

Research Article

Caloric Restriction Reverses Obesity-Induced Mammary Gland Inflammation in MicePriya Bhardwaj¹, Baoheng Du¹, Xi Kathy Zhou², Erika Sue¹, Michael D. Harbus¹, Domenick J. Falcone³, Dilip Giri⁴, Clifford A. Hudis⁵, Levy Kopelovich⁶, Kotha Subbaramaiah¹, and Andrew J. Dannenberg¹**Abstract**

Obesity is a risk factor for the development of hormone receptor–positive breast cancer in postmenopausal women. Estrogen synthesis is catalyzed by aromatase. Recently, we identified an obesity→inflammation→aromatase axis in mouse models and women. In mouse models of obesity, inflammatory foci characterized by crown-like structures (CLS) consisting of dead adipocytes encircled by macrophages were found in the mammary gland. CLS of the breast were found in most overweight and obese women. CLS were associated with adipocyte hypertrophy, activation of NF-κB, elevated levels of proinflammatory mediators and aromatase, and increased expression of the progesterone receptor (PR). Collectively, these findings provide a plausible explanation for the link between obesity, chronic inflammation, and postmenopausal breast cancer. Here, we investigated whether caloric restriction (CR) reversed the inflammatory state and related molecular changes in the mammary gland of obese mice. Obese ovariectomized C57BL/6J mice were subjected to 30% CR for 7 or 14 weeks. Findings in CR mice were compared with the results in mice fed a high-fat diet *ad libitum* or with control mice fed a low-fat diet. CR was associated with more than a 75% decrease in mammary CLS/cm². Reduced histologic inflammation following CR was associated with decreased adipocyte diameter and monocyte chemoattractant protein-1 (MCP-1) levels, reduced NF-κB binding activity, and normalization of levels of proinflammatory mediators, aromatase, and PR. In summary, obesity-related inflammation of the mammary gland and elevated aromatase and PR levels were reversed with CR. Our results provide a rationale for determining whether weight loss can reverse breast inflammation associated with obesity in women. *Cancer Prev Res*; 6(4); 282–9. ©2013 AACR.

Introduction

Obesity is a risk factor for the development of hormone receptor (HR)-positive breast cancer in postmenopausal women (1, 2). The development and growth of HR-positive breast cancers are commonly regulated by estrogens. Estrogen synthesis is catalyzed by aromatase, which is encoded by *CYP19* (3). Following menopause, peripheral aromatization of androgen precursors in adipose tissue is the key synthetic source of estrogen. The increased risk of developing HR-positive breast cancer in obese postmenopausal women has been attributed, in part, to increased circulating

levels of estrogen related to both excess adipose tissue and elevated aromatase expression (1, 2, 4, 5). In addition to increasing the risk of breast cancer, obesity is associated with a poor prognosis among breast cancer survivors (6–12). Obesity-related effects on hormones, adipokines, and proinflammatory mediators have been suggested to contribute to the worse prognosis of obese patients (13).

Subclinical inflammation is commonly found in visceral and subcutaneous white adipose tissue of overweight and obese women (2, 13–16). Macrophages infiltrate white adipose tissue and form characteristic crown-like structures (CLS) around dead adipocytes (15–18). These macrophages produce proinflammatory mediators (16, 19–21). Monocyte chemoattractant protein-1 (MCP-1) plays a significant role in the recruitment of macrophages to adipose tissue (22). Recently, we showed in both experimental models of obesity and obese women that CLS also occur in the white adipose tissue of the mouse mammary gland and human breast (CLS-B), respectively (23, 24). Breast inflammation, as determined by CLS-B, was paralleled by increased NF-κB-binding activity and elevated levels of proinflammatory mediators and aromatase. We concluded that the obesity→inflammation→aromatase axis may contribute to the increased risk of HR-positive breast cancer in postmenopausal women and the generally worse prognosis

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of obese patients with breast cancer through its impact on estrogen production. This provides a plausible explanation for the paradoxical observation that the incidence of HR-positive breast cancer rises after menopause when circulating levels of estrogen generally decline.

