Rosiglitazone and Pioglitazone Inhibit Estrogen Synthesis in Human Granulosa Cells by Interfering with Androgen Binding to Aromatase

Authors D. Seto-Young[1], D. Avtanski[1], G. Parikh[1], P. Suwandhi[1], M. Strizhevsky[1], T. Araki[1], Z. Rosenwaks[2], L. Poretsky[1]

Affiliations [1] G. J. Friedman Diabetes Institute and the Division of Endocrinology, Department of Medicine, Beth Israel Medical Center and Albert Einstein College of Medicine, New York, USA
[2] The Ronald O. Perelman and Claudia Cohen Center for Reproductive Medicine, Weill Medical College of Cornell University, New York, USA

Correspondence
D. Seto-Young, PhD
L. Poretsky, MD
Division of Endocrinology
Beth Israel Medical Center
317 East 17th Street
Fierman Hall, 7th Floor
NY 10003
New York
USA
Tel.: +1/212/420 4666
Fax: +1/212/420 2224
dyoung@chpnet.org
lporetsk@chpnet.org

Abstract
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The effects of rosiglitazone or pioglitazone (thiazolidinediones, TZDs) on estrogen production and aromatase activity in human ovarian cells were examined. Human granulosa cells were incubated in the tissue culture medium supplemented with androstenedione or testosterone, with or without insulin, TZDs, or type 1 17β-hydroxysteroid-dehydrogenase (17β-HSD) inhibitor. Estrogen concentrations in the conditioned medium, aromatase mRNA and protein expression in the cells and androgen substrate binding to aromatase were measured. With androstenedione as substrate, rosiglitazone or pioglitazone inhibited estrone production by up to 22 % (p < 0.012) while type 1 17β-HSD inhibitor enhanced this effect of rosiglitazone or pioglitazone by 37 % (p < 0.001) and by 67 % (p < 0.001), respectively. With testosterone as substrate, rosiglitazone or pioglitazone inhibited estradiol production by 32 % (p < 0.001). With ^{3}H-testosterone as substrate, rosiglitazone or pioglitazone inhibited the ^{3}H-tritiated water release by the cultured cells by 45 % and 35 %, respectively, thus directly demonstrating inhibition of aromatase. Rosiglitazone or pioglitazone, however, had no significant effect on aromatase mRNA or protein expression. Rosiglitazone or pioglitazone inhibited ^{125}I-androstenedione and ^{125}I-testosterone binding to aromatase by 38 % (p < 0.001). It was concluded that rosiglitazone or pioglitazone inhibit estrogen synthesis in human granulosa cells by interfering with androgen binding to aromatase.

Introduction
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Thiazolidinediones (TZDs – troglitazone, rosiglitazone, and pioglitazone) are peroxisome proliferator activated receptor-γ (PPAR-γ) agonists, which can be used in the treatment of type 2 diabetes because of their insulin-sensitizing properties. TZDs have been also used as therapeutic agents for women with polycystic ovary syndrome (PCOS) since they improve ovulatory rates and reduce androgen levels [1–4]. Although the effects of TZDs in the female reproductive system have been attributed to their systemic insulin-sensitizing effects and consequent reduction in hyperinsulinemia, TZDs also directly affect androgen and estrogen production in human ovarian cells [5].

Aromatase is an enzyme of cytochrome P450 super-family, which converts androgens to estrogens. Published reports of the TZD effects on aromatase activity in the ovary are controversial. Gasic et al. reported absence of troglitazone effects on aromatase in porcine granulosa cells [6], while others reported troglitazone-induced suppression of aromatase expression and activity in human granulosa cells and in granulosa carcinoma cell lines [7,8]. Our previous studies suggested that rosiglitazone or pioglitazone directly inhibit estrogen synthesis in human ovarian cell culture containing stromal, thecal, and granulosa cells [5]. The goal of the studies presented in this report was to determine the mechanisms of rosiglitazone or pioglitazone effect on estrogen synthesis by examining aromatase enzyme activity, mRNA, and protein expression in human granulosa cells.

Methods and Materials
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Institutional review boards at Beth Israel Medical Center and Weill Medical College of Cornel University approved all studies described in this report.

Human granulosa cell culture

Human granulosa cells obtained during in vitro fertilization (IVF) were pooled from several patients at a time to ensure adequate cell number for the experiments. Diagnoses included male factor, tubal factor and uterine factor infertility, endometriosis or anovulation.

