Research Article

Obesity Is Associated with Inflammation and Elevated Aromatase Expression in the Mouse Mammary Gland

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Abstract

Elevated circulating estrogen levels are associated with increased risk of breast cancer in obese postmenopausal women. Following menopause, the biosynthesis of estrogens through CYP19 (aromatase)-mediated metabolism of androgen precursors occurs primarily in adipose tissue, and the resulting estrogens are then secreted into the systemic circulation. The potential links between obesity, inflammation, and aromatase expression are unknown. In both dietary and genetic models of obesity, we observed necrotic adipocytes surrounded by macrophages forming crown-like structures (CLS) in the mammary glands and visceral fat. The presence of CLS was associated with activation of NF-κB and increased levels of proinflammatory mediators (TNF-α, IL-1β, Cox-2), which were paralleled by elevated levels of aromatase expression and activity in the mammary gland and visceral fat of obese mice. Analyses of the stromal-vascular and adipocyte fractions of the mammary gland suggested that macrophage-derived proinflammatory mediators induced aromatase and estrogen-dependent gene expression (PR, pS2) in adipocytes. Saturated fatty acids, which have been linked to obesity-related inflammation, stimulated NF-κB activity in macrophages leading to increased levels of TNF-α, IL-1β, and Cox-2, each of which contributed to the induction of aromatase in preadipocytes. The discovery of the obesity → inflammation → aromatase axis in the mammary gland and visceral fat and its association with CLS may provide insight into mechanisms underlying the increased risk of hormone receptor-positive breast cancer in obese postmenopausal women, the reduced efficacy of aromatase inhibitors in the treatment of breast cancer in these women, and their generally worse outcomes. The presence of CLS may be a biomarker of increased breast cancer risk or poor prognosis. Cancer Prev Res; 4(3); 329–46. ©2011 AACR.

Introduction

More than 40,000 women in the United States die each year of metastatic breast cancer. Approximately two-thirds of breast cancer patients have tumors that express estrogen receptors. Obese postmenopausal women are at significantly increased risk of developing hormone receptor (HR)-positive breast cancer (1). Estrogens are synthesized from androgens in a reaction catalyzed by cytochrome P450 aromatase, encoded by the CYP19 gene (2). After menopause, peripheral aromatization of androgen precursors in adipose tissue is largely responsible for estrogen biosynthesis (3). The increased risk of HR-positive breast cancer in obese postmenopausal women has been attributed, in part, to elevated levels of circulating estradiol related to both increased adipose tissue and elevated aromatase expression in subcutaneous adipose tissue (4–7). Whether obesity alone leads to changes in aromatase expression in breast tissue is uncertain.

Because of the significance of estrogen biosynthesis in the pathogenesis of HR-positive breast cancer, efforts have been made to elucidate the mechanisms that regulate the transcription of CYP19 (8). Multiple lines of evidence suggest a key role for proinflammatory mediators including TNF-α, IL-1β, and cyclooxygenase (Cox)-derived prostaglandin E2 (PGE2) in stimulating CYP19 transcription resulting in increased aromatase activity (9–17). Obesity causes inflammation and increased levels of proinflammatory mediators in adipose tissue (6, 18–20). In humans, a correlation has been observed between increased body mass index and elevated levels of aromatase in subcutaneous fat (7). This
constellation of findings suggested the possibility that obesity-related inflammation is causally linked to increased aromatase expression. Although studies have shown that obesity causes inflammation in both visceral and subcutaneous fat (20), it is unknown whether similar changes occur in the mammary gland.

In this study, we had several objectives. Our first goal was to determine whether obesity led to inflammation in the mammary gland in addition to visceral fat. Second it was important to establish whether obesity was associated with increased levels of both proinflammatory mediators and aromatase in the mammary gland and visceral fat. Our third objective was to develop a cellular model that could explain the link between obesity, inflammation, and increased aromatase expression. Here we show that obesity caused inflammation in the mammary glands (MG) and visceral fat of mice. Increased levels of aromatase mRNA and activity paralleled the elevated levels of TNF-α, IL-1β, and Cox-2 in the mammary gland and visceral fat of obese mice. Finally, we present evidence that activation of macrophages, a component of the inflammatory cell infiltrate in adipose and mammary tissues of obese mice, led to increased production of proinflammatory mediators which contributed, in turn, to the induction of aromatase. Collectively, these results provide potential insights into why obese postmenopausal women are at increased risk of developing HR-positive breast cancer. Moreover, overexpression of aromatase in the adipose tissue of obese mice offers a possible explanation for why the recommended doses of aromatase inhibitor are less effective in the treatment of HR-positive breast cancer in obese versus lean women (21). The discovery of the obesity → inflammation → aromatase axis provides the basis for developing mechanism-based strategies to reduce the risk of HR-positive breast cancer in this growing segment of the population.

Materials and Methods

Materials

Medium to grow visceral preadipocytes was purchased from ScienCell Research Laboratories. FBS was purchased from Invitrogen. Neutralizing antibodies and enzyme immunoassay (EIA) kits for TNF-α and IL-1β were purchased from R&D systems. Antibodies to phospho-p65, p65, histone H3, Cox-2, and β-actin were from Santa Cruz Biotechnology. Lowry protein assay kits, lipopolysaccharide, butylated hydroxytoluene (BHT), IFNγ, horseradish peroxidase–conjugated secondary antibody, collagenase type 1, bovine serum albumin (BSA), glucose-6-phosphate, glycerol, pepstatin, leupeptin, glucose-6-phosphate dehydrogenase, and rotenone were from Sigma. BAY11-7082 was purchased from InvivoGen. Celemocix was obtained from LKT Laboratories. Saturated fatty acids were obtained from Nu-Chek Prep. ECL Western blotting detection reagents were from Amersham Biosciences. Nitrocellulose membranes were from Schleicher & Schuell. 1β-[3H]-androstenedione and [32P]dCTP were from Perkin-Elmer Life Science. pSVβgal, electrophoretic mobility gel shift, and plasmid DNA isolation kits were from Promega. The F4/80 and aP2 cDNAs were obtained from Open Biosystems. The 18S rRNA cDNA was purchased from Ambion. RNaseasy mini kits were purchased from Qiagen. MuLV reverse transcriptase, RNase inhibitor, oligo (dT)16, and SYBR green PCR master mix were obtained from Applied Biosystems. Real-time PCR primers were synthesized by Sigma-Genosys. Luciferase assay substrates and cell lysis buffer were from BD Biosciences. PGE2, PGE2-d4, and PGE2 EIA kits were purchased from Cayman Chemicals. Protein assay kits were purchased from Bio-Rad. Chromatin immunoprecipitation (ChIP) assay kits were purchased from Millipore. Control siRNA and siRNAs for p65, TNF-α, IL-1β, and Cox-2 were purchased from Thermo Scientific.

