Regulation of catechol *O*-methyltransferase expression in granulosa cells: a potential role for follicular arrest in polycystic ovary syndrome

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Objective: To investigate the regulation of catechol *O*-methyltransferase (COMT) expression in granulosa cells and assess potential effects of 2-methoxyestradiol (2-ME_2) and COMT inhibitors on granulosa cell steroidogenesis and proliferation.

Design and Setting: Controlled experimental study in an academic research laboratory.

Intervention(s): JC410 porcine and HGL5 human granulosa cell lines were used for in vitro experiments. Effects of 2-ME_2 and COMT inhibitor treatment on DNA proliferation and steroidogenesis were assessed by using Hoechst dye and p450SCC-luciferase reporter assays. Effects of dihydrotestosterone (DHT), insulin, and all-*trans* retinoic acid (ATRA) on COMT messenger RNA expression were investigated by using COMTP1 promoter–luciferase reporter and Northern blot.

Main Outcome Measure(s): Granulosa cell steroidogenesis and proliferation following COMP inhibitor and 2-ME_2 treatment. Regulation of COMT expression with DHT, insulin, and ATRA.

Result(s): 2-Methoxyestradiol had a dual effect on granulosa cell proliferation and p450SCC- luciferase activity; low doses were stimulatory and high doses were inhibitory. Catechol *O*-methyltransferase inhibitor was associated with up to a 65% increase in JC410 cell number and a maximal 5.6-fold increase in p450SCC-luciferase activity at 20 μmol/L. Dihydrotestosterone, insulin, and ATRA all induced a dose-dependent increase in COMTP1-luciferase transactivation, as well as up-regulated COMT messenger RNA expression in granulosa cells.

Conclusion(s): Catechol *O*-methyltransferase expression in granulosa cells was up-regulated by insulin, DHT, and ATRA. Catechol *O*-methyltransferase product, 2-ME_2, decreased, whereas COMT inhibitor increased granulosa cell proliferation and steroidogenesis. These data suggest that COMT overexpression with subsequent increased level of 2-ME_2 may lead to ovulatory dysfunction. (Fertil Steril® 2008;89:1414–21. ©2008 by American Society for Reproductive Medicine.)

Key Words: Catechol *O*-methyltransferase expression, methoxyestrogen, granulosa cell steroidogenesis and proliferation, anovulation

The human ovary undergoes extensive remodeling during a woman's reproductive life. Several genes have been identified in the ovary and are postulated to have a role in ovarian physiology and in maintaining normal homeostasis in the ovarian cycle (1–3). Polycystic ovary syndrome (PCOS) is characterized by anovulation caused by the apparent arrest of antral follicle development at the 5- to 10-mm stage and, consequently, the failure to enter the preovulatory phase of the cycle (4). The underlying basis of PCOS is still obscure (4). Excessive androgen production is a universal finding in patients with PCOS, and studies have demonstrated that theca cells from polycystic ovaries produce significantly more androstenedione than theca cells from normal follicles (5); higher androstenedione concentrations have been found in polycystic follicles than in normal follicles (6, 7). Insulin resistance and hyperinsulinemia are recognized as being associated with reproductive abnormalities in women with PCOS (8). Lifestyle changes and pharmacologic intervention that improve insulin sensitivity can ameliorate these abnormalities (8). Wood et al. (9) recently demonstrated that PCOS theca cells synthesize increased amounts of all-*trans* retinoic acid (ATRA). They have also reported increasing levels of retinol dehydrogenase 2, which converts retinol to retinaldehyde, and aldehyde dehydrogenase 6 enzyme, which converts retinaldehyde to ATRA in PCOS theca cells compared with normal theca cells (10). The exact mechanisms by which these factors (androgen production, insulin, and ATRA) mediate follicle/granulosa cell atresia and follicular development arrest are unclear. In addition to granulosa cell/follicle atresia, the other main perturbed function of PCOS follicles is steroidogenesis. Mason et al. have indicated that the fluid from PCOS follicles contains less estrogen (E) than does that from size-matched normal follicles (6).

Catechol *O*-methyltransferase (COMT) is a ubiquitous enzyme that catalyzes the *S*-adenosyl-L-methionine–dependent methyl conjugation of the hydroxyl groups of catechol

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estrogens converting 2- and 4-hydroxyestrogen to their methoxyestrogen counterparts (11). Several reports have shown a strong inhibitory effect of 2-methoxyestradiol (2-ME_2), which is synthesized in the ovarian follicles and present in the follicular fluid, on granulosa cell proliferation in different assays (12–14). Shang et al. (12) demonstrated an additional strong antiangiogenic impact of 2-ME_2 on ovarian angiogenesis, adding another facet to mediate the antifollicular effect of this compound. The ovarian follicle is the site of 2-ME_2 production, with concentrations of 30 nmol/L (10.5 ng/mL) in humans and as high as 0.82 μmol/L (247 ng/mL) in mare follicles (12, 15). In addition, 2-ME_2 is lipophilic and may concentrate in cell membranes or cellular compartments at even higher concentrations. Thus, the highly specialized microenvironment of the ovarian follicle in vivo provided the physiologic situation where 2-ME_2 may act in an autocrine/paracrine action, independent of classic E receptor mechanism, to modulate angiogenesis, as well as other cellular processes, in any of the cell types of the follicle including the oocyte (12).

