The post-menopausal ovary displays a unique pattern of steroidogenic enzyme expression

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BACKGROUND: While menopause results in the loss of cyclic steroid production, evidence exists for persistent, albeit reduced, ovarian androgen production. In order to continue to synthesize ovarian androgens, the steroidogenic enzymes necessary for androgen biosynthesis must be present. Few studies have selectively analysed some of the steroidogenic enzymes present in the post-menopausal ovary (PMO), and a comprehensive study of this matter has never been undertaken. METHODS: RNA and protein were obtained from PMO, pre-menopausal ovarian stroma, corpora lutea (CL), ovarian follicles, placenta, and myometrium. Oligonucleotide microarray analysis was performed to compare the gene expression profiles of PMO with pre-menopausal ovarian stroma. Real-time RT–PCR was performed for LH/HCG receptor (LHCGR), steroidogenic acute regulatory (StAR), cholesterol side-chain cleavage (CYP11A), 3β-hydroxysteroid dehydrogenase type I (HSD3B1) and type II (HSD3B2, 3βHSD), 17α-hydroxylase (CYP17), cytochrome b5 (CytB5), and aromatase (CYP19). Western blot analysis was performed for StAR, CYP11A, CYP17, and 3βHSD. RESULTS: The PMO and pre-menopausal ovarian stroma had a similar pattern of steroidogenic enzyme expression. The PMO had persistent, but reduced, levels of LHCGR and most steroidogenic enzymes. CYP19 and HSD3B2 mRNA were greatly reduced in PMO in comparison with CL (50-fold and 2000-fold less respectively). HSD3B2 was not detectable in PMO by western analysis. CONCLUSIONS: This study supports the idea that the PMO retains some steroidogenic capacity. However, based on steroidogenic enzyme expression, the PMO has a unique pattern of steroidogenic enzyme expression that favors Δ5 steroid formation over Δ4 steroid formation.

Key words: HCG/ovary/post-menopausal/steroids

Introduction

Menopause results in the loss of cyclic estradiol and progesterone production and secretion by the human ovary. In contrast, there is some evidence that the post-menopausal ovary does retain the capacity to produce other steroids, specifically, androgens (Vermeulen, 1976; Adashi, 1994; Ushiroyama and Sugimoto, 1995). Some investigators have demonstrated that the post-menopausal ovary (PMO) continues to possess gonadotrophin binding sites (Peluso et al., 1976; Nakano et al., 1989), is steroidogenically responsive to gonadotrophins (Dennefors et al., 1982), and that serum androgen levels decrease in post-menopausal women treated with GnRH agonists (Dowsett et al., 1988). Since menopause results in the loss of ovarian follicles and the accompanying granulosa cell compartment, the PMO does not appear to remain responsive to FSH. In contrast, LH responsiveness is believed to persist in the ovarian hilus and stroma.

Both the ovary and the adrenal gland are responsible for androgen synthesis in women. Differentiating the quantitative and qualitative differences in the origin of the steroids requires an understanding of the steroidogenic capacity of each of these tissues. The adrenal gland produces predominantly the Δ5 steroids, dehydroepiandrosterone (DHEA) and its sulphated form DHEA-S. These steroids are then converted to more potent Δ4 androgens such as testosterone and dihydrotestosterone (DHT), through peripheral metabolism. In contrast, the ovary synthesizes negligible amounts of DHEA and DHEA-S, but does synthesize the Δ4 steroids androstenedione, testosterone, and DHT. These qualitative differences are due to the differences in steroidogenic enzyme expression.