Human granulosa cells obtained during IVF were purified on Percoll gradients and cultured as previously described [9]. Luteinized cells in the folliculicular fluid obtained from the IVF patients were immediately centrifuged at $338\,g$ (Hettich/Zentrifuge, 4723 swing out rotor) for 10 min at 4 °C. The cell pellets were resuspended in Hanks' Balanced salt solution (HBSS) supplemented with 10 µg/ml gentamicin and 250 ng/ml amphotericin (15 ml of HBSS per patient). 6 ml of the granulosa cell suspension were placed on top of the 5 ml of 50 % Percoll/HBSS. The mixture was centrifuged at $528\,g$ for 35 min at 4 °C. The granulosa cells were sedimented in the interface between 50 % Percoll/HBSS and the aqueous phase of HBSS. The purified granulosa cells were removed by small graduated transfer pipettes. The collected granulosa cells were purified once again with 50 % Percoll/HBSS gradient under the conditions described above, except that they were now resuspended at the buffer volume 7.5 ml per patient. 6 ml of the cell suspension were placed on top of the 5 ml of 50 % Percoll/HBSS and centrifuged again under the conditions described above. Purified human granulosa cells were then resuspended in M199 medium supplemented with 10 % FBS, 10 µg/ml gentamicin and 250 ng/ml amphotericin. 1 ml of 0.5×10^5 cells/ml suspension (placed in 24-well tissue culture plates) was used for steroid hormone experiments and 15 ml of 0.5×10^5 cells/ml suspension (placed in 100 mm^2 tissue culture plates) were used for immunoblot, RT-PCR and in the studies examining specific binding of androgens to aromatase.

Prior to the experiments, the purified granulosa cells were incubated for 48 h at 37 °C, 5 % CO_2, 90 % humidity in M199 tissue culture medium supplemented with 10 % FBS, 10 µg/ml gentamicin and 250 ng/ml amphotericin. After 48 h of incubation, the medium supplemented with 10 % FBS was replaced by medium with 2 % FBS and the cells were incubated for another 24 h. Thus, the granulosa cells were incubated in the tissue culture medium for 72 h before the experiments.

For the studies of TZD effect on estrogen synthesis, cells were incubated in tissue culture medium with or without 3 µM of testosterone or androstenedione as substrates, and in the presence or absence of insulin (0–10^3 ng/ml), with or without rosiglitazone or pioglitazone (25 µM), with or without type 1 17β-HSD inhibitor.

Method described by Balthazar et al. [10] was used to directly quantify aromatase activity by measuring ^{3}H-tritiated water production from 1β-^{3}H-testosterone. Purified human granulosa cells (0.1×10^3/ml) were incubated with 0.5 ml of 1β-^{3}H-testosterone (1 µCi/ml, 100 nM), with or without rosiglitazone or pioglitazone (25 µM) for 18 h. An amount of 0.4 ml of the conditioned tissue culture medium was placed on ice. Cold TCA and activated charcoal were added to the mixture to achieve final concentrations of 10 % TCA and 2 % activated charcoal. Samples were mixed and centrifuged at $9300\,g$ for 2 min. 350 µl of supernatant were filtered by cation-exchange spin columns prewashed with 400 µl NaOAc, pH 6. The filtrate was collected by centrifugation at $2300\,g$ for 5 min. The ^{3}H-tritiated water samples were counted in Beckman Liquid Scintillation counter (LS-500).

Cell viability count and cell toxicity

Human granulosa cells were incubated with or without rosiglitazone or pioglitazone (10, 25, and 50 µM). The treated cells were trypzinized, centrifuged, resuspended in tissue culture medium and counted in the hemocytometer using the trypan blue method. Rosiglitazone or pioglitazone had no significant effect on cell viability. Rosiglitazone or pioglitazone (25 µM and 50 µM) had no effect on the mRNA or protein expression of aromatase.

Protein measurement

Protein concentrations were determined by modification of Lowry protein assay.

Radioimmunoassay and immunosorbent assay

Estradiol concentration in the tissue culture medium was measured using radioimmunoassay (RIA, active-antibody coated tube). Estrone concentration was measured using enzyme-linked immunosorbent assay (ELISA) because the active-antibody coated tube RIA for estrone was not available.

Aromatase enzyme expression using immunoprecipitation and immunoblotting procedure

Rabbit anti-human aromatase antibodies (1.5 µg) or anti-human β-actin antibodies were added to 1 ml of cell lysate (15–20 mg of protein). Immunoprecipitation and immunoblotting procedures were performed as previously described [9]. Mouse anti-human aromatase antibodies (1/100 dilution) or mouse anti-human β-actin antibodies (0.5 µg/ml) were used for probing the expressed aromatase. The bands were detected by chemiluminescence.

Total RNA isolation and RT-PCR

Total RNA was isolated using RNAqueous-4PCR kit. Concentration of RNA was determined by reading the absorbance in spectrophotometer at 260 nm and 280 nm.

