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Adipose aromatase gene expression is greater in older women and is unaffected by postmenopausal estrogen therapy

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Abstract

Objective: Although natural menopause is associated with loss of ovarian estrogen production, this life phase is followed by a significant increase in estrogen-related cancers, namely breast and endometrial cancer. These tissues, as well as adipose, skeletal, and vascular tissues and the brain are important sites of postmenopausal estrogen production. Circulating C_{19} steroid precursors are essential substrates for extragonadal estrogen synthesis; however, the levels of these androgenic precursors decline markedly with advancing age. This implies an increase in capacity for extragonadal tissues to produce estrogen with age.

Design: To explore this, and the effects of the menopause transition and postmenopausal estrogen therapy on extragonadal estrogen biosynthesis, we have compared the expression of the aromatase gene and estrogen (ER) and androgen receptors (AR) in subcutaneous abdominal and gluteal fat taken from premenopausal (group 1: n = 11), postmenopausal (group 2: n = 10), and postmenopausal women taking estrogen therapy (group 3: n = 10). All subjects were of normal body mass index, euglycemic, and normolipemic.

Results: The postmenopausal women were older (group 1, 43.1 ± 5.0 vs groups 2 and 3, 57.9 ± 7.4 years, $P < 0.001$ and 56.1 ± 4.5 years, $P < 0.001$, respectively) and had lower serum estradiol levels (group 2, 22.2 ± 3.2 vs group 1, 442.5 ± 248.2 pmol/L, $P < 0.05$), which were restored to premenopausal levels with estrogen therapy. Expression analysis revealed that levels of transcripts encoding aromatase were greater in gluteal than abdominal depots in each group in postmenopausal versus premenopausal women ($P < 0.05$). Use of hormone therapy did not influence aromatase gene expression in either depot. No differences were detected in the expression of ER or AR between groups of between tissue depots.

Conclusion: Thus, the capacity of adipose tissue to produce estrogen seems to increase significantly with age at the time of menopause and to be unaltered by exogenous estrogen therapy. This difference in extragonadal estrogen production with age may play a pivotal role in the increase in estrogen-dependent malignancies in the postmenopausal years.

Key Words: Menopause – Adiposity – Hormone therapy.

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The primary estrogen during the reproductive years is estradiol produced by the ovaries, which functions as a circulating hormone to act on target tissues. However, throughout life, the ovaries and adrenals secrete androstenedione, testosterone, dehydroepiandrosterone (DHEA), and DHEA-sulfate (DHEAS), which may be converted to estradiol and estrone by the enzyme aromatase in peripheral tissues. After menopause, when the ovaries cease to produce estrogens, estradiol is no longer an endocrine factor. Instead it is primarily produced in extragonadal sites and acts locally at these sites as a paracrine or even intracrine factor.[1,2] These sites include the mesenchymal cells of adipose tissue, osteoblasts and chondrocytes of bone, the vascular endothelium and aortic smooth muscle cells, and numerous sites in the brain. Thus, circulating levels of estrogens in postmenopausal women reflect "spill over" into the circulation from the peripheral tissues in which estradiol is being produced and where it acts.

In postmenopausal women, the most abundant estrogen in circulation is estrone sulfate, levels of which have been measured at 10 to 25 times greater than levels of estrone and estradiol.[3] Estrone sulfate has a long plasma half-life and slow clearance rate and thus acts as a reservoir for the formation of estradiol and estrone in target tissues.[4]

Endometrial cancer, an estrogen-dependent malignancy, is rare before menopause, with 50% of cases being diagnosed in women more than 64 years of age.[5] Similarly almost 50% of breast cancer occurs in women after the age of 60.[5] Strong direct associations between circulating estrogens, androgens, and endometrial cancer in postmenopausal women, and an inverse association of SHBG levels with this cancer have been demonstrated.[6] The effect of elevated androstenedione and testosterone levels on disease risk may be mediated through their extragonadal conversion to estrogens.[6] Thus, the capacity for adipose tissue to produce estrogens after menopause appears to play a pivotal role in the development of estrogen-dependent cancers. However, levels of the circulating C_{19} steroid precursors, which are essential substrates for this extragonadal estrogen synthesis, decline markedly with advancing age.[7] There is preliminary evidence that the expression of the aromatase enzyme, which is responsible for estrogen biosynthesis, increases in fat with advancing age[8,9]; however, the effect of menopause per se or the use of postmenopausal estrogen therapy on adipose aromatase gene expression has not been studied. Thus, we have measured levels of transcripts encoding aromatase and the sex steroid receptors, estrogen (ER) and androgen (AR), in subcutaneous abdominal and gluteofemoral fat obtained from healthy premenopausal women and postmenopausal women who are nonusers or users of hormone therapy (HT). Our primary aim was to ascertain whether differences were apparent in association with the menopausal transition or the use of HT.