We recently demonstrated that COMT expression is regulated by steroid hormones and inflammatory cytokines, such as tumor necrosis factor-α, in human and rat leiomyoma cell lines (16) and in human myometrial cells (17). Our current hypothesis centers on the fact that COMT converts the 2-OH catechol estradiol (2-OHE_2) to 2-ME_2. It is well established that 2-ME_2 works as an antiproliferative and antiangiogenic factor against granulosa cells (12–14). Therefore, we hypothesize that there is elevated COMT expression in granulosa cells in PCOS ovaries, which is induced by the elevated levels of androgens, insulin, and/or ATRA. As the COMT levels/activity increases, a higher level of 2-ME_2 builds up in the ovarian follicular microenvironment. This build-up leads to slowed granulosa cell proliferation and decreased steroidogenesis, which, in turn, results in arrested folliculogenesis and anovulation—a hallmark of PCOS. The aims of this study were to investigate the effects of 2-ME_2 on proliferation and steroidogenesis in a well-characterized porcine granulosa cell line, as well as to investigate the attributes of COMT gene regulation by insulin, dihydrotestosterone (DHT), and ATRA in porcine and human granulosa cells. The study was approved by the Institutional Review Board of the University of Texas Medical Branch, Galveston, Texas.

MATERIALS AND METHODS

Cells and Cell Culture

The JC410 porcine granulosa cell is a spontaneously transformed steroidogenic cell line that was kindly provided by Dr. P. J. Chedrese, Department of Obstetrics, Gynecology and Reproductive Sciences, University of Saskatchewan, Saskatoon, Saskatchewan, Canada (18). The HGL5 human immortalized luteinized granulosa cell line was a kind gift from Dr. William Rainey, University of Texas Southwestern Medical Center, Dallas, Texas (19). Granulosa cells were maintained at 37°C in 5% CO_2/air in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), and 2 mmol/L L-glutamine. To test the effect of 2-ME_2 and COMT inhibitor treatments on proliferation and steroidogenesis, granulosa cells were grown in DMEM supplemented with 10% fetal bovine serum and 100 nmol/L of 17β-E_2. To test the effect of DHT, insulin, and ATRA on COMT expression, granulosa cells were grown in phenol red–free DMEM supplemented with 10% charcoal-stripped fetal bovine serum. Cells (2×10^6) were seeded on 100-mm culture dishes and incubated for 48 hours. The media were removed, and the cells were reincubated in fresh media with the indicated concentration of different hormones in 0.01% (vol/vol) ethanol. The cells were harvested for messenger RNA (mRNA) extraction at 6, 12, and 24 hours and for protein assays at 48 hours. Chemicals with the highest grade available were purchased from Sigma Chemical Co., St. Louis, Missouri.

Reporter Transactivation Assay

Logarithmically growing JC410 cells were seeded on 60-mm tissue culture dishes (1×10^6 cells per plate) the day before transfection. Cells were transfected with either p450SCC, pCOMTP1, or pCOMTP2-luciferase plasmid (2 μg per plate) and pSV-β-galactosidase (1 μg per plate) with use of Fugene 6 transfection reagent in triplicate according to manufacturer's protocol (Roche Diagnostics, Indianapolis, IN). The next day, the medium was changed and fresh medium was added. Cells were stimulated with the indicated agent at the indicated times and were then harvested for luciferase assay within 48 hours of transfection, as described previously (16). Luciferase activity was normalized to β-galactosidase activity.

Northern Blot Analysis

HGL5 human granulosa cells were grown to 70% confluence in charcoal-stripped media for 48 hours before treatment with DHT, insulin, or ATRA. The cells were harvested, and total RNA was isolated from the samples and control by using the RNAqueous kit (Ambion, Austin, TX). Twenty micrograms of the RNA samples was subjected to Northern blot analysis, as we have described previously (20). A well-characterized pTOB7 plasmid containing 1,213 base pairs of the human COMT complementary DNA (cDNA) (Open Biosystems, Huntsville, AL) was used as a template to generate COMT antisense cDNA riboprobe by polymerase chain reaction (PCR) using COMT complementary reverse primer (5′CCAAGCTTCAGGCTGGGTGAGAGAGC3′). A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was made with the end-labeling method with use of random hexamer primers. After gel electrophoresis, RNA was transferred to a nylon membrane, hybridized to COMT-labeled and GAPDH-P^{32}–labeled probes, and exposed to x-ray film. Northern blot hybridization signals that corresponded to COMT mRNA were quantified by laser densitometry. Each COMT band was normalized to the value that was obtained from GAPDH mRNA in the same lane.

Proliferation Assay

Porcine JC410 granulosa cells were plated into 12-well Corning plastic cell culture dishes (Sigma-Aldrich) at 7,000 to 8,000 cells per well in DMEM medium with 10% fetal bovine serum, and they were allowed to proliferate for 24 hours. The cells were incubated in fresh medium supplemented with 100 nmol/L of 17β-E_2 with the addition of a different concentration of 2-ME_2 or COMT inhibitors in 0.01 % (vol/vol) ethanol, per manufacturer instructions (Sigma). Ethanol was used as the vehicle control at 0.01% concentration. At each time point (0, 1, 2, 3, 4, 5, and 6 days), the medium was aspirated and the wells rinsed twice with 1× PBS. The number of cells were counted by using the Coulter counter (Beckmann Coulter Co., Fullerton, CA) and confirmed by measuring the fluorescence of Hoechst 33258 dye, as we have described previously (21). Two highly specific COMT inhibitors (Ro 41-0960 [2′-fluoro-3,4-dihydroxy-5-nitrobenzophone] and 3,5-dinitrocatechol; Sigma) were used, and all experiments were performed and confirmed with both compounds as we have described previously (22).

STATISTICS

The results are expressed as mean ± SEM for triplicate culture wells from two or more experiments. Statistical differences between groups were analyzed by analysis of variance (ANOVA). Specific differences between various treatments were determined by using Student's *t*-test. Northern blot data were analyzed with Kruskal-Wallis ANOVA on ranks with Dunn's post hoc test, and the expression of COMT mRNA in JC410 and HGL5 cells with different treatments was compared. All tests were assessed at the $P<.05$ level of significance. The SAS system, release 8.2 (SAS Institute Inc., Cary, NC), was used for all statistical computations.

