Chemerin Suppresses Ovarian Follicular Development and Its Potential Involvement in Follicular Arrest in Rats Treated Chronically With Dihydrotestosterone

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In the present study, we have investigated the cellular mechanisms of androgen-induced antral follicular growth arrest and the possible involvement of chemerin and its receptor chemokine-like receptor 1 (CMKLR1) in this process, using a chronically androgenized rat model. We hypothesize that hyperandrogenism induces antral follicle growth arrest via the action of chemerin and ovarian structural changes, resulting from granulosa cell and oocyte apoptosis and theca cell survival. Dihydrotestosterone (DHT) treatment resulted in increased expression of chemerin and CMKLR1 in antral follicles, absence of corpus luteum, and increased atypical follicles. Addition of chemerin to follicle cultures induced granulosa cell apoptosis and suppressed basal, FSH- and growth differentiation factor-9-stimulated follicular growth. DHT down-regulated aromatase expression and increased active caspase-3 content and DNA fragmentation in granulosa cells in vivo. These changes were accompanied by higher phosphatase and tensin homolog and lower phospho-Akt (Ser473) content in antral follicles and higher calpain expression and down-regulation of cytoskeletal proteins in atypical follicles, which were constituted predominantly of theca cells. DHT also activated granulosa cell caspase-3, decreased X-linked inhibitor of apoptosis protein, poly(ADP-ribose) polymerase, and phospho-Akt contents and induced apoptosis in vitro, responses readily attenuated by forced X-linked inhibitor of apoptosis protein expression. These findings are consistent with our hypothesis that antral follicular growth arrest in DHT-treated rats results from increased chemerin expression and action, as well as changes in follicular cell fate and structure, which are a consequence of dysregulated interactions of pro-survival and pro-apoptotic modulators in a cell-specific manner. Our observations suggest that this chronically androgenized rat model may be useful for studies on the long-term effects of androgens on folliculogenesis and may have implications for the female reproductive disorders associated with hyperandrogenism. (*Endocrinology* 154: 2912–2923, 2013)

The mammalian ovary is a complex and highly organized structure, and the follicle is its functional unit, consisting of an oocyte surrounded by granulosa and theca cells (1). Follicular development is tightly regulated by gonadotropins, cytokines, and growth factors via cross talk between granulosa cells, theca cells, and the oocyte. Follicular cell interaction is critical for folliculogenesis and steroidogenesis (2–4). Thal androgens stimulate gran...
ulosa and theca cell proliferation and promote preantral/early antral follicle growth in the primate ovary (5), but high concentrations of these steroids can disturb late-stage follicular development through induction of granulosa cell apoptosis and theca hypertrophy, symptoms often associated with the complex ovarian dysregulation present in hyperandrogenic anovulation (6, 7). Although hyperandrogenism is known to be associated with follicular growth arrest at the antral stage, the cellular mechanisms involved are not completely understood.

Chemerin, a novel adipokine associated with obesity and polycystic ovary syndrome (PCOS) (8, 9), is known to act through its G protein-coupled receptor chemokine-like receptor 1 (CMKLR1). It is synthesized as an 18-kDa prochemerin protein and undergoes serine protease cleavage to form an active 16-kDa protein (10, 11). Our recent studies have shown that ovarian and circulating chemerin levels are elevated in a chronically androgenized rat model and that chemerin suppresses FSH-induced steroidogenesis (12). However, whether and how chemerin is involved in antral follicular growth arrest has not been reported.

The objectives of the present study were to better understand the cellular mechanisms of androgen-induced antral follicular growth arrest and the possible involvement of chemerin and its receptor CMKLR1 in this process, using a chronically androgenized rat model. Our studies have demonstrated that chronic androgen administration increases chemerin and CMKLR1 expression, which is associated with suppressed antral follicle development. The latter response is characterized by dysregulated interactions of survival and proapoptotic modulators in a cell-specific manner, marked changes in follicle structure, apoptotic deletion of granulosa cells and oocytes, and the survival and retention of theca cells. These findings support the notion that our rat model is a useful tool, not only for studies on the long-term effects of androgens on folliculogenesis, but also has implications for the female reproductive disorders with hyperandrogenism.

