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Measurement of Sex Steroid Hormones in Breast Adipocytes: Methods and Implications

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Abstract

Background—The lack of validated methods for measuring sex steroid hormones in breast tissue has limited our knowledge of their role in the development of breast cancer. We explored the feasibility of measuring hormones in breast adipocytes for epidemiologic and clinical studies by refining an existing assay procedure and assessing the reliability of hormone measurements using the modified assay. This report presents the reproducibility of measurements of androstenedione (A), testosterone (T), estrone (E₁) and estradiol (E₂), using breast adipose tissue samples obtained from women undergoing surgical resection for a variety of pathologic conditions.

Methods—Breast adipose tissue was obtained from 20 women. Measurements of the steroid hormones were carried out by harvesting oil from adipocytes following enzymatic digestion of the adipose tissue, extracting and chromatographing the steroids, and quantifying them by RIA. The study was conducted in 3 phases: first, samples from 5 women were used to assess the assay procedure; following this, tissues from an additional 5 women were assayed repeatedly to determine reproducibility of the hormone measurements. Finally, using samples from 10 women undergoing surgical resection of a breast tumor, we evaluated hormone concentrations in samples distal and proximal to the tumor. The assay coefficient of variation (CV) and intraclass correlation coefficient (ICC) were used to assess hormone reproducibility.

Results—The within batch CVs ranged from 5% to 17%, and between batch estimates were between 2% and 10%, suggesting that E₁, E₂, A, and T can be reliably measured in breast adipocytes. Among samples obtained from women undergoing surgical resection of a breast tumor, hormone levels did not differ between adipose tissue fragments that were distal or proximal to the tumor, with the possible exception of E₁, where levels were 10% higher in distal fragments.

Conclusion—These data support the feasibility of measuring steroid hormone concentrations in breast adipocytes in epidemiologic studies. Future investigations that include the measurement of hormones in the breast microenvironment may have value in understanding breast carcinogenesis, developing prevention strategies and assessing hormonal treatments.

Keywords

breast; adipose tissue; tumor; sex steroid hormones; RIA

Introduction

Understanding the actions of sex steroid hormones in mammary carcinogenesis is critical for developing improved methods of preventing and treating breast cancer, however, the lack of validated methods for measuring hormones within breast tissues has been limiting. The importance of studying intra-mammary hormone levels is supported by several observations. First, the doubling of breast cancer risk associated with elevated serum levels of estrogens and androgens seems lower than expected given that hormones represent a key etiologic factor in the genesis of this tumor (1). Second, limited data suggest that steroid hormone levels in breast tissues, including neoplastic, glandular and adipose tissues, differ dramatically from blood levels, particularly among postmenopausal women (2). Further, within the breast, hormone levels between premenopausal and postmenopausal women are comparable (3-7). The source of high levels of breast tissue estrogens in postmenopausal women has been attributed both to the uptake of circulating hormones and to the local synthesis and metabolism of these steroids in the breast itself (8). A growing body of evidence demonstrates that enzymes involved in hormone metabolism (aromatase, sulfatase, sulfotransferase, 17 β -hydroxysteroid dehydrogenase) are expressed and functional in normal and neoplastic breast tissues (8,9). Thus, the breast, as a site of uptake, biosynthesis and metabolism of circulating hormones, has the capacity to modulate its microenvironment.

Methodologic challenges have limited studies of breast tissue hormone concentrations (9). Breast tissue varies in relative epithelial, stromal and fat composition, making it difficult to develop a standardized metric for measuring hormone levels in pulverized specimens and compare results between subjects. In addition, some laboratory assays have suffered from lack of specificity and limited sensitivity. Understanding differences between intracellular and interstitial fluid hormone levels is another important issue. Given that mammary fat has a relatively homogeneous cellular makeup and is an important site for hormone production and/or sequestration, particularly after the menopause, we refined and validated methods for measuring steroid hormones in breast adipocytes.

This report presents results from a proof-of-performance study designed to refine an existing assay procedure and to assess the reproducibility of hormone measurements obtained by the modified assay. Breast adipose tissue specimens were collected from women undergoing surgical resections for a spectrum of pathologic conditions ranging from non-invasive breast lesions to invasive carcinoma. These samples were used to measure levels of androstenedione (A), testosterone (T), estrone (E₁) and estradiol (E₂) in adipocytes.

