used unafected ovarian tissue, our cells may have been influenced by pathological processes for which oophorectomies were performed (28). In each of our experiments, however, a specimen from a single patient was used for both control and experimental cell samples. The experiments were repeated with cells from four to ten patients, and although the absolute amount of steroid hormones and IGFBP-1 produced by the cells varied among the patients, similar effects were observed in tissue samples obtained from different patients. Furthermore, in some patients, the pathology for which oophorectomy was performed involved only the uterus.

The concentrations of insulin that we used ranged from physiological (1–10 ng/ml), to moderately supraphysiological (10–100 ng/ml), to significantly supraphysiological (up to 10^4 ng/ml). The significance of studying physiological concentrations of insulin is quite clear, because insulin participates in the regulation of normal ovarian function, including steroidogenesis and ovulation (1). Moderately supraphysiological circulating concentrations of insulin are observed in patients with PCOS (1), whereas significantly supraphysiological concentrations are present in patients with the syndromes of extreme insulin resistance and acanthosis nigricans (1–4). Thus, our data regarding the response of cultured ovarian cells to insulin are relevant for both physiological and pathological conditions in the ovary.

The concentrations of rosiglitazone and pioglitazone that we used were chosen in dose-response experiments based on the optimal cell responses. Circulating concentrations of pioglitazone in patients receiving this compound are approximately 5 μM (29), whereas for rosiglitazone, these concentrations range from 0.25–2.5 μM (30). Target tissue concentrations of these TZDs, however, are 10–20 times higher than circulating concentrations (Dr. Ronald Law, Takeda Pharmaceuticals, personal communication), thus approximating concentrations used in our studies. Furthermore, concentrations of rosiglitazone or pioglitazone used in other reported in vitro studies ranged from 10–25 μM (18,31) and are therefore comparable to the concentrations used by us (20–50 μM).

At the concentrations that we examined, pioglitazone appeared to be more potent than rosiglitazone in its ability to inhibit estradiol or testosterone production, whereas rosiglitazone appeared to be more potent than pioglitazone in its ability to stimulate insulin-independent IGFBP-1 production. Pioglitazone and rosiglitazone exhibited similar potency in their effects on progesterone and on insulin-induced inhibition of IGFBP-1 production. Differences in the potency of different TZDs are commonly observed when these agents are used in vivo and may be due to their different binding characteristics in relation to PPAR-γ (32) or, possibly, due to different signaling mechanisms used by TZDs for their specific effects.

In our studies, TZDs had a clear stimulatory effect on progesterone production. It appears that under our experimental conditions, TZDs alone were capable of maximizing progesterone response. Once this response was maximized, insulin had no additional effect, although it had a potent independent effect on progesterone production in the absence of TZDs. The stimulatory effect of TZDs on progesterone production in our cell system is in agreement with in vivo studies, which demonstrated normalization of the reduced circulating progesterone levels in patients with PCOS by TZDs (13).

We demonstrated that pioglitazone inhibited basal and FSH-stimulated estradiol production, whereas both rosiglitazone and pioglitazone abolished insulin-dependent stimulation of estradiol production in the presence of FSH. These findings may be either a result of the direct effect of TZDs on estradiol production or due to TZD-induced inhibition of testosterone production, because testosterone serves as a substrate for aromatase-dependent estradiol production. Additionally, TZDs may have direct inhibitory effect on aromatase (16). In our system, we would not expect testosterone concentration in the tissue culture medium to increase, even if aromatase activity is inhibited, because in the studies of estradiol production, we used a high concentration of testosterone as substrate (30 μM), whereas the concentration of estradiol as the end product was approximately 6 nM. Under these conditions, a small rise in the testosterone concentration in the conditioned tissue culture medium because of the inhibition of aromatase would not be detected.

In vivo experiments (19), in agreement with our studies, pioglitazone inhibited FSH-stimulated estradiol production.
In the same study, pioglitazone also prolonged the estradiol peak observed in the course of the ovulatory cycle, an effect that could not be examined in our in vitro system. As we will discuss below, in vitro and in vivo TZD effects can be quite different because of the differences between the mechanisms involved in the direct TZD action and their systemic insulin-sensitizing activity.

TZDs in our cell culture system had a significant direct inhibitory effect on testosterone production, in agreement with in vivo observations in patients with PCOS (9–11). Our studies suggest, however, that inhibition of testosterone production observed in PCOS patients treated with TZDs may be caused not only by a reduction of hyperinsulinemia (which occurs because of systemic insulin-sensitizing activity of TZDs) but also because of the direct action of TZDs in the ovary, specifically because of their direct inhibitory effect on testosterone synthesis.