Materials and Methods

Reagents and antibodies

Dimethyl sulfoxide, PBS tablets, phenylmethyl-sulfonyl fluoride, hematoxylin solution, eosin Y solution, paraformaldehyde, Hoechst-33258, Triton X-100, Tween 20, and phosphatase inhibitor cocktail were obtained from Sigma-Aldrich (Oakville, Ontario, Canada). Fetal bovine serum, nonessential amino acids, penicillin, streptomycin, and fungizone were obtained from Life Technologies (Gaithersburg, Maryland). Anticalpain, antivimentin, antiphosphatase and tensin homolog (PTEN), anticathepsin, anti-CMKLR1, antigrowth differentiation factor-9 (GDF9), blocking peptides for chemerin, CMKLR1 and GDF9, and normal rabbit IgG and normal mouse IgG antibodies were from Santa Cruz Biotechnology (Santa Cruz, California). Anticleaved caspase-3, antifodrin, antipoly (ADP-ribose) polymerase (PARP), anti-Akt and anti-phospho-Akt (Ser473) were from Cell Signaling Technology, Inc (Beverly, Massachusetts). Z-val-ala-aspartic (OMe)-FMK inhibitor (Z-VAD-FMK) for pan-caspase inhibition was purchased from Tocris Bioscience (Ellisville, Missouri). Anti-rabbit and mouse IgG conjugated with horseradish peroxidase were purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). Fluorescein isothiocyanate-conjugated secondary antibodies, Alexa Fluor-488 goat antimouse and antirabbit IgG and Alexa Fluor-594 goat antimouse and anti-rabbit IgG were from Invitrogen (Burlington, Ontario, Canada).

Animals and dihydrotestosterone (DHT)-treated rat model

Female Sprague Dawley rats were obtained from Charles River Canada (Montreal, Quebec, Canada) and maintained under standard conditions. All animal procedures were carried out in accordance with the guidelines of the Canadian Council on Animal Care and approved by the Ottawa Hospital Research Institute Animal Care Committee.

Preparation of DHT-treated rats was performed as described by Mannerås (13) with some modifications. Briefly, rats at 21 days of age were randomly divided into 2 experimental groups (control [CTL, n = 12], DHT [n = 12]) and implanted sc with 90-day continuous-release SILASTIC capsules (Dow Corning Corp., Midland, Michigan) containing 7.5 mg DHT (daily dose, 83 μg; empty SILASTIC capsule as CTL). CTLs received identical pellets lacking the steroid. Rats were monitored twice daily in the first 3 days and once daily thereafter. Animals were weighed weekly to monitor weight gain and euthanized at 12 weeks after implantation. Ovaries were collected for analysis.

Ovarian morphology and follicle types

Ovarian sections from individual animals (10 in CTL group and 12 in DHT group) were used for the morphologic analysis and follicle-counting experiments. Whole ovary samples were fixed in 10% neutral-buffered formalin, paraffin embedded, and then serially sectioned at 4 μm, before mounting on positively charged glass slides. Technical services for hematoxylin-eosin and periodic acid-Schiff (PAS) staining were provided by the Department of Pathology and Laboratory Medicine, University of Ottawa, Ontario, Canada. For measurements and photographs, the slides were scanned with a Scan-Scope (Aperio Technologies, Vista, California) and analyzed with ImageScope virtual microscopy software (Aperio Technologies). The growing follicles in every sixth ovary sections (90–135 slides per ovary were examined per experimental group, depending on ovarian size) were scored according to the following categories: primary, preantral, antral and preovulatory stages, and corpus luteum. Follicles in which the oocyte nucleus was present (the largest cross-section) were scored. Most atypical follicles contained no oocyte, as determined by serial sections.

Ovarian follicle isolation and culture

Large preantral and early antral follicles (diameter, 150–180 μm) from 14- to 15-day-old rats were isolated in Leibowitz L-15