Decreased calorie intake is a commonly recommended lifestyle change to reduce excess adiposity and its consequences. In experimental models, caloric restriction (CR) can prolong life, reduce tumor growth and metastases, and reverse endothelial dysfunction (25–30). In the current study, we investigated whether CR could reverse histologic inflammation and related molecular changes in the mammary gland of obese mice. Our results suggest that obesity-related inflammation in the mammary gland including activation of NF- κ B, elevated levels of proinflammatory mediators and aromatase can all be attenuated by CR. These findings strengthen the rationale for evaluating whether weight loss can reverse white adipose tissue inflammation in high-risk women, which could, in turn, reduce breast cancer risk. Finally, the current results suggest that it will be worthwhile to determine whether agents with CR-mimetic properties possess antiinflammatory activity.

Materials and Methods

Materials

Lowry protein assay kits, glucose-6-phosphate, glycerol, pepstatin, leupeptin, glucose-6-phosphate dehydrogenase, and rotenone were from Sigma. 1β -[3 H]-androstenedione and [γ - 32 P]ATP were from Perkin-Elmer Life Science. Electrophoretic mobility gel shift assay kits were from Promega. RNeasy 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 (RT-PCR) primers were synthesized by Sigma-Genosys.

Animal model

At 5 weeks of age, ovary intact and ovariectomized (OVX) C57BL/6J female mice (Jackson Laboratories) were randomized ($n = 9$ –14/group) to receive either low-fat (LF) or high-fat (HF) diets (Fig. 1). The low-fat (12450Bi) and high-fat (D12492i) diets contain 10 kcal% fat and 60 kcal% fat, respectively (Research Diets). The low-fat group was fed *ad libitum* for 24 weeks until sacrifice. All 4 high-fat fed groups received a minimum of 10 weeks of *ad libitum* food intake. One high-fat fed group was sacrificed after 10 weeks of *ad libitum* food intake. A second high-fat fed group was sacrificed after 24 weeks of *ad libitum* food intake. Two other high-fat fed groups received 10 weeks of high-fat feeding *ad libitum* before being 30% calorically restricted for 7 or 14 weeks, respectively. These 2 groups received 70% of the amount consumed by the high-fat group that was fed *ad libitum* for 24 weeks. To achieve this goal, *ad libitum* food intake of the high-fat group was measured daily, and on the next day, 70% of that amount was given to the 2 CR groups. Following sacrifice, mammary glands were snap frozen in liquid nitrogen and stored at -80°C for molecular analysis

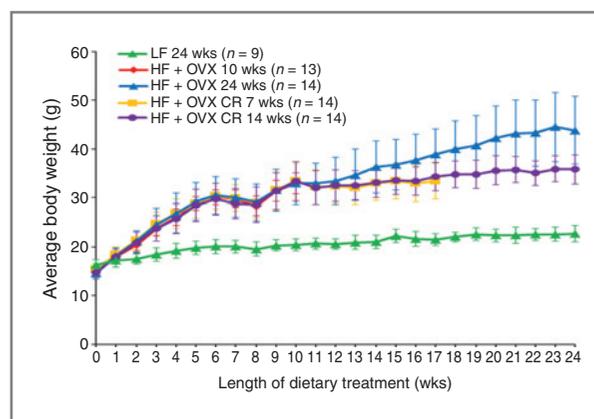


Figure 1. Caloric restriction suppresses weight gain. Body weight gain of mice in different treatment groups. The low-fat diet group was fed *ad libitum* for 24 weeks until sacrifice. All 4 high-fat fed groups received a minimum of 10 weeks of *ad libitum* food intake. One high-fat fed group was sacrificed after 10 weeks of *ad libitum* food intake. A second high-fat fed group was sacrificed after 24 weeks of *ad libitum* food intake. Two other high-fat fed groups received 10 weeks of high-fat feeding *ad libitum* before being 30% calorically restricted for 7 or 14 weeks, respectively. These 2 groups received 70% of the amount consumed by the high-fat group that was fed *ad libitum* for 24 weeks. The number of mice in each group varied as indicated from 9 to 14.

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 CLS per cm^2 of adipose tissue.

Adipocyte diameter

Adipocyte diameter was quantified as previously described (24).

