Increased Levels of COX-2 and Prostaglandin E₂ Contribute to Elevated Aromatase Expression in Inflamed Breast Tissue of Obese Women

Kotha Subbaramaiah¹, Patrick G. Morris², Xi Kathy Zhou³, Monica Morrow⁴, Baoheng Du¹, Dilip Giri⁵, Levy Kopelovich⁶, Clifford A. Hudis¹², and Andrew J. Dannenberg¹

ABSTRACT

Obesity is a risk factor for hormone receptor-positive breast cancer in postmenopausal women. Estrogen synthesis is catalyzed by aromatase, which is encoded by CYP19. We previously showed that aromatase expression and activity are increased in the breast tissue of overweight and obese women in the presence of characteristic inflammatory foci (crown-like structures of the breast [CLS-B]). In preclinical studies, proinflammatory prostaglandin E₂ (PGE₂) is a determinant of aromatase expression. We provide evidence that cyclooxygenase (COX)-2–derived PGE₂ stimulates the cyclic AMP (cAMP)→PKA signal transduction pathway that activates CYP19 transcription, resulting in increased aromatase expression and elevated progesterone receptor levels in breast tissues from overweight and obese women. We further demonstrate that a measure of in-breast inflammation (CLS-B index) is a better correlate of these biologic end points than body mass index. The obesity→inflammation→aromatase axis is likely to contribute to the increased risk of hormone receptor-positive breast cancer and the worse prognosis of obese patients with breast cancer.

SIGNIFICANCE: We show that obesity-associated inflammatory foci in the human breast are associated with elevated COX-2 levels and activation of the PGE₂→cAMP→PKA signal transduction pathway resulting in increased aromatase expression. These findings help to explain the link among obesity, low-grade chronic inflammation, and breast cancer with important clinical implications. Cancer Discov; 2(4): 356–65. © 2012 AACR.
INTRODUCTION

Obesity is a risk factor for the development and progression of hormone receptor (HR)-positive breast cancer in postmenopausal women (1, 2). The formation and growth of HR-positive breast cancers is regulated by estrogens. The rate-limiting enzyme for the synthesis of estrogen is aromatase, a member of the P450 family of enzymes. Aromatase is encoded by CYP19 and catalyzes the conversion of androgens to estrogens (3). Aromatase expression is regulated in a complex manner. The upstream portion of the CYP19 gene contains multiple tissue-specific promoters that direct transcription of alternative first exons resulting in aromatase mRNA species with unique 5′-untranslated first exons (3). The downstream region of the gene is comprised of 9 coding exons. Each promoter-specific mRNA gives rise to the same aromatase enzyme. Distinct signaling pathways regulate each promoter. In normal breast tissue, aromatase mRNA is derived in large part from promoter I.4, but, in breast cancer, promoters I.3, II, and I.7 are activated, leading to a marked increase in aromatase expression and enhanced estrogen production (4). Quantification of aromatase transcripts derived from specific promoters can provide insight into the pathways that control CYP19/aromatase gene transcription in obese women.

Subclinical inflammation is commonly found in visceral and subcutaneous white adipose tissue of overweight and obese women (2, 5–8). Macrophages infiltrate white adipose tissue and form characteristic crown-like structures (CLS) around necrotic adipocytes (6, 7, 9, 10). These macrophages also produce proinflammatory mediators (7, 11–13). Recently, we showed both in experimental models of obesity and in obese women that CLS also occur in the white adipose tissue of the mammary gland and breast (CLS-B), respectively (14, 15). Breast inflammation, as determined by CLS-B, was associated with increased NF-κB binding activity and elevated levels of aromatase mRNA and aromatase activity.

Numerous studies have attempted to elucidate the mechanisms by which CYP19 expression is regulated (3). Several findings suggest a significant role for cyclooxygenase (COX)-derived prostaglandin E2 (PGE2) in stimulating CYP19 transcription leading to increased aromatase activity. PGE2 stimulates the cyclic AMP (cAMP)→protein kinase A (PKA) signal transduction pathway resulting in coordinated activation of promoters I.3 and II and enhanced CYP19 transcription in vitro (16–18). In mice that express a mammary-targeted COX2 transgene, increased PGE2 and aromatase levels were observed (19). A positive correlation was found between levels of COX and aromatase in human breast cancer specimens (20, 21). Use of non-steroidal anti-inflammatory drugs, prototypic inhibitors of COX-derived PGE2 synthesis, has also been associated with reduced circulating estradiol levels (22). Finally, some studies have shown that use of aspirin, a COX inhibitor, is associated with a reduced risk of HR-positive but not HR-negative breast cancer (23, 24).