Materials and Methods

Tissue collection and storage

Anonymized adipose tissue samples that were not required for diagnosis were systematically collected from breast specimens removed for clinical indications. Age and final pathologic diagnosis based on the most severe lesion found in the resection or preceding biopsy were recorded as follows: invasive carcinoma (ductal or lobular); in-situ carcinoma (ductal or lobular); atypical hyperplasia (ductal or lobular); ductal hyperplasia or non-specific fibrocystic changes. We collected two fat samples from each breast specimen. For specimens containing grossly identifiable invasive carcinoma, we removed one fragment adjacent to the tumor edge and one as far distant from the tumor edge as possible. For specimens that did not contain

identifiable invasive carcinoma, we collected one fragment from the center of the specimen and the other from near the margin of the resection. Sampling was performed to obtain the highest percentage of pure fat based on gross inspection; areas of adipose tissue intimately intermixed with fibroglandular tissue were avoided. Tissues were frozen at -80°C within one hour following removal. This project was conducted under an exemption granted by Review Boards of participating institutions.

Study Design

The study was conducted in three steps. In step I, two fat specimens from 5 women were evaluated to assess the feasibility of extracting and measuring steroid hormones using a modified assay procedure. In step II, we evaluated the reproducibility of the measurements using tissue obtained from an additional 5 women. In step II, one adipose fragment from each woman was divided into 4 samples and measured in 2 batches. Each batch contained 2 aliquots per subject. In step III, which was restricted to specimens containing invasive cancer, we preliminarily assessed whether hormone levels in adipose tissue differ by proximity to the breast tumor. Specifically, we compared measurements from proximal and distal adipose tissue samples obtained from 10 women undergoing surgical excision of a breast cancer. For each woman in step III, 2 aliquots were obtained from the proximal and distal fragments. The schema for this component of the study is shown in Figure 1. For each woman, 2 extracts were assayed in the same batch, one obtained from the proximal and one from the distal fragment. Samples were kept at -80°C at the laboratory until analyzed.

Laboratory Methods

Quantification of steroids in adipocytes was conducted using a modification of the methods described by Rodbell (10) and O'Brien et al (11), in which oil was separated from the more dense stromal cells by flotation. Approximately 0.4 gram portions of breast adipose tissue were minced and incubated in a 20 ml glass vial with 1ml of 1mg/mL collagenase (type 1A; Sigma Chemical Co., St Louis, MO) in Krebs Ringer bicarbonate buffer (4% BSA) (Sigma Chemical Co., St. Louis, Mo) at 37°C for 50 min in a shaking water bath. During enzymatic disaggregation, significant lysis of the adipocytes occurred, and oil droplets were formed. The floating oil layer was separated by centrifugation in a micro-centrifuge at room temperature for 2 min at 10,000 rpm, and the oil was weighed. A, T, E_1 and E_2 were then quantified by radioimmunoassay (RIA) with preceding purification steps. Internal standards ($^3\text{H-A}$, $^3\text{H-T}$, $^3\text{H-E}_1$ and $^3\text{H-E}_2$), were first added to the oil and incubated at 50°C for 10 min to follow procedural losses of each steroid. The steroids were then extracted from the oil with 80% MeOH/ H_2O and the extract was evaporated. The residue was then redissolved in 0.4 mL of distilled water and an additional extraction was carried out with 3 ml of hexane:ethyl acetate (3:2). This extraction step was repeated and the solvents were evaporated to dryness and redissolved in 1mL of isooctane. The isooctane was transferred to a Celite partition chromatography column, and A, T, E_1 and E_2 were eluted sequentially and quantified by RIA as described previously (12-15). Results are expressed per gm of oil.

From the residual aqueous fraction after extraction, a 0.1 ml aliquot was taken to measure estrone sulfate (E_1S) by direct immunoassay using a commercial kit (Diagnostics Systems Laboratories, Webster, TX).

Statistical Methods

We assessed assay reproducibility using a nested, within-person analysis of variance (ANOVA) model with data transformed to the natural logarithmic scale. Variance components methods were used to estimate the variability in the laboratory measurements. We estimated the variability among subjects (σ^2_a), variability between batches for a given subject (σ^2_b), and variability associated with different aliquots of the same extracted tissue (σ^2) using the SAS

procedure Proc VARCOMP (16). From the variance estimates we computed an estimate of the ICC as $(\sigma_a^2/[\sigma_a^2 + \sigma_b^2 + \sigma^2])$, and an estimate of the assay CV (17). Geometric means are presented.

Results

Step I - Feasibility of Measuring Hormones in Mammary Fat

Initially, assays were conducted on adipose tissue obtained from 5 women, of whom 4 were undergoing reduction mammoplasty, and one was undergoing surgical excision of ductal carcinoma in-situ. The women ranged in age from 23-59 years (mean 44 years), and two were postmenopausal. Two fragments of adipose tissue were removed from the same region of each breast, and the steroid hormones were measured in adipocytes from each fragment.