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 reverse transcribed using murine leukemia virus reverse transcriptase and oligo (dT)₁₆ primer. The resulting cDNA was then used for amplification. With the exception of MCP-1, primer sequences have been reported previously (23). For MCP-1, the forward and reverse primers used were 5'-AGG-TCCCTGTCATGCTTCTG-3' and 5'-GCTGCTGGTGATCC-TCTTGT-3'. Glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous normalization control. RT-PCR was conducted using $2\times$ SYBR green PCR master mix on a 7500 Fast Real-time PCR system (Applied Biosystems). Relative fold induction was determined using the $\Delta\Delta C_t$ (relative quantification) analysis protocol.

Electrophoretic mobility shift assay

Nuclear extracts were prepared from mouse mammary glands using an EMSA kit (24). For binding studies, oligonucleotides containing NF- κ B sites (Active Motif) were used. The complementary oligonucleotides were annealed in 20 mmol/L Tris (pH 7.6), 50 mmol/L NaCl, 10 mmol/L MgCl₂, and 1 mmol/L dithiothreitol. The annealed oligonucleotide was phosphorylated at the 5'-end with [γ -³²P]ATP and T4 polynucleotide kinase. The binding reaction was conducted by incubating 5 μ g of nuclear protein in 20 mmol/L HEPES (pH 7.9), 10% glycerol, 300 μ g of bovine serum albumin, and 1 μ g of poly(dI-dC) in a final volume of 10 μ L for 10 minutes at 25°C. The labeled oligonucleotides were added to the reaction mixture and allowed to incubate for an additional 20 minutes at 25°C. The samples were electrophoresed on a 4% non-denaturing polyacrylamide gel. The gel was then dried and subjected to autoradiography at -80°C.

Aromatase activity

To determine aromatase activity, microsomes were prepared from tissues by differential centrifugation. Aromatase activity was quantified by measurement of the tritiated water released from 1 β -[³H]-androstenedione (31). Aromatase activity was normalized to protein concentration.

Statistics

For mouse weight at the baseline, the end of first period (10 weeks), and the end of study, differences across study groups were examined using ANOVA. Pairwise comparisons were carried out using Tukey method, which adjusts the *P* values for multiple comparisons by controlling the experiment-wise error rate. To examine mouse growth rates across study groups in each of the 2 study periods (i.e., the growth period and the intervention period), a linear mixed-effects model was used to fit the growth curves of the mice in each time period. This model takes into account between- and within-mice variation in baseline weights and growth rates. Difference in growth rates across experimental groups was examined using simultaneous tests for general linear hypotheses (32). *P* values were adjusted for multiple comparisons by controlling the false discovery rate (FDR). For the levels of various biomarkers, differences across experimental groups were examined using the nonparametric Kruskal-Wallis test. Wilcoxon rank-sum test was used to compare biomarker levels in experimental groups pairwise. *P* values were adjusted for multiple comparisons by controlling for FDR.

Results

Effects of CR on weight gain and inflammation

Initially, we investigated the effects of the different diets on the weights of mice. Treatment of ovary intact mice with a low-fat diet for 24 weeks led to a slow and gradual increase in weight (Fig. 1). To mimic the obese postmenopausal state, 4 groups of OVX mice were fed a high-fat diet for a minimum of 10 weeks. As shown in Fig. 1, a marked

increase in weight occurred in the high-fat OVX mice versus the low-fat ovary intact group. One group of high-fat OVX mice was sacrificed after 10 weeks of high-fat feeding for histologic and molecular analyses. Three other groups of high-fat OVX mice continued to be fed the high-fat diet. One group was fed *ad libitum* for an additional 14 weeks (24 weeks of high-fat feeding). This group of obese mice continued to gain weight throughout the study. The other 2 high-fat OVX groups received the high-fat diet for 10 weeks *ad libitum* followed by 30% CR (compared with mice that received the high-fat diet *ad libitum*) for either 7 or 14 weeks. In contrast to the high-fat OVX mice that received the high-fat diet *ad libitum* for 24 weeks, weight gain was suppressed in both CR groups (*P* < 0.001).