In the current study, we had 3 main objectives. First, we investigated whether activation of the COX-2→PGE2→cAMP →PKA signal transduction pathway occurs in inflamed human breast tissue. Second, we investigated whether activation of this pathway may account for increased levels of aromatase in the breast tissue of overweight and obese women. Third, we determined whether the CLS-B index, a measure of breast inflammation, was a better correlate of several biologic end points than measurements of body mass index (BMI). Collectively, our results suggest that the obesity→inflammation→aromatase axis contributes to the increased risk of HR-positive breast cancer in postmenopausal women and the worse prognosis of obese patients with breast cancer. Our findings also help to explain why aromatase inhibitors reduce the risk of breast cancer in overweight or obese women (25).

RESULTS

Levels of COX-2 and PGE2 Are Increased in Inflamed Breast Tissue and Correlate With Aromatase Levels and Activity

As previously described, 30 women of median age 50 years (range, 26–70 years) and median BMI 26.3 kg/m2 (range, 19.3–45.6 kg/m2) were enrolled (15). Two had ipsilateral invasive cancer; the remaining 28 did not have invasive cancer (Table 1). CLS-B were found in 47% (14 of 30) of cases (Fig. 1A, 1B). Increased levels of aromatase mRNA (Fig. 1C) and activity (Fig. 1D) were found in inflamed breast tissue. Increased levels of aromatase mRNA and activity were also present in breast tissue from overweight/obese women versus normal weight women but only the difference in aromatase activity achieved statistical significance (Fig. 1C, 1D). Levels of COX-2 and PGE2 were also quantified in breast tissue. Levels of COX-2 mRNA (Fig. 2A), COX-2 protein (Fig. 2B), and PGE2 (Fig. 2C) were increased in inflamed breast tissue. The correlations for both COX-2 and PGE2 were stronger with the CLS-B index than with BMI (Table 2). Consistent with the known catalytic activity of COX-2, a positive correlation was also observed between levels of COX-2 protein and PGE2 (Table 2). Levels of COX-2 and PGE2 also correlated with both aromatase expression and activity (Table 2).

cAMP Levels and PKA Activity Are Increased in Inflamed Breast Tissue and Correlate With Levels of PGE2 and Aromatase

Preclinical studies using adipose stromal cells suggest that PGE2 can stimulate the cAMP→PKA pathway leading to increased CYP19 transcription and elevated levels of aromatase. Hence, we next quantified levels of cAMP in breast tissue. Levels of cAMP were increased in association with elevated BMI and breast inflammation (CLS-B−) (Fig. 3A). Once again, the positive correlation was stronger for breast inflammation (p = 0.83, P < 0.001) than BMI (p = 0.41, P = 0.03). Interestingly, a strong correlation was also observed between PGE2 and cAMP levels (Table 2). Levels of cAMP also correlated with levels of both aromatase mRNA and aromatase activity (Table 2). Similar to the findings for PGE2 and cAMP, PKA activity was higher in inflamed breast...
tissue (Fig. 3B). Strong correlations were found between levels of PKA activity and levels of PGE$_2$, cAMP, and aromatase activity (Table 2).

**Aromatase mRNA Expression Derived From Promoters I.3 and II and Progesterone Receptor Levels Correlate With Elevated Levels of COX-2 and PGE$_2$**

Exogenous PGE$_2$ can stimulate CYP19 transcription through promoters I.3 and II resulting in increased aromatase transcripts and aromatase activity. We therefore investigated use of CYP19/aromatase promoters I.3 and II in breast tissue. Levels of promoter I.3 and promoter II-derived aromatase transcripts were increased in inflamed breast tissue (CLS-B$^+$ vs. CLS-B$^-$) and correlated with the CLS-B index (Fig. 4A, 4B; Table 2). Increased levels of promoter I.3 and promoter II-derived aromatase transcripts were also observed in breast tissue from overweight/obese versus normal weight subjects, but the significant differences were less pronounced than found in inflamed versus uninflamed breast tissue (Fig. 4A, 4B). Levels of aromatase transcripts arising from promoters I.3 and II also correlated with levels of COX-2, PGE$_2$, cAMP, PKA activity, and aromatase activity (Table 2).