For all women, levels of A were highest, followed by E_1 , T, and E_2 . This ranking was the same for samples from premenopausal and postmenopausal women, but absolute levels of A, T, and E_2 in premenopausal samples were approximately double those of postmenopausal samples. Concentrations of E_1 were similar for both groups. The within subject CVs ranged between 20%-32% for the four hormones evaluated (Table 1). No E_1S was detectable in the samples.

Step II – Reproducibility of Hormone Measurements from Breast Adipocytes

Next we evaluated the reproducibility of the laboratory measurements using extracts obtained from a single tissue fragment from 5 additional women aged 26-45 years (mean 36 years). These samples were obtained from a younger group than those in step I. For each woman, a total of 4 aliquots were obtained from the adipose fragment, two of which were assayed in one batch and the remaining two assayed in a second batch. The steroid hormones were measured in adipocytes from each fragment.

The hormones in these samples showed a similar pattern and levels were of similar magnitude as observed in Step I, with concentrations of A being highest and E_2 levels lowest (Table 2). The overall CVs were lower than those found initially, ranging from 8-19%, with the within-batch CVs ranging from 5.1% to 16.7% and between batch CVs all less than 10%. The ICCs were quite high, with values of 80% or greater for all of the analytes measured.

Step III - Breast Adipocyte Hormone Levels in Proximity to Breast Tumor

For the final component of the study, adipose fragments distal and proximal to the breast tumor were evaluated. Samples were extracted from adipose tissues in 9 of the 10 women who provided samples and ranged in age from 39-84 years, with a mean of 61.3 years. Five women were postmenopausal at the time of surgical excision of the tumor. The pattern of relative concentrations of the different hormones was similar to that found in the previous steps; however, the absolute values were lower. Hormone levels were similar for adipose tissue fragments that were distal vs. proximal to the tumor, with the possible exception of E_1 , where levels were 10% higher in distal fragments.

Discussion

In this study, the steroid assay method yielded within batch CVs ranging from 5-17%, between batch CVs ranging from 2-10% and intra-class correlation coefficients of 80-95% for A, T, E_1 and E_2 in breast adipocytes. While sex steroids may be found in adipocytes in an unesterified form or esterified to fatty acids (lipoidal estrogens), the assay method used to measure the steroid hormones in breast adipocytes in the present study predominantly detected unesterified estrogens, as esterified estrogens are not likely to be removed during the chromatography procedure and require hydrolysis to free the estrogens (18). Also, conjugated steroids, such as

E₁S, have low lipid solubility and thus, would not be expected to be sequestered in breast adipocytes, as confirmed in this study. The present data support the feasibility of measuring unconjugated steroid hormone concentrations in breast adipocytes in epidemiologic and clinical studies.

After menopause, incidence rates of estrogen receptor positive breast carcinoma continue to rise despite falling serum estrogen levels, suggesting that intra-mammary hormone synthesis may be an important mechanism in carcinogenesis. The aromatization of androgens to estrogens in peripheral adipose tissue is a primary source of circulating estrogen in the post-menopause, and recent data suggests that extragonadal aromatase expression increases with age following menopause (19). Adipocytes, adipose stromal cells and vascular cells are the primary cell constituents of adipose tissue, with the largest proportion being adipocytes (20). Within the breast, aromatase expression has been reported in both tumor and adipose tissue (20); furthermore, within the breast adipose, aromatase expression is higher in stromal cells compared to adipocytes, yet estrogen receptor α expression is greater in adipocytes (21,22).

In the present study, we measured steroid hormones in oil derived from adipocytes. Among the hormones measured, we found that A was most abundant, followed by E₁, T and E₂, with the latter hormones at concentrations ranging from 2% to 13% of that found for A. This ranking was similar for pre- and post-menopausal adipose samples. Given that our data are expressed per gram of oil as opposed to per ml of serum, it is difficult to interpret findings with respect to hormone levels in the circulation. However, the relative concentrations of the adipocyte hormones were similar to their ranking in serum. During the follicular and luteal phases of the menstrual cycle, average serum levels of A are 20- to 30-fold higher than those of E₁, whereas average serum T levels are 3- to 8-fold higher than E₂ (23). In the present study, we found that levels of A were approximately 10-fold higher than E₁ in both pre- and post-menopausal adipose extracts; for T, levels were about twice that of E₂ in premenopausal samples and more than 7-fold higher in the postmenopausal fat. However, it is notable that A was at least 23 times higher than T in the adipocytes, as compared to a 3- to 5-fold difference in female serum. This suggests that the availability of substrate for E₁ formation by aromatase in adipose tissue is high.