Animal models

Both dietary and genetic models of obesity were used. In the dietary model, at 5 weeks of age, ovariectomized (OVX) C57BL/6J female mice (Jackson Laboratories) were randomized (n = 10/group) to receive either low fat (LF) or high fat (HF) diets. The LF (D12450Bi) and HF (D12492i) diets contain 10 kcal% fat and 60 kcal% fat, respectively (Research Diets) and are commonly used in studies of obesity (22). Mice were fed these diets ad libitum for 10 weeks before being sacrificed. In the genetic model, female ob/ob and control C57BL/6J mice were obtained at 8 weeks of age (Jackson Laboratories) and fed PicoLab Rodent Diet 20, #5053 (W.F. Fisher & Son) ad libitum for 3 weeks prior to sacrifice. Following sacrifice, MG and visceral fat were snap frozen in liquid nitrogen and stored at −80°C for molecular analysis or formalin fixed for histological and immunohistochemical analyses. In experiments to separate the adipose and stromal-vascular fractions (SVF) of the mammary gland, tissues were directly subjected to cell fractionation. The animal protocol was approved by the Institutional Animal Care and Use Committee at Weill Cornell Medical College.

Light microscopy and immunohistochemistry

Four-micron thick sections were prepared from formalin-fixed, paraffin-embedded mammary gland tissue and visceral fat and stained with hematoxylin and eosin. The total number of inflammatory foci per section was quantified by a pathologist (RKY) and the amount of tissue present on each slide was recorded, to determine the number of inflammatory foci per cm² of tissue. Immunohistochemical stains against F4/80 were performed using standard techniques. Briefly, 4-μm formalin-fixed, paraffin-embedded tissue sections of mammary gland were deparaffinized and rehydrated prior to antigen retrieval in citrate buffer, pH 6.0 (Dako). Endogenous peroxidase activity was quenched with 0.3% H2O2, and nonspecific binding blocked with 0.5% Tween-20 in 5% BSA prior to overnight incubation with rat anti-mouse F4/80 primary antibody (Serotec). Horseradish peroxidase-labeled goat anti-rat IgG (Jackson ImmunoResearch) and NovaRed substrate (Vector Labs) were used for detection.
Separation of stromal-vascular and adipocyte fractions

To fractionate mammary gland tissue into SVF and adipocyte fractions, we utilized a modified version of an existing protocol (23). Mammary gland tissue was minced into small pieces and placed in sterile plastic tubes with Krebs-Ringer buffer containing 25 mmol/L NaHCO₃, 11 mmol/L glucose, 25 mmol/L Hepes (pH 7.4), 2% BSA, and 1.5 mg/mL collagenase type I. The ratio between adipose tissue mass and incubation solution was 1:4 (w/v). The tissue suspension was incubated at 37°C for 45 to 60 minutes. Once digestion was completed, samples were passed through a sterile 250-mL collagenase type I. The ratio between adipose tissue mass and incubation solution was 1:4 (w/v). The tissue suspension was incubated at 37°C with gentle shaking for 45 to 60 minutes. Once digestion was completed, samples were passed through a sterile 250-μm nylon mesh (VWR). The suspension was centrifuged at 200 x g for 10 minutes; the pellet was resuspended as SVF and the floating cells were considered the adipocyte-enriched fraction. The adipocytes were washed twice with Krebs-Ringer-bicarbonate-Hepes-BSA buffer and centrifuged as before. The SVF was resuspended in erythrocyte lysis buffer consisting of 0.154 mol/L NH₄Cl, 10 mmol/L KHCO₃, and 0.1 mmol/L EDTA, and incubated at room temperature for 10 minutes. The erythrocyte-depleted SVF was centrifuged at 400 x g for 5 minutes. After washing, the SVF and adipocyte fractions were subjected to analysis or cultured.

Tissue culture

Human visceral preadipocytes (ScienCell) were grown in preadipocyte medium containing 10% FBS. 3T3-L1 cells (ZenBio) were grown in DMEM supplemented with 10% calf bovine serum. THP-1 cells (ATCC) were maintained in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS. These cells were treated with phorbol 12-myristate 13-acetate (10 ng/mL) overnight to differentiate them into macrophages. Human monocytes (Astarte Biologics) were activated with IFNγ (15 ng/mL) and lipopolysaccharide (15 ng/mL) in RPMI-1640 medium for 4 days. THP-1 cells and blood monocyte–derived macrophages were then treated with saturated fatty acids. To prepare conditioned medium (CM), these cells were treated with saturated fatty acids for 12 hours in medium comprised of RPMI-1640 and preadipocyte medium at a 1:1 ratio. Following treatment with fatty acids, the medium was removed and cells were washed thrice with PBS to remove fatty acids. Subsequently, fresh medium was added for 24 hours. This CM was then collected and centrifuged at 4,000 rpm for 30 minutes to remove cell debris. CM was then used to treat preadipocytes.

Immunoblot analysis

Lysates were prepared by treating cells with lysis buffer (150 mmol/L NaCl, 100 mmol/L Tris, pH 8.0, 1% Tween 20, 50 mmol/L diethylidithiocarbamate, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL trypsin inhibitor, and 10 μg/mL leupeptin). Lysates were sonicated for 20 seconds on ice and centrifuged at 10,000 x g for 10 minutes to sediment the particulate material. The protein concentration of the supernatant was measured by the method of Lowry and colleagues (24). SDS-PAGE was done under reducing conditions on 10% polyacrylamide gels. The resolved proteins were transferred onto nitrocellulose sheets and then incubated with primary antisera. Antibodies to phospho-p65, p65, histone H3, Cox-2, and β-actin were used. Secondary antibody to IgG conjugated to horseradish peroxidase was used. The blot was probed with the ECL Western blot detection system.