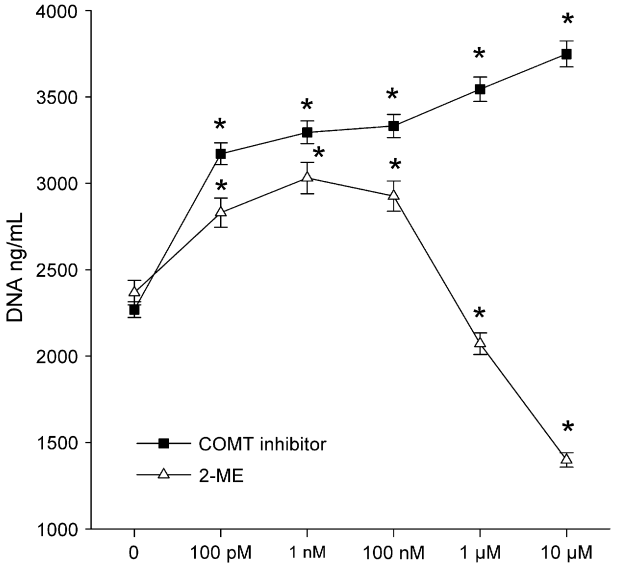
RESULTS

Growth Inhibition of Granulosa Cells by 2-ME_2 and Growth Potentiation by COMT Inhibitors

We investigated the direct effect of 2-ME_2, as well as highly specific inhibitors of the COMT activity, on the growth of the porcine JC410 granulosa cell line. The 2-ME_2 treatment caused a biphasic effect on granulosa cell growth. At lower concentrations (100 pmol/L, 1 nmol/L, 100 nmol/L), there was a dose-dependent increase in cell proliferation (19% ± 3.9%, 28% ± 4.2%, 23% ± 2.2%, respectively; $P < .001$) compared with the untreated control. At higher doses (1 μmol/L–10 μmol/L), however, the 2-ME_2 treatment caused marked dose-dependent attenuation of cell growth. At concentrations of 1 μmol/L and 10 μmol/L, 2-ME_2 attenuated granulosa cell proliferation by 13% ± 4% and 41% ± 11% of untreated control, respectively ($P<.001$, Fig. 1). In contrast, the Ro 41-0960 highly specific COMT inhibitor induced a significant dose-dependent increase in granulosa cell proliferation at all tested doses (100 pmol/L–10 μmol/L). The increase ranged from 39% ± 5.7% to 65% ± 0.9% (Fig. 1). It was statistically significant at all tested doses ($P<.001$).

FIGURE 1

Effect of 2-ME_2 and COMT inhibitor on granulosa cell proliferation. Porcine JC410 granulosa cells were treated with increasing concentrations of COMT inhibitor Ro 41-0960 or 2-ME_2. Cell numbers were examined by measuring DNA contents with use of the Hoechst method 48 hours later and were compared with cells exposed to medium alone. Each value is the mean ± SEM of triplicate wells in two independent experiments. *$P<.05$ compared with untreated controls.



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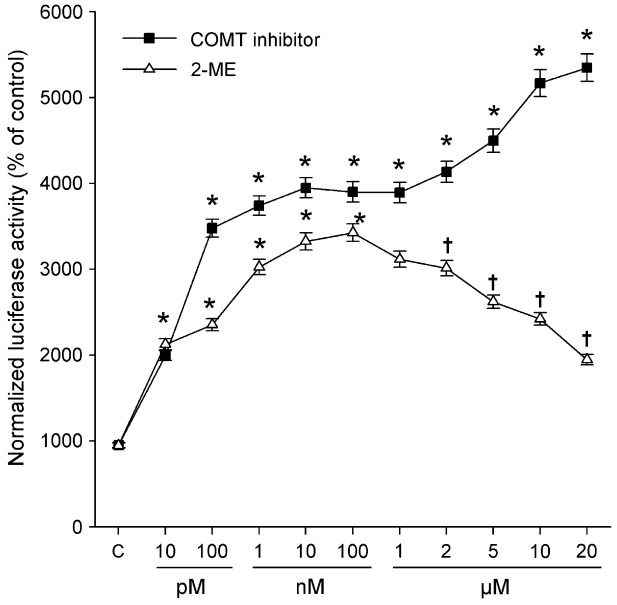
Effect of 2-ME_2 and COMT Inhibitors on Steroidogenesis

We assessed the effect of 2-ME_2 and the specific COMT inhibitor Ro 41-0960 on steroidogenesis in the JC410 porcine granulosa cell line. In lieu of measuring steroid hormone production in the culture media, we used a modified JC410 cell line that has been stably transfected with the p450 side chain cleavage gene promoter region cloned upstream from a luciferase reporter gene (p450SCC-luciferase) (23). The p450SCC enzyme is one of the key enzymes involved in steroid biosynthesis in granulosa cells, and we have reported on the validity of this cell line previously (24). The E metabolite 2-ME_2 had a dual effect on p450SCC- luciferase transactivation and, therefore, on steroidogenesis. As shown in Figure 2, lower levels of 2-ME_2 (10 pmol/L–100 nmol/L) exhibited a dose-dependent increase in p450SCC-luciferase transactivation; the maximal increase of 3.6-fold ± 1.1 was achieved at 100 nmol/L 2-ME_2 ($P<.001$). The additional increase in 2-ME_2 concentrations induced a marked inhibition of steroidogenesis; a maximal inhibitory effect of 43% ± 13% (compared with maximum stimulation) was reached at 20 μmol/L 2-ME_2 ($P<.001$). The specific COMT inhibitor Ro 41-0960 demonstrated a dose-dependent stimulatory effect on p450SCC-luciferase transactivation and, therefore,

FIGURE 2

Effect of 2-ME_2 and COMT inhibitor on porcine JC410 granulosa cells stably transfected with p450SCC-luciferase reporter. Cells were exposed to the indicated concentration of 2-ME_2 or COMT inhibitor Ro 41-0960. After 48 hours incubation, cells were collected and assayed for luciferase activity. Each value is the mean ± SEM of triplicate wells in two independent experiments. *$P<.05$ compared with untreated control. †$P<.05$ compared with peak 2-ME_2 stimulation at 100 nmol/L.