METHODS

Study subjects

Women were recruited from the Jean Hailes Foundation Clinic and the general community via approved advertisements. Inclusion criteria included a body mass index (BMI) greater than 22 and less than 30 kg/m^2 and being able to be classified as premenopausal (group 1, aged 18 to 45 years with regular menstrual cycles and not on steroidal contraception, nonpregnant, and nonlactating; samples were taken during days 1 to 14 of the cycle), postmenopausal women (group 2, >12 months amenorrhea, FSH >30 or >55 years of age and no HT within 3 months), and postmenopausal women taking HT (group 3, as for group 2, but users of HT for >3 months; if on cyclical progestin, samples were taken during the estrogen-only phase).

Women with elevated fasting insulin, glucose, or lipids, known acute or chronic renal or liver disease, acute cardiovascular event in the preceding 6 months, unstable hypertension, use of: multiple hypoglycemic agents, oral glucocorticosteroids, lipid-lowering agents within the preceding 8 weeks, use of anticoagulants in the preceding 1 month or therapeutic antiplatelet agents in the preceding 2 weeks, cigarette smoking, alcohol consumption more than 30 g per day, use of oral contraceptive pills within 8 weeks of enrollment or use of other systemic progestin contraception, untreated hypo/hyperthyroidism, weight loss within 3 months before enrollment of more than 5% of total body weight induced by either diet or appetite suppressant medication, or women immediately seeking fertility and pregnancy; pregnant and lactating women were excluded.

The study was approved by the Human Research and Ethics Committee of Southern Health, Clayton, Victoria and all women provided written, informed consent to participate in the study.

Lipid and hormone profile

A fasting blood sample was collected to determine insulin, glucose, triglycerides, total cholesterol, HDL cholesterol and LDL cholesterol. Estradiol, FSH, testosterone, androstenedione, sex hormone-binding globulin (SHBG), and dehydroepiandrosterone (DHEAS)

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were also measured. Hormone analyses were performed by the Department of Clinical Pathology, Southern Health, Clayton, Australia. LDL cholesterol and VLDL were calculated by Friedewald formula.[10] Total cholesterol, HDL cholesterol, and triglycerides were measured by standard commercial enzymatic assays using a DADE Boehring Dimension RxL Chemistry Analyser (Dade Behring Diagnostics, Sydney, Australia) with coefficient of variations (CV) being 1.9%, 4.4%, and 7.4%, respectively. Serum estradiol was measured using a double-antibody competitive immunoassay (RIA) (Diagnostic Products Corporation, Los Angeles, CA) method on a LKB Wallac 1277 Gamma master Automatic Gamma Counter (Wallac 3 Oy, Turku, Finland) with an interassay CV at 61 pmol/L of 14.9% and follicle stimulating hormone (FSH) was measured based on Microparticle Enzyme Immunoassay (MEIA) technology performed on an Abbott Diagnostics AxSym (Abbott, IL) with an interassay CV of 4.4%. Total testosterone was measured using the Bayer ACS 180 Automated Chemiluminescence System with an interassay CV at 1.7 nmol/L (50 ng/dL) of 13.8%. SHBG and DHEAS were measured using a solid-phase, two-site chemiluminescent enzyme immunometric assay using the IMMULITE automated analyser (IMMULITE, Diagnostic Products Corporation, Los Angeles, CA), interassay CV, 8.7% for SHBG, and 8.1% to 15% for DHEAS. The free androgen index (FAI) was calculated as total testosterone nmol/L divided by SHBG nmol/L $\times$ 100. Insulin was measured using the Abbott Axsym immunoassay (Abbott, IL) and glucose by the glucose oxidase method (Dade Dimension, IL).

Adipose biopsy

After an overnight fast, 300 to 400 mg of subcutaneous adipose tissue was obtained using the following procedure. Ice packs were applied to the prospective sites for approximately 15 minutes to numb the region and minimize bruising. Local anesthetic (1% Xylocaine) was injected in a fan-style pattern and the ice packs reapplied for several minutes. The biopsy was then taken using a 15-gauge needle attached to a 20-mL syringe containing 10-mL sterile saline. The abdominal sample was taken from the lower abdominal region, randomly from the left or right side lateral to and below the umbilicus, and the gluteal sample was taken from the upper lateral thigh or buttock. After negative-pressure aspiration of fat, the contents of the syringe were filtered through a sterile strainer over a sterile jar to wash away excess blood and clot with extra saline. The sample was placed immediately into liquid nitrogen and stored at $-80°C$ for subsequent transcriptional analysis.