Northern blotting

Total RNA was prepared from SVF and adipocytes derived from the mammary gland using an RNA isolation kit. Ten micrograms of total RNA/lane were electrophoresed in a formaldehyde-containing 1% agarose gel and transferred to nylon-supported membranes. F4/80, aP2 and 18S rRNA probes were labeled with [32P]dCTP by random priming. The blots were probed as described previously (25).

Quantitative real-time PCR

Total RNA was isolated using the RNeasy mini kit. For tissue analyses, poly A RNA was prepared with an Oligotex mRNA mini kit (Qiagen). Poly A RNA was reversed transcribed using murine leukemia virus reverse transcriptase and oligo (dT)₁₄,₁₈ primer. The resulting cDNA was then used for amplification using primers listed in Supplementary Table S1. Glycereraldehyde 3 phosphate dehydrogenase (GAPDH) and β-actin were used as endogenous normalization controls for tissue and cell culture analyses, respectively. Real-time PCR was performed using 2x SYBR green PCR master mix on a 7500 Real-time PCR system (Applied Biosystems). Relative fold induction was determined using the ddCt (relative quantification) analysis protocol.

Transient transfections

NF-κB-luciferase (Panomics) and pSV-fgal were transfected into THP-1 cells using the Amaxa system. After 24 hours of incubation, the medium was replaced with basal medium. The activities of luciferase and β-galactosidase were measured in cellular extracts. For siRNA transfections, THP-1 cells were plated at 60% confluence and transfected with nontargeting siRNA or siRNA targeting TNF-α, IL-1β, Cox-2, and p65 using DharmaFect 4 for 36 hours. Subsequently, the cells were treated with vehicle or saturated fatty acids.

ChIP assay and qPCR

ChIP assays were performed with a kit according to the manufacturer’s instructions. A total of 3.5 × 10⁶ cells were cross-linked in a 1% formaldehyde solution for 10 minutes at 37°C. Cells were then lysed in 200 μL of SDS buffer and sonicated to generate 200 to 1,000-bp DNA fragments.
After centrifugation, the cleared supernatant was diluted 10-fold with ChIP buffer and incubated with 1.5 ng of the indicated antibody at 4°C. Immune complexes were precipitated, washed, and eluted as recommended. DNA-protein cross-links were reversed by heating at 65°C for 4 hours, and the DNA fragments were purified and dissolved in 50 μL of water. A total of 10 μL of each sample was used as a template for PCR amplification. The following PCR primers were used: TNF-α promoter, forward 5’-GATCCCG-GAGGAGATTCCTTTA-3’ and reverse 5’-ACACTCCAGG-CCTTAAAGGTCCCGACATAGT-3’ (−255 to −655); IL-1β promoter, forward 5’-CGTTGGAAAAATCCAGTATTGT-3’ and reverse 5’-CAAATGTATCACCATTGCAAAATATGC-3’ (−490 to −190), and COX-2 promoter forward 5’-AAGCTATGTATGTGTGCTGCAT-3’ and reverse 5’-ACCTCCAGTATGCTCCTTTG-3’ (−7 to −621). PCR was performed at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds for 30 cycles. The PCR products generated from the ChIP template were sequenced, and the identity of TNF-α, IL-1β, and COX-2 promoters was confirmed. Real-time PCR was performed as described above.

**Electrophoretic mobility shift assay**

Nuclear extracts were prepared from mouse MGs using an electrophoretic mobility shift assay (EMSA) kit. For binding studies, oligonucleotides containing NF-κB sites (Active Motif) were used. The complementary oligonucleotide-binding studies, oligonucleotides containing NF-κB sites were reverse transcribed and then subjected to the analysis using established methods (12). The mass spectrometer was operated in positive ion mode.

**Statistics**

Data generated in this study include mouse weights, the number of inflammatory foci, tissue TNF-α, IL-1β, Cox-2, and aromatase mRNA levels and aromatase activity. In general, endpoints that conform to normality assumption, such as the mouse weight data, were summarized in terms of mean ± SD and compared across multiple groups using ANOVA. Pair-wise comparison of mouse weights were then carried out using Tukey’s test to identify pairs of groups with significant difference in weights and adjusted for
multiple comparisons. Endpoints that are usually not normally distributed were summarized in terms of median and range and with box-plots graphically. Differences in these endpoints were examined across multiple groups using the nonparametric Kruskal–Wallis test. The Wilcoxon rank-sum test was then used for pair-wise comparisons. P values were adjusted for multiple comparisons using the conservative Bonferroni method. To examine the magnitude of difference between 2 experimental groups in different fractions of mammary gland tissue, linear mixed-effects models were used to take into account both the within and between mouse variation. Data were log-transformed where appropriate to ensure that the underlying normality assumption was satisfied. For in vitro studies, comparisons between groups were made using Student’s t test. A difference between groups of $P < 0.05$ was considered significant.

Results

Obesity causes inflammation and elevated aromatase expression in the MGs and visceral fat of mice

Initially, we investigated the effects of dietary fat and ovarian function on weight gain in female mice. Ovary intact or OVX mice were fed LF or HF diets for 10 weeks. Both ovariectomy and feeding a HF diet led to weight gain (Fig. 1A). Thus, mice that were both fed a HF diet and subjected to ovariectomy gained the most weight. Previously, inflammatory lesions known as crown-like structures (CLS) have been identified in the adipose tissue of obese mice and humans (18, 19, 29). Hence, we next investigated whether CLS (Fig. 1B) were present in the MGs and visceral fat of obese mice. A marked increase in the number of these inflammatory lesions was found in the mammary gland and visceral fat of mice that gained the