GeneAmp-EZrTth RNA PCR kit was used for RT-PCR reaction. Thermo-stable recombinant DNA polymerase was used as both a reverse transcriptase and as a DNA polymerase in a single PCR reaction. Aromatase forward primer was 5′-ACC-CTT-CTG-CGT-CGT-GTC-A-3′ and reverse primer was 5′-GAA-CTT-CTA-TGG-CAT-CTT-TCA-AAT-CC-3′. 3 µg purified total RNA were used. Concentration of aromatase primers was 0.6 µM, and concentrations of deoxyribose nucleotides and of rTth DNA polymerase were used as described in the manufacturer's recommendations (Applied Biosystems, Branchburg, NJ, USA). Reverse transcription was performed by incubating the reaction mixture at 42 °C for 5 min, ramping to 65 °C over 5 min (42 °C for 1 min, 50 °C for 1 min, 55 °C for 1 min, 60 °C for 1 min, 65 °C for 1 min) and 65 °C for 40 min in GeneAmp PCR System 9 700. PCR reaction was carried out at 94 °C for 1 min, followed by 30 cycles of a 2 temperature PCR (94 °C for 15 s and 62 °C for 40 s), ending with 60 °C for 7 min and on hold at 4 °C. To rule out genomic DNA contamination in the RNA preparation, reverse transcription step was omitted in a separate sample. Because band signal intensity was used to demonstrate the amount of aromatase mRNA under different conditions, 28 s rRNA and 18 s rRNA were used to examine the relative abundance of total RNA under these conditions. RNA agarose gels were performed as described in the manufacturer's recommendations (Ambion Inc., Austin, Texas, USA).

cDNA was separated on 2.5 % agarose gel in TBE buffer (89 mM Tris, pH 8.3, 89 mM boric acid, and 2 mM EDTA). Agarose gel was

stained with 2 µg/ml ethidium bromide and DNA bands were visualized on UV transilluminator.

Purification of PCR DNA fragments

The 208 bp cDNA fragments were eluted from agarose gel by cutting a well below the 208 bp position. The 3× TBE buffer was placed into the well and electrophoresed for 5–10 min until the 208 bp cDNA fragments were eluted into the well. cDNA fragments were collected and 2 volumes of 100% ethanol, 0.1 volume of 5 M ammonium acetate, and 0.02 volume of linear acrylamide were added. This mixture was placed at −20°C for 2 h. Precipitated cDNA was incubated with 2 units of *BamHI* restriction enzyme for 2 h at 37°C. One volume of DNA loading buffer was added and the mixture was electrophoresed on 3% agarose gel in TBE buffer. Restriction cDNA map was examined under the UV transilluminator.

Androstenedione and testosterone binding experiments

Aromatase enzyme was immunoprecipitated as described above. Purified aromatase bound to protein A-agarose was added to 250 µl of solution containing the final concentration of 1 µM testosterone (2 µCi of ^{125}I/ml testosterone, 857 µCi/nmol) or androstenedione (3.5 µCi of ^{125}I/ml androstenedione, 430 µCi/nmol), with or without insulin (0–10^3 ng/ml) and with or without 25 µM rosiglitazone or pioglitazone. This mixture was incubated at room temperature for 30 min and centrifuged at 6 000 rpm for 3 min. Pellets were washed 2 times with 400 µl Dulbecco's phosphate-buffered saline. Nonspecific binding was examined as described above, except that aromatase was not added to the protein A-agarose. Bound testosterone or androstenedione were expressed as % of total hormone.

Materials

M199 medium, HBSS, FBS, gentamicin, and amphotericin were from Invitrogen Corp., Carlsbad, CA; androstenedione and testosterone were from Sigma-Aldrich Corp., St. Louis, MO; ^{125}I-androstenedione and ^{125}I-testosterone were from MP Biomedicals, LLC, Solon, OH; 1β-^{3}H-testosterone was from New England Nuclear, Boston MA; rosiglitazone and troglitazone were from Cayman Chemical, Ann Arbor, MI; pioglitazone was from Takeda Pharmaceuticals America, Inc. Lincolnshire, IL; type 1 17β-hydroxysteroid dehydrogenase (17β-HSD) inhibitor [*N*-methyl, *N*-butyl-6β-(thiaheptamamide)estradiol] was a gift from Dr. Donald Poirier, Laval University Medical Research Center, Quebec, Canada; Lowry protein assay kit was from Thermo Scientific, Rockford, LI; estrone ELISA kits were from Alpco Diagnostics, Salem, NH; estradiol RIA kits were from Diagnostic Systems Laboratories, Webster, TX; rabbit anti-human aromatase antibodies, and rabbit and mouse anti-human β-actin antibodies were from Abcam, Inc. Cambridge, MA; mouse anti-human aromatase antibodies were from abD Serote, Inc., Kingston, NH; RNAqueous-4PCR kits were from Ambion Inc., Austin, Texas.

Statistical analysis

2-way analysis of variance (ANOVA) was used to compare mean values according to insulin concentrations in the presence or absence of TZDs, and with or without type 1 17β-HSD inhibitor. Statistical interactions between the sets of data obtained with or without TZDs, and with or without type 1 17β-HSD inhibitor were examined. Pairwise Bonferroni-adjusted contrasts were used to determine statistical significance. Analysis of covariance (ANCOVA) was used to assess statistical significance of mean differences among the sets of data according to insulin concentrations in the presence or absence of TZDs, and with or without type 1 17β-HSD inhibitor. Adjustments were made for initial inhibition of estradiol or estrone production induced by TZDs in the absence of insulin.