In order for the post-menopausal ovary to continue to produce androgens, it must maintain the ability to express the enzymes necessary for androgen biosynthesis, and additionally, retain the ability to respond to gonadotrophins, specifically through activation of the LH/HCG receptor (LHCGR) by LH. While research to date has been limited on this subject, initial studies had demonstrated by northern analysis that the post-menopausal ovary possessed the capacity to express the steroidogenic enzymes necessary for the initiation of ovarian steroidogenesis, but only ovariectomy of post-menopausal women with endometrial hyperplasia or cancer expressed all the enzymes necessary for androgen synthesis (Nagamani and Urban, 2003). A recent analysis of...
Sequences of primers and probes used for quantification of gene expression by real-time RT–PCR. CL was identified separately from follicles by gross morphological appearance, menstrual dating, and endometrial histology to differentiate between the two groups of tissues. Myometrial tissues were obtained from non-pregnant patients, to ensure that placental tissue was not present. The PMO and pre-menopausal ovarian stroma was isolated by removing 1–2 mm of the surface epithelium free from the cortex. PMO were identified in women with absence of menses >1 year and/or FSH >30 IU/l. Tissues were either stored in RNAlater (Ambion, Inc., Austin, TX, USA) at −20°C, or snap-frozen in liquid nitrogen and stored at −80°C prior to RNA or protein isolation. All tissues were collected in accordance with the Institutional Review Board of the University of Texas Southwestern Medical Center at Dallas and with informed consent from women undergoing surgery.

**RNA preparation**

Tissues were pulverized in liquid nitrogen and then homogenized in guanidinium isothiocyanate. Total RNA was isolated from pre-menopausal and post-menopausal ovarian stroma, corpora lutea, ovarian follicles, placenta, and myometrium as previously described (Chigrwin et al., 1979), followed by deoxyribonuclease I treatment (Ambion). Concentration of RNA was measured and purity confirmed by spectroscopy.

**Microarray analysis**

Pools of RNA from pre-menopausal ovarian stroma (n = 2) and a post-menopausal ovarian stroma sample (n = 1) were hybridized to an Affymetrix Human Genome U133 plus 2.0 oligonucleotide two-microarray set containing >54,000 probe sets representing >38,500 human genes (Affymetrix, Santa Clara, CA, USA). The arrays were scanned at high resolution using an Affymetrix GeneChip Scanner 3000 located at the University of Texas Southwestern Microarray Core Facility. Results were analysed using GeneSpring version 7 software (Silicon Genetics, Redwood City, CA, USA) to identify genotypic differences between pre-menopausal and post-menopausal human ovarian stroma.

**Real-time RT–PCR**

Two micrograms of total RNA were reverse-transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Primers and probes for real-time RT–PCR were designed using the Primer Express computer program (Applied Biosystems) (Table I). All real-time RT–PCR were in a 30 μl reaction volume. For LHCGR, StAR, CYP11A1, cytochrome b5 (CytB5), and CYP19 quantitation, SYBR Green I (Applied Biosystems) was used along with SYBR Green Universal PCR Master Mix (1x) (Applied Biosystems) and 0.1 μmol/l of each primer. CYP17, 3β-hydroxysteroid dehydrogenase type I (HSD3B1), HSD3B2 and 18S quantification were performed using TaqMan Ribosomal RNA Reagent kit (1x) (Applied Biosystems). For CYP17, the final concentrations of primer and probe used were 0.1 μmol/l each. For HSD3B1 and HSD3B2, the final concentrations of primer and probe used were 0.4 and 0.2 μmol/l respectively. For 18S, the final concentrations of primer and probe were 0.05 and 0.1 μmol/l respectively. The TaqMan primer/probe pairs were only used when

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provides the problem that arises, or when there were difficulties in designing specific primers due to significant sequence homology (HSD3B1 and HSD3B2). The reverse transcription product from 40 ng RNA was used as template for all real-time RT–PCR reactions. All real-time RT–PCR were carried out in two steps, using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) and the dissociation protocol. Step 1: 50°C for 2 min followed by 95°C for 10 min, one cycle. Step 2: 95°C for 15 s, followed by 60°C for 60 s, 40 cycles. Standard curve cDNA plasmids for all genes were used to quantify transcript levels. As an internal standard, each individual sample was normalized to its 18S ribosomal RNA content. mRNA levels were expressed as attomoles per microgram 18S rRNA.