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medium with BSA (0.1%, wt/vol), using 28.5-gauge needles (Becton Dickinson and Co., Franklin Lakes, New Jersey) under a microscope. Follicles with intact basement membranes and theca layers were individually cultured in a 96-well plate (Sarstedt, Newton, North Carolina) in 100 μL of α-MEM supplemented with HEPES (10 mM), BSA (0.1%, wt/vol), bovine insulin (5 μg/mL), transferrin (2 μg/mL), ascorbic acid (25 μg/mL), sodium selenite anhydrous (2 ng/mL), l-glutamine (3 mM), sodium pyruvate (100 μg/mL), streptomycin (100 μg/mL), and penicillin (100 U/mL). Rats at 14-15 days of age, but not older, were selected for these experiments because follicles isolated exhibit minimal granulosa cell apoptosis and atresia. Follicular diameter was measured daily as the average distance between the outer edges of the basement membrane in 2 perpendicular planes and results were expressed as the change in follicular volume. Follicular volume was calculated according to the formula for the volume of a sphere: volume = 4πr³/3, where r is radius. The percent change of follicular volume on day n of culture is defined as the volume difference between day n and day 0 (the day of follicle isolation) expressed as a percentage of the volume at day 0. The culture medium was changed every other day. The expression of X-linked inhibitor of apoptosis protein (XIAP) and GDF9 in cultured follicles was examined by IF and immunohistochemistry (IHC), respectively, as previously described (16, 17).

In situ localization of apoptosis (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling [TUNEL] and immunolocalization of active-caspase-3)

In situ TUNEL was carried out on ovarian sections using a TUNEL kit (Roche, Laval, Ontario, Canada) in accordance with the manufacturer’s instructions. After TUNEL staining, tissue sections were incubated with anti-cleaved-caspase-3 (1:100) overnight at 4°C. For secondary antibody reactions, the sections were incubated with a fluorescence-conjugated secondary antibody (Alexa Fluor-488 goat antirabbit IgG) at room temperature (RT) for 2 hours, and then incubated with ProLong gold antifade reagent and 4,6-diamino-2-phenylindole (DAPI, blue, nuclear stain).

IHC and immunofluorescence (IF) microscopy

Ovarian sections from at least 5 CTL or 5 DHT-treated rats were used to investigate the expression of target proteins by IHC or IF. Tissues were fixed with 4% paraformaldehyde, washed with PBS, and incubated with 0.2% Triton X-100 before incubation with the appropriate primary antibody in 3% BSA at RT. Sections were incubated with fluorescence-conjugated secondary antibody (anti-Akt [1:1000], phospho-Akt [Ser473, 1:1000], PTEN [1:2000], β-actin [1:10 000], XIAP [1:2000]) or PARP [1:5000]), followed by horseradish peroxidase-conjugated rabbit or mouse secondary antibodies (1:2000-1:10,000; 1 h, RT). Signal intensity (enhanced chemiluminescence kit [Amersham Pharmacia Biotech, Arlington Heights, Illinois]) was assessed densitometrically using Molecular Analyst software, version 1.4 (Bio-Rad Laboratories). β-Actin was used as a loading control.

IF quantification of granulosa cells with active caspase-3

Isolated granulosa cells were plated on poly-D-lysine (0.05% wt/vol; Sigma) coated 8-well glass culture slides (Becton, Dickinson and Co.) and cultured in M199 growth media (48 h) before DHT treatment. The cells were fixed in paraformaldehyde 4% (1 h, RT), washed in PBS, and blocked with 1% BSA and 1% goat serum. Active caspase-3 was detected using an anticleaved caspase-3 antibody (1:100; Cell Signaling Technology) and Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (1:100; Invitrogen). Actin was detected with red fluorescent Texas Red-X phalloidin (1:100; Invitrogen) as a cytosolic pro-
tein. Cells were incubated with ProLong gold antifade reagent containing DAPI (blue, nuclear stain). At least 1000 cells per treatment group were counted in randomly selected fields with the counter blinded to the sample group to avoid experimental bias. Cells with active caspase-3 were expressed as a percentage of total cells counted.

Statistical analysis
All data were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc, San Diego, California). The results are presented as mean ± SEM of at least 3 independent replicates as detailed in the figure legends. Data were analyzed by Student’s t test and 1-way or 2-way ANOVA. A 2-way ANOVA was used to assess the effects and interactions of 2 variables, and multiple comparisons were achieved using the Bonferroni post hoc test. Statistical significance was defined at P < .05 (*).