The histology of the mammary gland was assessed to quantify the severity of inflammation (Fig. 2). A typical CLS is shown in Fig. 2A. In comparison with the low-fat fed ovary intact mice, a significant increase in mammary gland inflammation (CLS/cm²) was found in the high-fat OVX mice following either 10 or 24 weeks of high-fat feeding (Fig. 2B). Notably, high-fat feeding for 10 weeks followed by CR for either 7 or 14 weeks was associated with significant decrease in the severity of mammary gland inflammation compared with the 2 high-fat fed groups. Obesity-induced adipocyte hypertrophy has been suggested to contribute to adipocyte death triggering CLS formation. Hence, adipocyte diameter was determined in each of the 5 dietary treatment groups. In comparison with the low-fat fed ovary intact mice, mammary gland adipocyte diameter was markedly increased following high-fat feeding for either 10 or 24 weeks (Fig. 2C). Following feeding a high-fat diet *ad libitum* for 10 weeks, CR for 7 or 14 weeks was associated with a small decrease in the diameter of adipocytes in the mammary gland. Because MCP-1 plays a role in the recruitment of macrophages to adipose tissue leading to CLS, levels in the mammary gland were quantified. As shown in Fig. 2D, feeding a high-fat diet to OVX mice for either 10 or 24 weeks was associated with increased levels of MCP-1, an effect that was reversed by CR for either 7 or 14 weeks.

Obesity-related induction of inflammatory mediators is attenuated by CR

Previously, activation of NF- κ B was found in the mammary glands of obese mice and the inflamed breast tissue of obese women (23, 24). Hence, we next evaluated the effects of CR on NF- κ B binding activity in the mammary gland. Feeding OVX mice a high-fat diet for either 10 or 24 weeks was associated with a marked increase in NF- κ B binding activity compared with low-fat diet fed mice (Fig. 3). Consistent with the marked improvement in histologic inflammation, CR for either 7 or 14 weeks led to a significant reduction in NF- κ B binding activity. We also quantified levels of several proinflammatory mediators [TNF- α , interleukin (IL)-1 β , and Cox-2] in the mammary gland (Fig. 4). The