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**Figure 1.** Diet-induced obesity causes inflammation in the mammary gland and visceral fat. Ovary intact or OVX female mice ($n = 10$ / group) were fed either a LF or HF diet for 10 weeks. A, weight of mice in the diet-induced obesity experiment, shown as mean ± SD. A significant difference in average weights was observed across groups ($P < 0.001$, ANOVA). In particular, OVX mice fed an HF diet had a significantly higher average weight compared with the other groups ($P_{adj} < 0.001$, Tukey’s test). B, hematoxylin and eosin-stained slides were evaluated for the presence of inflammatory foci containing macrophages that surrounded necrotic adipocytes (arrow, 200×). C and D, box-plots of the number of inflammatory foci in MGs and visceral fat of mice in the different treatment groups. Significant differences were observed across the 4 experimental groups for both tissue types ($P < 0.001$, Kruskal–Wallis test). In pair-wise comparisons, OVX mice fed the HF diet showed significantly greater number of inflammatory foci in mammary gland compared with those in the LF diet groups ($P_{adj} = 0.01$, Wilcoxon rank-sum test, $P$ values were adjusted for multiple comparisons with Bonferroni method), and in visceral fat compared with those in the other groups ($P_{adj} < 0.01$). E, inflammatory foci contain F4/80-positive macrophages that formed a typical crown around adipocytes (200×). F, box-plots of PGE2 levels in MGs of mice in the different treatment groups. Significant differences were observed across the 4 experimental groups ($P < 0.001$, Kruskal–Wallis test). In pair-wise comparisons, OVX mice fed the HF diet showed significantly greater PGE2 levels compared with those in the LF diet groups ($P_{adj} = 0.01$, Wilcoxon rank-sum test, $P$ values were adjusted for multiple comparisons with Bonferroni method).
most weight (Fig. 1C and D). To determine whether macrophages were present in these inflammatory lesions, immunohistochemistry was performed to assess F4/80, a macrophage marker. These studies revealed that F4/80-positive cells were abundant in the CLS (Fig. 1E). Because PGE2 levels are increased in a variety of inflammatory conditions, levels of PGE2 were determined in the MGs of mice in each of the treatment groups. Consistent with the histological evidence of inflammation, obesity was associated with elevated levels of PGE2 (Fig. 1F).

Real-time PCR was used to quantify levels of proinflammatory mediators in mammary gland and visceral fat. Obesity-related inflammation was associated with elevated levels of TNF-α, IL-1β, and Cox-2 in both the mammary gland and visceral fat (Fig. 2). Importantly, each of these proinflammatory molecules is a known inducer of CYP19 transcription and aromatase activity (8–17). Accordingly, we next quantified aromatase mRNA levels and activity in the MGs and visceral fat of the different treatment groups. Remarkably, the changes in aromatase expression and activity paralleled the changes in levels of TNF-α, IL-1β, and Cox-2 mRNAs in both the mammary gland (Fig. 2D and E) and visceral fat (Fig. 2I and J). Taken together, these data suggest that diet-induced obesity causes inflammation in the mammary gland and visceral fat resulting in increased aromatase expression and activity.

To confirm that the aforementioned findings were not unique to the diet-induced obesity model, ob/ob mice were used as a second model of obesity. Ob/ob mice are leptin-deficient and have been widely used in studies of obesity. At 11 weeks of age, the ob/ob mice weighed 54.3 ± 2.2 gm whereas lean wild-type mice weighed 19.2 ± 0.8 gm (P < 0.001). Consistent with the findings in the diet-induced model of obesity, significant increases in the number of inflammatory foci and levels of proinflammatory mediators (TNF-α, IL-1β, Cox-2) were observed in the MGs and visceral fat of ob/ob vs. wild-type mice (Table 1). Importantly, levels of aromatase mRNA and activity were also significantly increased in both the MGs and visceral fat of ob/ob mice (Table 1). Once again, the elevated levels of aromatase paralleled the increased amounts of proinflammatory mediators.

The SVF of the mammary gland is a source of proinflammatory mediators that induce aromatase

Previous studies have suggested that obesity-related inflammation is associated with increased levels of
model which includes mice subjected to OVX because of morbidity. We focused on the diet-induced obesity led to increased levels of proinflammatory mediators and aromatase in the SVF or adipocyte fractions of the mammary gland. We compared mice to induce aromatase in preadipocytes. As a result, obesity was associated with increased expression of both PR and pS2 (Fig. 3D).

Moreover, weight gain was more modest in this model because mice in these 2 groups exhibited the greatest obesity (HF vs. LF) was associated with markedly increased levels of aromatase mRNA and activity in 3T3-L1 cells. Experiments were carried out to determine if cross-talk between macrophages and preadipocytes can explain the increased aromatase levels found in the adipocyte fraction of the MG of obese versus lean mice. Notably, treatment with CM-derived from the SVF of the HF + OVX versus LF group led to higher levels of aromatase mRNA and activity in mouse preadipocyte 3T3-L1 cells (Fig. 4E). Experiments were then carried out to evaluate the importance of each of the SVF-derived inflammatory mediators (TNF-α, IL-1β, and PGE2 for inducing aromatase. Antibodies were used to neutralize TNF-α and IL-1β in CM-derived from the HF + OVX mammary gland SVF. Neutralizing either TNF-α (Fig. 4F) or IL-1β (Fig. 4G) attenuated CM-mediated induction of aromatase in 3T3-L1 cells. In addition, treatment of macrophage-enriched SVF with celecoxib, a selective Cox-2 inhibitor, blocked the release of PGE2 into the CM (Fig. 4H). Following Cox-2 inhibition, the ability of SVF-derived CM to induce aromatase mRNA and aromatase activity in 3T3-L1 cells was markedly attenuated (Fig. 4I). Taken together, these data suggest that SVF isolated from the MG of obese versus lean mice (HF + OVX versus LF) produces increased levels of TNF-α, IL-1β, and Cox-2-derived PGE2, each of which contributes, in turn, to the induction of aromatase in adipocytes.