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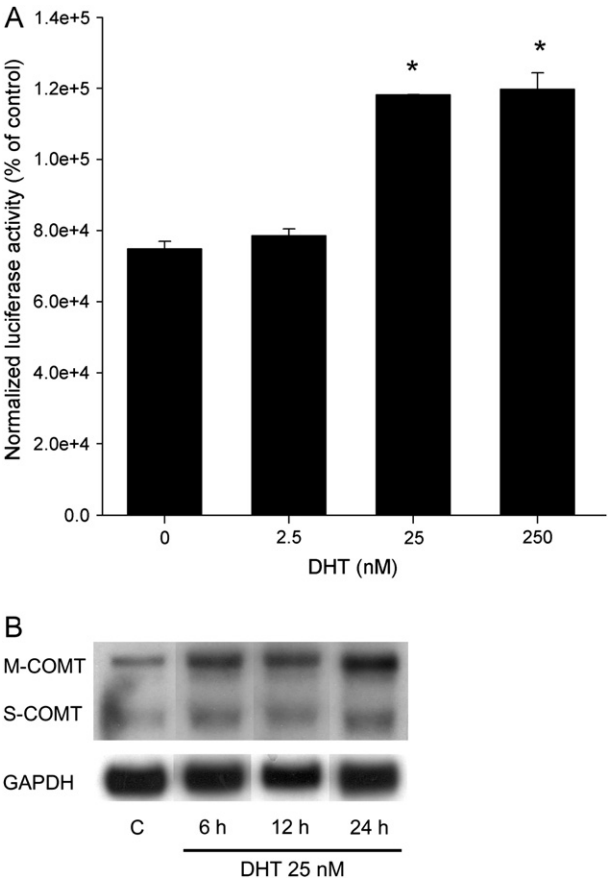
on steroidogenesis at all tested doses; an increase of 3.1-fold ± 0.05 ($P<.001$) was attained at 100 nmol/L, and a maximal increase of 5.6-fold ± 1.7 ($P<.001$) was attained at 20 μmol/L of COMT inhibitor (Fig. 2).

Dihydrotestosterone Increases COMT mRNA Expression in Porcine and Human Granulosa Cells

Androgen is a key player in PCOS pathogenesis (5–7). We wanted to assess the effect of androgen treatment on COMT expression in granulosa cells. Because the porcine JC410 granulosa cells possess aromatase activity (17), we used the nonaromatizable androgen DHT to ensure that any modulation of COMT expression was not due to aromatization of the androgen added to the E counterpart. We have created separate COMTP1-luciferase and COMTP2-luciferase constructs. Catechol *O*-methyltransferase P1 represents the proximal COMT promoter (P1), which codes for a 1.3 kb transcript that encodes the soluble COMT polypeptide (S-COMT, 25 kd). Catechol *O*-methyltransferase P2 represents the distal COMT promoter (P2), which codes for a 1.5 kb transcript that encodes the membrane-bound COMT polypeptides (MB-COMT, 30 kd) (16, 25). These reporter gene

FIGURE 3

Dihydrotestosterone (*DHT*) up-regulates COMT expression in porcine and human granulosa cells. (**A**) DHT increased COMTP1-luciferase reporter transactivation in JC410 porcine granulosa cell line. The cells were transfected with pCOMTP1. After the addition of various doses of DHT, luciferase production was analyzed 48 hours later. The stimulatory effect of DHT was statistically significant ($P<.01$). Each value is the mean ± SEM of triplicate wells in two independent experiments. (**B**) Northern blot analysis of the effect of DHT on COMT mRNA expression in HGL5 human granulosa cells at the indicated dose and exposure time. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is the internal loading control. C = vehicle control; S-COMT = soluble catechol-*O*-methyl transferase; MB-COMT = membrane-pound catechol-*O*-methyl transferase.



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constructs were transiently cotransfected into JC410 granulosa cells with use of pSV-β-galactosidase as an internal control (see Materials and Methods for details). Luciferase assays were then used to test the response of these promoters to DHT. As shown in Figure 3A, DHT induced a dose-dependent increase in COMTP1-luciferase transactivation with an

optimal increase of 60% ± 8% achieved at DHT 250 nmol/L ($P<.01$). Catechol *O*-methyltransferase expression also remained elevated at a higher concentration of DHT, but there was no further increase (data not shown). Dihydrotestosterone exerted a minimal effect on COMTP2- luciferase transactivation (data not shown). We also wanted to confirm the reporter gene assay observation at the RNA level using real-time PCR or Northern blot analysis. Unfortunately, the sequence of the published porcine COMT cDNA was not long enough to develop a reliable real-time PCR assay. Further, the human COMT cDNA probe did not hybridize to porcine RNA in Northern blot analysis even at low stringency (data not shown). We then shifted this test to the human HGL5 granulosa cell line (19, 26, 27). Dihydrotestosterone up-regulated the human COMT mRNA level in a time-dependent manner in human granulosa cells, which concurs with the reporter-gene and protein expression findings (Fig. 3B).