Results

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In the absence of a substrate

In the absence of a substrate, the purified human granulosa cells (0.5×10^5 cells) produced approximately 5×10^4 ng/mg/ml of estrone and 1×10^5 ng/mg/ml estradiol. With androstenedione or testosterone as a substrate, a 20–25-fold increase of estrone or estradiol production was observed (data not shown).

Androstenedione as substrate (◦ Fig. 1)

A. In the absence of type 1 17β-HSD inhibitor: Insulin was used because it can modify effects of TZDs on estrogen synthesis [5,11,12]. In the absence of type 1 17β-HSD inhibitor, insulin stimulated estrone production by 14% (p < 0.001) while rosiglitazone or pioglitazone inhibited estrone production by 22% compared to baseline (p < 0.012) (◦ Fig. 1a).

B. Type 1 17β-HSD inhibitor: Conversion of androstenedione to estrone by aromatase is followed by conversion of estrone to estradiol by type 1 17β-HSD (◦ Fig. 1e). Type 1 17β-HSD inhibitor was used to prevent the conversion of estrone to estradiol, thus allowing us to use estrone concentration as a true reflection of aromatase activity when androstenedione is used as substrate.

In the presence of type 1 17β-HSD inhibitor, estrone production in human granulosa cells was stimulated by 87% (p < 0.001) and estradiol production was inhibited by 22% (p < 0.01) (◦ Fig. 1b). In the presence of type 1 17β-HSD inhibitor, rosiglitazone inhibited estrone production by 59% (p < 0.016) and pioglitazone by 89% (p < 0.02) (◦ Fig. 1c). Thus, the interaction between rosiglitazone or pioglitazone and estrone was enhanced in the presence of type 1 17β-HSD inhibitor.

Testosterone as substrate (◦ Fig. 2)

Insulin stimulated estradiol production by 24% (p < 0.001) (◦ Fig. 2a) while rosiglitazone or pioglitazone inhibited estradiol production by 20% (p < 0.001) in the absence of insulin and by 32% (p < 0.001) in the presence of insulin (◦ Fig. 2a). When it was attempted to use the available type 2 17β-HSD inhibitor to prevent the conversion of estradiol to estrone, we unexpectedly discovered that this agent also produced significant inhibition of aromatase activity (data not shown). Therefore, we used the ^{3}H-tritiated water method, which examines directly conversion of testosterone to estradiol, to confirm the above results. When ^{3}H-testosterone was used as substrate, rosiglitazone or pioglitazone inhibited the ^{3}H-tritiated water release by 45% and 35% respectively (p < 0.001) (◦ Fig. 2b).

Effects of TZDs on aromatase mRNA and protein expression (◦ Fig. 3, ◦ Fig. 4)