Collectively, the aforementioned findings suggest that obesity causes inflammation that is characterized by macrophage-enriched CLS in the mammary gland. Elevated levels of proinflammatory mediators were found in the SVF; these proinflammatory mediators are likely to induce CYP19 transcription and aromatase expression together with aromatase activity in other cell type(s) including adipocytes via a paracrine mechanism. Therefore, a series of experiments was performed to evaluate this possibility. Initially, SVF isolated from the MG of mice in the HF + OVX versus LF groups was cultured to quantify the production of proinflammatory mediators. SVF derived from the HF + OVX group produced markedly increased levels of TNF-α, IL-1β, and PGE2 compared with SVF from the LF group (Fig. 4A–C). Consistent with the difference in PGE2 production, higher levels of Cox-2 protein were found in SVF of the HF + OVX versus LF group (Fig. 4D). Preadipocytes express higher levels of aromatase than mature adipocytes and are often used as a model cell system in studies of aromatase (31). Next experiments were carried out to determine if cross-talk between macrophages and preadipocytes can explain the increased aromatase levels found in the adipocyte fraction of the MG of obese versus lean mice. Notably, treatment with CM-derived from the SVF of the HF + OVX versus LF group led to higher levels of aromatase mRNA and activity in mouse preadipocyte 3T3-L1 cells (Fig. 4E). Experiments were then carried out to evaluate the importance of each of the SVF-derived inflammatory mediators (TNF-α, IL-1β, and PGE2 for inducing aromatase. Antibodies were used to neutralize TNF-α and IL-1β in CM-derived from the HF + OVX mammary gland SVF. Neutralizing either TNF-α (Fig. 4F) or IL-1β (Fig. 4G) attenuated CM-mediated induction of aromatase in 3T3-L1 cells. In addition, treatment of macrophage-enriched SVF with celecoxib, a selective Cox-2 inhibitor, blocked the release of PGE2 into the CM (Fig. 4H). Following Cox-2 inhibition, the ability of SVF-derived CM to induce aromatase mRNA and aromatase activity in 3T3-L1 cells was markedly attenuated (Fig. 4I). Taken together, these data suggest that SVF isolated from the MG of obese versus lean mice (HF + OVX versus LF) produces increased levels of TNF-α, IL-1β, and Cox-2-derived PGE2, each of which contributes, in turn, to the induction of aromatase in preadipocytes.

<table>
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<tr>
<th>Endpoint</th>
<th>MG</th>
<th>VF</th>
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<tr>
<td>Wild-type</td>
<td>ob/ob</td>
<td>P</td>
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<td>Inflammatory foci</td>
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<td>13.8 (8.5, 20.4)</td>
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<tr>
<td>Relative TNF-α expression</td>
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<td>4.9 (0.7, 9.7)</td>
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<tr>
<td>Relative IL-1β expression</td>
<td>1.0 (0.4, 2.3)</td>
<td>2.9 (0.3, 9.7)</td>
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<tr>
<td>Relative Cox-2 expression</td>
<td>1.0 (0.4, 2.5)</td>
<td>3.8 (1.2, 5.7)</td>
</tr>
<tr>
<td>Relative aromatase expression</td>
<td>1.2 (0.2, 5.6)</td>
<td>6.1 (0.04, 7.6)</td>
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<tr>
<td>Aromatase activity</td>
<td>90 (68,112)</td>
<td>210 (146, 278)</td>
</tr>
</tbody>
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**Table 1.** Significantly greater inflammation, proinflammatory molecule levels, and aromatase expression were found in both the MG and visceral fat (VF) of ob/ob compared with wild-type mice.

**NOTE:** Inflammatory foci, number of inflammatory foci per cm² of tissue; real-time PCR was used to quantify relative TNF-α, IL-1β, Cox-2, and aromatase transcript levels; aromatase activity, femtomoles/μg protein/hour. Values are summarized in median (range), P values are based on Wilcoxon rank-sum test, n = 10/gp.
Figure 3. Diet-induced obesity is associated with elevated levels of proinflammatory mediators and aromatase in different compartments of the mammary gland. Adipocyte (Adipo) and SVF were prepared from MGs of ovary intact mice fed a low fat (LF) diet or mice that were subjected to ovariectomy (OVX) and fed an HF diet for 10 weeks. A, RNA from SVF and adipocyte fractions was isolated from 3 MGs and subjected to northern blotting. Blots were probed for F4/80 and aP2 as indicated. B-D, Real-time PCR was used to quantify mRNA levels for TNF-α, IL-1β, Cox-2, aromatase, PR and pS2. B, box-plots of the TNF-α, IL-1β, and Cox-2 mRNA levels in the SVF and the adipocyte fractions of MGs (n = 10/group) are shown. In comparison to lean mice (LF), obese mice (HF + OVX) showed significantly higher expression levels of all 3 proinflammatory mediators in the SVF but not in the adipocyte fraction of the mammary gland (P < 0.001). C, box-plots of relative aromatase mRNA levels and aromatase activity in the SVF and adipocyte fractions of MGs (n = 10/group) are shown. aromatase activity is expressed as femtomoles/µg protein/hour. D, relative progesterone receptor (PR) and pS2 mRNA expression in the SVF and adipocyte fractions of the mammary gland (n = 10/group) are shown.
Figure 4. Proinflammatory mediators derived from the SVF of the mammary gland of obese mice induce aromatase in preadipocytes. SVF cells derived from the mammary gland of mice in the LF and HF + OVX groups were grown overnight in DMEM. Conditioned medium (CM) was then collected and analyzed or used to treat 3T3-L1 preadipocytes. Levels of TNF-α (A), IL-1β (B), and PGE₂ (C) in CM were determined by enzyme immunoassay. D, Cox-2 protein abundance was determined by immunoblotting of whole-cell lysates. β-actin was used as a loading control. E, 3T3-L1 cells were treated with CM derived from the mammary gland SVF of LF or HF + OVX mice for 24 hours. Relative aromatase mRNA levels and aromatase activity were then determined. F and G, neutralizing either TNF-α (F) or IL-1β (G) suppressed the ability of CM derived from HF + OVX SVF to induce aromatase in 3T3-L1 cells. In both F and G, the bar labeled Control represents 3T3-L1 cells that received DMEM medium alone; the bar labeled CM represents 3T3-L1 cells that received CM derived from HF + OVX SVF. In addition, CM from HF + OVX SVF was incubated with control IgG, TNF-α IgG, or IL-1β IgG overnight at 4 °C to neutralize TNF-α (F) and IL-1β (G), respectively. 3T3-L1 cells were then treated as indicated for 24 hours prior to measurements of relative aromatase mRNA levels and aromatase activity. H, SVF cells derived from the mammary gland of mice in the HF + OVX group were grown overnight. The cells were then treated with fresh DMEM containing vehicle (HF + OVX) or 5 μmol/L celecoxib (HF + OVX + celecoxib) for 6 hours. CM was collected and analyzed for PGE₂ levels (H) or used to treat 3T3-L1 preadipocytes (I). I, inhibiting Cox-2 in SVF cells derived from HF + OVX mammary gland attenuated the ability of CM to induce aromatase in 3T3-L1 cells. The bar labeled Control represents 3T3-L1 cells that received DMEM medium; the bar labeled CM represents 3T3-L1 cells that received CM derived from HF + OVX SVF; the bar labeled CM + celecoxib represents 3T3-L1 cells that received CM derived from HF + OVX SVF treated with 5 μmol/L celecoxib. 3T3-L1 cells were treated as indicated for 24 hours prior to measurements of relative aromatase mRNA levels and aromatase activity. Aromatase activity is expressed as femtomoles/μg protein/minute. Columns, means (n = 3); bars, SD. *, P < 0.05.
Figure 5. Treatment of macrophages with saturated fatty acids causes dose-dependent induction of proinflammatory mediators. THP-1 cells were treated with the indicated concentrations of saturated fatty acid (LA, MA, PA, and SA) for 24 hours. Real-time PCR was used to quantify TNF-α, IL-1β, and Cox-2 mRNAs (A, C, E). Levels of TNF-α protein, IL-1β protein and PGE₂ in the culture medium were determined by enzyme immunoassay (B, D, G). Cox-2 protein abundance was determined by immunoblotting of whole-cell lysates. β-actin was used as a loading control (F). Columns, means (n = 6); bars, SD. *, P < 0.05.
Saturated fatty acids stimulate the production of proinflammatory mediators by macrophages leading to increased aromatase levels