Because aromatase is a rate-limiting enzyme for estrogen biosynthesis, we also quantified levels of the progesterone receptor (PR), a prototypic estrogen-inducible gene that may play a role in inflammation (26, 27). Levels of PR were increased in the breast tissue of overweight/obese women and in inflamed breast tissue (Fig. 4C). Here, too, the correlation for PR was stronger with the CLS-B index than with BMI (Table 2). A positive correlation was also observed between each component of the COX-2→PGE$_2$→cAMP→PKA→aromatase signal transduction pathway and PR levels (Table 2).

**COX-2–Derived PGE$_2$ Regulates the cAMP→PKA→Aromatase Axis**

Obesity is associated with increased lipolysis. CLS-B are comprised of macrophages surrounding a necrotic adipocyte. Previously, we showed that saturated fatty acids activate macrophages leading to increased production of proinflammatory mediators that then induce aromatase in preadipocytes through a paracrine mechanism. Although COX-2–derived PGE$_2$ was causally linked to the induction of aromatase, the underlying mechanism was not completely defined. Hence, we carried out a series of in vitro experiments to further elucidate the mechanism by which PGE$_2$ induced aromatase. Treatment of THP-1 cells, a human macrophage cell line, with stearic acid led to a severalfold increase in PGE$_2$ production, an effect that was abrogated by silencing COX-2 (Fig. 5A). Conditioned medium (CM) from stearic acid-treated THP-1 cells induced cAMP, PKA activity, aromatase mRNA, and aromatase activity in preadipocytes (Fig. 5B–E). Each of these stimulatory effects of CM was attenuated by silencing COX-2 in THP-1 cells. Treatment with

### Table 1. Characteristics of patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Overall (n = 30)</th>
<th>No evidence of CLS-B (n = 16)</th>
<th>CLS-B demonstrated (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>50 (26–70)</td>
<td>48.5 (26–70)</td>
<td>51.5 (38–68)</td>
</tr>
<tr>
<td>Menopausal status, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premenopausal</td>
<td>16</td>
<td>9 (56)</td>
<td>7 (44)</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>14</td>
<td>7 (50)</td>
<td>7 (50)</td>
</tr>
<tr>
<td>Body mass index (kg/m$^2$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>26.3 (19.3–45.6)</td>
<td>22.5 (19.3–35.7)</td>
<td>29.2 (22.1–45.6)</td>
</tr>
<tr>
<td>BRCA status, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Known mutation</td>
<td>7</td>
<td>5 (71)</td>
<td>2 (29)</td>
</tr>
<tr>
<td>No known mutation</td>
<td>9</td>
<td>6 (67)</td>
<td>3 (33)</td>
</tr>
<tr>
<td>Unknown</td>
<td>14</td>
<td>5 (36)</td>
<td>9 (64)</td>
</tr>
<tr>
<td>Breast surgery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ipsilateral breast cancer</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Contralateral breast cancer</td>
<td>14</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Carcinoma in situ</td>
<td>12</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>No breast cancer history</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: CLS-B, crown-like structures of the breast.
Figure 1. Increased levels of aromatase are found in inflamed breast tissue. Crown-like structure of the breast (CLS-B) is shown in A and B. A, hematoxylin and eosin demonstrating an inflammatory focus containing macrophages surrounding an adipocyte (×200). B, immunohistochemical stain of CD68 showing the same lesion (×200). In A and B, arrows indicate CLS-B. Levels of aromatase mRNA (C) and activity (D) are increased in inflamed breast tissue (CLS-B⁺ vs. CLS-B⁻). Box plots are shown for subjects (n = 30) varying in weight and breast inflammation status.