In an interesting display of the differences between insulin-independent and insulin-sensitizing effects of TZDs, pioglitazone and rosiglitazone stimulated IGFBP-1 production by ovarian cells in the absence of insulin but enhanced insulin-induced inhibition of IGFBP-1 production. The latter effect could presumably increase bioavailability of IGFs, thus enhancing the role of IGF in the development of ovulatory follicles (1, 33). In PCOS patients treated with rosiglitazone, plasma levels of IGFBP-1 were reported to increase (34). This observation is unlikely to be a result of a direct stimulatory effect of TZDs on IGFBP-1 production in the ovary but rather due to the systemic insulin-sensitizing action of TZDs: with a reduction of hyperinsulinemia, circulating levels of IGFBP-1 will be expected to rise because IGFBP-1 synthesis in the liver is under inhibitory control of insulin (1, 35).

The opposite (stimulation or inhibition) direct effects of TZDs on specific steroidogenic pathways and the differences between their insulin-dependent vs. insulin-independent effects may occur because of variable signaling pathways used by TZDs for their specific effects. Similarly, a variety of signaling mechanisms has been demonstrated to exist for the ovaries of insulin. For example, phosphatidylinositol-3 kinase (36) or MAPK (37) activation is not necessary for the insulin effects on steroidogenesis, indicating that classical insulin-signaling pathways, used for glucose transport and for mitogenic activity, differ from those involved in insulin effects on steroidogenesis. Furthermore, unlike steroidogenic effects of insulin, IGF-I effects on steroidogenesis, which are mediated by type I IGF receptors, are MAPK dependent (37). The latter observation is important because in supraphysiological concentrations, insulin up-regulates and activates type I IGF receptors in the ovary (1, 38). In summary, in analogy with insulin and IGFs, TZDs may employ multiple postreceptor signaling pathways, thus explaining a variety of their effects in the ovary.

The multiplicity of TZD effects in the ovary can be best understood in terms of their direct effects (both insulin-independent and insulin-sensitizing) vs. the indirect effects, which are caused by the systemic insulin-sensitizing action of the TZDs leading to a reduction in hyperinsulinemia (Table 1). Direct effects can be demonstrated in vitro and may be present in vivo, although in some cases, they may be minimized or even abolished by the systemic insulin-sensitizing TZD action. For example, TZDs increase IGFBP-1 production by ovarian cells (direct effect demonstrated in vitro) but enhance insulin-induced inhibition of IGFBP-1 production (direct insulin-sensitizing effect, also demonstrated in vitro). In vivo, because IGFBP-1 production in the liver is under inhibitory control of insulin (1, 35) and because circulating insulin levels fall under the influence of TZDs, TZDs produce an increase in circulating IGFBP-1 levels (34).

Another example of the interaction between the direct and indirect TZD effects would be their influence on the circulating testosterone levels. The direct effect of TZDs leads to a reduction of both insulin-independent and insulin-dependent testosterone production in the ovary. In vivo, in addition, circulatory free testosterone levels are reduced because of systemic insulin-sensitizing action of TZDs: since the production of SHBG in the liver is under the inhibitory control of insulin (1, 39), reduction of hyperinsulinemia leads to a rise of SHBG and a fall in circulating free testosterone levels (1, 8).

The multiplicity of TZD effects, combined with the potential multiplicity of signaling mechanisms employed by TZDs, provide for a complex novel system of ovarian regulation by PPAR-γ.

We conclude that, in cultured human ovarian cells, TZDs 1) enhance progesterone production, 2) inhibit estradiol and testosterone production, 3) completely abolish insulin-induced stimulation of testosterone production, 4) completely abolish insulin-induced stimulation of estradiol production in the presence of FSH, 5) stimulate insulin-independent IGFBP-1 production, and 6) enhance insulin-induced inhi-

| TABLE 1. Effects of TZDs related to ovarian function |

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<th>Direct: can be observed in vitro; may be present in vivo</th>
<th>Indirect: observed in vivo; are due to systemic insulin-sensitizing action and reduction of hyperinsulinemia</th>
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<td>† IGFBP-1 production</td>
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†, Increased; ↓, decreased. Please see the text for references.
bition of IGFBP-1 production. Thus, PPAR-γ represents a novel regulatory system in the human ovary.

Acknowledgments

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