Lipolysis is increased in obesity (32). Moreover, saturated fatty acids released from adipocytes can activate macrophages resulting in an inflammatory response (30). Hence, our next goal was to determine whether the elevated levels of proinflammatory mediators in the SVF of the mammary gland of obese versus lean mice might be explained by the effects of saturated fatty acids on macrophages. Experiments were carried out to determine if saturated fatty acids stimulated the production of proinflammatory molecules by macrophages leading, in turn, to elevated aromatase expression in preadipocytes. Saturated fatty acids ranging in chain length from C12 to C18 including lauric acid (LA), myristic acid (MA), palmitic acid (PA), and stearic acid (SA) were used. Treatment with each of these saturated fatty acids caused dose-dependent induction of TNF-α, IL-1β, and Cox-2 in THP-1 cells, a cell line with properties of human monocyte–derived macrophages (Fig. 5). Induction of Cox-2 mRNA by saturated fatty acids was associated with a corresponding increase in Cox-2 protein and PGE2 production (Fig. 5F and G). Saturated fatty acids also induced proinflammatory mediators in human blood monocyte–derived macrophages (Supplementary Fig. 1). Treatment with CM derived from either saturated fatty acid–treated THP-1 cells or human blood monocyte–derived macrophages induced aromatase mRNA and activity in human preadipocytes (Fig. 6). Neutralizing either TNF-α or IL-1β attenuated CM-mediated induction of aromatase in preadipocytes (Fig. 7). Use of siRNA to silence either TNF-α or IL-1β in THP-1 cells led to similar suppressive effects (Supplementary Fig. 2). In addition, either silencing of Cox-2 or treatment of THP-1 cells with celecoxib blocked saturated fatty acid–mediated induction of PGE2 release into the CM (Fig. 7 and Supplementary Fig. 2). Following Cox-2 inhibition, the ability of macrophage-derived CM to induce aromatase mRNA or aromatase activity in preadipocytes was attenuated. Taken together, these data suggest that saturated fatty acid–mediated induction of TNF-α, IL-1β, and Cox-2 in macrophages contributes to the induction of aromatase in preadipocytes.

Activation of NF-κB contributes to increased aromatase levels in obesity

NF-κB plays a significant role in regulating the expression of proinflammatory mediators in macrophages (33).
Hence, experiments were performed to investigate the potential role of this transcription factor in saturated fatty acid–mediated induction of TNF-α, IL-1β, and Cox-2 in macrophages. Transient transfections were performed with an NF-κB-luciferase reporter construct. Saturated fatty acids induced NF-κB-luciferase activity in THP-1 cells (Fig. 8A). EMSA indicated that saturated fatty acids stimulated binding of nuclear protein to a 32P-labeled NF-κB consensus sequence (Fig. 8B). This binding was abrogated when a large excess of cold probe was used (data not shown). Supershift assays indicated that p65, a component of NF-κB, was present in the binding complex (Fig. 8C). Consistent with the activation of NF-κB, saturated fatty acids stimulated the phosphorylation of p65 and its translocation from cytosol to nucleus (Fig. 8D and E). To further evaluate the potential role of NF-κB in regulating the expression of TNF-α, IL-1β and Cox-2 in macrophages, it was important to determine if saturated fatty acids stimulated the binding of p65 to the
promoters of each of these proinflammatory genes. ChIP assays were carried out. Each of the saturated fatty acids stimulated phospho-p65 binding to the promoters of the proinflammatory genes (Fig. 8F).

To further evaluate the importance of p65 in regulating the production of both proinflammatory mediators and the induction of aromatase, THP-1 cells were treated with small interfering RNA to p65 (Fig. 9A). Silencing of p65