When androstenedione or testosterone was used as substrate, 25 µM rosiglitazone or pioglitazone had no effect on aromatase mRNA expression (◦ Fig. 3a). Similar results were observed
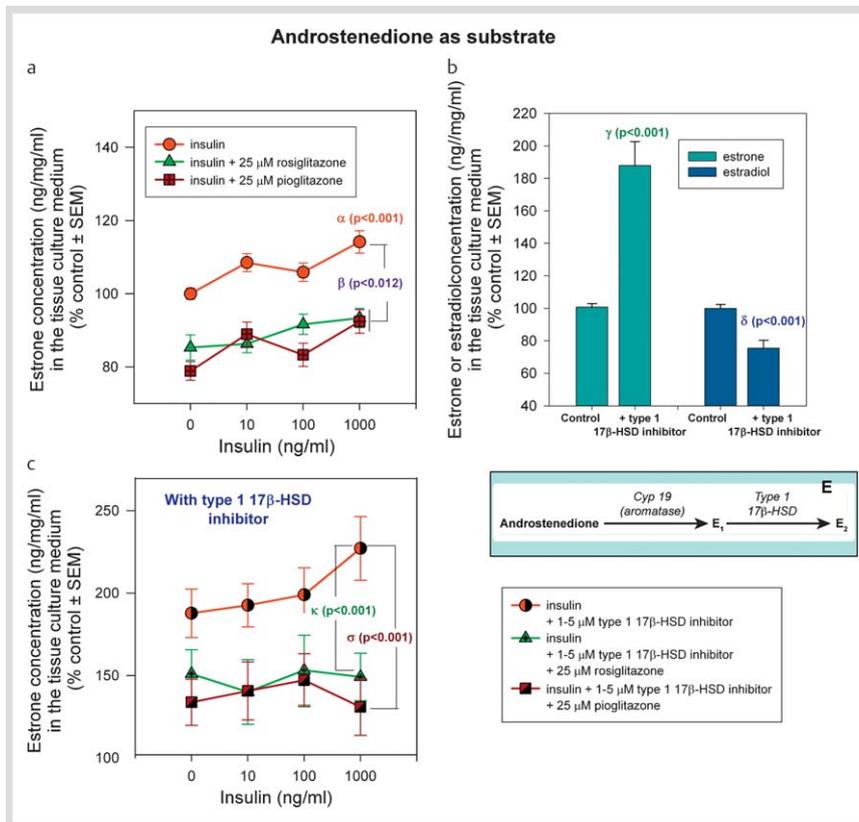
Fig. 1 Effects of rosiglitazone or pioglitazone and type 1 17β-HSD inhibitor on estrone production in human granulosa cells, with androstenedione as a substrate. **a**: Effects of TZDs on estrone production; α, p<0.001 (n=17), compared to baseline (absence of insulin, rosiglitazone or pioglitazone); β, p<0.012 (n=14), compared to control (in the presence of insulin and in the absence of rosiglitazone or pioglitazone). **b**: Effects of type 1 17β-HSD inhibitor on estrone and estradiol production; γ, p<0.001 (n=7) and δ, p<0.001 (n=8), compared to control (in the absence of type 1 17β-HSD inhibitor). **c**: Effects of TZDs on estrone production in the presence of type 1 17β-HSD inhibitor; κ, p<0.001 (n=7) and σ, p<0.001 (n=8), compared to control (in the presence of type 1 17β-HSD inhibitor and in the absence of rosiglitazone or pioglitazone). **e**: Conversion of androstenedione to estrone and estradiol. To convert ng/ml insulin to pmol/l, multiply by 167.
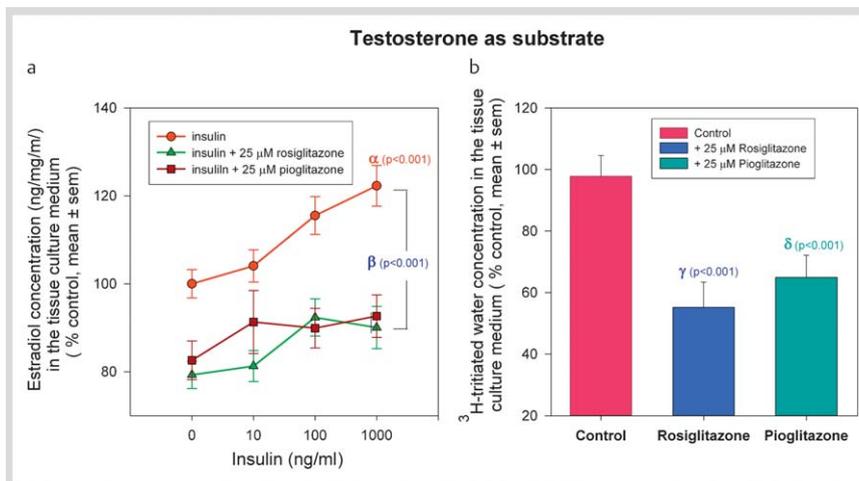
Fig. 2 Effects of rosiglitazone or pioglitazone on estradiol production in human granulosa cells, with testosterone or ^{3}H-testosterone as substrate. **a**: Effects of rosiglitazone or pioglitazone on estradiol production, α, p<0.001 (n=12), compared to baseline (absence of insulin, rosiglitazone or pioglitazone); β, p<0.001 (n=12), compared to control (presence of insulin and absence of rosiglitazone or pioglitazone). **b**: rosiglitazone or pioglitazone effect on ^{3}H-tritiated water release with ^{3}H-testosterone used as substrate; γ, p<0.001 (n=16); δ, p<0.001 (n=16). To convert ng/ml insulin to pmol/l, multiply by 167.

when 28 s rRNA and 18 s rRNA were used to demonstrate the relative abundance of total RNA under each set of conditions (○ **Fig. 3a**). The supraphysiological concentration of 50 μM rosiglitazone or pioglitazone had no effect on aromatase mRNA expression (data not shown).

Purified 208 bp aromatase PCR fragments began at 313 bp and ended at 510 bp of aromatase gene. These cDNA fragments contained single *BamHI* restriction site at 362 bp. A 148 bp DNA fragment was observed when *BamHI* restriction enzyme was added (○ **Fig. 3b**), proving that RT-PCR generated fragments corresponded to the position of aromatase gene. No genomic DNA contamination was observed in the total RNA preparation (○ **Fig. 3c**).

In contrast to the experiments utilizing rosiglitazone or pioglitazone, 10 μM troglitazone (used as control), inhibited aromatase mRNA expression, as previously described [7,8] (○ **Fig. 3d**), thus

confirming the validity of our technique. The abundance of 28 s rRNA and 18 s rRNA was similar under each set of conditions (○ **Fig. 3d**).

When androstenedione or testosterone was used as substrate, 25 μM rosiglitazone or pioglitazone had no effect on aromatase enzyme protein expression (○ **Fig. 4**). Similar results were observed when house protein β-actin was used to demonstrate the relative abundance of protein under each set of conditions (○ **Fig. 4**). The concentration of 50 μM rosiglitazone or pioglitazone had no effect on aromatase enzyme protein expression (data not shown).