Few studies have explored hormone concentrations in breast tissues (8), most of which measured hormones in tumor tissue, tissue surrounding the tumor and/or distal glandular tissue (3,4,6,24,25). Analyses of steroid hormones in breast adipocytes have been even more restricted. In one study that used a different purification procedure than ours, but similar RIAs to measure hormone levels (26), the ranking of A, E₁, T and E₂ concentrations in breast adipose tissue from postmenopausal women was similar to our findings. However, the values were expressed as pmol/g tissue, precluding direct comparisons of absolute levels. Reported absolute values in this study (pmol/g tissue) were: A=16, E₁=0.67, T=0.62 and E₂=0.20. The similarity in ranking between these studies despite differences in methods is encouraging.

Some previous reports have found that promoter switching may increase aromatase activity in tissues adjacent to carcinoma, however, we did not find topographical differences in adipose hormone levels in relation to tumor. This could reflect our selection of pure adipose tissue samples based on gross examination, and it is possible that adipose tissue that is located within microscopic distances of tumor as opposed to macroscopically removed might show hormone elevations. Similar findings have been reported in another study that compared tumor and adipose tissue from premenopausal and postmenopausal breast cancer cases (27), where little difference in hormone concentrations between adipose tissue samples at various distances from the breast tumor were observed.

Further, in the previously described study (27), low but measurable quantities of E₁S and estradiol sulfate were found in breast adipose tissue (142 and 59 fmol/g, respectively). Explanations for the contradictory findings regarding the sulfated estrogen are not clear, but may rest in the assay methodology. Sulfated molecules are highly polar, thus E₁S in the fat should be negligible. Detection of sulfated compounds could represent contamination of adipose tissue with steroids from interstitial fluids or blood, either within the tissue vessels or from hemorrhage into the tissue.

Addressing the importance of local hormone metabolism in mammary carcinogenesis will require future inter-disciplinary studies in which epidemiologic factors are combined with detailed pathologic and biological tissue characterizations. Research is needed to develop methods for defining tissue content and for standardizing metrics for expression of steroid hormone concentrations. Advances in this area may permit the improved etiologic understanding of hormonal carcinogenesis, which in turn holds promise for developing new prevention and treatment strategies and better monitoring of therapies.

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Figure 1. Study design to assess hormone concentrations in fat samples from areas adjacent to and remote from breast tumor

Table 1
Hormone Concentrations in Breast Adipocytes - Assay Feasibility

Hormone	Mean Concentration *		Overall	CV**
	Premenopausal	Postmenopausal		
Androstenedione	9247	4197	6738	19.7
Testosterone	393	181	298	23.5
Estradiol	211	51	114	26.4
Estrone	889	910	897	32.2

* Values are pg per gram of oil.

** Overall CV expressed as percent

Adipose tissue obtained from 5 women, 2 of whom were postmenopausal.

Surgery was performed for benign conditions (reduction mammoplasty), except for one woman with IDC.

Ages ranged from 23-59; mean 44.1 years.

For each woman, 2 tissue fragments were obtained; the steroid hormones were analyzed in each fragment.

Table 2
Batch Reproducibility of Hormone Measurements in Breast Adipocytes

Hormone	Mean Concentration	ICC**	CV	Percent Difference	
				within batch	between batch
Androstenedione	6144	85.3	8.24	0.29	1.43
Testosterone	317	79.9	19.23	3.24	12.20
Estradiol	96	94.4	14.85	2.42	6.18
Estrone	771	84.3	12.37	2.72	11.90

* Values are pg per gram of oil

** ICC and CV are percent values

A single tissue fragment was obtained from each of 5 women, ranging in age from 26–45 (mean=35.6).

Four aliquots were created for each woman, with 2 aliquots assayed in batch 1 and the remaining 2 in batch 2.

Table 3
Hormone Concentration in Breast Adipocytes by Distance to Breast Tumor Tissue

Hormone	Mean concentration *		Percent Difference	
	Proximal	Distal	between batch	between location
Androstenedione	4876	4551	1.96	7.19
Testosterone	149	149	3.12	0.09
Estradiol	34	34	2.33	4.17
Estrone	354	374	31.7	10.1

* Values are pg per gram of oil

Two adipose tissue fragments were obtained and successfully extracted from 9 women at time of surgical excision of the breast tumor.

Extractions were performed on each fragment separately.

Women ranged in age from 39-84, mean of 61.3. Five women were postmenopausal.

One fragment was proximal to the tumor; the second from was distal to tumor.