Insulin and ATRA Increase COMT mRNA Expression in Porcine and Human Granulosa Cells

Insulin and ATRA have also been presented as key factors in modulating ovarian function, which leads to the anovulatory phenotype of PCOS (8–10). We tested the effect of these two factors on COMT expression in the JC410 granulosa cell line. Insulin induced a dose-dependent increase in COMTP1-luciferase transactivation at doses of 50 μIU/mL to 200 μIU/mL (Fig. 4A). The increase ranged from 16% ± 3% at 50 μIU/mL insulin to 71% ± 9% at 200 μIU/mL ($P<.001$, Fig. 4A). All-*trans* retinoic acid had a similar effect with maximal stimulation of 28% ± 7% at a 10 nmol/L concentration ($P<.03$, Fig. 5A). There was no observed effect on COMTP2-luciferase activity (data not shown). These data were normalized against pSV-β-galactosidase. We also performed a Northern blot analysis on mRNA isolated from the human HGL5 granulosa cell line. Both insulin and ATRA increased human COMT RNA transcript levels compared with untreated cells (Figs. 4B and 5B).

DISCUSSION

Although PCOS is the most common cause of anovulatory infertility, the mechanism of follicular arrest in this complex disorder remains unknown (28, 29). Androgen, insulin, and ATRA are all increased in PCOS and seem to be key players in the pathogenesis of this complex disorder (28–31).

Several possibilities have been put forward to explain how androgens might mediate anovulation. Agarwal et al. (32) suggested that androgens probably act as inhibitors of the aromatase enzyme. Others suggested that androgens augment LH-induced cyclic adenosine 3′:5′ monophosphate production, leading to premature luteinization of the follicles (33, 34). In this work, we propose another potential pathway to mediate the deleterious effect of increased androgen on granulosa cell proliferation and steroidogenesis. Increased follicular androgens up-regulate COMT expression in granulosa cells, which leads to augmented methylation of the 2-OHE_2 into 2-ME_2 (Fig. 6). The elevated 2-ME_2 levels in the follicular microenvironment would be associated with inhibited steroidogenesis (Fig. 2) and granulosa cell arrest (Fig. 1), as well as reduced angiogenesis (12–14).

FIGURE 4

Insulin up-regulates COMT expression in porcine and human granulosa cells. (**A**) Insulin increased COMTP1-luciferase reporter transactivation in the JC410 porcine granulosa cell line. The cells were transfected with pCOMTP1. After the addition of various doses of insulin, luciferase production was analyzed 48 hours later. The stimulatory effect of insulin was statistically significant ($P<.001$). Each value is the mean ± SEM of triplicate wells in two independent experiments. (**B**) Northern blot analysis of the effect of insulin on total mRNA expression in HGL5 human granulosa cells at the indicated dose and exposure time. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is the internal loading control. C = vehicle control; S-COMT = soluble catechol-*O*-methyl transferase; MB-COMT = membrane-pound catechol-*O*-methyl transferase.

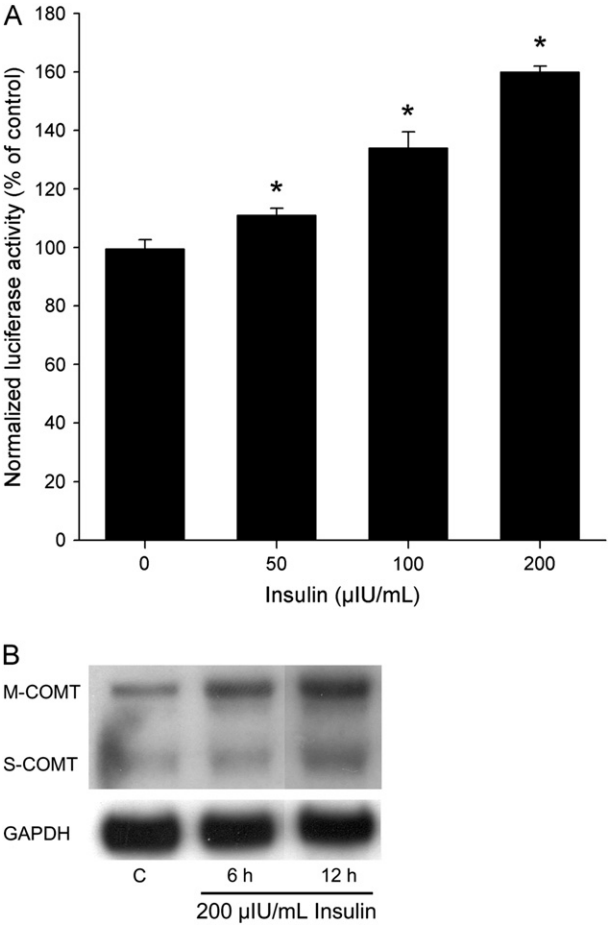

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FIGURE 5

All-*trans* retinoic acid (*ATRA*) up-regulates COMT expression in porcine and human granulosa cells. (**A**) ATRA increased COMTP1-luciferase reporter transactivation in JC410 porcine granulosa cell line. The cells were transfected with pCOMTP1. After the addition of various doses of ATRA, luciferase production was analyzed 48 hours later. The stimulatory effect of ATRA was statistically significant ($P<.03$). Each value is the mean ± SEM of triplicate wells in two independent experiments. (**B**) Northern blot analysis of the effect of ATRA on total mRNA expression in HGL5 human granulosa cells at the indicated dose and exposure time. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is the internal loading control. C = vehicle control; S-COMT = soluble catechol-*O*-methyl transferase; MB-COMT = membrane-pound catechol-*O*-methyl transferase.