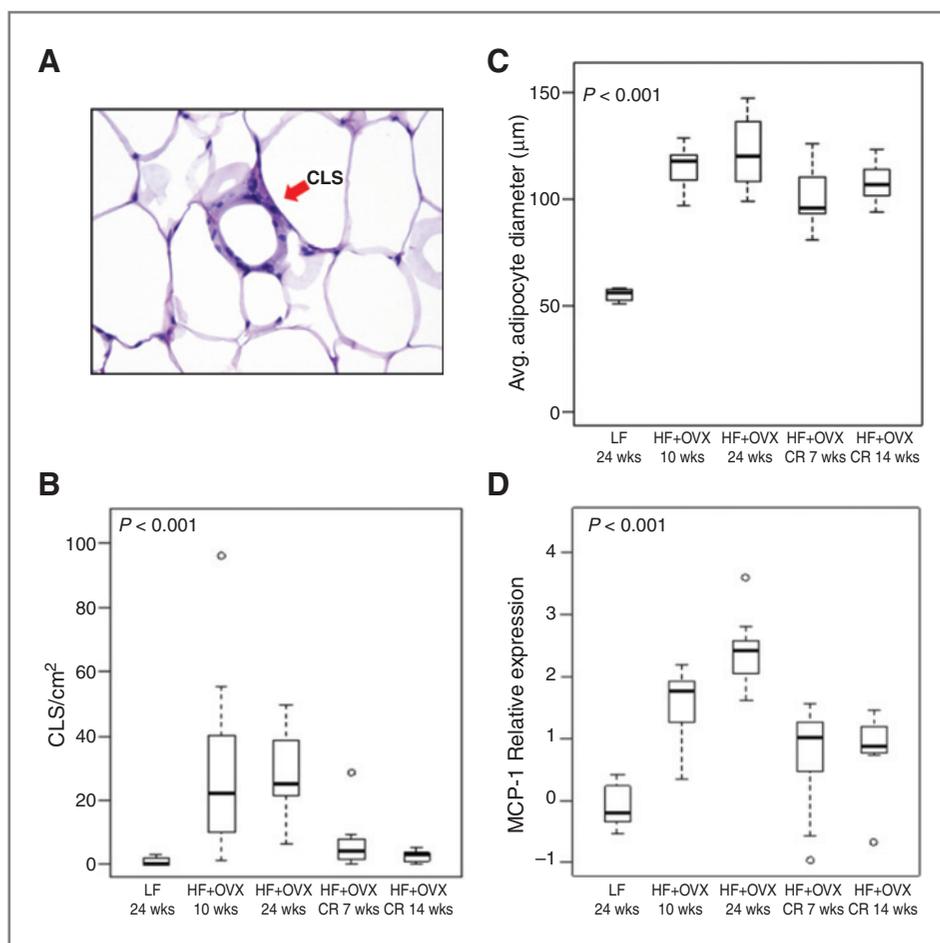


Figure 2. Mammary gland inflammation is reversed by caloric restriction. A, hematoxylin and eosin–stained section showing a CLS of the mammary gland. B, box-plot of the number of inflammatory foci per cm^2 in mammary glands of mice in the different treatment groups. Significant differences were observed across the 5 experimental groups ($P < 0.001$, Kruskal–Wallis test). In pairwise comparisons, CR for either 7 or 14 weeks led to a resolution of inflammation. Specifically, the number of CLS per cm^2 was statistically significantly decreased compared with that in mice with either 10 weeks of high-fat feeding ($P_{\text{adj}} = 0.02$ and 0.008 , respectively) or 24 weeks of high-fat feeding ($P_{\text{adj}} = 0.008$ and 0.003 , respectively). C, box-plot of average mammary gland adipocyte diameter in different groups. Significant differences were observed across the 5 experimental groups ($P < 0.001$, Kruskal–Wallis test). Specifically, mammary gland adipocytes in mice with either 10 or 24 weeks of high-fat feeding were significantly larger compared with mice with 24 weeks of low-fat feeding ($P_{\text{adj}} = 0.01$ and 0.01 , respectively). CR for 7 weeks led to small yet statistically significant decrease in mammary gland adipocyte diameter compared with mice with either 10 or 24 weeks of high-fat feeding ($P_{\text{adj}} = 0.03$ and 0.02 , respectively). D, box-plot of MCP-1. Significant differences were observed across the 5 experimental groups ($P < 0.001$, Kruskal–Wallis test). The high-fat +OVX 10 and 24 week groups had higher MCP-1 levels than the low-fat group ($P_{\text{adj}} = 0.01$). In pairwise comparisons, CR for 7 weeks was associated with significantly lower MCP-1 levels compared with mice in the high-fat+OVX 10 week group ($P_{\text{adj}} = 0.015$). Similarly, CR for 14 weeks was associated with significantly lower MCP-1 expression compared with mice in the high-fat+OVX 24 week group ($P_{\text{adj}} = 0.001$).

increased levels found after high-fat feeding for either 10 or 24 weeks were attenuated by CR.

Effect of CR on aromatase and PR levels

In comparison to low-fat fed ovary intact mice, increased levels of aromatase expression and activity were found in the mammary gland of OVX mice following high-fat feeding for 10 or 24 weeks (Fig. 5). CR reversed the inductive effects of the high-fat diet. Because aromatase is the rate-limiting enzyme for estrogen synthesis, we also quantified levels of *progesterone receptor (PR)*, a prototypic estrogen-inducible gene (Fig. 6). Consistent with the effects on aromatase levels, CR for either 7 or 14 weeks reversed the inductive effects of high-fat feeding on PR levels.

Discussion

Chronic inflammation of several tissues including the colon, liver, stomach, pancreas, and esophagus has been linked to an increased risk of cancer. We showed that subclinical inflammation manifested as CLS-B occurs in breast white adipose tissue of most overweight and obese women (24). CLS-B was associated with adipocyte hypertrophy, activation of NF- κ B, increased levels of proinflammatory mediators, elevated aromatase activity, and enhanced expression of PR (24, 33). Remarkably, each of these findings in human breast tissue was predicted by earlier work in a diet-induced mouse model of obesity (23). We concluded that the mouse model accurately predicts the human condition and that the