Results
DHT-treated rats exhibit marked ovarian structural abnormalities
In the present study, we have investigated the molecular and cellular events associated with antral follicle arrest that also occur in DHT-treated rats. In contrast to normal ovarian structure containing follicles at different stages of development (preantral and large antral) and normal appearance of theca and granulosa cell layers, and the presence of corpora lutea (Figure 1A), DHT treatment in vivo resulted in a marked reduction in overall ovarian size, the appearance of atypical follicles (with the absence of oocytes and reduced granulosa cell numbers but intact thecal layer), and no corpus lutea (Figure 1A). Follicle counting revealed that DHT-treated rat ovaries harbored a lower percentage of follicles in the preantral to preovulatory stages but increased numbers of condensed atypical follicles (0.1–0.4 mm diameter) (Figure 1B). In order to examine whether the observed DHT-induced changes in follicular morphology were related to the induction of granulosa cell apoptosis, we determined the levels of active caspase-3 and assessed DNA fragmentation in the DHT groups. As shown in Figure 1C, DHT induced a varying degree of granulosa cell apoptosis in antral follicles (Figure 1C, b, c, g, and h), whereas no apoptosis was evident in atypical follicles (Figure 1C, d and i), implying that atypical follicles were depleted of granulosa cells and composed mainly of theca cells. Antral follicles exhibit initially lower levels of granulosa cell apoptosis (Figure 1C, b and g) in DHT-treated rats, which increases with the progres-
sion of the atypical follicles (Figure 1C, c and h), until all granulosa cells were depleted (Figure 1C, d and i). In this context, DHT-treated rats exhibited distinct reductions in the antral volume or absence of the antrum, as well as decreased granulosa cell numbers and retention of the theca layer in antral follicles and the basement membrane in the inner core of the atypical follicles (Supplemental Figure 1 published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). In addition, NR5A1 (also known as steriodogenic factor-1), expressed predominantly in the theca cells of healthy pre-antral and early antral follicles (19), was localized in atypical follicles of DHT-treated rats (Supplemental Figure 2).

Estrogen is synthesized from androgens by the p450 cytochrome aromatase and is responsible for granulosa cell proliferation and the ovarian follicular development (20, 21). We then investigated the relationship between granulosa cell p450 aromatase expression and the occurrence of apoptosis by double immunostaining in the ovaries from CTL and DHT-treated rats. Supplemental Figure 3 shows the granulosa cell response to DHT treatment: aromatase expression is down-regulated (Supplemental Figure 3, a and e) while apoptosis increases, as evidenced by higher active caspase-3 level in the follicles of DHT-treated rats (Supplemental Figure 3, b and f). Strong aromatase expression is clearly visible in the granulosa cells of antral follicles in CTL rats (Supplemental Figure 3a). H&E staining showed considerable granulosa cell detachment and nuclear fragmentation in antral follicles of DHT-treated rats, indicative of extensive atresia (Supplemental Figure 3h).

PTEN protein content is markedly up-regulated in granulosa cells of DHT-treated rats, whereas phospho-Akt (Ser473) content is decreased

The phosphoinositide 3-kinase (PI3K)/Akt (protein kinase B) pathway plays a pivotal role in regulation of granulosa cell proliferation and survival and is negatively regulated by phosphatase and tensin homolog (PTEN) (22, 23). To determine whether the increased levels of granulosa cell apoptosis after DHT treatment in vivo is associated with dysregulation of the PI3K, follicular phospho-Akt (Ser473) and PTEN contents in CTL and DHT-treated rats were examined by double-label fluorescent IHC. Granulosa cells of DHT-treated rats exhibited considerably higher PTEN levels and significantly decreased Akt phosphorylation, whereas theca phospho-Akt levels were relatively higher (Figure 2A). Interestingly, PTEN expression was limited to the granulosa cells and was not detected in the theca layer. Assessment of total Akt levels revealed no difference between granulosa and theca cells of antral follicles in CTL and DHT-treated rats (Figure 2B). In addition, we examined the influence of DHT on the content of phospho-Akt and PTEN in granulosa cells in vitro. Consistent with our in vivo IF data, DHT increased PTEN levels and down-regulated phospho-Akt content but not total Akt in granulosa cells (Figure 2C). A time course experiment show that DHT suppressed p-AKT contents at 12 and 24 hours without changing total-Akt content (Figure 2D).