**Protein determination and western blot analysis**

Cells were solubilized in 1× Passive Lysis Buffer (Promega, Madison, WI, USA) and stored frozen at ~20°C. Subsequently, protein content was determined by bicinchoninic acid protein assay, using the BCA assay kit (Pierce, Rockford, IL, USA). Polyacrylamide gel electrophoresis (PAGE) was carried out using pre-cast 4–12% bis–Tris NuPage gels (Novex, San Diego, CA, USA) using a NuPAGE 1x MOPS running buffer (Invitrogen, Carlsbad, CA, USA). After electrophoresis, proteins were electrophoretically transferred onto polyvinylidene difluoride membranes for 1 h at 25 V. After transfer and 1 h blocking with 5% w/v milk/TBS–0.1% Tween solution, immunoblotting was performed with rabbit polyclonal antibodies. Antibody sources were: human CYP17 antibody (M.Waterman, Vanderbilt University Medical Center, Nashville, TN, USA), StAR antibody (D.Buck Hales, University of Illinois at Chicago, Chicago, IL, USA), human 3βHSD antibody (J.Ian Mason, University of Edinburgh, Edinburgh, UK), and human CYP11A antibody (Bong-Chu Chung, Institute of Molecular Biology Taiwan, China). The membranes were incubated with StAR (1:5000), CYP11A (1:2000), CYP17 (1:2000) and 3βHSD (1:2500) antibodies overnight at 4°C. The primary antibody incubation was followed by incubation with an anti-rabbit Ig, and horseradish peroxidase-linked F (ab')2 fragment from donkey (Jackson ImmunoResearch, West Grove, PA, USA). Immunoreactive bands were visualized using enhanced chemiluminescence western blotting detection reagents from Amersham Biosciences (Piscataway, NJ, USA) on X-Omat Blue XB-1 Scientific Imaging Film (Kodak, Rochester, NY, USA). Two independent PMO samples (PMO 1 and PMO 2), two independent pre-menopausal ovarian stroma samples (ovarian stroma 1 and ovarian stroma 2) and two independent CL samples (corpus luteum 1 and corpus luteum 2) were compared with myometrium as a negative control. Western blot analyses were repeated once or twice to confirm the initial results.

**Statistical analysis**

Data were analysed using one-way analysis of variance on ranks. Dunn’s test was used for multiple comparisons testing. Significance level was $P < 0.05$. Data were displayed as mean ± SE. Data analysis was performed using SigmaStat version 3.0 (SPSS, Chicago, IL, USA).

**Results**

**Gene expression profiles for ovarian steroidogenic enzymes and LHCG in pre-menopausal and post-menopausal ovarian stroma**

In order to determine whether there are any significant differences in LHCG or steroidogenic enzyme expression in post-menopausal ovarian stroma compared with the steroidogenic pre-menopausal ovarian stroma, microarray analysis was performed (Figure 1). Comparison of the gene expression profiles of these two tissues demonstrated similar expression levels of the genes encoding the steroidogenic enzymes, with the exception of CYP19 (33-fold greater in pre-menopausal stroma) and HSD3B2 (8-fold greater in pre-menopausal stroma). In order to confirm the findings of the oligonucleotide microarray analysis, real-time RT–PCR and western blot analyses were performed to validate the gene expression profile results.

**LHCGr expression in steroidogenic tissues**

Analysing LHCGr mRNA expression was necessary to determine whether the post-menopausal ovarian stroma retains the ability to respond to gonadotrophins, as seen in pre-menopausal theca-interstitial cells. As shown in Figure 2A, both the PMO and pre-menopausal ovarian stroma expressed ~100-fold higher LHCG transcript levels than myometrium ($P < 0.005$). In contrast, in comparison with other steroidogenic tissue, CL levels were ~30-fold greater than PMO, and follicles ~8-fold greater than PMO or pre-menopausal ovarian stroma (Figure 2B).