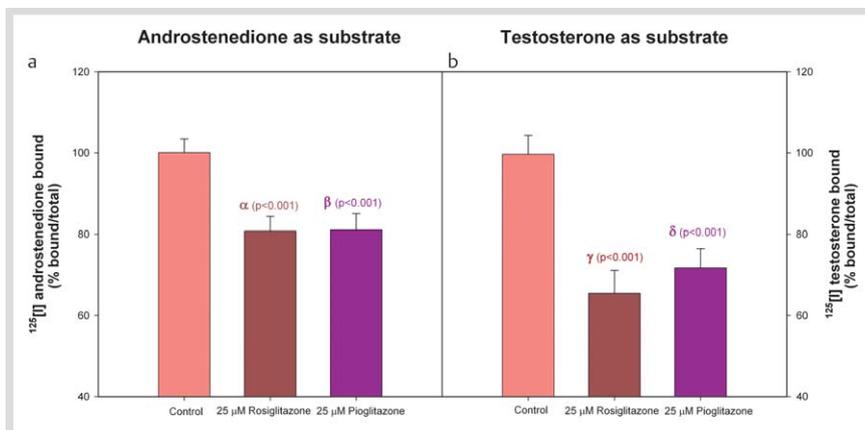### Effects of rosiglitazone or pioglitazone on androgen binding to aromatase (○ **Fig. 5**)

In the presence of 25 μM rosiglitazone or pioglitazone, ^{125}I-androstenedione binding to aromatase enzyme was inhibited by
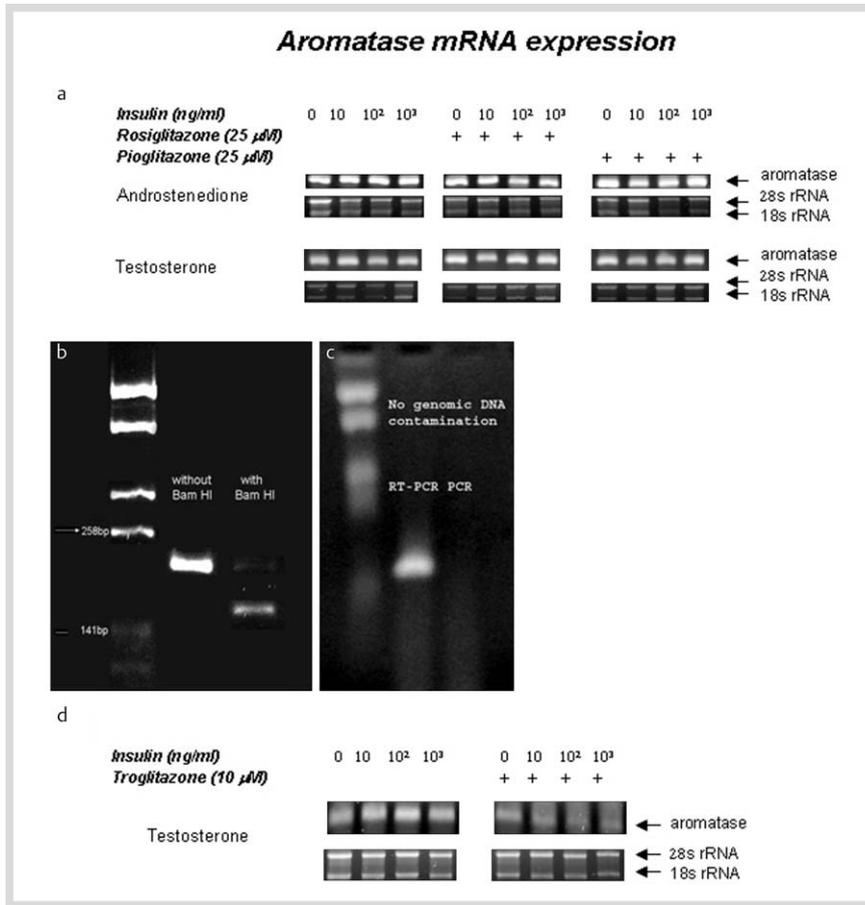
Fig. 3 Effects of TZDs on aromatase mRNA expression. **a**: Aromatase mRNA expression: representative RT-PCR analysis of aromatase mRNA, 18 s rRNA and 28 s rRNA expression in the presence of androstenedione or testosterone and various concentrations of insulin, with or without rosiglitazone or pioglitazone. **b**: restriction enzyme map for the purified cDNA fragments of aromatase. **c**: assessment of genomic DNA contamination – representative RT-PCR and PCR analysis of aromatase. **d**: aromatase mRNA expression in the absence or presence of troglitazone – representative RT-PCR analysis of aromatase mRNA, 18 s rRNA and 28 s rRNA expression in the presence of testosterone and various concentrations of insulin. To convert ng/ml insulin to pmol/l, multiply by 167.