DISCUSSION

In this study, we made several observations that help to explain the link among obesity, inflammation, and breast cancer. The regulation of CYP19 transcription is complex (3, 28). In cell models, COX-2–derived PGE₂ induces CYP19 transcription resulting in increased aromatase activity (19, 29, 30). In preclinical models of obesity, COX-2 was overexpressed in inflamed mammary tissue in association with elevated levels of PGE₂ and aromatase (14). Consistent with these previous findings, we found higher levels of COX-2 and PGE₂ in the breast tissue of obese versus lean women. Importantly, levels of COX-2 and PGE₂ correlated with the severity of inflammation, aromatase expression, and activity. Taken together, these findings suggest that PGE₂ may play a significant role in mediating the induction of aromatase in inflamed breast tissue. Because several in vitro studies have suggested that PGE₂ stimulates the cAMP→PKA pathway leading to enhanced CYP19 transcription (16, 19, 30), both cAMP levels and PKA activity were measured. Levels of both cAMP and PKA activity were higher in inflamed breast tissue. Similar to the findings for COX-2 and PGE₂, cAMP levels and PKA activity correlated with aromatase expression and activity. These data strongly support the possibility that obesity-associated breast inflammation leads to upregulation of COX-2 and activation of the PGE₂→cAMP→PKA pathway resulting in enhanced CYP19 transcription and increased aromatase activity. Based on our preclinical findings (14), it is likely that the upregulation of COX-2 and increased PGE₂ synthesis occur in the stromal vascular fraction of the inflamed breast that is enriched in macrophages.

Obesity is associated with increased lipolysis and saturated fatty acids possess proinflammatory effects (31, 32).
Figure 2. Increased levels of COX-2 mRNA, COX-2 protein, and PGE₂ are found in inflamed breast tissue. Levels of COX-2 mRNA (A), COX-2 protein (B), and PGE₂ (C) were compared in breast tissue of normal weight versus overweight/obese women and in breast tissue with or without evidence of inflammation (CLS-B⁻ vs. CLS-B⁺). A, quantitative real-time PCR was used to determine COX-2 expression. B, immunoprecipitates were subjected to Western blot analysis. Representative results (top of panel) for COX-2 and β-actin are shown versus the CLS-B index for 17 cases. Densitometry (bottom panel) was used to quantify COX-2 protein. C, PGE₂ was quantified by enzyme immunoassay. A–C, box plots are shown for subjects (n = 30) varying in weight and breast inflammation status.

We therefore carried out in vitro studies and demonstrated that COX-2-derived PGE₂ from stearic acid-activated macrophages stimulated the cAMP→PKA pathway resulting in enhanced CYP19 transcription and increased aromatase activity in preadipocytes. Future studies are warranted to elucidate the mechanisms by which COX-2 is induced in the breast tissue of obese women. Possibly, levels of proinflammatory cytokines, known inducers of COX-2, will also be increased in inflamed breast tissue. This seems likely because NF-κB is activated in inflamed breast tissue (14, 15). We also note that proinflammatory mediators in addition to PGE₂, including TNF-α, interleukin (IL)-1β, and IL-6 could play a role in mediating the induction of aromatase (4, 27, 33, 34). Although this study highlights the relationship between breast inflammation, COX-2, and elevated levels of aromatase, we acknowledge that inflammation is likely to contribute to breast carcinogenesis by a variety of additional mechanisms.

In cultured cells, PGE₂ stimulates CYP19 transcription through promoters I3 and II (28). Consequently, we evaluated the use of these 2 CYP19 promoters in breast tissue that varied in severity of inflammation. Levels of aromatase transcripts derived from each of these promoters correlated with the CLS-B index. Importantly, levels of transcripts from both promoters correlated with levels of COX-2, PGE₂, cAMP, and PKA activity. Collectively, these findings suggest that obesity-related breast inflammation is associated with elevated COX-2 expression, which leads to activation of the PGE₂→cAMP→PKA pathway resulting in elevated CYP19 transcription. The fact that levels of PR, an estrogen-inducible gene, correlated with aromatase levels suggests that the observed effects were of functional importance. Although COX-2 is known to be overexpressed in a subset of human breast cancers (35, 36), to our knowledge, this study is the first to suggest that obesity-associated breast inflammation is associated with elevated COX-2 levels. Importantly, PGE₂, cAMP, and PKA can modulate numerous aromatase-independent processes, which may also contribute to the increased risk of breast carcinogenesis in obese women (37).

