Estrogen Protects against Obesity-Induced Mammary Gland Inflammation in Mice

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Abstract

Obesity is a risk factor for the development of hormone receptor (HR)–positive breast cancer in postmenopausal women. Obesity causes subclinical inflammation in white adipose tissue (WAT), characterized by macrophages surrounding dead or dying adipocytes forming crown-like structures (CLS). Estrogen synthesis is catalyzed by aromatase. Previously, we demonstrated CLS and elevated levels of proinflammatory mediators and aromatase in the mammary glands of obese mice and breast tissue of obese women. Here, we tested the hypothesis that supplemental estrogen could prevent or reverse WAT inflammation (WATi) and related molecular changes in the mammary gland. C57BL/6 mice were ovariectomized (OVX) to simulate the postmenopausal state. Supplementation with 17β-estradiol (E2) protected against high fat diet (HFD)-induced weight gain and mammary glands WATi. Expression of proinflammatory mediators (Cox-2, TNFα, IL1β) and aromatase were also reduced in the mammary glands of mice that received supplemental E2. Next, to determine whether E2 supplementation can reverse WATi, obese OVX mice were treated with E2 or placebo and then continued on HFD. E2 supplementation induced weight loss, reversed mammary gland inflammation, and downregulated expression of proinflammatory mediators and aromatase. Finally, we determined whether the protective effects of E2 were mediated by estrogen receptor-α (ERα). Knocking out ERα in ovary intact mice fed a HFD led to weight gain, WATi and elevated levels of proinflammatory mediators and aromatase mimicking the effects of OVX. Taken together, our findings indicate that estrogen via ERα protects against weight gain, WATi and associated increases in proinflammatory mediators and aromatase in the mammary gland. Cancer Prev Res; 8(8); 751–9. ©2015 AACR.

Introduction

Obesity is a risk factor for the development of hormone receptor (HR)–positive breast cancer in postmenopausal women (1, 2). In addition to increasing risk, obese patients with breast cancer have a worse prognosis (3–5). The development and growth of HR-positive breast cancers are commonly regulated by estrogens. Aromatase, a member of the cytochrome P450 superfamily of enzymes, catalyzes estrogen biosynthesis (6, 7). Inhibitors of aromatase that block the synthesis of estrogens reduce the risk of breast cancer and suppress the recurrence of HR-positive breast cancers (8). After menopause, peripheral aromatization of androgen precursors in adipose tissue is a key source of estrogen. A key unanswered question concerns the relative importance of breast adipose itself versus peripheral adipose tissue in the synthesis of estrogens that stimulate the formation and growth of HR-positive tumors. Although modestly increased levels of circulating estrogen are found in obese postmenopausal women (9), the levels are low and the importance of this source of estrogen in the development of breast cancer is unclear. In fact, The Women’s Health Initiative Estrogen-alone Trial found that supplemental estrogen appeared to reduce the risk of breast cancer in postmenopausal women (10). The results of this clinical trial raise additional uncertainty about the link between mildly elevated circulating estrogen levels in obese postmenopausal women and increased breast cancer risk.

Clinically occult inflammation is commonly found in the visceral and subcutaneous white adipose tissue (WAT) of obese women (11, 12). Macrophages infiltrate the WAT and form crown-like structures (CLS) around dead or dying adipocytes (11, 13). These macrophages produce a variety of proinflammatory mediators. Monocyte chemoattractant protein-1 (MCP-1) plays a significant role in the recruitment of these macrophages to the adipose tissue and may also stimulate macrophage proliferation within the CLS (14, 15). We demonstrated in both experimental models of obesity and in obese women that CLS occur in WAT of the mammary gland and breast, respectively (16–18). Ovariectomy (OVX) sensitized mice to both weight gain and mammary gland inflammation (16). Furthermore, both the incidence and severity of breast white adipose inflammation are greater in postmenopausal than in premenopausal women suggesting an anti-inflammatory role for estrogen (19). Inflammation, as determined by the presence of CLS, was associated with elevated levels of proinflammatory mediators and aromatase suggesting that the obesity—Inflammation—Aromatase axis may
contribute to the increased risk of HR-positive breast cancer in obese women (16, 17).