**Steroidogenic enzyme expression in steroidogenic tissues**

The first step in ovarian steroidogenesis involves the transport of cholesterol to the inner mitochondrial membrane and metabolism to pregnenolone. This step involves the cholesterol transport protein, StAR, and the cholesterol-metabolizing enzyme CYP11A. PMO was found to have StAR transcript levels 3000-fold greater than myometrium ($P < 0.001$) (Figure 3A). StAR mRNA expression appeared to be similar between all steroidogenic tissues studied, with levels in the PMO comparable to CL (Figure 3B). Western analysis confirmed the presence of the StAR protein in PMO, pre-menopausal ovarian stroma, but not in myometrium (Figure 3C). Comparing CYP11A mRNA expression in the PMO with ovarian stroma and myometrium, the PMO had similar transcript levels as pre-menopausal ovarian stroma, but had ~300-fold greater CYP11A transcript levels than myometrium ($P < 0.005$) (Figure 4A). In contrast, in comparison with other steroidogenic tissues, CL and placenta expression levels were significantly greater than the PMO, with a 70-fold and 900-fold difference respectively (Figure 4B). Finally western analysis indicated that CYP11A protein was still present in PMO, pre-menopausal ovarian stroma, and CL, but not present in myometrium (Figure 4C).

The next step in ovarian steroidogenesis involves the 17α-hydroxylase and 17,20-lyase reactions that occur under the control of one enzyme encoded by the CYP17 gene. This results in the conversion of pregnenolone to DHEA. The PMO and ovarian stroma had 200-fold greater CYP17 transcript levels than myometrium ($P < 0.005$) (Figure 5A). In contrast, when compared with other steroidogenic tissues, CL CYP17 mRNA levels were ~20-fold greater than levels in PMO (Figure 5B). Western analysis indicated that CYP17 protein was still present in pre- and post-menopausal ova ries, as in CL, but not present in myometrium (Figure 5C). CytB5, an
accessory protein that preferentially enhances the 17,20-lyase activity of the CYP17 enzyme, had 5-fold greater mRNA levels in PMO compared with myometrium (P < 0.05), but PMO mRNA levels of CytB5 were similar to the other steroidogenic tissues studied (not shown).

The predominant androgen directly produced by the pre-menopausal ovary is the weak Δ4 androgen, androstenedione, produced through the conversion of the Δ5 steroid DHEA by the enzyme encoded by HSD3B2. The PMO expressed significantly higher HSD3B2 transcript levels than myometrium, where HSD3B2 levels were undetectable (P < 0.005) (Figure 6A). While it did not reach significance, the pre-menopausal ovarian stroma HSD3B2 levels were 6-fold greater than the PMO, consistent with the microarray results. As seen with other steroidogenic enzymes, HSD3B2 expression levels in CL were 2000-fold greater than PMO (Figure 6B). In agreement with the HSD3B2 mRNA levels, HSD3B2 protein was detected in CL, but not in post- or pre-menopausal ovarian stroma (not shown). Additionally, HSD3B1 mRNA was undetectable in the post-menopausal ovary (not shown).

The final step in ovarian steroidogenesis involves conversion of androgens to estrogens through aromatization of C19 steroids to C18 steroids by the enzyme encoded by the CYP19 gene. Once again, the PMO has significantly elevated (50-fold) CYP19 transcript levels when compared with myometrium, although it was a less striking difference than some of the other transcripts (Figure 7A) (P < 0.006). When comparing PMO to pre-menopausal ovarian stroma, there was no significant difference in CYP19 expression, which was in contrast with the microarray results. Finally, CL and placenta CYP19 mRNA levels were significantly greater than PMO, which were ~50-fold greater in CL and 600-fold greater in placenta (Figure 7B).

Discussion

The findings of this study demonstrate that the post-menopausal ovary retains the steroidogenic enzymes necessary for persistent ovarian steroid production. The negligible CYP19 and HSD3B2 transcript levels suggest that the PMO lacks the
ability to produce estrogens or Δ⁴ steroids, respectively. Although low levels of HSD3B2 transcripts were detected in PMO, the absence of HSD3B2 protein likely reflects the less sensitive detection methods of western blot analysis. While the microarray results suggested that the PMO and pre-menopausal ovarian stroma have a similar steroiogenic phenotype, the finding of greater (although not significant) CYP19 expression in PMO by real-time RT–PCR was in direct contradiction to the microarray findings. While this may reflect sample heterogeneity due to the small number of tissue samples used for the microarray analysis, the finding of greater CYP19 expression in PMO is still surprising, due to the lack of granulosa cells in the PMO. In contrast, these levels were still significantly lower than in CL and placenta, suggesting that the aromatase activity of the post-menopausal ovary is almost negligible.