Whether the use of nonsteroidal anti-inflammatory drugs (NSAIDs), including aspirin and selective COX-2 inhibitors, in reducing the risk of breast cancer varies according to BMI and the CLS-B index should be determined. Use of either traditional NSAIDs or selective COX-2 inhibitors has been associated with toxicity (38). Therefore, in the absence of prospectively collected clinical data demonstrating that aspirin-like molecules reduce the risk of breast cancer, the role of these agents in reducing the risk of breast cancer remains uncertain.

Obesity has a range of clinical consequences. For example, not all obese individuals have metabolic syndrome. We previously found that breast inflammation manifested as CLS-B occurred in most but not all overweight and obese women (15). We showed that levels of COX-2, PGE₂, cAMP, and PKA activity correlated more strongly with the CLS-B index (severity of breast inflammation) than with BMI. Aromatase levels and activity also correlate more strongly with the CLS-B index than with BMI (15). These results imply that obesity-related breast inflammation, as indicated by the presence of CLS-B,
Table 2. Correlation matrix of mediators that regulate aromatase expression

<table>
<thead>
<tr>
<th></th>
<th>COX-2 mRNA</th>
<th>COX-2 protein</th>
<th>PGE$_2$ level</th>
<th>cAMP level</th>
<th>PKA activity</th>
<th>Aromatase expression</th>
<th>Aromatase PI.3</th>
<th>Aromatase PI</th>
<th>Aromatase activity</th>
<th>PR protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>0.43*</td>
<td>0.36†</td>
<td>0.50*</td>
<td>0.41*</td>
<td>0.37*</td>
<td>0.42*</td>
<td>0.28‡</td>
<td>0.35*</td>
<td>0.50*</td>
<td>0.54*</td>
</tr>
<tr>
<td>CLS-B index</td>
<td>0.60</td>
<td>0.83</td>
<td>0.85</td>
<td>0.83</td>
<td>0.79</td>
<td>0.75</td>
<td>0.66</td>
<td>0.71</td>
<td>0.88</td>
<td>0.91</td>
</tr>
<tr>
<td>COX-2 protein</td>
<td>0.69</td>
<td>—</td>
<td>0.85</td>
<td>0.83</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PGE$_2$ level</td>
<td>0.62</td>
<td>0.83</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>cAMP level</td>
<td>0.64</td>
<td>0.89</td>
<td>0.96</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PKA activity</td>
<td>0.63</td>
<td>0.82</td>
<td>0.94</td>
<td>0.90</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Aromatase expression</td>
<td>0.60</td>
<td>0.62</td>
<td>0.74</td>
<td>0.62</td>
<td>0.65</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Aromatase PI.3</td>
<td>0.42*</td>
<td>0.57*</td>
<td>0.54*</td>
<td>0.51*</td>
<td>0.51*</td>
<td>0.58*</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Aromatase PI</td>
<td>0.45*</td>
<td>0.62</td>
<td>0.59</td>
<td>0.60</td>
<td>0.61</td>
<td>0.73</td>
<td>0.87</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Aromatase activity</td>
<td>0.57*</td>
<td>0.75</td>
<td>0.82</td>
<td>0.85</td>
<td>0.76</td>
<td>0.60</td>
<td>0.63</td>
<td>0.64</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PR protein</td>
<td>0.41*</td>
<td>0.71</td>
<td>0.78</td>
<td>0.73</td>
<td>0.73</td>
<td>0.60</td>
<td>0.52†</td>
<td>0.55*</td>
<td>0.73</td>
<td>1.0</td>
</tr>
</tbody>
</table>

NOTE: Each number represents the Spearman’s rank correlation coefficient of the levels of the 2 corresponding mediators in the study population. Most of the correlation coefficients are statistically significantly different from 0 (P < 0.001, *P < 0.01, †P < 0.05, ‡P ≥ 0.05).