DHT down-regulates XIAP expression and activates caspase-3 in the induction of granulosa cell apoptosis

We observed that DHT-induced granulosa cell apoptosis in vivo is associated with caspase-3 activation, and in vitro DHT treatment significantly induces the activation of caspase-3 in granulosa cells (Supplemental Figure 4). To investigate the mechanism responsible for granulosa cell death, we examined the effect of DHT in vitro on the content of the antiapoptotic protein XIAP, an important intracellular protein involved in the regulation of granulosa cell proliferation and apoptosis (16), and PARP in granulosa cells. DHT decreased granulosa cell XIAP and PARP contents in a concentration-dependent manner (Figure 3A) and in different time intervals (Figure 3B). The activation of caspase-3 and granulosa cell apoptosis was directly related to decreased XIAP expression and increased XIAP fragmentation, whereas forced expression of XIAP attenuated DHT-induced granulosa cell caspase-3 activation (Figure 3D) and PARP down-regulation (Figure 3C). Finally, we found evidence for the role of caspase-3 activation on DHT-induced apoptosis using the pan-caspase inhibitor zVAD-fmk. Pretreatment with zVAD-fmk significantly suppressed DHT-induced down-regulation of XIAP and PARP (Figure 3E) and caspase-3 activation (Figure 3F).

Chemerin and its receptor are overexpressed in the ovarian follicles in DHT-treated rats and suppress follicular growth by down-regulation of granulosa cell XIAP expression and induction of apoptosis

Recently, we have demonstrated that ovarian mRNA abundance and protein contents of chemerin and its receptor CKMLR1 were higher in DHT-treated rats compared with CTLs (12). However, whether and how chemerin and CKMLR1 are involved in the dysregulation of follicular development in this chronically androgenized rat model is unknown. As shown in Supplemental Figure 5A, Chemerin and CKMLR1 were expressed in granulosa cells, theca cells, and oocytes at different stages of normal rat follicular development. GDF9 was expressed only in the oocyte, with expression increasing with follicular mat-
Figure 2. Effect of DHT on PTEN and phospho-Akt (Ser473) Protein Levels in Vivo and in Vitro. A, Immunolocalization of PTEN and phospho-Akt in CTL and DHT-treated rats. Ovarian tissue sections were immunostained (magnification, ×200) for PTEN (red, a and e) and phospho-Akt (green, b and f) and selected areas of merged images (c and g) with PTEN and phospho-Akt are shown at increased magnification (d and h). PTEN content was up-regulated in granulosa cells but undetectable in the theca cells of DHT-treated rats. Phospho-Akt was significantly down-regulated in the granulosa cells of DHT-treated rats compared with CTLs, whereas its expression in the theca cells was relatively higher. B, There was no difference in total Akt levels in granulosa and theca cells between CTL and DHT-treated rats. C and D, DHT increased PTEN levels but decreased contents of phospho-Akt, but not total Akt, in vitro. Granulosa cells from antral follicles were cultured with various concentrations of DHT (0–10 μM, 48 h) or with 10 μM DHT for the indicated time periods (0–24 h). Contents of PTEN, phospho-Akt, and Akt were examined by Western blot. Actin was used as a loading control. Results are expressed as mean ± SEM (n = 3 independent experiments); data were analyzed by 1-way ANOVA followed by Bonferroni post hoc test (*, P < .05; **, P < .01; ***, P < .001 compared with CTL groups). NS, not significant; P = .754; GCs, granulosa cells; TCs, theca cells.
duration (Supplemental Figure 5B). Whereas the expression of chemerin and CKMLR1 was higher in granulosa cells and theca cells in antral follicles of DHT-treated rats compared with that in CTL groups (Figure 4), oocyte-derived growth factor GDF9 was down-regulated in DHT-treated rats (Supplemental Figure 5C).

To examine whether chemerin regulates rat follicular growth, ovarian follicles (diameter, 150–180 μm) were cultured with chemerin (0–1000 ng/mL) for 4 days in vitro. As shown in Figure 5A, chemerin significantly suppressed basal follicle growth in a concentration-dependent manner. Whereas 50 ng/mL chemerin significantly suppressed basal follicular growth by 50%, maximal suppression was observed at 100 ng/mL of the adipokine. Because GDF9 plays a critical role in the regulation of early follicular development and granulosa cell proliferation (17, 24), we then examined the influence of chemerin on GDF9 expression and action. Chemerin suppressed GDF9 expression in the oocyte of follicles in a concentration-dependent manner (Supplemental Figure 5D), suggesting that chemerin-induced follicle growth arrest may be associated, in part, with GDF9 down-regulation.

In addition, chemerin significantly suppressed GDF9- and FSH-stimulated follicle growth (Figure 5, B and C). These responses were associated with down-regulation of XIAP (Figure 5, E and F) and a significant level of granulosa apoptotic cell death (Figure 5D), suggesting that XIAP down-regulation by chemerin may be responsible for the induction of granulosa cell apoptosis and follicular growth arrest.