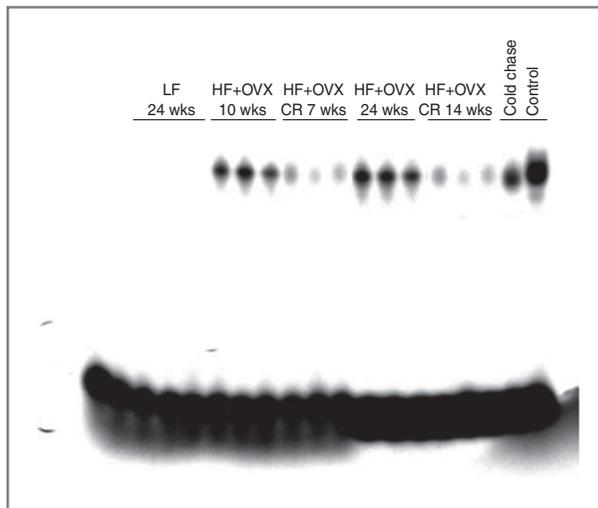


Figure 3. Obesity-related activation of NF- κ B in the mammary gland is attenuated by caloric restriction. Binding of nuclear protein from mammary glands of 15 mice (3 mice/group) in the indicated treatment groups. A total of 10 μ g of nuclear protein was incubated with a 32 P-labeled oligonucleotide containing NF- κ B binding sites. Control and cold chase, nuclear protein was incubated with labeled oligonucleotide in the absence (control) and presence (cold chase) of a 50 \times excess of cold probe. The protein-DNA complexes that formed were separated on a 4% polyacrylamide gel.

obesity \rightarrow inflammation \rightarrow aromatase axis was likely to contribute to the increased risk of HR-positive breast cancer in postmenopausal women and the worse prognosis of obese women with breast cancer.

Importantly, the availability of a mouse model, which mimics human disease, provides the opportunity to both explore the obesity \rightarrow inflammation \rightarrow aromatase axis and develop and test interventions with the goal of reversing

obesity-related inflammation of the mammary gland. In the current study, we replicated our initial findings by showing that diet-induced obesity was associated with histologic inflammation, hypertrophy of mammary adipocytes, increased NF- κ B binding activity, and elevated levels of proinflammatory mediators (TNF- α , IL-1 β , and Cox-2). Each of these proinflammatory mediators is a known inducer of aromatase (31, 34–39). Consistent with these effects, levels of aromatase mRNA, aromatase activity, and PR were all increased in the mammary glands of obese mice. In an effort to reverse this inflammatory process, we investigated the effects of CR in obese mice. Remarkably, 30% CR for either 7 or 14 weeks was associated with a marked improvement of inflammation and related molecular changes. Resolution of histologic inflammation was associated with significant reductions in NF- κ B binding activity, and reduced levels of proinflammatory mediators, aromatase, and PR. In obesity, secretion of MCP-1 by adipocytes triggers the recruitment of macrophages to adipose tissue (22). The fact that levels of MCP-1 were increased in the inflamed mammary white adipose tissue of obese mice is consistent with this concept. Moreover, the decrease in MCP-1 levels following CR provides further evidence that this factor is critical for the development of CLS because the decline was associated with reduced mammary gland inflammation.

CR prevented weight gain and led to a significant improvement in inflammation even though the decrease in mammary adipocyte size was very modest. Nonetheless, we cannot exclude the possibility that the small decrease in adipocyte size mediated by CR is important for the resolution of white adipose tissue inflammation. Previous studies have suggested that adipocyte hypertrophy predisposes to cell death and the formation of CLS (15). Hypertrophic