Abbreviations: BMI, body mass index; cAMP, cyclic AMP; CLS-B, crown-like structures of the breast; COX, cyclooxygenase; PKA, protein kinase A; PR, progesterone receptor.

is critical for the induction of aromatase, whereas obesity alone may not be. In future studies that aim to elucidate the link between obesity and breast cancer or its treatment, measurements of CLS-B should be carried out. Possibly, measurements of CLS-B will provide prognostic information that is superior to simple measurements of BMI.

Overexpression of aromatase in the murine mammary gland stimulates carcinogenesis (39). Our studies are the first to show that obesity-mediated breast inflammation is associated with elevated aromatase activity, which may drive estrogen synthesis thereby increasing the risk of postmenopausal HR-positive breast cancer. In the first series of adjuvant trials testing aromatase inhibitors, reduced rates of second breast cancers were observed. Recently, Exemestane, an aromatase inhibitor, was shown to reduce the risk of breast cancer in postmenopausal women without histories of breast cancer. Importantly, the aromatase inhibitor appeared to be effective even in women who were overweight or obese (25). The discovery of the obesity→inflammation connection provides a mechanistic rationale for developing additional risk reduction strategies. Studies are underway with the goal of developing dietary, behavioral (weight loss, exercise), or pharmacologic strategies to disrupt the inflammatory process and thereby reduce the risk of breast cancer or improve outcomes in breast cancer survivors.

METHODS

Cell Culture and Reagents

Human visceral preadipocytes (ScienCell) were grown in preadipocyte medium containing 10% fetal bovine serum (FBS). THP-1 cells were obtained from American Type Culture Collection and 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 induce macrophage differentiation. Human monocytes (Astarte Biologics) were activated with IFN-γ (15 ng/ml) and lipopolysaccharide (15 ng/ml) in RPMI-1640 medium for 4 days. To prepare CM, THP-1 cells or blood monocyte-derived macrophages were treated with vehicle or 10 μM stearic acid for 12 hours in medium comprised of RPMI-1640 and preadipocyte medium at a 1:1 ratio. The medium was then removed and cells were washed twice with phosphate-buffered saline. 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. Separate experiments were not done to confirm the authenticity of the THP-1 cell line.

Lowry protein assay kits, bovine serum albumin, glucose-6-phosphate, glyceral, peptatin, leupeptin, T4 polynucleotide kinase, and glucose-6-phosphate dehydrogenase were obtained from Sigma. 1β-[3H]androstenedione was from Perkin-Elmer Life Science. MuLV reverse transcriptase, RNase inhibitor, oligo(dT)$_{18}$, and SYBR green PCR master mix were obtained from Applied Biosystems. Real-time PCR primers were synthesized by Sigma-Genosys. Medium to grow visceral preadipocytes was purchased from ScienCell Research Laboratories. FBS was purchased from Invitrogen. Stearic acid was obtained from Nu-Chek Prep. Enhanced chemiluminescence Western blotting detection reagents were from Amersham Biosciences. Nitrocellulose membranes were from Schleicher & Schuell. RNAeasy mini-kits were purchased from Qiagen. Lipofectamine 2000 was purchased from Invitrogen. Control small interfering RNA (siRNA) and siRNAs for COX-2 were purchased from Thermo Scientific. PKA constructs were obtained from Dr. M. Montminy (Salk Institute for Biological Science).
**Figure 3.** Increased levels of cAMP and protein kinase A (PKA) activity are found in inflamed breast tissue. Levels of cAMP (A) and PKA activity (B) were compared in breast tissue of normal weight versus overweight/obese women and in breast tissue with or without evidence of inflammation (CLS-B− vs. CLS-B+). Box plots are shown for subjects (n = 30) varying in weight and breast inflammation status.

**Study Population and Samples**

Approval for this study was obtained from the Institutional Review Boards of Memorial Sloan-Kettering Cancer Center and Weill Cornell Medical College. Detailed methods have been previously described (15). In brief, breast tissue was obtained from 30 women undergoing mastectomy at Memorial Sloan-Kettering Cancer Center who gave informed consent under a standard tissue acquisition protocol. BMI was defined using standard definitions: normal, BMI 18.5 to 24.9 kg/m²; overweight, BMI 25 to 29.9 kg/m²; and obese, BMI ≥ 30 kg/m².