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**Figure 8.** Saturated fatty acids activate NF-κB in THP-1 cells. A, cells were transiently transfected with NF-κB-luciferase and pSV-β-gal constructs. Cells were then treated with 10 μmol/L LA, MA, PA, or SA for 24 hours. Activities represent data that have been normalized to β-galactosidase activity. Columns, mean (n = 6); bar, SD. B and C, 10 μg of nuclear protein was incubated with a 32P-labeled oligonucleotide containing NF-κB binding sites. B, lanes 1 to 4 represent binding with nuclear protein from cells treated with the indicated concentration of SA for 1 hour. C, lanes 1 to 4 represent binding with nuclear protein from cells treated with vehicle (lane 1) or 10 μmol/L SA (lanes 2–4) for 1 hour. Lanes 2 to 4 represent nuclear protein incubated with normal IgG (lane 2) or antibodies to p65 (2 μL, lane 3; 1 μL, lane 4). B and C, the protein-DNA complexes that formed were separated on a 4% polyacrylamide gel. D, cells were treated as indicated with 0 to 10 μmol/L LA, MA, PA, or SA for 30 minutes. The abundance of phospho-p65 and p65 protein in cell lysates was determined by immunoblotting. E, cells were treated with vehicle (control) or 10 μmol/L SA for 30 minutes. The abundance of phospho-p65 was determined by immunoblotting in cytosolic (C) and nuclear (N) preparations. β-actin and histone H3 represent cytosolic and nuclear markers, respectively. F, cells were treated with vehicle (Control) or 10 μmol/L LA, MA, PA, and SA for 3 hours. ChIP assays were performed. Chromatin fragments were immunoprecipitated with antibodies against phospho-p65 and the TNF-α, IL-1β, and Cox-2 promoters were amplified by real-time PCR. DNA sequencing was carried out, and the PCR products were confirmed to be correct promoters. These promoters were not detected when normal IgG was used or when antibody was omitted from the immunoprecipitation step (data not shown). Means ± SD are shown; n = 3.
inhibited the production of proinflammatory mediators (TNF-α, IL-1β, PGE2) in response to treatment with saturated fatty acids (Fig. 9, B-D). Silencing of p65 in THP-1 cells also suppressed CM-mediated induction of aromatase expression and activity in preadipocytes (Fig. 9E and F). We also evaluated the effects of BAY11-7082, a pharmacological inhibitor of NF-κB. Treatment of THP-1 cells with BAY11-7082 suppressed both SA-mediated activation of NF-κB and the production of increased amounts of TNF-α, IL-1β, and PGE2 (Supplementary Fig. 3). Following NF-κB inhibition, the ability of macrophage-derived CM to induce aromatase expression and activity was suppressed (Supplementary Fig. 3, D and E).

Because of the important role that NF-κB was found to play in regulating proinflammatory mediator production and aromatase expression in vitro, complementary studies were carried out on tissues from the diet-induced model of obesity. EMSAs were performed using nuclear extracts isolated from mammary gland tissue in the different treatment groups. Consistent with the elevated levels of TNF-α, IL-1β, and Cox-2 in MGs of obese mice (Fig. 2), NF-κB binding activity was increased in this group (Fig. 10A). Once again, p65 was found in the binding complex (Fig. 10B). As shown previously, higher levels of TNF-α, IL-1β, and Cox-2 message were found in the SVF versus adipocyte fraction of MGs derived from obese mice (Fig. 3B). Notably, higher levels of NF-κB binding activity were also found in the SVF versus adipocyte fractions prepared from the MGs of obese mice (Fig. 10C). Consistent with the in vitro findings (Fig. 8), supershift assays indicated that p65 was present in the binding complex (Fig. 10D). Taken together, our findings suggest that NF-κB plays a key role in regulating the production of proinflammatory mediators in macrophages leading, in turn, to the induction of aromatase in the adipocyte fraction of the mammary gland.

Figure 9. Silencing of p65 inhibits saturated fatty acid (FA)-mediated induction of proinflammatory mediators and aromatase. A, THP-1 cells were transfected with control siRNA or siRNA to p65. The abundance of p65 protein was determined by immunoblotting in cell lysates. β-actin was used as a loading control. B–D, THP-1 cells were untreated or transfected with control siRNA or p65 siRNA. Subsequently, cells were treated with 0 or 10 μmol/L LA, MA, PA, or SA as detailed in the Materials and Methods sections to generate CM. Enzyme immunoassays were used to quantify levels of TNF-α (B), IL-1β (C), and PGE2 (D) in the CM. E and F, preadipocytes were treated with THP-1 cell-derived CM for 24 hours prior to measurements of aromatase mRNA and activity. Activity is expressed as femtomoles/μg protein/minute. Columns, means (n = 6); bars, SD. *, P < 0.05.
Discussion

Obese postmenopausal women are at increased risk of developing HR-positive breast cancer compared with lean postmenopausal women. This increased risk has been attributed, in part, to elevated levels of circulating estrogens that reflect both increased adipose tissue mass and enhanced aromatase expression in subcutaneous fat (4–7). Given the link between estrogen biosynthesis and the development and progression of HR-positive breast cancer, we sought to determine whether obesity-induced inflammation led to increased aromatase expression in the mammary gland in addition to adipose tissue.

Previous studies of adipose tissue indicate that obesity is associated with adipocyte death leading to increased levels of circulating estrogens that reflect both increased adipose tissue mass and enhanced aromatase expression in subcutaneous fat (4–7). The macrophages are aggregated in CLS that form around individual necrotic adipocytes in the visceral and subcutaneous fat of obese humans and mice (18–20, 29, 34). Here we observed crowns of macrophages surrounding adipocytes in both the mammary gland and visceral fat of obese mice. To our knowledge, this is the first report of CLS in the mammary gland. Consistent with the histological evidence of CLS, representing foci of inflammation, increased levels of TNF-α, IL-1β, and Cox-2 were found in the mammary gland and visceral fat in both dietary and genetic models of obesity. The increased levels of proinflammatory mediators occurred in the macrophage-enriched SVF of the mammary gland. These findings are consistent with prior evidence that macrophages are a major source of proinflammatory mediators in adipose tissue in obese mice and humans (20, 34).