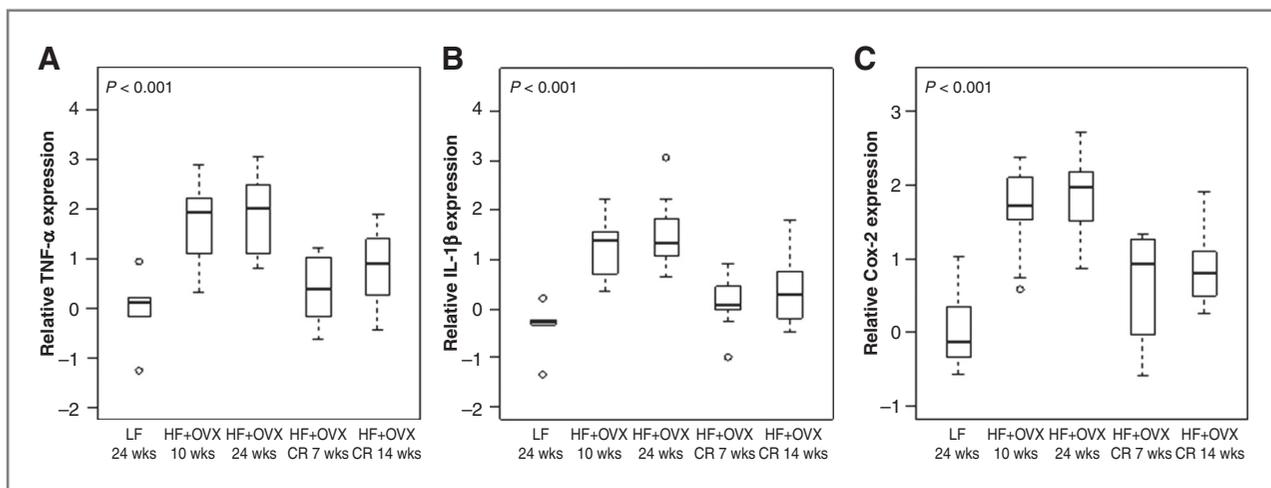


Figure 4. Obesity-related increased expression of proinflammatory mediators is reversed by CR. Real-time PCR was carried out on RNA isolated from the mammary glands ($n = 5$ –10/group) of mice in each of the 5 groups. Box-plots of TNF- α (A), IL-1 β (B), and Cox-2 (C) mRNA expression in mammary glands. Significant differences were observed across the 5 experimental groups for each proinflammatory mediator ($P < 0.001$). In pairwise comparisons, CR for 7 weeks was associated with significantly lower expression of the 3 genes in mammary gland compared with mice in the high-fat+OVX 10-week group ($P_{\text{adj}} = 0.02, 0.006, \text{ and } 0.02$, respectively). Similarly, CR for 14 weeks was associated with significantly lower expression of the 3 genes compared with mice in the high-fat+OVX 24 week group ($P_{\text{adj}} = 0.05, 0.01, \text{ and } 0.02$, respectively).

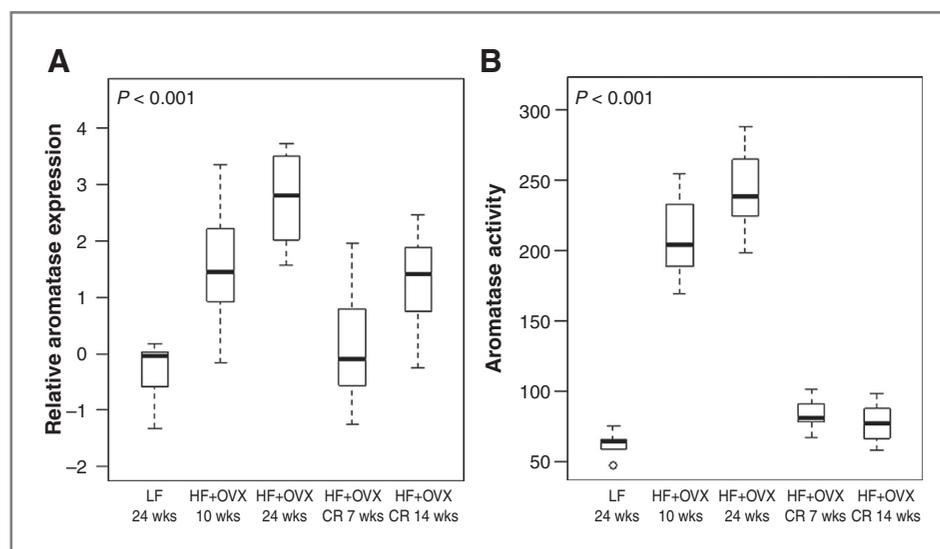


Figure 5. Caloric restriction reverses the elevated levels of aromatase found in the mammary glands of obese mice. Box-plots of relative aromatase mRNA levels (A) and activity (B) in mammary glands of mice in each of the 5 groups. Significant differences were observed across the 5 experimental groups for aromatase expression and activity ($P < 0.001$). In pairwise comparisons, caloric restriction for 7 weeks was associated with lower levels of aromatase mRNA ($P_{\text{adj}} = 0.02$) and activity (femtomoles/ μg protein/hour; $P_{\text{adj}} < 0.001$) compared with the high-fat+OVX 10-week group. CR for 14 weeks was associated with significantly lower expression of aromatase mRNA ($P_{\text{adj}} = 0.01$) and activity ($P_{\text{adj}} < 0.001$) compared with mice in the high-fat+OVX 24-week group.