RNA extraction and quantification

Total RNA was isolated from 100 mg of frozen adipose tissue using the phenol/chloroform extraction method (Ultraspec RNA, Fisher Biotec, Perth, Australia). RNA was treated with ribonuclease-free deoxyribonuclease (Ambion, Inc., Austin, TX). Total RNA was quantified using a spectrophotometer (DU-530 Beckman, CA), and RNA integrity was confirmed via 1% agarose gel electrophoresis.

RNA expression

RNA (1 μg) was reverse transcribed as described.[11] cDNA was diluted five times and amplified by real-time PCR in the Lightcycler (Roche, Germany), using Fast Start Master SYBR Green I (Roche, Germany) and specific oligonucleotide pairs designed to amplify a transcript that spans a minimum of two exons to avoid genomic DNA contamination. A measurement of each transcript was conducted in three separate, replicate experiments. Real-time PCR data were normalized to ribosomal 18S RNA. Within each experiment results were reported on an arbitrary scale, with all values standardized to the lowest value (which was the value in premenopausal-abdominal tissue).

Qualitative and statistical analysis

Data were analyzed using SPSS (Statistical Package for Social Sciences 10.0, Chicago, IL). Subject characteristic and hormone and lipid profile variables are presented as mean $\pm$ SD. Comparisons between groups were made using univariate analysis of variance, and Tukey post hoc test was used to determine significance.

When two sets of data were obtained from one individual (eg, abdominal vs gluteal from a premenopausal woman), the data were not independent and comparisons were made using paired analysis. However, where data from two different sets of individuals (eg, premenopausal woman vs postmenopausal woman) were compared, independent analysis was conducted. Due to the nature of the biopsy, the quantity and quality of RNA was variable. The experimental process itself also used up tissue. Therefore, in repeating experiments, group sizes were not always maintained. Given the small number of these groups, it was difficult to establish that the distribution of levels of transcripts followed a normal distribution. It was then decided that a more robust approach would be to use nonparametric analyses that do not assume normal distribution of the data. To assess the statistical significance of differences in levels of transcripts between groups of women, the nonparametric, unpaired, Mann-Whitney rank test was

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performed. To confirm significance of comparisons between groups, an additional nonparametric median test was performed. Within groups, abdominal and gluteal differences were compared using nonparametric paired, Wilcoxon analyses. In all of these analyses, significance was accepted with a P value less than 0.05.

RESULTS

Subject characteristics

Thirty-one healthy women completed the study. Their characteristics are presented in Table 1. The postmenopausal women (groups 2, n = 10, and group 3, n = 10; 46-71 years of age) were significantly older (group 1 vs group 2, $P < 0.001$; group 1 vs group 3, $P < 0.001$) than the premenopausal women (group 1, n = 11; 38-55 years of age). There were no differences in body weight or BMI between the groups consistent with the selection criteria. Of the postmenopausal women using HT, seven were taking oral estrogen (micronized estradiol 2 mg, n = 3; estrone sulphate 1.25 mg, n = 2; conjugated equine estrogens 0.625 mg, n = 2), including five women who also took progestin (norethindrone, n = 2; dydrogesterone, n = 1; medroxyprogesterone acetate, n = 2), two were using an estradiol transdermal 25-μg patch only and one the estradiol 50-μg norethindrone 250-μg patch. One woman had undergone bilateral oophorectomy. All were normolipemic and euglycemic (data not shown).

FSH was higher ($P < 0.001$) and estradiol ($P < 0.005$) and DHEAS ($P < 0.05$) were lower in postmenopausal women. In postmenopausal women taking HT, estradiol levels were not different from those of premenopausal women. Although no differences were observed for total testosterone and androstenedione between groups, SHBG was greater in postmenopausal women using HT than in premenopausal women ($P < 0.05$). The calculated free androgen index did not differ among the three groups.

Levels of transcripts encoding aromatase and hormone receptors

Levels of transcripts encoding aromatase were greater in gluteal fat than abdominal fat within each of the three groups (groups 1 and 2, $P < 0.05$; group 3, $P < 0.069$; Fig. 1). Moreover, levels in abdominal fat were greater in groups 2 and 3 compared with group 1 ($P < 0.01$) with levels in gluteal fat also being greater in group 3 versus group 1 ($P < 0.05$). No statistically significant differences were apparent for levels of transcripts encoding the AR (Fig. 2A) or ERα (Fig. 2B). Transcript levels of ERβ were also measured and detected; however, the levels of these transcripts were minimal and therefore no differences can be reported (data not shown).