Follicles in DHT-treated rats exhibit high calpain expression and down-regulated cytoskeletal proteins

DHT-treated rats exhibited markedly increased numbers of condensed atypical follicles and dramatic changes in the structure of the ovary (Figure 1). To determine whether the structural changes observed in the condensed atypical follicles of DHT-treated rats are associated with changes in cytoskeleton-remodeling enzymes, the expression of calpain and its substrates vimentin, fodrin, and α-tubulin in DHT-treated rats was analyzed by fluorescence IHC. Antral and atypical follicles exhibited higher calpain expression in DHT-treated rats compared with nontreated CTLs (Figure 6A). The inter-
mediate filaments, vimentin and fodrin, as well as the microtubule α-tubulin were significantly down-regulated in atypical follicles in DHT-treated rats (Figure 6B, a–c). In contrast, actin, which is not a substrate of calpain, remained unaffected (Figure 6B, d).

Discussion

In the present study, we have investigated the role of chemerin and the cellular mechanisms involved in androgen-induced antral follicular growth arrest, using a chronically androgenized rat model. Our studies demonstrate that chronic androgen administration increases chemerin and CKMLR1 expression that is involved in the induction of antral follicle growth arrest. The latter response is characterized by dysregulated interactions of survival (p-Akt, XIAP, PARP) and proapoptotic (PTEN, caspase-3) factors in a cell-specific manner. This hyperandrogenic state is also accompanied by marked changes in follicle structure, including up-regulation of calpain expression and decreased cytoskeletal proteins, apoptotic deletion of granulosa cells and oocytes, and the survival and retention of theca cells.

Aromatase catalyzes the conversion of androgens to estrogens, and estrogens are known to promote granulosa cell proliferation and follicular growth in vivo (20, 21). In the present study, active caspase-3 level and nuclear fragmentation in granulosa cells were markedly increased in DHT-treated rats, a response accompanied by down-regulation of aromatase expression. In addition, it is also known that PI3K/Akt signaling is important for aromatase expression during granulosa cell differentiation (22). Our results reveal that DHT-treated rats exhibit decreased granulosa cell Akt phosphorylation that was accompanied by higher PTEN level, raising the possibility that they may be involved in the regulation of granulosa cell demise. PTEN, as a phosphatase, is known to dephosphorylate phosphatidylinositol (3,4,5)-trisphosphate, thereby negatively regulating PI3K/Akt signaling (23). A recent study reports that PTEN mutant mice exhibit enhanced ovulation, decreased apoptosis, and increased proliferation in granulosa cells (25).

Moreover, our current study also suggests that the dysregulated follicular growth in DHT-treated rats is controlled by complex interactions and dysregulation of survival (down-regulation of p-Akt, XIAP, and PARP) and proapoptotic (up-regulation of PTEN and caspase-3) modulators in a cell-specific manner. The mechanism by which phospho-Akt down-regulation influences granulosa cell apoptosis in the DHT-treated rat model remains unknown. It is possible that phospho-Akt down-regulation reduces the expression and stability of XIAP. We have previously demonstrated the antiapoptotic role of XIAP in granulosa cells (16, 18, 26), and here we show that physiologically relevant concentrations of DHT decrease XIAP and phospho-Akt contents and enhance active caspase-3 level in granulosa cells in vitro. Because Akt phosphorylates XIAP and prevents its auto-ubiquitination and proteasomal degradation (27), it is conceivable that the decreased granulosa cell phospho-Akt content may lead to XIAP destabilization and caspase-3 activation. This was further supported by the observation that forced expression of XIAP significantly attenuated DHT-induced caspase-3 activation and PARP down-regulation. These findings suggest that the interaction of survival and antiapoptotic factors plays a critical role in regulating DHT-induced granulosa cell apoptosis.