Menopause-associated reductions in estrogen are linked to a significant increase in the incidence of obesity (20). In mouse models, exogenous estrogen attenuates OVX-induced weight gain (21). In this study, we investigated whether supplemental estrogen protected against or reversed histologic inflammation and related molecular changes in the mammary glands of mice. Our results suggest that obesity-related inflammation in the mammary glands, including elevated levels of proinflammatory mediators and aromatase, can be attenuated by supplemental estrogen. These findings strengthen the rationale for evaluating whether supplemental estrogen can affect white adipose inflammation in postmenopausal women or women who have undergone surgical removal of the ovaries.

Materials and Methods

Materials

Lowry protein assay kits, glucose-6-phosphate, glyceraldehyde 3-phosphate dehydrogenase, and rotenone were purchased from Sigma. 17β-estradiol was purchased from Calbiotech Inc. 17β-estradiol ELISA kit was purchased from Calbiotech Inc. 17β-estradiol (E2) and placebo pellets were purchased from Innovative Research of America.

Animal models

Estrogen prevention study. At 5 weeks of age, sham ovariectomized (sOVX) and OVX C57BL/6J female mice (The Jackson Laboratory) were fed either low fat diets (LFD) or high fat diets (HFD). The LFD (12450Bi) and HFD (D12492i) contain 10 kcal% fat and 60 kcal% fat, respectively (Research Diets). The sOVX mice were fed LFD for 10 weeks until sacrifice at 15 weeks of age. The OVX mice were fed HFD for 2 weeks and then randomized (n = 11/gp) to receive either a placebo pellet or an active 0.1 mg E2 pellet (60-day continuous release). The pellets were implanted subcutaneously via 10-gauge trochar. The mice then continued on HFDs for an additional 8 weeks until sacrifice at 15 weeks of age. The weights of the mice and calories consumed were monitored throughout the experiment.

Figure 1.

Supplemental estrogen protects against HFD-induced weight gain and mammary gland inflammation. A, study schema. A group of ovary intact mice (n = 12) was fed a LFD for 10 weeks until sacrifice at 15 weeks of age. Mice that were subjected to OVX were fed HFD for 2 weeks and then randomized (n = 11/gp) to either receive a placebo pellet or an active 0.1 mg E2 pellet (subcutaneous implantation, 60-day continuous release). The mice then continued on HFDs for an additional 8 weeks until sacrifice at 15 weeks of age. B, body weights of mice in different treatment groups. E2 supplementation prevented HFD-induced weight gain. C, caloric consumption was monitored weekly in mice in each of the three groups. D, in pair-wise comparisons, at sacrifice, mice given E2 supplementation had significantly lower body weights (P < 0.001), mammary gland inflammation as defined by presence of CLS (CLS/cm²; P < 0.001), and smaller adipocyte diameter (P < 0.001) compared with HF mice treated with placebo. Inset, example of a CLS in the mammary glands as shown by hematoxylin and eosin stain.
Estrogen treatment study. At 5 weeks of age, sOVX and OVX C57BL/6J female mice (The Jackson Laboratory) were fed either LFDs or HFDs. The sOVX mice were fed LFD and randomized to one of two groups (n = 10/gp). One group was sacrificed at 15 weeks of age after 10 weeks on LFD while the other group was sacrificed at 23 weeks of age after 18 weeks on LFD. The OVX mice were fed HFD for 10 weeks to induce obesity and were then randomized to one of three groups (n = 8–10/gp). One group was sacrificed at 15 weeks of age after 10 weeks on HFD. The second group received a placebo pellet and the third group received a 0.1 mg E2 pellet. Pellets (60-day continuous release) were implanted subcutaneously. The two groups that received placebo or E2 were continued on HFD for an additional 8 weeks until sacrifice at 23 weeks of age. The weights of the mice and calories consumed were monitored throughout the experiment.