Fig. 4 Effects of TZDs on aromatase protein expression. Representative immunoblot analysis of aromatase protein and β-actin house protein expression in the presence of androstenedione or testosterone, with various concentrations of insulin, with or without rosiglitazone or pioglitazone. To convert ng/ml insulin to pmol/l, multiply by 167.

Fig. 5 Effects of rosiglitazone or pioglitazone on ^{125}I-androstenedione and ^{125}I-testosterone binding to aromatase enzyme. **a**: androstenedione as substrate α, $p < 0.001$ (n = 5) and β, $p < 0.001$ (n = 5) compared to control (absence of rosiglitazone or pioglitazone). **b**: testosterone as substrate, γ, $p < 0.001$ (n = 5) and δ, $p < 0.001$, compared to control (absence of rosiglitazone or pioglitazone).

20 % (p < 0.001) (Fig. 5a). Similarly, rosiglitazone inhibited ^{125}I-testosterone binding to aromatase enzyme by 38 % (p < 0.001) (Fig. 5b) and pioglitazone by 32 % (p < 0.001) (Fig. 5b). Rosiglitazone or pioglitazone had no effect on the non-specific binding (protein A-agarose) of either ^{125}I-androstenedione or ^{125}I-testosterone or (data not shown).

Discussion
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Thiazolidinediones are insulin-sensitizing agents used in the treatment of type 2 diabetes. Their action, however, extends beyond their effects on glucose metabolism. For example, TZDs are known to affect female reproductive system and bone metabolism [13–15]. Understanding these nonglycemic effects of TZDs is important not only for the full assessment of their safety and effectiveness in patients with diabetes, but also in order to develop potential therapeutic application of TZDs in other populations. Indeed, the use of TZDs has been shown to be beneficial in women with polycystic ovary syndrome [1–5]. Although initially the effects of TZDs in female reproductive system were attributed to their insulin-sensitivity properties, it is clear that TZDs also exhibit direct effects in the human ovary [5,11].

In cultured human ovarian cells, TZDs stimulate progesterone production, inhibit testosterone and estradiol production and completely abolish insulin-induced testosterone production and insulin/FSH-induced estradiol production [5], suggesting an effect on aromatase. However, the literature regarding the effects of TZDs on aromatase is controversial [1,5–8]. The goal of our studies was to examine the effect of rosiglitazone or pioglitazone on aromatase activity in human ovarian cells.

We utilized human granulosa cells obtained in the course of IVF because of their availability and because granulosa cells contain significantly higher aromatase activity than other types of ovarian cells [5,9]. Human granulosa cells obtained during IVF, however, have been exposed to high concentrations of gonadotropins in vivo and therefore are highly luteinized. We minimized this problem by incubating granulosa cells ex vivo for 72 h in tissue culture medium prior to using them in the experiments.

We have previously examined a range of rosiglitazone or pioglitazone concentrations (10–50 μM) for their effect on steroid hormone production [5]. TZD concentrations of 20–30 μM resulted in the maximum effect on steroid hormone production. Rosiglitazone and pioglitazone (30 μM) had a stimulatory effect on progesterone production [5]. In this report, we demonstrated that rosiglitazone and pioglitazone (25 μM or 50 μM) had not effect on the mRNA, protein expression and the cell viability. These data also showed that the concentrations of rosiglitazone or pioglitazone utilized in our experiments had no significant effect on the cell toxicity.

We hypothesized that rosiglitazone or pioglitazone inhibit aromatase activity. This hypothesis was supported by our data which demonstrated inhibition of both estrone and estradiol production in human granulosa cells in the presence of either rosiglitazone or pioglitazone. To further confirm that TZDs inhibit aromatase activity, we utilized type 1 17β-HSD inhibitor. Type 1 17β-HSD inhibitor catalyzes the reaction of estrogen metabolism chain immediately following aromatase, conversion of estrone to estradiol. In the presence of type 1 17β-HSD inhibitor, the inhibitory effect of rosiglitazone or pioglitazone on estrogen production was enhanced, as one would expect if this effect were indeed mediated by aromatase.

Type 2 17β-HSD inhibitor catalyzes conversion of estradiol to estrone. Because the available type 2 17β-HSD inhibitor unexpectedly inhibited not only the type 2 17β-HSD but also the aromatase activity (data not shown), we examined TZD effect on aromatase activity using the ^{3}H-tritiated water method. Indeed, TZDs significantly inhibited tritiated water release when ^{3}H-testosterone was used as substrate. Thus, inhibition of aromatase activity by TZDs was established in our studies using 3 methods: measurements of estrogen production by RIA; use of type 1 17β-HSD inhibitor; and use of ^{3}H-tritiated water method.