It has been demonstrated that chemerin levels in the circulation are elevated in obese women (8) and PCOS patients (9). In the current studies, we observed a higher ovarian chemerin and CMKLR1 expression in the DHT-treated rats when compared with CTLs. Our unpublished data indicated that the levels of chemerin and CMKLR1 are elevated in ovarian follicles of human PCOS subjects.
Chemerin Induces Follicular Growth Arrest Which Is Associated with the Induction of Granulosa Cell Apoptosis and Down-Regulation of XIAP

A–C, Chemerin suppressed basal and GDF9- and FSH-stimulated rat follicle growth in vitro. Follicles (diameter, 150–180 μm) isolated from 14-day-old rats were cultured with different concentrations of chemerin (A; chem, 0, 10, 50, 100, and 1000 ng/mL), chemerin (B; chem, 0, 100, and 1000 ng/mL) GDF9 (100 ng/mL) or (C; chem, 0, 100, and 1000 ng/mL) FSH (10 ng/mL) for 4 days. Follicular diameter was determined and changes in follicular volume were calculated as we reported earlier (17). n = 5 replicate experiments each with 10 follicles per group. (Data were analyzed by 2-way ANOVA followed by Bonferroni post hoc test. In panel A: *, P < .05; **, P < .01; ***, P < .001 compared with CTL group. In panels B and C: *, P < .05; **, P < .01; ***, P < .001 compared with GDF9 or FSH only group). D, Follicles were cultured with chemerin (0–1000 ng/mL) for 4 days and in situ detection of apoptotic cells (TUNEL staining) was performed. A positive CTL (DNase, 3 μg/mL) and a negative CTL (TUNEL buffer without terminal deoxynucleotidyl transferase) are shown. Images were merged with DAPI staining. Chemerin induced significantly granulosa cells apoptosis. (n = 10 follicles per group); data were analyzed by 1-way ANOVA followed by Bonferroni post hoc test (*, P < .05; **, P < .01 compared with CTL group). E, Follicles were cultured with and without chemerin (100 ng/mL) 4 days. Follicles tissue sections were immunostained for XIAP and normal rabbit IgG (as a negative CTL). Images were merged with DAPI staining. XIAP fluorescence intensity was analyzed by Image J. Chemerin suppressed XIAP expression in granulosa cells of follicles. (n = 10 follicles per group); data were analyzed by an unpaired Student t test (*, P < .05 compared with CTL group). F, Granulosa cells were cultured with chemerin (0–1000 ng/mL) for 4 days. XIAP content was examined by Western blot and actin was used as a loading control. Chemerin down-regulated XIAP protein levels. Results are expressed as mean ± SEM (n = 3 independent replicates); data were analyzed one-way ANOVA followed by Bonferroni post hoc test (*, P < .05 compared with CTL group). Chem, chemerin.
**Figure 6.** A, Fluorescent immunolocalization of calpain and cytoskeletal proteins (vimentin, fodrin, α-tubulin, and actin) in CTL and DHT-treated rats (magnification, ×200). Ovarian tissue sections were immunostained for calpain (a–c) or normal rabbit IgG (negative CTL, d). Panels e–h show DAPI staining. The expression of calpain in granulosa and theca cells was considerably higher in the antral and atypical follicles of DHT-treated rats when compared with CTL. B, Immunoreactivities of the intermediate filament proteins fodrin (a) and vimentin (b), microtubular protein α-tubulin (c), and microfilamental protein actin (d) in DHT-treated rats. Vimentin, fodrin, and α-tubulin were significantly down-regulated in atypical follicles of DHT-treated rats. In contrast, actin (which is not a substrate of calpain), remained unaffected by DHT treatment. C, A hypothetical model illustrating the cellular events involved in dysregulated ovarian follicular growth under chronic hyperandrogenization in the rat. Excess androgen increases the levels of chemerin and CMKLR1 and decreases granulosa cell aromatase expression, a response accompanied by increased active caspase-3 content and DNA fragmentation. These events result in apoptotic clearance of the oocyte and granulosa cells but survival and retention of the theca cell layer. The latter collapses with increased calpain expression and activity down-regulates the cytoskeletal substrates vimentin, fodrin, and α-tubulin in the atypical follicles. D, Proposed signaling pathways involved in chemerin-induced follicular growth arrest in a chronically androgenized rat model showing up-regulation of intracellular proapoptotic mediators PTEN and caspases-3 and down-regulation of antiapoptotic factors XIAP, PARP, and phospho-Akt in granulosa cells and oocyte-expressing GDF9, with phospho-Akt being up-regulated for theca cell survival. F, follicle; CL, corpus luteum; GCs, granulosa cells; TCs, theca cells; OO, oocyte; An, antrum.
compared with the CTLs. In addition, chemerin levels in human follicular fluid are 8-fold higher than those in serum (Wang, Q., and K. Xue, unpublished data). These observations suggest that chemerin may be a potential intraovarian regulator of ovarian follicular development. For the first time, we demonstrate that chemerin down-regulates XIAP expression and induces apoptosis in granulosa cells, thereby suppressing follicle growth. It is known that the oocyte-derived factor GDF9 promotes granulosa cell proliferation and preantral/early antral follicle growth (17, 24). In this study, we also observed that chemerin suppresses GDF9 expression and GDF9-induced follicular growth in vitro. Whereas GDF9 levels in oocytes were reduced in DHT-treated rats, an aberrant GDF9 expression (28) and 5 novel missense GDF9 mutations have been reported in PCOS patients (29). Therefore, defining the interplay between chemerin and GDF9 expression and action could provide important insights into the dysregulated follicular development in this complex syndrome.