Estrogen receptor-α (ERα) knockout study. At 5 weeks of age, sOVX and OVX C57BL/6J female mice (The Jackson Laboratory) were fed either LFDs or HFDs. One group of sOVX mice was fed LFD for 10 weeks until sacrifice (n = 10, same group described in the Treatment Study). The OVX mice were fed HFD for 10 weeks until sacrifice (n = 10, same group described in the Treatment Study). Another group of sOVX mice was fed HFD for 10 weeks until sacrifice (n = 10). The last group of sOVX mice was homozygous for deletion of estrogen receptor-α (ERα-/-, n = 10. B6.129P2-Esr1tm{dEα}) and was fed HFD for 10 weeks until sacrifice. The ERα-/- mice had been backcrossed to C57BL/6J mice for 10 generations. All mice were sacrificed at 15 weeks of age. The weights of the mice and calories consumed were monitored throughout the experiment.

Following sacrifice, mammary glands were snap-frozen in liquid nitrogen and stored at −80°C for molecular analysis or formalin fixed for histologic analyses. The animal protocol was approved by the Institutional Animal Care and Use Committee at Weill Cornell Medical College (New York, NY).

Light microscopy

Four-micron-thick sections were prepared from formalin-fixed, paraffin-embedded mammary gland tissue and stained with hematoxylin and eosin. The total number of CLS per section was quantified by a pathologist (D. Giri) and the amount of adipose tissue present on each slide was determined using NIH ImageJ. Inflammation was quantified as number of CLS/cm² of adipose tissue.

Adipocyte diameter

Adipocyte diameter was quantified as previously described (17).

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)₁₆ primer. The resulting cDNA was then used for amplification. Primer sequences have been reported previously (16, 22). GAPDH was used as an endogenous normalization control. Real-time PCR was performed using 2 × Fast SYBR green PCR master mix on a 7500 Fast Real-time PCR system (Applied Biosystems). Relative

Figure 2.

Estrogen supplementation suppresses the increased levels of proinflammatory mediators and aromatase found in the mammary glands of obese mice. Real-time PCR was carried out on RNA isolated from the mammary gland (MG) of mice in each of the three groups (n = 11–12/gp). EIA was used to quantify PGE₂. Box plots of F4/80, MCP-1, Cox-2, PGE₂, TNFα, and IL1β are shown (A–F). In pair-wise comparisons, HF OVX = E2 mice compared with HF OVX = placebo mice had significantly lower mammary gland levels of all biomarkers (P < 0.05) except TNFα (P = 0.09). G and H, mammary gland aromatase expression and activity were also significantly decreased in HF OVX mice supplemented with E2 compared with placebo (P = 0.003 and P < 0.001, respectively).
fold-induction was determined using the ddCt (relative quantification) analysis protocol.

**Aromatase activity**

To determine aromatase activity, microsomes were prepared from mammary gland tissue by differential centrifugation. Aromatase activity was quantified by measurement of the tritiated water released from [1β-[3H]-androstenedione (23)]. Aromatase activity was normalized to protein concentration.

**PGE2 levels**

PGE2 levels were quantified using an EIA kit from Cayman Chemical. Protein levels were determined by the method of Lowry (24). Levels of PGE2 were normalized to protein concentrations and expressed as pg/mg protein.

**Serum estradiol concentration**

Blood was collected from mice by cardiac puncture after sacrifice and allowed to clot at room temperature for 20 minutes. The blood was then centrifuged at 8,000 rpm at 4°C and serum was isolated and stored at –80°C until assayed. E2 concentration in serum was determined using an ELISA kit. Absorbance was measured at 450 nm on an absorbance microplate spectrophotometer.

**Statistical analysis**

In the mouse experiments, endpoints of interest include mouse weight over time, mouse caloric consumption over time, and various biomarker measurements obtained from mouse mammary glands, including number of CLS/cm², average adipocyte diameter, relative mRNA expression level and aromatase activity, and PGE2 levels. Mouse growth rates were calculated under linear growth assumption for defined period of time based on study design. For endpoints that conform to normality assumption (including mouse weight at a specific time point, mouse growth rate and caloric consumption during a specific periods of time, and average adipocyte diameter), ANOVA was used to examine the differences across experimental groups. Pairwise comparisons were carried out using the Tukey method that adjusts the P values for multiple comparisons by controlling experiment-wide error rate. For biomarker data including number of CLS/cm², relative mRNA expression level, aromatase activity and PGE2 levels, the nonparametric Kruskal–Wallis test was used to examine the difference in biomarker levels across experimental groups.