DISCUSSION

After menopause, extragonadal estrogen synthesis becomes the most important source of circulating estrogen, primarily as estrone. Two earlier studies, which involved a small number of women across a broad age range, have indicated that the expression of aromatase appears to increase with age and is greater in gluteal than abdominal subcutaneous fat.[8,9] Similarly, in our study, we found that gluteal fat aromatase expression exceeds that of subcutaneous abdominal fat and that there are significantly higher levels of aromatase gene expression in postmenopausal women. Due to the small sample size, these findings should be interpreted with some caution and ideally reconfirmed in a larger study. However, it may be difficult to recruit large numbers of women to such a study because of the

TABLE 1. *Characteristics and hormone profiles of participants*

	Premenopausal women	Postmenopausal women	Postmenopausal women + HT
N	11	10	10
Age (y)	43.1 ± 5.0 (38–55)[a,c]	57.9 ± 7.4 (50–75)	56.1 ± 4.5 (46–61)
Weight (kg)	70.1 ± 6.4	70.5 ± 9.3	72.3 ± 10.3
Height (cm)	165.8 ± 5.7	163.0 ± 7.5	161.3 ± 8.2
Body mass index (kg/m^2)	25.5 ± 2.2	26.7 ± 3.0	27.3 ± 2.4
Estradiol (pmol/L)	442.5 ± 248.2[a]	22.2 ± 3.2[b]	321.2 ± 217.6
FSH (IU/L)	6.4 ± 3.0[a]	68.3 ± 21.9[b]	25.4 ± 17.0
Total testosterone (nmol/L)	1.2 ± 0.7	1.6 ± 1.0	1.2 ± 0.6
SHBG (nmol/L)	49.2 ± 22.1[c]	57.7 ± 24.9	94.3 ± 50.2
Free androgen index	3.0 ± 2.4	2.6 ± 1.6	1.4 ± 1.1
Androstenedione (nmol/L)	2.0 ± 1.0	1.6 ± 0.8	2.0 ± 1.1
DHEAS (μmol/L)	4.3 ± 1.9[a,c]	2.0 ± 0.9	1.9 ± 1.2

Values are mean ± SD.
[a] $P < 0.05$ Pre/Post, [b] $P < 0.05$ Post/Post + HT, [c] $P < 0.05$ Pre/Post + HT.

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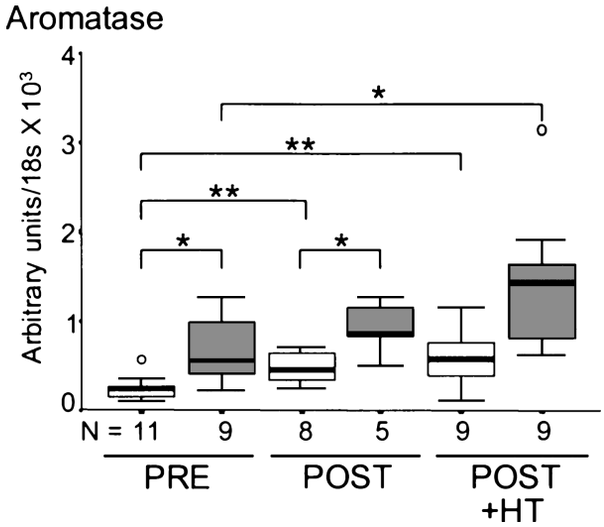

FIG. 1. Real-time PCR analysis of transcripts encoding aromatase in subcutaneous abdominal and gluteal adipose tissue depots of premenopausal (PRE), postmenopausal (POST), and postmenopausal women taking hormone therapy (POST+HT). Results are presented as box and whisker plots, where the center line represents the median, the boxes represent the interquartile range, and the whiskers represent the full range of data excluding outliers and extreme values, which are represented individually. *, $P < 0.05$; **, $P < 0.01$.