For each of the 30 study cases, paraffin blocks and frozen samples were prepared. Frozen samples were stored in the presence or absence of RNA later (Ambion). From each available tissue block, 2 sections (5 μm thick and approximately 2 cm in diameter) were stained by hematoxylin and eosin and for CD68 (mouse monoclonal KP1 antibody; Dako; dilution 1:4000), a macrophage marker, to identify CLS-B. As previously reported (15), CD68 was a more sensitive measurement for the detection of CLS-B and all analyses are based on CD68 staining. All cases were reviewed by a breast histopathologist. Light microscopy was used to assess for evidence of CLS-B. A CLS-B index (0–1.0) was defined using the following formula: (number of slides with evidence of CLS-B)/(number of slides examined). The results of the CLS-B analysis for these 30 women have been reported previously (15).

**Aromatase Activity**

To determine aromatase activity, microsomes were prepared from tissue and cell lysates by differential centrifugation. Aromatase activity was quantified by measurement of the tritiated water released from 18[1H]-androstenedione (17). Aromatase activity was normalized to protein concentration.

**Quantitative Real-Time PCR Analysis**

Total RNA was isolated from frozen breast tissue using the RNeasy mini kit (Qiagen). Poly A RNA was prepared with an Oligotex mRNA mini kit (Qiagen). One hundred nanograms 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. The volume of the PCR was 20 μL and contained 5 μL of cDNA with the following primers: for CYP19/aromatase coding region, the forward and reverse primers used were 5′-CATATGCCATATAAGCTC-3′ and 5′-CCAGATCCAGACTTGGAGTG-3′; for COX-2, the forward and reverse primers used were 5′-GCCTGCTATGTATCTAGTGC-3′ and 5′-GGCCCTGCTATGTATCTAGTGC-3′; for PR, the forward and reverse primers used were 5′-TCCTATTAGTTGAGCAGTCTGT-3′ and 5′-CCAGACTTTCTAGGCGACA-3′. Promoter-specific aromatase mRNA levels were determined using primer sequences that amplified specific regions within aromatase promoters L3 and P1 in combination with primers that amplified a sequence within the CYP19/aromatase coding region (18). For CYP19 promoter L3, the forward and reverse primers used were 5′-GTCATGTAAACAGAAGCTTAAATGCCC-3′ and 5′-GCAAAACTT CTTGTTGTCTTTTG-3′; for CYP19 promoter II, the forward and reverse primers used were 5′-CCCTTGGGTGGTGCTCAGAGAC-3′ and 5′-GATGCGGTTTCAGAGCTTCCT-3′. Real-time PCR was performed using 2× SYBR green PCR master mix on a 7500 HT real-time PCR system (Applied Biosystems) with glyceraldehyde-3-phosphate dehydrogenase (forward 5′-TCTTT TTGCCTCGCAGCGCA-3′ and reverse 5′-GTGACGGGCGGCCC3 TACGA-3′) serving as an endogenous normalization control. Expression was determined using the ddCt analysis protocol.
**Figure 4.** Levels of aromatase transcripts derived from promoters I.3 and II and PR are increased in association with breast inflammation. Expression levels of (A) promoter I.3- and (B) promoter II-specific aromatase transcripts were quantified by real-time PCR and compared in breast tissue of normal weight versus overweight/obese women and in breast tissue with or without evidence of inflammation (CLS-B− vs. CLS-B+). Immunoprecipitates were subjected to Western blot analysis. Representative results (top of panel) for PR and β-actin are shown versus the CLS-B index for 16 cases. Densitometry (bottom of panel) was used to quantify PR protein. A–C, box plots are shown for subjects (n = 30) varying in weight and breast inflammation status.

**Transfections**

THP-1 cells and blood monocyte-derived macrophages were plated at 60% confluence and transfected with nontargeting siRNA or siRNA targeting COX-2 using DharmaFect 4 for 36 hours. Subsequently, the cells were treated with vehicle or searic acid. Preadipocytes were transfected with wild-type or dominant negative PKA expression vectors using LipoFectamine 2000. Cells that overexpressed PKA were then selected for using hygromycin.