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**Figure 3.**

Supplemental estrogen reverses HFD-induced weight gain and mammary gland inflammation. A, study schema. Ovary intact mice were fed LFD for 10 or 18 weeks until sacrifice (n = 10/group). The mice that had been subjected to OVX were fed HFD for 10 weeks and were then randomized to one of three groups (n = 10/group). One group was sacrificed immediately after 10 weeks on HFD. The second group received placebo and the third group received 0.1 mg E2 pellet (subcutaneous implantation, 60-day continuous release). Both groups continued on HFD for an additional 8 weeks. B, body weight was monitored weekly in the different treatment groups. E2 supplementation reversed HFD-induced weight gain. C, caloric consumption was monitored weekly in the HFD fed mice following implantation of E2 or placebo and compared with the group that received the LFD. D, in pair-wise comparisons, at sacrifice, mice treated with E2 had significantly lower body weight (*, P < 0.001), mammary gland inflammation (CLS/cm², †, P = 0.02), and average adipocyte diameter (*, †, P < 0.001) compared with HFD fed mice given placebo.
Estrogen Inhibits Breast Tissue Inflammation

Results

Estrogen protects against diet-induced obesity and associated histologic and molecular changes in the mammary gland

To determine whether estrogen supplementation prevented OVX mice from becoming obese with HFD feeding, HF OVX mice were implanted subcutaneously with E2 or placebo pellets. Following implantation, the mice continued on HFD for 8 weeks (Fig. 1A). Ovary intact mice fed a LFD for 10 weeks served as lean controls. At sacrifice, serum estradiol levels were similar in ovary intact mice and OVX mice that received supplemental E2 (data not shown). As shown in Fig. 1B, a marked increase in weight occurred in the placebo group over time while the group supplemented with E2 failed to gain weight with HFD feeding. Consistent with the suppression of weight gain, caloric consumption was also initially reduced in the E2-treated group compared with the placebo group (Fig. 1C). Histologic analysis of mammary glands tissue was conducted to determine whether the inhibition of weight gain in the E2 group was associated with decreased WAT inflammation. Treatment with the HFD led to a significant increase in mammary gland inflammation, as defined by the number of CLS/cm², an effect that was attenuated by supplemental E2 (Fig. 1D). Adipocyte size correlates with the number of CLS in both mice and women (17, 22). Accordingly, we next measured adipocyte diameters in all treatment groups. In accord with the inflammation findings, supplemental E2 blocked the increase in adipocyte diameter found in the mammary glands of OVX mice fed the HFD (Fig. 1D).

Because supplemental E2 suppressed HFD-mediated increases in the number of CLS/cm² in the mammary glands, we next conducted real-time PCR to assess expression of the macrophage marker F4/80 in the mammary glands (Fig. 2A). Consistent with the CLS findings, treatment with E2 attenuated the increase in F4/80 mRNA levels mediated by the HFD (Fig. 2A). MCP-1 plays a role in the recruitment of blood monocytes that become macrophages in adipose tissue. Given the ability of E2 to suppress the increase in macrophages in the mammary glands of mice fed the HFD, levels of MCP-1 were quantified. As shown in Fig. 2B, supplemental E2 also blocked the increase in MCP-1 levels in mice fed the HFD. Next, we assessed levels of proinflammatory mediators. Here too supplemental E2 suppressed the increase in levels of proinflammatory mediators (Cox-2, PGE₂, TNFα, IL1β) found in the mammary glands of mice fed the HFD (Fig. 2C–F). Because these proinflammatory mediators can modulate aromatase expression, we also assessed both aromatase mRNA levels and aromatase activity. The increase in aromatase in the mammary glands of mice fed the HFD was attenuated by supplemental E2 (Fig. 2G and H).