The predominant circulating Δ⁵ androgens, DHEA and DHEA-S, are produced mainly in the adrenal gland, which serve as precursors for peripheral metabolism into more potent steroids (Labrie, 1991, 2004). The findings from this study suggest that the post-menopausal ovary may have a qualitative production of Δ⁵ androgens similar to that of the adrenal. However, further metabolism of DHEA to the weakly estrogenic Δ⁴-androstenediol may also occur in the post-menopausal ovary (Labrie et al., 2003). These Δ⁵ androgens can then be further converted to androstenedione, testosterone, and estradiol in peripheral tissues (Labrie et al., 2001; Simpson, 2003). As a result, the post-menopausal ovary acts as a contributor to this steroid pool destined for peripheral metabolism (Figure 8).

This study is the first to demonstrate the presence of all the enzymes necessary to synthesize androgens by the post-menopausal ovaries. Previous studies have not identified the presence of the CYP17 mRNA or protein that is necessary for androgen synthesis (Couzin et al., 2001; Jabara et al., 2003; Nagamani and Urban, 2003). This may be a result of the use of less sensitive detection methods such as immunohistochemistry and northern blot analysis, or a phenotypic change from long-term in vitro cell culture. Additionally, it is thought that the ovarian stroma is a heterogeneous tissue with the steroiogenic secondary interstitial cells being distributed throughout the stroma (Erickson et al., 1985). As a result, CYP19 expression may demonstrate significant variability throughout the post-menopausal ovary, and in the population of cells that divide once placed in monolayer culture. Nonetheless, our study definitively demonstrated both CYP17 mRNA and protein in the post-menopausal ovary. Furthermore, this is the first study to demonstrate persistent CytB5 mRNA production in the post-menopausal ovary. CytB5 is important in augmenting the 17,20-lyase reaction of CYP17 (Katagiri et al., 1995) and has recently been shown to be present in the adult pre-menopausal ovary in the follicle and corpus luteum, as well as scattered throughout the stroma (Dharia et al., 2004). Presence of this enzyme in the post-menopausal ovary would enhance the conversion of 21-carbon (C₂¹) steroids, such as pregnenolone, to C₁₉ androgens.

The absence of HSD3B2 expression in the post-menopausal ovary is consistent with some studies (Couzin et al., 2001; Jabara et al., 2003), but not others, where it was identified in dispersed interstitial cells (Dupont et al., 1992). While HSD3B2 was not detectable by western analysis, and mRNA was 2000-fold less than in the CL, the ability of the post-menopausal ovary to produce androstenedione and testosterone at low levels in vitro suggests persistence of low levels of this enzyme (Nagamani et al., 1992). At the levels detected in this study, it is likely that the Δ⁴ steroid production is minimal. This is further supported by the absence of HSD3B1 transcripts in the post-menopausal ovary.

While it has long been accepted that menopause results in the loss of cyclic ovarian estrogen and progesterone production, the residual steroiogenic capacity of the post-menopausal ovary is not completely understood. Initial studies suggested that the post-menopausal ovary retains the ability to produce ovarian androgens both in vivo (Judd et al., 1974; Maroulis and Abraham, 1976; Vermeulen, 1976) and in vitro (Mattingly and Huang, 1969; Nagamani et al., 1992). These studies also demonstrated a lack of ovarian estrogen production, suggesting that the post-menopausal ovary is predominantly an androgen-producing gland. The relative deficiency of CYP19 mRNA production in our study supports these earlier clinical studies, and also the immunohistochemical studies of others.
Sasano et al., 1990; Couzinet et al., 2001), although CYP19 has been identified in occasional clusters of cells in some post-menopausal ovaries in one study (Inkster and Brodie, 1991). Furthermore, one study demonstrated an ovarian/peripheral venous estradiol gradient (Aiman et al., 1986), but is in disagreement with others (Judd et al., 1974; Greenblatt et al., 1976).