Proinflammatory mediators including TNF-α, IL-1β, and Cox-2-derived PGE2 are known inducers of CYP19 transcription resulting in increased aromatase activity (10, 13, 14, 16, 17, 26, 35). In this study, elevated levels of proinflammatory mediators were paralleled by increased levels of aromatase in the mammary gland and visceral fat of obese mice suggesting a causal relationship. In support of this possible mechanism, SVF isolated from the MG of obese mice produced increased levels of TNF-α, IL-1β, and Cox-2-derived PGE2 compared with SVF from lean mice. Importantly, each of these proinflammatory molecules contributed to the ability of SVF-derived CM to induce aromatase (16). Macrophage-derived cytokines have been suggested to stimulate lipolysis in adipocytes leading to increased release of saturated fatty acids (37). These saturated fatty acids activate TLR4 signaling in macrophages and thereby stimulate the production of proinflammatory mediators (38, 39). Therefore, in obesity, a paracrine loop involving

Figure 10. NF-κB is activated in the SVF of the mammary gland of obese mice. A–D, 10 μg of nuclear protein was incubated with a 32P-labeled oligonucleotide containing NF-κB binding sites. A, binding of nuclear protein from unfractionated MGs of 17 mice in the indicated treatment groups. B, lanes 1 to 7 represent binding of protein from MGs in the HF + OVX group. Lane 1, binding reaction without nuclear protein; lane 2, binding of nuclear protein to labeled oligonucleotide; lanes 3 to 5, nuclear protein incubated with normal IgG (lane 3) or phospho-p65 antibody (2 μL, lane 4; 1 μL, lane 5); lanes 6 and 7, nuclear protein was incubated with labeled oligonucleotide and a 10× excess (lane 6) or 50× excess of cold probe (lane 7). C, binding of nuclear protein isolated from the adipocyte or SVF fractions of the mammary gland of 5 mice in the HF + OVX group. D, binding of nuclear protein from SVF isolated from a mammary gland in the HF + OVX group. Lane 1, binding of nuclear protein to labeled oligonucleotide; lane 2, nuclear protein was incubated with labeled oligonucleotide and a 50× excess of cold probe; lanes 3 and 4, nuclear protein incubated with normal IgG (lane 3) or 2 μL of phospho-p65 antibody (lane 4). In A–D, the protein-DNA complexes that formed were separated on a 4% polyacrylamide gel.

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We present evidence that obesity led to activation of NF-κB in the mammary gland. This finding can explain the observed increase in proinflammatory gene expression and the resulting increase in aromatase expression. In support of this conclusion, silencing of p65 or use of a pharmacological inhibitor of NF-κB blocked saturated fatty acid–mediated induction of proinflammatory mediators in macrophages and thereby suppressed CM-mediated induction of aromatase in preadipocytes. Previously, tumor cell NF-κB was suggested to play a role in breast carcinogenesis including in models of HR-negative breast cancer (42). The current results suggest that stromal NF-κB plays a key role in regulating aromatase expression via a paracrine mechanism (Fig. 11). Hence, NF-κB may play a role in both HR-positive and HR-negative breast cancer. Future studies will be required to determine whether single agents or combinations of agents that inhibit the activation of NF-κB will suppress the increased levels of aromatase found in the mammary gland and visceral fat of obese mice. Interventions that disrupt the obesity → inflammation → aromatase axis may prove useful in reducing the risk of HR-positive breast cancer in postmenopausal women.

In the dietary model of obesity, we found that a HF diet caused more severe inflammation, greater proinflammatory cytokine expression and increased aromatase levels in the mammary gland and visceral fat of OVX compared with ovary intact mice. Rodent OVX has been used as a model of human menopause because it provides the means to study the metabolic consequences that arise through the loss of ovarian function (43). Studies have consistently shown that OVX causes expansion of adipose tissue, insulin resistance, and inflammation (43). These effects, which are associated with increased breast cancer risk in postmenopausal women, can be reversed by systemic administration of estradiol (44, 45). Consequently, it appears that circulating estradiol plays a critical role in preventing obesity and inflammation. However, obesity in postmenopausal women and its attendant increase in breast cancer risk are associated with an increase in serum estradiol, although the actual concentrations are much lower than that found in premenopausal women (46). It is possible, therefore, that very high estradiol concentrations can be formed in the breast tissue of obese postmenopausal women and that this is responsible for the increased risk of HR-positive breast cancer rather than circulating estradiol. If so, circulating estradiol in postmenopausal women might only be a biomarker for breast cancer risk and not the primary mediator of the increased risk. The answers to these questions could have profound implications for the chemoprevention of HR-positive breast cancer in postmenopausal women.

Our data also raise important questions concerning the relationship between obesity and the progression of breast cancer. Numerous studies have shown that obesity is associated with poor prognosis including an increased risk of distant metastasis and cancer-related death (47, 48). This is true for both premenopausal and postmenopausal breast cancer patients. A variety of mechanisms have been suggested to explain the relationship between obesity and poor

Figure 11. Paracrine interactions between adipocytes and macrophages can explain the elevated levels of aromatase in the mammary gland and visceral fat of obese mice. In obesity, lipolysis is increased resulting in increased concentrations of free fatty acids. Saturated fatty acids trigger the activation of NF-κB in macrophages resulting in enhanced production of proinflammatory mediators (PGE2, TNF-α, IL-1β). Each of these proinflammatory mediators contributes to the induction of aromatase in preadipocytes and adipocytes.
Obesity is Associated with Mammary Gland Inflammation

prognosis. For example, increased levels of estrogens, insulin or proinflammatory mediators can potentially drive tumor progression (6, 49). Here, we present the first evidence that obesity leads to activation of NF-κB, increased levels of proinflammatory mediators and elevated aromatase expression in the mammary gland. In a different context, each of these effects has been linked to tumor progression. Based on the current results, additional studies are warranted to further interrogate the mechanisms underlying the link between obesity and breast cancer progression. It’s possible that agents that suppress the obesity → inflammation axis will have a role in inhibiting tumor progression. Finally, we note that the efficacy of anastrozole, an aromatase inhibitor, in the adjuvant treatment of HR-positive breast cancer is reduced in obese compared to lean postmenopausal women (21). It has been theorized that a standard dose of anastrozole is insufficient to completely suppress the elevated estrogen levels resulting from obesity. Our finding that obesity causes elevated aromatase expression in adipose tissue provides a mechanism that may contribute to the reduced efficacy of an aromatase inhibitor in obese women given standard therapeutic doses. Future studies are warranted to determine the dose of aromatase inhibitor that is required to completely suppress tissue estrogen biosynthesis in obese women.

Disclosure of Potential Conflicts of Interest

Andrew J. Dannenberg is a member of the Scientific Advisory Board of Tragara Pharmaceuticals Inc., a company that is developing a selective COX-2 inhibitor. The other authors disclosed no potential conflicts of interest.

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Obesity Is Associated with Inflammation and Elevated Aromatase Expression in the Mouse Mammary Gland

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