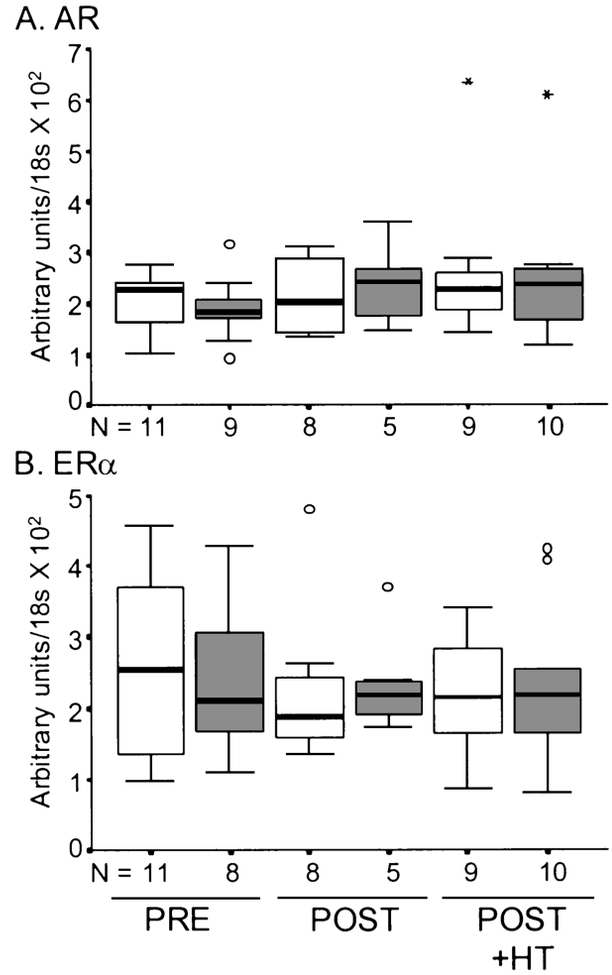

FIG. 2. Real-time PCR analysis of transcripts encoding the androgen receptor (AR) and the estrogen receptor α (ER α) in subcutaneous abdominal and gluteal adipose tissue depots of premenopausal (PRE), postmenopausal (POST), and postmenopausal women taking hormone therapy (POST+HT). Results are presented as box and whisker plots, where the center line represents the median, the boxes represent the interquartile range, and the whiskers represent the full range of data excluding outliers and extreme values, which are represented individually.

discomfort the procedure involves and bruising that results. The menopause transition coincides with the life phase at which there is a marked increase in the prevalence of both breast and endometrial cancer.[5] Our aim in this study was to disentangle the impact of age and change in menopausal status on aromatase gene expression. This was not possible comparing only premenopausal and postmenopausal women as these two groups differ both in age and menopause status. By showing that there is a difference in aromatase gene expression in premenopausal and postmenopausal women adequately estrogenized by being on exogenous estrogen, one can conclude that the difference observed is more likely to be due to age than the menopause transition. If we had chosen a premenopausal versus an all surgically menopausal group, the study would have been confounded by the lack of any residual ovarian sex steroid production. Hormone levels were measured to confirm the menopausal status of the study subjects and to exclude pathology. The women studied were healthy, euglycemic and normolipemic, and of normal BMI and despite the small sample size, our findings are likely to be generalizable to the healthy population. In our study, the use of HT by postmenopausal women did not influence this difference in aromatase gene expression with age, suggesting that the difference is age-related rather than due to menopausal estrogen insufficiency. This is consistent with the earlier studies of Hemsell and coworkers[12] who demonstrated that the fractional conversion of circulating androstenedione to estrone increased with age, but with no obvious break at the menopausal transition.

It is not known what underlies the apparent increase in aromatase expression because known stimulators of this gene such as the glucocorticoid receptor, interleukin-6, and tumor necrosis factor α[13] were constant over all three groups and between fat depots (data not shown).

Obese transgenic mouse models suggest that the AR and the ER also regulate adipogenesis.[14-16] In our study,

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levels of transcripts encoding AR and ERα did not vary with estrogen availability in our women. This may be due to the limitation of a small sample size. Joyner and colleagues[17] reported more AR in visceral fat depots compared with subcutaneous depots, suggesting that the AR may not exert its effects in the subcutaneous fat depots measured in our study. Also, it is possible that the effect of AR on adipogenesis is sexually dimorphic because only a male-specific adipogenic effect was reported in AR knockout mice.[14] The lack of change in ERα transcripts in this study supports the work of Pedersen et al,[18] whereby expression of ERα mRNA was equal in subcutaneous gluteal, subcutaneous abdominal and intra-abdominal adipose depots.

CONCLUSION

In conclusion, we report an apparent increase in capacity for adipose tissue to produce estrogen with age at midlife and this may have important clinical implications in terms of the increasing incidence of both endometrial and breast cancer beyond the middle years.

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