Surprisingly, in our studies rosiglitazone or pioglitazone had no effect on either aromatase mRNA or protein expression. Fan et al. [7] and Mu et al. [8] found that troglitazone inhibited aromatase mRNA expression. Therefore, we examined the effect of troglitazone on aromatase mRNA expression and confirmed findings of Fan and Mu, also validating our method in the process. Thus, it appears that troglitazone indeed inhibits aromatase mRNA expression, while rosiglitazone and pioglitazone do not. This discrepancy can be explained, at least in part, by the fact that troglitazone receptor affinity and action differ from those of rosiglitazone and pioglitazone [16,17].

In addition to the lack of the effect of rosiglitazone or pioglitazone on aromatase mRNA expression, aromatase protein expression was not affected by either rosiglitazone or pioglitazone. Thus, neither rosiglitazone nor pioglitazone had an effect on transcription or translation of aromatase gene and yet inhibited aromatase activity.

In order to understand this paradox, we examined the effects of rosiglitazone or pioglitazone on binding of androstenedione or testosterone to aromatase. Binding studies demonstrated that rosiglitazone or pioglitazone reduced substrate binding to aromatase, and the degree of rosiglitazone or pioglitazone-induced inhibition of estrogen production (22–32 %) was comparable to the inhibitory effects of rosiglitazone or pioglitazone on the binding of androgen to aromatase enzyme (22–38 %). In our preliminary kinetic studies both rosiglitazone and pioglitazone inhibited the V_{max} and K_m acting as uncompetitive inhibitors (data not shown).

In summary, rosiglitazone or pioglitazone did not affect transcription or translation of aromatase gene, but rather interfered with substrate (androgen) binding to aromatase. In vivo, in addition to reducing aromatase activity, rosiglitazone or pioglitazone directly inhibit androgen synthesis and reduce hyperinsulinemia due to their systemic insulin-sensitizing action [2–5,11]. These 2 effects would result in an additional decline in estrogen production due to a reduction in available aromatase substrate (Fig. 6).

Clinically available aromatase inhibitors suppress biosynthesis of estrogens and, therefore, reduce the negative feedback on the hypothalamic-pituitary axis. These results in increased secretion of FSH, in turn, can lead to follicle selection, maturation and ultimately, to ovulation [18]. TZDs have been used in PCOS for ovulation induction [1–4]. It is possible that, in addition to their insulin-sensitizing properties, rosiglitazone or pioglitazone may improve ovulation by inhibiting aromatase.

Beyond their potential role in treating diabetes and PCOS, inhibition of estrogen synthesis by TZDs may make them useful in the therapy of estrogen-dependent diseases such as breast cancer, gynecomastia, uterine fibroids, and endometrial cancer [19–21]. Inhibition of aromatase activity by TZDs may also help explain their negative effect on bone density in older or postmenopausal women [13,14].
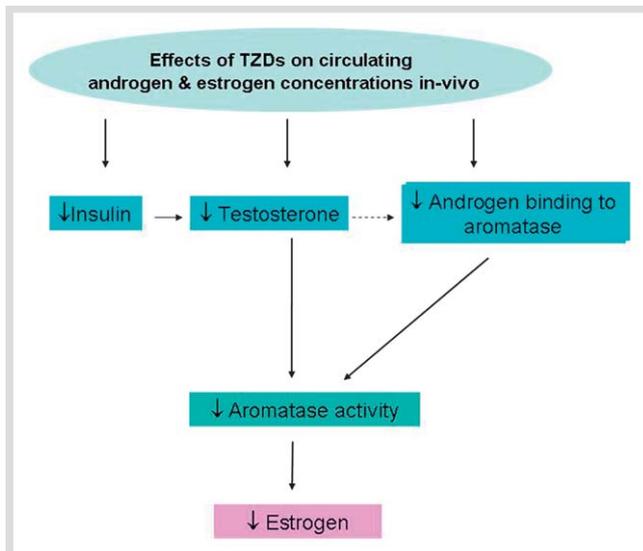
Fig. 6   Proposed in vivo effects of rosiglitazone or pioglitazone on circulating androgen and estrogen concentrations in women. Rosiglitazone or pioglitazone reduces circulating insulin level through their insulin-sensitizing action, which in turn leads to a reduction of circulating testosterone levels. In addition, rosiglitazone or pioglitazone directly inhibits testosterone production in human ovarian cells [5], producing a reduction of aromatase activity because of the kinetics of substrate/enzyme interaction. Finally, rosiglitazone or pioglitazone inhibits aromatase activity by interfering with androgen binding to aromatase. In summary, estrogen production is inhibited by rosiglitazone or pioglitazone in vivo both because of substrate (androgen) depletion and reduction of aromatase activity.

We conclude that in human granulosa cells, rosiglitazone or pioglitazone inhibit estrone and estradiol production by interfering with androstenedione or testosterone binding to aromatase. Inhibition of aromatase activity by TZDs may help explain their therapeutic effects in PCOS and negative effect on the bone density in postmenopausal women with diabetes. Inhibition of aromatase activity by TZDs may be therapeutically useful in a variety of estrogen-dependent diseases.

Acknowledgements
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