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Research Article

Subcellular Localization of Cyclic AMP-Responsive Element Binding Protein-Regulated Transcription Coactivator 2 Provides a Link between Obesity and Breast Cancer in Postmenopausal Women

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Abstract

Epidemiologic evidence supports a correlation between obesity and breast cancer in women. AMP-activated protein kinase plays an important role in energy homeostasis and inhibits the actions of cyclic AMP-responsive element binding protein-regulated transcription coactivator 2 (CRTC2). In postmenopausal women, the cyclic AMP-responsive element binding protein-dependent regulation of aromatase is a determinant of breast tumor formation through local production of estrogens. The present work aimed to examine the effect of adipokines on aromatase expression and identify additional mechanisms by which prostaglandin E_2 causes increased aromatase expression in human breast adipose stromal cells. Treatment of human adipose stromal cells with forskolin and phorbol 12-myristate 13-acetate (PMA), to mimic prostaglandin E_2, resulted in nuclear translocation of CRTC2. Aromatase promoter II (PII) activity assays showed that CRTC2 in addition to forskolin/PMA treatment significantly increased PII-induced activity. CRTC2 binding to PII was examined by chromatin immunoprecipitation, and forskolin/PMA treatment was associated with increased binding to PII. Treatment of human adipose stromal cells with leptin significantly up-regulated aromatase expression associated with nuclear translocation of CRTC2 and increased binding of CRTC2 to PII. Adiponectin treatment significantly decreased forskolin/PMA-stimulated aromatase expression, consistent with the decreased nuclear translocation of CRTC2 and the decreased binding of CRTC2 to PII. The expression and activity of the AMP-activated protein kinase LKB1 was examined and found to be significantly decreased following either forskolin/PMA or leptin treatment. In contrast, adiponectin significantly increased LKB1 expression and activity. In conclusion, the regulation of aromatase by CRTC2, in response to the altered hormonal milieu associated with menopause and obesity, provides a critical link between obesity and breast cancer. [Cancer Res 2009;69(13):5392–9]

Introduction

There is now substantial epidemiologic evidence to support the conclusion that obesity is linked to the increased risk of several forms of cancer such as colon and breast cancer (1). Given the obesity problem worldwide, the potential significance of this conclusion is that tens of millions more women may develop breast cancer in their senior years than was previously believed to be the case. The problem is compounded by the fact that breast cancer risk increases with aging. In postmenopausal life, this is due primarily to an increased capacity of adipose tissue to synthesize estrogens as a function of age (2–4). At this time, the cellular and molecular mechanisms underlying the increased risk of breast cancer associated with obesity and aging are poorly understood.

In recent years, the concept has been developed that, in postmenopausal women, when the ovaries cease to make estrogens, the major source of estrogens driving breast cancer development is local aromatase expression within the breast (5, 6). This is consistent with the efficacy of aromatase inhibitors as endocrine therapy for breast cancer. We believe that inflammatory factors such as prostaglandin E_2 (PGE_2) produced by the tumorous epithelium activate aromatase expression in breast adipose stromal cells (the cell type in adipose tissue where aromatase is expressed) via the E prostanoid 2 receptor, which results in stimulation of adenylyl cyclase, and the E prostanoid 1 receptor, which stimulates diacylglycerol and inositol triphosphate formation (6). This is an example of the role of epithelial/mesenchymal interactions in carcinogenesis. There is also an absolute requirement for a monomeric orphan member of the nuclear receptor family to bind to a nuclear receptor half-site downstream of the cyclic AMP-responsive elements (CRE) on the aromatase promoter II (PII), that is, liver receptor homologue-1 (7). Hence, these stimulatory pathways work in concert to facilitate tumor-driven aromatase expression in the breast (8).