**Immunoprecipitation and Western Blotting**

Tissue lysates were prepared from each of the samples of frozen human breast tissue. Five hundred micrograms of tissue lysate protein prepared from each of the 30 cases was subjected to immunoprecipitation followed by Western blotting as previously described (35). A cocktail of 3 COX-2 antibodies (Santa Cruz, Transduction Labs, Cayman) or antibodies to PR or β-actin (Santa Cruz) was used for immunoprecipitation. The immunoprecipitates were then subjected to Western blotting using COX-2, PR, or β-actin antisera (Santa Cruz). Densitometry was performed to quantify the COX-2, PR, or β-actin signal using Adobe Photoshop. β-actin levels were similar across samples of study subjects. Results were expressed as arbitrary units. Western blotting for COX-2 or PR in cell lysates was carried out as described previously (14).

**PGE, and Cyclic AMP Levels**

PGE, and cAMP levels were quantified using enzyme immunoassay kits from Cayman Chemical and Biomol-Enzo Life Sciences, respectively. Protein levels were determined by the Lowry method (40). Levels of PGE, and cAMP were normalized to protein concentrations and expressed as pg/μg protein.

**Protein Kinase A Activity**

A kit from Calbiochem was used to quantify PKA activity according to the manufacturer’s instructions. PKA activity was normalized to protein concentration and expressed as optical density/μg protein.

**Statistical Analyses**

The variables of interest in the study included CLS-B index defined as the percent of blocks with positive CD68 staining for each case, BMI, aromatase mRNA levels, aromatase activity, COX-2 mRNA and protein levels, PGE, levels, cAMP levels, PKA activity, and PR protein levels. The strength of correlation between CLS-B intensity and levels of each biomolecule, between BMI and each biomolecule, and between levels of pairs of biomolecules were quantified using the Spearman’s rank correlation coefficient. Correlation coefficients were tested against the null hypothesis that the correlation coefficients were 0. Results with P values < 0.05 were considered statistically significant. Correlation was considered as strong, moderate, or weak if the correlation coefficient was ≥ 0.75, ≥ 0.45 and < 0.75, or < 0.45, respectively. The difference in the level of a biomolecule between subjects in different weight categories or with different CLS-B status was evaluated using the Wilcoxon rank-sum test. Distributions of the biomolecule levels in subjects of different BMI categories or CLS-B status were illustrated using box plots.

**Disclosure of Potential Conflicts of Interest**

A.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.
Acknowledgments

We thank the patients who consented to have tissue used in this study. We are also grateful to the staff of the pathology and tissue procurement service of Memorial Sloan-Kettering Cancer Center. In particular, we thank Muzaffar Akram for his assistance with immunohistochemistry.

Grant Support

This work was supported by National Cancer Institute R01CA154481 and N01-CN-43302 (to A.J. Dannenberg), the Breast Cancer Research Foundation (to A.J. Dannenberg and C.A. Hudis), the Botwinick-Wolfensohn Foundation [in memory of Mr. and Mrs. Benjamin Botwinick (to A.J. Dannenberg)], the Kochavi Breast Cancer Prevention Fund (to C.A. Hudis), and the Metastasis Research Center of Memorial Sloan-Kettering Cancer Center (to C.A. Hudis). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the NIH.

Received September 21, 2011; revised January 13, 2012; accepted January 20, 2012; published OnlineFirst January 27, 2012.

REFERENCES

COX-2 and Aromatase in Inflamed Breast Tissue of Obese Women


16. Zhao Y, Agarwal VR, Mendelson CR, Simpson ER. Estrogen biosynthesis proximal to a breast tumor is stimulated by PGE1 via cyclic AMP, leading to activation of promoter II of the CYP19 (aromatase) gene. Endocrinology 1996;137:5739–42.


Increased Levels of COX-2 and Prostaglandin E\textsubscript{2} Contribute to Elevated Aromatase Expression in Inflamed Breast Tissue of Obese Women

Kotha Subbaramaiah, Patrick G. Morris, Xi Kathy Zhou, et al.