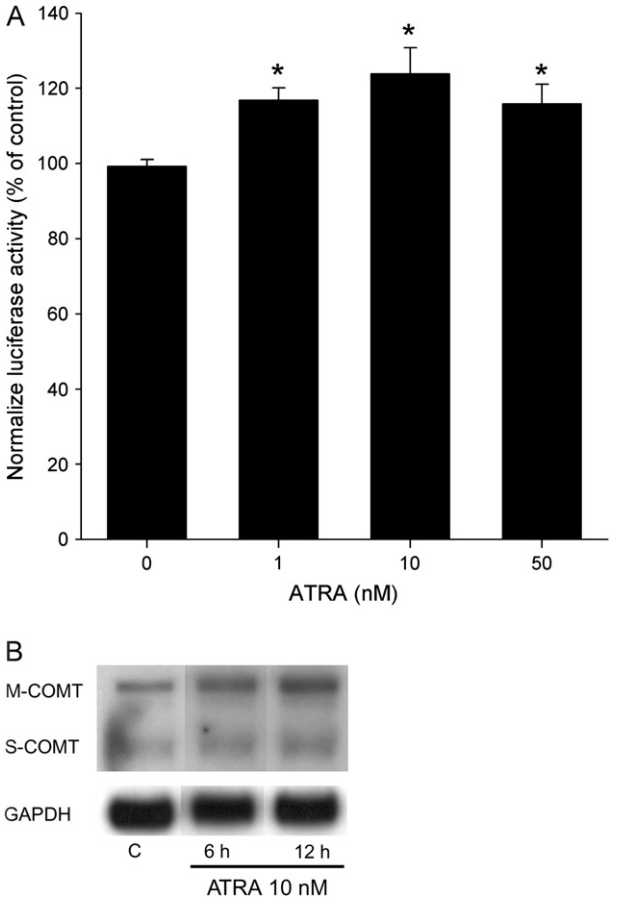

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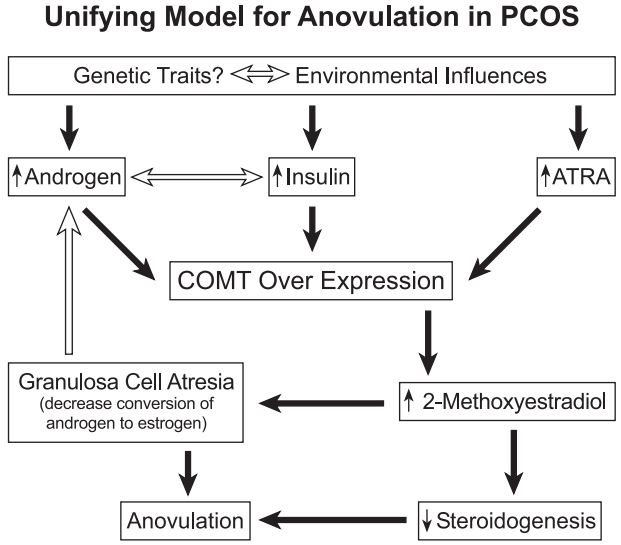
FIGURE 6

Proposed model depicting the potential role of COMT in PCOS pathogenesis. Increased androgen/insulin/ATRA levels result in the up-regulation of COMT in granulosa cells early in the follicular cycle. The higher COMT expression will increase cellular levels of 2-ME_2, a known apoptotic and antiangiogenic agent in the ovary that also inhibits steroidogenesis. The decreased granulosa cell proliferation and steroidogenesis will lead to follicular arrest, augmenting the perturbation of androgen aromatization to E. This will lead to decreased levels of E and increased levels of androgens in the follicle microenvironment, creating a vicious loop that will result in anovulation.



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The diminished granulosa cell number would lead to limited androgen aromatization and eventually create a vicious loop of accumulating androgen and follicular atresia, leading to the PCOS ovarian phenotype (Fig. 6).

In addition to high androgens, hyperinsulinemia and insulin resistance are important features of PCOS (35, 36). Insulin has specific actions on steroidogenesis, which is effected through its own receptor (37). These actions seem to be preserved in insulin-resistant states, and the ovary does not appear to be insulin resistant in women with PCOS (38). The association between hyperinsulinemia and ovarian polycystic phenotype is unclear. Some reports have proposed that effects of insulin could be secondary to its ability to stimulate androgen production from theca cells (5, 39). This effect might then lead to a high intrafollicular concentration of androgens, which may impair the stimulatory effect of insulin on granulosa cell E production (34). This work proposes a potential model that links insulin and increasing androgen production to the pathogenesis of follicular atresia in PCOS ovaries. Insulin up-regulates COMT expression in granulosa cells, leading to augmented methylation of the 2-OHE_2 into 2-ME_2 (Fig. 6). The elevated 2-ME_2 levels in the follicular microenvironment would be associated with inhibited steroidogenesis (Fig. 2) and granulosa cell arrest (Fig. 1).

The mechanism is unclear by which ATRA might contribute to the PCOS phenotype of arrested follicle development. In an attempt to understand the potential role of ATRA in PCOS pathogenesis, Woods et al. (9) demonstrated that ATRA increased dehydroepiandrosterone (DHEA) synthesis. The increased DHEA synthesis was correlated with increased expression of CYP11A1 and CYP17 mRNA. This effect, however, was similar between normal and PCOS theca cells (10). Our model suggests that ATRA may partially inflict its deleterious effect on granulosa cell proliferation and follicle development via up-regulation of COMT expression (Fig. 6). All-*trans* retinoic acid induced a significant increase in COMTP1-reporter transactivation (Fig. 5A), as well as an increase in COMT transcription in human granulosa cells (Fig. 5B). This increased COMT expression leads to higher production of 2-ME_2, which mediates its established inhibitory effect on granulosa cell proliferation and ovarian angiogenesis. Although this pathway might be part of physiologic ovarian homeostasis under normal conditions (40, 41), its hyperactivity can, at least in part, explain the PCOS phenotype of arrested follicle development (9).