In addition to its role in the control of ovarian follicular growth, chemerin is important in the regulation of follicular steroidogenesis (12). We have previously reported that chemerin inhibits FSH-induced aromatase expression and estrogen secretion in granulosa cells and that this influence is mediated through increased expression and action of the mitochondrial protein (12, 30). This observation, together with our present finding that elevated chemerin levels and down-regulated aromatase expression are positively related to increased granulosa cell apoptosis in DHT-treated rats, supports the hypothesis that chemerin plays a paracrine and/or autocrine-regulatory role in the ovary and contributes to the dysfunction of the ovarian function. Further studies on the downstream signaling pathway triggered by chemerin in the dysregulation of follicular development may provide important clues in the understanding of the precise role of chemerin in follicle growth arrest.

In the present study, we have shown that DHT-treated rats display dysregulated antral follicular growth and have begun to define the cellular mechanisms involved in its structural changes. Our observation that the ovarian weight in DHT-treated rats was decreased compared with that in CTLs is in good agreement with other PCOS rodent models induced by androgen or estrogen treatment (13, 31–33) but is in contrast to the human phenotype. The mechanisms responsible for the reduced ovarian weight in these rodent models remain unknown. We have demonstrated here that DHT administration induces shrinkage of the follicular structure, the appearance of atypical follicles with oocytes absent, a marked reduction in granulosa cell number and follicular fluid volume, and retention of the theca layer. It is possible that the observed changes in cytoskeletal proteins and structural perturbations in atypical follicles could decrease the overall permeability of critical areas required for fluid influx. The calcium-dependent protease calpain degrades cytoskeletal components such as microtubule and intermediate filament proteins and is a critical regulator of cytoskeletal reorganization, cell morphology, and apoptosis (34–36). In the present studies, antral and atypical follicles of DHT-treated rats exhibited markedly higher calpain expression compared with CTLs, an observation associated with decreased expression of the cytoskeletal substrates, vimentin, fodrin, and α-tubulin, but not of actin (not a calpain substrate). Taken together, these findings demonstrate, for the first time, that increased calpain expression, with concomitant down-regulation of cytoskeletal substrates, is closely associated with the follicle collapse and development of atypical follicles, the final stage of follicular dysregulation in DHT-treated rats.

In conclusion, using a chronically androgenized rodent model, we have defined the cellular and molecular events leading to the dysregulation of ovarian follicular growth and offered a new insight into the mechanism of ovarian follicular growth arrest in the pathology of hyperandrogenism. Notably, we have provided novel evidence that chemerin is an important intraovarian regulator and may contribute to the dysregulation of follicular development. To facilitate future investigations, we hypothesize that antral follicle growth promoted by gonadotropin and GDF9 is arrested in DHT-treated rats as a result of enhanced chemerin expression and action, as well as of ovarian structural changes following apoptotic clearance of the oocyte and granulosa cells and the survival and retention of the theca cell layer. The follicle collapses with increased calpain expression and down-regulation of its cytoskeletal substrates (Figure 6C). We also propose that the dysregulated ovarian follicular development in DHT-treated rats is a consequence of chemerin-induced up-regulation of intracellular proapoptotic mediators (PTEN and caspases-3) and down-regulation of antiapoptotic factors (XIAP and phospho-Akt) and their complex interactions (Figure 6D), many of which remain to be elucidated. Although inherent limitations exist in all animal models developed for human diseases (37), the present studies significantly contribute to the current understanding of the chronic influence of excess androgen on ovarian follicular growth as well as the pathology of female reproduction.
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