Caloric Restriction Reverses Obesity-Induced Mammary Gland Inflammation in Mice

Priya Bhardwaj, Baoheng Du, Xi Kathy Zhou, Erika Sue, Michael D. Harbus, Domenick J. Falcone, Dilip Giri, Clifford A. Hudis, Levy Kopelowich, Kotha Subbaramaiah, and Andrew J. Dannenberg

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-kB, 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-kB 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; 1–8. ©2013 AACR.
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-kB, 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, peptatin, leupeptin, glucose-6-phosphate dehydrogenase, and rotenone were from Sigma. Iβ-[3H]-androstenedione and [γ-32P]ATP were from Perkin-Elmer Life Science. Electrophoretic mobility gel shift assay kits were from Promega. 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 (RT-PCR) primers were synthesized by Sigma-Genosys.

Animal model
At 5 weeks of age, ovary intact and ovariectomized (OVX) C57BL/6 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. The number of mice in each group varied as indicated from 9 to 14.

Relative fold induction was determined using the校验 quantification) analysis protocol. 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-TCCCTGTATGCTTG-3' and 5'-GCCCTGCTGGTGATCC-3'. Glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous normalization control. RT-PCR was conducted using 2× SYBR green PCR master mix on a CFX96 Real-Time System (BioRad) for 24 weeks. The number of mice in each group varied as indicated from 9 to 14.

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.

Materials and Methods

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

Animal model
At 5 weeks of age, ovary intact and ovariectomized (OVX) C57BL/6 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. The number of mice in each group varied as indicated from 9 to 14.

Relative fold induction was determined using the校验 quantification) analysis protocol. 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-TCCCTGTATGCTTG-3' and 5'-GCCCTGCTGGTGATCC-3'. Glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous normalization control. RT-PCR was conducted using 2× SYBR green PCR master mix on a CFX96 Real-Time System (BioRad) for 24 weeks. The number of mice in each group varied as indicated from 9 to 14.

Materials and Methods

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

Animal model
At 5 weeks of age, ovary intact and ovariectomized (OVX) C57BL/6 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. The number of mice in each group varied as indicated from 9 to 14.

Relative fold induction was determined using the校验 quantification) analysis protocol. 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-TCCCTGTATGCTTG-3' and 5'-GCCCTGCTGGTGATCC-3'. Glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous normalization control. RT-PCR was conducted using 2× SYBR green PCR master mix on a CFX96 Real-Time System (BioRad) for 24 weeks. The number of mice in each group varied as indicated from 9 to 14.
Electrophoretic mobility shift assay

Nuclear extracts were prepared from mouse mammary glands using an EMSA kit (24). For binding studies, oligonucleotides containing NF-κB 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 poly nucleotide kinase. The binding reaction was conducted by incubating 5 μg of nuclear protein in 20 mmol/L HEPES (pH 7.9), 10% glycerol, 300 μg of bovine serum albumin, and 1 μg of poly(dl-dC) in a final volume of 10 μL 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-[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 each 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, repeated measures ANOVA was performed to determine differences 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-κB 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-κB 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-κB 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-κB binding activity. We also quantified levels of several proinflammatory mediators (TNF-α, interleukin (IL)-1β, and Cox-2) in the mammary gland (Fig. 4). The
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-κB, 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...
obesity—inflammation—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—inflammation—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-κB binding activity, and elevated levels of proinflammatory mediators (TNF-α, IL-1β, 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-κB 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 3. Obesity-related activation of NF-κB 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 μg of nuclear protein was incubated with a 32P-labeled oligonucleotide containing NF-κB binding sites. Control and cold chase, nuclear protein was incubated with labeled oligonucleotide in the absence (control) and presence (cold chase) of a 50× excess of cold probe. The protein–DNA complexes that formed were separated on a 4% polyacrylamide gel.

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-1β (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 (Padj = 0.02, 0.006, 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 (Padj = 0.05, 0.01, and 0.02, respectively).
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
Discrimination of Obesity and Breast Cancer: The Estrogen Connection

Introduction

Obesity—Inflammation Axis in the Mammary Gland of Oophorectomized Mice

Methods

Results

Discussion

Conclusion

Acknowledgments

References

Appendix

Author Contributions

Disclosure of Potential Conflicts of Interest

Grant Support

Received November 27, 2012; accepted February 4, 2013; published OnlineFirst February 19, 2013.
36. Zhao Y, Agarwal VR, Mendelson CR, Simpson ER. Estrogen biosynthesis proximal to a breast tumor is stimulated by PGE2 via cyclic AMP, leading to activation of promoter II of the CYP19 (aromatase) gene. Endocrinology 1996;137:5739–42.
Caloric Restriction Reverses Obesity-Induced Mammary Gland Inflammation in Mice

Priya Bhardwaj, Baoheng Du, Xi Kathy Zhou, et al.

*Cancer Prev Res* Published OnlineFirst February 19, 2013.