Our current report confirms previous reports that 2-ME_2 induces apoptosis in granulosa cells (12–14). The dual effect of 2-ME_2 on granulosa cell growth may suggest a potential physiologic role for this E metabolite in follicle homeostasis; under normal conditions, its level is low (in the stimulatory range) in early folliculogenesis, and the level rises (probably to the inhibitory range) with the augmented E production in the fully developed dominant follicle. This report also shows for the first time that both membrane COMT and soluble COMT are expressed in porcine and human granulosa cells. Modulation of COMT activity and, therefore, the concentration of 2-ME_2 might be part of the ovarian physiologic apparatus. It also suggests that COMT could be a step where pathologic alteration might lead to major perturbation of folliculogenesis. We have recently demonstrated significantly higher COMT immunoreactivity in various compartments of the ovaries from women with PCOS compared with matched controls (42). In the same cohort, there was significantly less urinary 2-OHE_2 excretion in patients with PCOS compared with age-matched healthy controls (Salih et al., unpublished data). Computer-assisted homology analysis identified multiple androgen response elements {TGTTCT (−1201: −1206) and TGACCT (−969: −975; −1474: −1480)}, retinoic acid response elements {ACCAGA (−333: −338) and TGACCT (−969: −975; −1474: −1480)}, and insulin response elements {CCCGCCTC (−132: −140)} in the COMTP1 region (43). These findings suggest that COMT expression might be under intricate regulation by androgen, ATRA, insulin, and other factors.

Catechol *O*-methyltransferase overexpression and increased levels of 2-ME_2 in ovarian granulosa cells might represent a novel mechanism for the abnormalities of steroidogenesis, follicular arrest, and anovulation that characterize women with PCOS. Highly selective COMT inhibitors were able to induce porcine granulosa cell proliferation (Fig. 1), as well as p450SCC transactivation (Fig. 2). Preliminary work in rats suggested that COMT inhibitors exerted a strong inhibitory effect on ovarian apoptosis (44). We are currently investigating the regulation of COMT expression in the whole reproductive process including ovulation, conception, and implantation.

Caution should be exercised when extrapolating these in vitro results on immortalized granulosa cell lines to the clinical and pathologic situation in PCOS. Nevertheless, our data are compatible with the notion that increased levels of androgen, insulin, and ATRA may compromise the development of ovarian follicles and contribute to protracted maturation of oocytes and anovulation in women with PCOS through up-regulation of the COMT enzyme.

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REFERENCES

1. Palumbo A, Yeh J. Apoptosis as a basic mechanism in the ovarian cycle: follicular atresia and luteal regression. J Soc Gynecol Investig 1995;2: 565–73.
2. O'Shea JD, Hay MF, Cran DG. Ultrastructural changes in the theca interna during follicular atresia in sheep. J Reprod Fertil 1978;54:183–7.
3. Hurwitz A, Adashi EY. Ovarian follicular atresia as an apoptotic process: a paradigm for programmed cell death in endocrine tissues. Mol Cell Endocrinol 1992;84:C19–23.
4. Frank S, Mason HD, Polson DW. Mechanism and management of ovulatory failure in women with polycystic ovary syndrome. Hum Reprod 1988;3:351–4.
5. Gilling-Smith C, Willis DS, Beard RW, Franks S. Hypersecretion of androstenedione by isolated thecal cells from polycystic ovaries. J Clin Endocrinol Metab 1994;79:1158–65.
6. Mason HD, Willis DS, Beard RW, Winston RM, Margara R, Franks S. Estradiol production by granulosa cells of normal and polycystic ovaries: relationship to menstrual cycle history and concentrations of gonadotropins and sex steroids in follicular fluid. J Clin Endocrinol Metab 1994;79: 1355–60.
7. Franks S, Mason H, White D, Willis D. Etiology of anovulation in polycystic ovary syndrome. Steroids 1998;63:306–7.
8. The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group. Revised 2003 consensus on diagnostic criteria and long-term health risk related to polycystic ovary syndrome (PCOS). Hum Reprod 2004;19:41–7.
9. Wood JR, Ho CK, Nelson-Degrave VL, McAllister JM, Strauss JF III. The molecular signature of polycystic ovary syndrome (PCOS) theca cells defined by gene expression profiling. J Reprod Immunol 2004;63: 51–60.
10. Wood JR. The molecular phenotype of polycystic ovary syndrome (PCOS) theca cells and new candidate PCOS genes defined by microarray analysis. J Biol Chem 2003;278:26380–90.
11. Lachman HM, Papolos DF, Saito T, Yu YM, Szumlanski CL, Weinshilboum RM. Human catechol-O-methyltransferase pharmacogenetics: description of a functional polymorphism and its potential application to neuropsychiatric disorders. Pharmacogenetics 1996;6:243–50.
12. Shang W, Konidari I, Schomberg DW. 2-Methoxyestradiol, an endogenous estradiol metabolite, differentially inhibits granulosa and endothelial cell mitosis: a potential follicular antiangiogenic regulator. Biol Reprod 2001;65:622–7.
13. Spicer LJ, Hammond JM. Regulation of ovarian function by catecholestrogens: current concepts. J Steroid Biochem 1989;33:489–501.
14. Spicer LJ, Hammond JM. Catecholestrogens inhibit proliferation and DNA synthesis of porcine granulosa cells in vitro: comparison with