Comparing ovarian vein with peripheral vein estradiol levels demonstrated negligible ovarian estrogen production. However, ovarian/peripheral androstenedione and testosterone gradients were observed, suggesting persistent ovarian androgen production (Judd et al., 1974; Ushiroyama and Sugimoto, 1995). Post-menopausal women with intact ovaries have been shown to have 40% greater testosterone levels and 10% greater androstenedione levels than age-matched women who had previously undergone oophorectomy (Laughlin et al., 2000), findings that have recently been confirmed (Davison et al., 2005). The results of our study do not support the ability of the post-menopausal ovary to synthesize Δ5 androgens de novo, suggesting that it is more likely that the decrease in testosterone seen after oophorectomy reflects the loss of Δ5 androgen precursors from the ovary that are peripherally metabolized.

The role of androgens in post-menopausal women remains unclear, but some data suggest that it may improve bone density (Davis et al., 1995; Barrett-Connor et al., 1999) and libido (Shifren et al., 2000; Davis and Tran, 2001). While these studies examined exogenous androgen use in post-menopausal women, the loss of endogenous androgens in menopause after oophorectomy revealed conflicting results. The Rancho Bernardo Study did not demonstrate any differences in bone loss in post-menopausal women with intact ovaries when compared with age-matched oophorectomized women (Kritz-Silverstein et al., 2004a). Furthermore, the Rancho Bernardo Study detected no differences in quality of life between post-menopausal women with intact ovaries when compared with age-matched oophorectomized women, and controlling for estrogen use (Kritz-Silverstein et al., 2004b). However, in women with hypoactive sexual desire, those receiving androgen replacement in conjunction with estrogen replacement had a greater increase in libido than women receiving estrogen replacement alone (Lobo et al., 2003). Although the post-menopausal ovary may continue to produce ovarian androgens, these conflicting results suggest that their role in women’s health and well-being in menopause is unclear.

The results of this study do support the long-held belief that the post-menopausal ovary retains the steroidogenic capacity to produce ovarian androgens. The lack of CYP19 expression also supports the notion that the post-menopausal ovary does not possess the ability to synthesize estrogens de novo, but rather serves as a producer of steroids acting as precursors for estrogen production at target tissues. While the post-menopausal ovary possesses the enzymes necessary for predominantly Δ5

![Figure 3. Steroidogenic acute regulatory (StAR) transcript levels and protein in steroidogenic tissues. Real-time RT-PCR was used to quantify StAR mRNA levels.](https://academic.oup.com/humrep/article-abstract/21/1/309/570742/1742)
Figure 4. CYP11A transcript levels and protein in steroidogenic tissues. Real-time RT–PCR was used to quantify CYP11A mRNA levels. (A) Tissue CYP11A transcript levels shown for post-menopausal ovarian stroma (PMO), pre-menopausal ovarian stroma (Ovarian Stroma) and myometrium. (B) Tissue CYP11A transcript levels in corpora lutea (CL), ovarian follicles, placenta, and PMO. (C) Western blot analysis performed to detect CYP11A protein. Amount of protein loaded per lane is shown above the lane. *P < 0.005 compared with myometrium.

Figure 5. CYP17 transcript levels and protein in steroidogenic tissues. Real-time RT–PCR was used to quantify CYP17 mRNA levels. (A) Tissue CYP17 transcript levels shown for post-menopausal ovarian stroma (PMO), pre-menopausal ovarian stroma (Ovarian Stroma) and myometrium. (B) Tissue CYP17 transcript levels in corpora lutea (CL), ovarian follicles, placenta, and PMO. (C) Western blot analysis performed to detect CYP17 protein. Amount of protein loaded per lane is shown above the lane. *P < 0.005 compared with myometrium.
steroid production, the quantitative contribution to the steroid pool remains to be determined. The physiological and clinical significance of these ovarian androgens also remains to be determined.

References