Obesity-related mammary gland inflammation and elevated aromatase levels are reversed by estrogen treatment

Next, we conducted a treatment study to determine whether estrogen supplementation could reverse obesity-related
mammary gland inflammation. OVX mice were made obese with HFD feeding for 10 weeks (Fig. 3A). Mice were then either sacrificed or implanted with a placebo or E2 pellet and then continued on HFD for an additional 8 weeks. Ovary intact mice fed a LFD for 10 or 18 weeks served as lean controls. At sacrifice, serum estradiol levels were similar in ovary intact mice and OVX mice that received supplemental E2 (data not shown). Immediately after implantation with E2, there was a significant decrease in body weight while the placebo group continued to gain weight normally (Fig. 3B). Consistent with the decreased body weight, E2 supplementation also caused a significant decrease in calorie consumption (Fig. 3C). Histologic analysis of mammary gland tissue also showed decreased CLS/cm² and smaller adipocyte diameters compared with the placebo group (Fig. 3D). In fact, both the number of CLS/cm² and adipocyte diameters were also reduced compared with the HFD group that was sacrificed prior to pellet implantation. Therefore, E2 treatment led to weight loss that was associated with both reduced adipocyte size and decreased mammary gland inflammation.

Levels of F4/80, MCP-1, and proinflammatory mediators (Cox-2, PGE₂, and TNFα) were all significantly decreased in HFD-fed mice treated with E2 compared with placebo (Fig. 4A–E). There was also a trend toward decreased expression of IL1β following E2 treatment (Fig. 4F). As in in the E2 prevention study, aromatase expression and activity paralleled the changes in levels of the proinflammatory mediators (Fig. 4G and H).

**Estrogen acts via ERα to inhibit weight gain and inflammation in the mammary glands**

ERα plays a key role in both adipose tissue biology and metabolic processes. Therefore, an experiment was carried out to determine whether knocking out ERα mimicked the effects of OVX. As indicated in Fig. 5A, we compared the effects of HFD in OVX mice, ovary intact mice and ovary intact ERα knockout mice. Ovary intact mice fed a LFD served as lean controls. Weight gain and calorie consumption were increased in ovary intact ERα knockout mice fed a HFD compared with ovary intact wild-type mice fed a HFD (Fig. 5B and C). In fact, the weight increase observed in ERα knockout mice mimicked the increase found in OVX mice, suggesting that E2 acts via ERα to suppress weight gain. Consistent with the weight gain that was observed in ERα knockout mice fed a HFD, both adipocyte size and the number of CLS/cm² were increased in the mammary glands of ERα knockout mice (Fig. 5D). In addition, levels of F4/80, MCP-1, Cox-2, PGE₂, TNFα, IL1β, and aromatase were all significantly increased in the ERα knockout versus wild-type control mice (Fig. 6A–H). The fact that both the histologic changes and related molecular endpoints found in ERα knockout mice mirrored the changes induced by OVX suggests that the protective effects of estrogen are mediated via ERα.
Estrogen Inhibits Breast Tissue Inflammation

Discussion

Chronic inflammation of several organs increases the risk of epithelial malignancies (25). Previously, we demonstrated that subclinical inflammation of the breast manifested as CLS-B occurs in most obese and overweight women. Importantly, menopause is an independent determinant of CLS-B (19). Histologic inflammation was associated with adipocyte hypertrophy and elevated levels of proinflammatory mediators and aromatase (17, 18). We concluded that the obesity—inflammation—aromatase axis was likely to contribute to the development of HR-positive breast cancer in postmenopausal women. These findings in women were predicted by earlier work in a diet-induced mouse model of obesity (18). In the mouse model, OVX sensitized the mice to HFD-induced obesity, adipose inflammation, and related molecular changes in the mammary glands. The fact that ovary intact mice were resistant to these effects led us to focus on the role of estrogen in the current study.