estradiol, 5 alpha-dihydrotestosterone, gonadotropins and catecholamines. Mol Cell Endocrinol 1989;64:119–26.
15. Dehennin L, Blacker C, Reiffsteck A, Scholler R. Estrogen 2-, 4-, 6- or 16-hydroxylation by human follicles shown by gas chromatography–mass spectrometry associated with stable isotope dilution. J Steroid Biochem 1984;20:465–71.
16. Salama SA, Ho SL, Wang HQ, Tenhunen J, Tilgmann C, Al Hendy A. Hormonal regulation of catechol-O-methyl transferase activity in women with uterine leiomyomas. Fertil Steril 2006;86:259–62.
17. Wentz MJ, Jamaluddin M, Garfield RE, Al Hendy A. Regulation of catechol-o-methyltransferase expression in human myometrial cells. Obstet Gynecol 2006;108:1439–47.
18. Chedrese PJ, Rodway MR, Swan CL, Gillio-Meina C. Establishment of a stable steroidogenic porcine granulosa cell line. J Mol Endocrinol 1998;20:287–92.
19. Rainey WH, Sawetawan C, Shay JW, Michael MD, Mathis JM, Kutteh W, et al. Transformation of human granulosa cells with the E6 and E7 regions of human papillomavirus. J Clin Endocrinol Metab 1994;78:705–10.
20. Al-Hendy A, Toivanen P, Skurnik M. The effect of growth temperature on the biosynthesis of Yersinia enterocolitica O:3 lipopolysaccharide: temperature regulates the transcription of the rfb but not of the rfa region. Microb Pathog 1991;10:81–6.
21. Al-Hendy A, Lee EJ, Wang HQ, Copland JA. Gene therapy of uterine leiomyomas: Adenovirus-mediated expression of dominant negative estrogen receptor inhibits tumor growth in nude mice. Am J Obstet Gynecol 2004;191:1621–31.
22. Al-Hendy A, Salama SA. Catechol-O-methyltransferase polymorphism is associated with increased uterine leiomyoma risk in different ethnic groups. J Soc Gynecol Investig 2006;13:136–44.
23. Swan CL, Agostini MC, Bartlewski PM, Feyles V, Urban RJ, Chedrese PJ. Effects of progestins on progesterone synthesis in a stable porcine granulosa cell line: control of transcriptional activity of the cytochrome p450 side-chain cleavage gene. Biol Reprod 2002;66:959–65.
24. Smida AD, Valderrama XP, Agostini MC, Furlan MA, Chedrese J. Cadmium stimulates transcription of the cytochrome p450 side chain cleavage gene in genetically modified stable porcine granulosa cells. Biol Reprod 2004;70:25–31.
25. Tenhunen J, Salminen M, Lundstrom K, Kiviluoto T, Savolainen R, Ulmanen I. Genomic organization of the human catechol O-methyltransferase gene and its expression from two distinct promoters. Eur J Biochem 1994;223:1049–59.
26. Copland JA, Zlatnik MG, Ives KL, Soloff MS. Oxytocin receptor regulation and action in a human granulosa-lutein cell line. Biol Reprod 2002;66:1230–6.
27. Havelock JC, Rainey WE, Carr BR. Ovarian granulosa cell lines. Mol Cell Endocrinol 2004;228:67–78.
28. Franks S. Polycystic ovary syndrome: a changing perspective. Clin Endocrinol (Oxf) 1989;31:87–120.
29. Franks S. Polycystic ovary syndrome. N Engl J Med 1995;333:853–61.
30. Dunaif A, Segal KR, Futterweit W, Dobrjansky A. Profound peripheral insulin resistance, independent of obesity, in polycystic ovary syndrome. Diabetes 1989;38:1165–74.
31. Latronico AC, Anasti J, Arnhold IJ, Mendonca BB, Domenice S, Albano MC, et al. A novel mutation of the luteinizing hormone receptor gene causing male gonadotropin-independent precocious puberty. J Clin Endocrinol Metab 1995;80:2490–4.
32. Agarwal SK, Judd HL, Magoffin DA. A mechanism for the suppression of estrogen production in polycystic ovary syndrome. J Clin Endocrinol Metab 1996;81:3686–91.
33. Harlow CR, Shaw HJ, Hillier SG, Hodges JK. Factors influencing follicle-stimulating hormone-responsive steroidogenesis in marmoset granulosa cells: effects of androgens and the stage of follicular maturity. Endocrinology 1988;122:2780–7.
34. Greisen S, Ledet T, Ovesen P. Effects of androstenedione, insulin and luteinizing hormone on steroidogenesis in human granulosa luteal cells. Hum Reprod 2001;16:2061–5.
35. San Roman GA, Magoffin DA. Insulin-like growth factor binding proteins in ovarian follicles from women with polycystic ovarian disease: cellular source and levels in follicular fluid. J Clin Endocrinol Metab 1992;75:1010–6.
36. Dunaif A. Insulin resistance and the polycystic ovary syndrome: mechanism and implications for pathogenesis. Endocr Rev 1997;18:774–800.
37. Willis D, Franks S. Insulin action in human granulosa cells from normal and polycystic ovaries is mediated by the insulin receptor and not the type-I insulin-like growth factor receptor. J Clin Endocrinol Metab 1995;80:3788–90.
38. Willis D, Mason H, Gilling-Smith C, Franks S. Modulation by insulin of follicle-stimulating hormone and luteinizing hormone actions in human granulosa cells of normal and polycystic ovaries. J Clin Endocrinol Metab 1996;81:302–9.
39. Nestler JE, Strauss JF III. Insulin as an effector of human ovarian and adrenal steroid metabolism. Endocrinol Metab Clin North Am 1991;20:807–23.
40. Clagett-Dame M, DeLuca HF. The role of vitamin A in mammalian reproduction and embryonic development. Annu Rev Nutr 2002;22:347–81.
41. Tilly JL, Hirshfield AN. The molecular basis of ovarian cell death. In: Chang RJ, ed. Polycystic ovarian syndrome. Sacramento (CA): Springer, 1995:37–69.
42. Salih S, Nagamani M, Duleba A, Al-Hendy A. Reduced levels of urinary 2 hydroxy estrogens in polycystic ovary syndrome, an insight into pathogenesis of follicular atresia [abstract]. J Soc Gynecol Investig 2006;13:266A.
43. O'Brien RM, Streeper RS, Ayala JE, Stadelmaier BT, Hornbuckle LA. Insulin-regulated gene expression. Biochem Soc Trans 2001;29:552–8.
44. Al-Hendy A, Zaiden M, Eltorky M, Salama S, Wang HQ, Nagamani M. Inhibitors of catechol-O-methyl transferase enzyme exhibit estrogen antagonist effects on female reproductive tract [abstract]. J Soc Gynecol Investig 2005;12:208A.