adipocytes are subjected to multiple cytotoxic stressors including hypoxia, endoplasmic reticulum stress, reactive oxygen species, and free fatty acids (15, 40–42). Stress-related mechanisms of adipocyte death may be augmented

by macrophage-derived proinflammatory mediators including TNF- α . Obesity-related microcirculatory defects could also contribute to the death of hypertrophic adipocytes. In support of this possibility, treatment with a prostacyclin analogue that has potent vasodilating effects protected against CLS formation in high-fat diet-induced obese mice (43). On the basis of this constellation of findings, we posit that adipocytes can expand in response to energy excess until a threshold is exceeded leading to cell death and inflammation. It is possible, therefore, that CR led to a small, but physiologically significant, decrease in adipocyte size that was sufficient to protect against cell death.

Previous studies of obese humans have shown that both diet and surgically induced weight loss lead to reduced macrophage infiltration and decreased proinflammatory gene expression in subcutaneous white adipose tissue (14, 44). In addition, CR-mediated weight loss with or without exercise reduced biomarkers of inflammation in postmenopausal women (45). Although none of the previous human studies evaluated breast tissue, our findings in the murine mammary gland are certainly consistent with the results of these prior studies. The inclusion of biomarkers of inflammation such as CLS is likely to be helpful for selecting overweight or obese patients with breast cancer who are most likely to benefit from weight loss interventions. At the same time, the results of the current study raise other significant questions. It is uncertain, for example, whether endogenous or exogenous factors that suppress appetite and calorie intake will have the same beneficial effects as CR. For example, estrogen can suppress both calorie intake and inflammation (46). On the basis of our CR findings, it will be worthwhile to determine whether exogenous estrogen or other pharmacologic interventions can suppress calorie consumption and reverse the

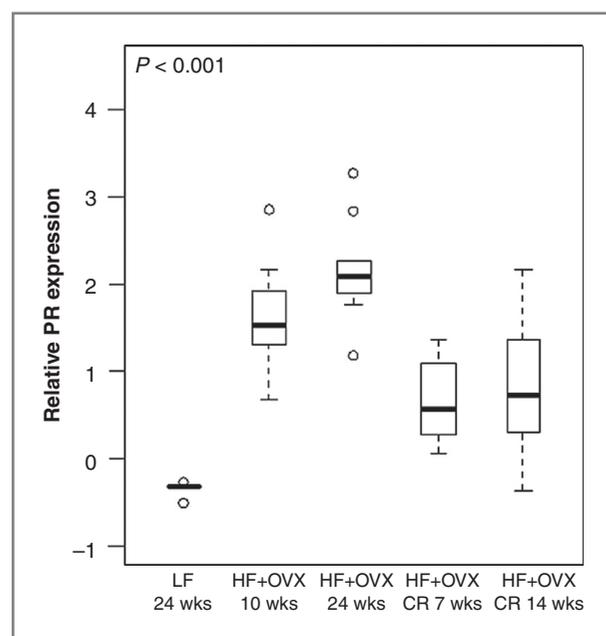


Figure 6. Obesity-related increased levels of PR in the mammary gland are reversed by caloric restriction. Box-plots of relative PR mRNA levels are shown. Significant differences were observed across the 5 experimental groups for relative PR levels ($P < 0.001$). In pairwise comparisons, CR for 7 weeks was associated with significantly lower PR levels compared with mice in the high-fat+OVX 10-week group ($P_{\text{adj}} = 0.008$). Similarly, CR for 14 weeks was associated with significantly lower PR expression compared with mice in the high-fat+OVX 24-week group ($P_{\text{adj}} = 0.007$).

obesity→inflammation axis in the mammary gland of oophorectomized mice. Ultimately, strategies that combine modest weight loss, exercise, and use of agents that mimic some of the biologic effects of CR may provide a path forward for mitigating some of the harmful effects of obesity (47).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: P. Bhardwaj, E. Sue, L. Kopelovich, K. Subbaramaiah, A.J. Dannenberg

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Bhardwaj, B. Du, E. Sue, M.D. Harbus, D.J. Falcone, D. Giri, K. Subbaramaiah, A.J. Dannenberg

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