Initially, we determined whether supplemental estrogen protected against diet-induced obesity and associated histologic and molecular changes in the mammary glands of OVX mice. Supplemental E2 suppressed caloric consumption and weight gain. Moreover, HFD-induced adipocyte hypertrophy, white adipose inflammation, and increased levels of proinflammatory mediators and aromatase were suppressed by supplemental E2 in OVX mice. To complement this prevention study, we treated obese OVX mice with E2. Here, treatment with E2 led to weight loss with associated decreases in adipocyte size, white adipose inflammation, and levels of proinflammatory mediators and aromatase.

Although these effects of exogenous estrogen on the mammary glands were previously unknown, the findings are consistent with the known biologic effects of estrogen. More specifically, supplemental estrogen has been reported to suppress weight gain, protect against adipocyte hypertrophy, and attenuate adipose inflammation in other fat depots in mice (21, 26, 27). Our results are also consistent with findings in women where loss of ovarian function due to natural aging or surgical removal of the ovaries is associated with weight gain and increases in adipose tissue (20, 28). The observed anti-inflammatory effects of E2 in the mammary glands could be a consequence of its ability to inhibit weight gain or stimulate weight loss. However, estrogen is also known to possess anti-inflammatory activity. For example, estrogen can block lipopolysaccharide-mediated induction of proinflammatory pathways in macrophages (29, 30). NF-κB activation can be blocked by estrogen (30, 31). Hence, it is unclear if treatment with E2 led to improved white adipose inflammation exclusively due to reductions in weight or if there were additional anti-inflammatory effects. The fact that the findings in ERα knockout mice mirrored the effects of OVX mice reflected the effects of OVX is a clear indication that the effects of E2 were mediated by ERα. ERα can mediate its effects via the nucleus, the plasma membrane or both (32). Future studies are warranted to determine whether one or both of these functions is important for regulating energy balance and inflammation. Our results also raise the possibility of a feedback loop whereby loss of ovarian function leads to upregulation of aromatase. This increase in aromatase was blunted by treatment with exogenous E2. Given the significance of estrogen in mediating a multitude of important biologic effects, such a feedback loop...
could well exist. Energy affects energy balance via effects on both appetite and metabolism (33, 34). We showed that E2 suppressed caloric intake that helps to explain the observed changes in weight. This finding fits with prior reports that higher levels of estradiol during the menstrual cycle and pregnancy lead to decreased food intake and fat accumulation (35). In contrast, OVX and menopause led to increased food intake, an effect that is reversed by treatment with estradiol. Estradiol has anorexigenic functions in the brain (35). More specifically, E2 has direct actions in the hypothalamus that affect feeding behavior. In fact, silencing of ERα in the ventromedial nucleus of the hypothalamus of mice leads to obesity (36). Overall, our results suggest that E2 via ERα suppressed caloric intake leading to weight changes. Because estradiol can also affect energy expenditure leading to weight changes (33, 34), future studies are warranted to determine whether E2-mediated changes in metabolism contribute to the observed suppressive effects on adipose inflammation and related molecular changes in the mammary glands.

It is important to consider the possible clinical implications of this study. As mentioned above, we recently showed that meno-
apause is associated with both an increased incidence and severity of breast white adipose inflammation (19). The current preclinical results raise the possibility that supplemental estrogen will either protect against or reverse white adipose inflammation and related molecular changes in the breast and other adipose depots. Because estrogen stimulates endometrial hyperplasia, this type of question will be best addressed in women who have undergone a hysterectomy. Our results also support the goal of developing agents that target estrogen delivery to the brain as an approach to improving the therapeutic index. Such an agent might prove useful in modulating weight and secondarily breast adipose inflammation. In support of this possibility, a glucagon-like peptide-1-estrogen conjugate was recently reported to improve energy balance, glucose and lipid metabolism in the absence of the hallmark side effects of estrogen such as reproductive endocrine toxicity (37). A limitation of this study is that we did not study the effects of supplemental estrogen in lean mice. This was purposeful because the WAT is uninnflamed in lean mice. Hence, our preclinical findings are potentially relevant to the subset of women with occult adipose inflammation. Taken together, our data raise the intriguing possibility that supplemental estrogen will be beneficial in modulating white adipose inflammation, a process linked to insulin resistance, cardiovascular disease and possibly breast cancer (12, 38).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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