Thiazolidinediones decrease vascular endothelial growth factor (VEGF) production by human luteinized granulosa cells in vitro

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Objective: To determine the effect of thiazolidenedione derivatives (TZDs) on vascular endothelial growth factor (VEGF) production by human luteinized granulosa cells and the morphologic development of murine embryos.
Design: Prospective, experimental, in vitro and in vivo study.
Setting: Research laboratory.
Patient(s): Follicular aspirates from 10 women undergoing oocyte retrieval.
Intervention(s): Isolated human granulosa cells were treated with a dimethyl sulfoxide (DMSO) control or ciglitazone, in the presence and absence of an hCG stimulus. Embryos extracted from superovulated B6C3F1 female mice were cultured in the presence of DMSO or pioglitazone.
Main Outcome Measure(s): Vascular endothelial growth factor concentrations at 24 and 48 hours. Morphologic development of murine embryos at 96 hours.
Result(s): Following an hCG stimulus, treatment with 20 μM or 40 μM ciglitazone decreased VEGF production in a statistically significant manner at both time intervals. Blastocyst development at 96 hours did not significantly differ between untreated zygotes and those treated with pioglitazone.
Conclusion(s): Ciglitazone significantly decreased VEGF production by human granulosa cells in an in vitro model. Pioglitazone did not adversely impact the development of cultured murine embryos. Although mechanistic evidence is not provided, the pivotal role of VEGF in ovarian hyperstimulation syndrome prompts investigation of TZDs as a novel treatment for this condition. (Fertil Steril® 2010;93:2042–7. ©2010 by American Society for Reproductive Medicine.)

Key Words: Thiazolidinediones, PPAR-gamma, VEGF, granulosa cells, OHSS

The female reproductive tract is one of a few adult systems in which angiogenesis occurs as a normal process, one that is essential for menstrual cyclicity, placentation, and support of a growing embryo (1). In contrast to that observed during pathologic tumor growth, the angiogenic process in female reproductive tissues is limited, and therefore must be tightly regulated. Although a number of potential regulators of angiogenesis have been identified, vascular endothelial growth factor (VEGF) has emerged as one of the most significant.

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Evidence supports the regulatory function of VEGF in nearly all stages of follicular angiogenesis—from development of the antral follicle to formation and maintenance of the corpus luteum (2–5). Despite VEGF's integral role in physiologic ovarian function, its overproduction may result in pathologic conditions such as ovarian hyperstimulation syndrome (OHSS). In its most severe form, OHSS is characterized by massive cystic enlargement of the ovaries associated with increased capillary permeability and a third space fluid shift—leading to the classic triad of ascites, pleural effusion, and hemoconcentration (2). Some groups have focused on the use of peripheral blood VEGF concentration as a predictor of OHSS during in vitro fertilization cycles (6). Serum and follicular fluid VEGF concentrations have been shown to be significantly elevated in patients who developed severe OHSS compared with those who did not, suggesting a role for VEGF in the pathogenesis of this condition (7).

Peroxisome proliferator activated receptors (PPAR) are a newly characterized class of ligand-activated transcription factors belonging to the nuclear receptor family. Three PPAR isotypes have been identified, and are commonly designated PPARα, PPARβ, and PPARγ. Peroxisome proliferator activated receptor γ is found in adipose tissue, liver, spleen,

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colon, adrenal glands, and muscle tissue (8), where its actions were originally thought to be limited to the control of lipid metabolism and glucose homeostasis. Recent studies, however, have identified PPAR isotypes in cells of the immune system as well as those of the reproductive tract, including the ovary, mammary glands and the uterus (9). In the ovary, PPARγ is expressed most strongly in the granulosa cells, and less strongly in theca cells and corpora lutea (10).

Thiazolidinedione derivatives (TZDs), such as pioglitazone, are synthesized ligands of PPARγ that are approved as oral antihyperglycemic agents as therapy for noninsulin-dependent diabetes mellitus. More recently, TZDs have been found to decrease VEGF production by activating PPARγ in tumor endothelial cells, thereby inhibiting tumor growth and metastasis (11). Thiazolidinedione derivatives have been shown to similarly suppress VEGF gene expression in human endometrial cells (12).

The inhibition of VEGF expression by PPARγ ligands in these other cell types prompted us to investigate if TZDs can similarly mitigate VEGF expression in human granulosa cells. We predicted that VEGF concentrations would be diminished in the media collected from luteinized granulosa cells subjected to treatment with the commercially available TZD ciglitazone, compared with placebo-treated controls. For TZDs to realistically be used as a prophlyaxis or therapy for OHSS, their safety profile with respect to embryogenesis would have to be established. We therefore investigated the safety of the US Food and Drug Administration (FDA)-approved TZD pioglitazone on the morphologic development of murine embryos in an in vitro model.

MATERIALS AND METHODS

Granulosa Cell Isolation and Culture

Ovarian follicular aspirates were obtained from 10 women undergoing oocyte retrieval as part of an in vitro fertilization program. Ovulation was induced by sequential treatment with recombinant human FSH (Gonal F), followed by administration of hCG. Follicles were aspirated 36 hours after hCG administration and the follicular fluid donated to our study. The use of discarded follicular fluid and associated granulosa cells had been given exemption from continuing review by the University of Michigan Medical institutional review board based on Federal Regulations 45 CFR 46.101 (b). The follicular fluid from each individual was pooled and centrifuged at 500 × *g* for 5 minutes. The supernatant was removed, and the cell pellets resuspended in 4 mL McCoy's 5A medium, pH 7.4 (Invitrogen, Grand Island, NY). The suspension was layered over 3 mL Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) and centrifuged at 500 × *g* for 30 to 40 minutes to isolate the granulosa cells from the sample (13). The granulosa cells were removed from the interface and washed twice. Cells were counted with a hemocytometer, and cell viability determined using Trypan Blue exclusion, which was found to be consistently >90%. Isolated granulosa cells from each individual were cultured in triplicate in separate 24-well tissue culture plates at a density of 50,000 viable cells in 1 mL McCoy's 5A medium supplemented with 10% fetal calf serum, glutamine (0.0015%), gentamycin (50 μg/mL), and nystatin (2 U/mL) (13, 14).

Vascular Endothelial Growth Factor Measurement

Culture media was replaced with a serum-free media 24 hours after plating. On day 2 of culture, the cells were treated with a dimethyl sulfoxide (DMSO) control, 10 μM, 20 μM, or 40 μM ciglitazone (Cayman Chemical, Ann Arbor, MI). Molar concentrations of the commercially available ciglitazone were calculated to reflect standard clinical dosing of pioglitazone, with a 40 μM concentration of ciglitazone corresponding to serum levels achieved with a 45 mg daily dose of pioglitazone (15). Cells were cultured both in the presence and absence of 2.7 μM hCG CR 127 (purchased from Dr. Al Parlow, University of California Los Angeles, through NIDDK hormone distribution program) in a manner consistent with the published literature (3, 13). Medium was harvested 24 and 48 hours posttreatment and frozen at -20°C (11). Vascular endothelial growth factor protein concentrations were measured in duplicate by enzyme-linked immunosorbent assay (ELISA) using R&D Systems Quantikine kits (R&D Systems, Minneapolis, MN) with 1 part assay diluent to 4 parts sample as per the manufacturer's instructions.

Cell Proliferation Assay

Isolated granulosa cells from four individual patients were plated at a standard concentration of 50,000 cells per well and treated with a DMSO control, 10 μM, 20 μM, or 40 μM ciglitazone as described above. Mean cell numbers were assessed using Trypan Blue exclusion after 48 hours of treatment.

Murine Embryo Isolation and Culture

Six- to 8-week-old B6C3F1 female mice (Charles River Laboratories, Wilmington, MA) were superovulated by an intraperitoneal administration of 10 IU of pregnant mare serum gonadotropin (Sigma-Aldrich, St. Louis, MO), followed 44 hours later with an injection of 10 IU of human chorionic gonadatropin (Sigma-Aldrich). After hCG injection, females were caged individually with mature B6C3F1 male mice of known fertility. Presumptive zygotes were collected 21 hours later by dissecting oviducts in HEPES-buffered human tubal fluid (HTF-H) medium (Irvine Scientific, Santa Ana, CA), supplemented with 0.1% hyaluronidase to remove surrounding cumulus cells. Zygotes were washed three times in HTF-H medium supplemented with 0.3% of bovine serum albumin (BSA) (Fisher BioReagents, Pittsburgh, PA). All procedures performed on animals were approved by the University of Michigan Animal Care and Use Committee.

Embryos were cultured in 50-μL drops of Potassium Simplex Optimized Media (KSOM + AA) (Chemicon International, Billerica, MA) overlaid with mineral oil (Irvine