Obesity is characterized by increased adipose tissue mass, which results in alterations in the hormonal milieu that has been suggested to influence breast cancer risk (9, 10). Two adipokines, leptin and adiponectin, have been examined in this regard. Leptin synthesis and plasma levels increase with obesity and recent work has shown that higher leptin levels were significantly associated with an increase in breast cancer (11). Moreover, there is a report that leptin stimulates aromatase expression in MCF-7 cells (12, 13). By contrast, adiponectin levels in the serum decrease with increased obesity and the three reported epidemiologic studies of adiponectin have all shown an inverse association between serum adiponectin levels and breast cancer risk (reviewed in ref. 14). Studies have also shown inhibition of growth of MCF-7 cells by

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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adiponectin (15). Furthermore, the adiponectin receptor AdipoR1 is highly expressed in human adipose tissue (16) and both AdipoR1 and AdipoR2 are expressed in MCF-7 and MDA-MB-231 breast cancer cells (15).

AMP-activated protein kinase (AMPK) is recognized to be a master regulator of energy homeostasis and a nexus for the convergence of endocrine signals including leptin, adiponectin, estradiol, androgens, and phytoestrogens (17–19). AMPK activity is regulated covalently through phosphorylation of the α catalytic subunit at T172 by upstream kinases LKB1 and CaMKK; however, in most tissues, LKB1 appears to predominate. Furthermore, phosphorylation of the α catalytic subunit of AMPK at S485 (α1) or S491 (α2) by protein kinase A reduces its catalytic activity by reducing the accessibility of the T172 phosphorylation site (20).

The possibility of a link between the LKB1/AMPK pathway and aromatase expression in the breast arose from an unexpected source, the rare condition of Peutz-Jeghers syndrome. Boys with this condition develop florid gynecomastia at age 6 or 7 years due to the formation of Sertoli cell tumors. These tumors have very high rates of aromatase expression driven by PII as shown by us (21), thus explaining the gynecomastia in boys with this condition. The link with the LKB1/AMPK pathway was revealed when it was shown that Peutz-Jeghers syndrome was due to mutations in the LKB1 gene, *STK11* (22).

Recently, a new family of CRE binding protein (CREB) coactivators called CREB-regulated transcription coactivators (CRTC; previously known as transducers of regulated CREB) has been shown to increase the expression of cyclic AMP-responsive genes. Under basal conditions, CRTCs are sequestered in the cytoplasm through phosphorylation by members of the AMPK family and binding of CRTCs to 14-3-3 proteins via phosphorylation-dependent mechanisms. In the absence of AMPK activity, CRTC2 is dephosphorylated and translocates to the nucleus where it associates with CREB and increases target gene expression (23). Because aromatase and peroxisome proliferator-activated receptor-γ coactivator-1α are both CREB target genes (24), this provides a mechanism whereby the LKB1/AMPK pathway can inhibit expression of aromatase in the breast. Therefore, we hypothesized that the LKB1/AMPK pathway inhibits aromatase expression in the breast via the proximal PII.

Materials and Methods

Plasmids. The *CYP19A1* PII-516 and PII-516-CLS$_{mut}$ reporter constructs were generated as described previously (25). The Flag-CRTC2-pcDNA vector was obtained from Mark Montminy (Salk Institute), generated as described previously (26). The CRTC2-pEF-GFP plasmid was generated by amplifying the CRTC2 coding sequence from the above-mentioned plasmid and using primers CRTC2-*Asc*I-F: 5-ACTTGGCGCGCCAAGGAAGATGGCGACGTCGGGGGCGAA-3′ and CRTC2-*Asc*I-R: 5′-CTTTACTCTGGCGCGCCACTTGGAGCCGGTCACTGCGGA-3′. These primers incorporate *Asc*I restriction enzyme cleavage sites, and cleavage results in the removal of the stop codon. The coding region was then inserted into the pEF-GFP vector described previously (27) resulting in a COOH-terminal GFP tag. The LKB1-pcDNA vector was generated using primers LKB1-F: 5′-GGGACTCCAGGACCCTGGGTCCA-3′ and LKB1-R: 5′-CCAGCCTCACTGCTGCTTGCAGG-3′, subcloned into pGEM-T easy (Promega), digested with *Eco*RI, and subcloned into pcDNA3.1+ (Invitrogen Australia). Correct orientation was confirmed by sequencing.

Human tissue, cell culture, transfection, and reporter gene assays. Human breast adipose stromal cells were isolated by collagenase digestion from breast reduction procedures as described previously (28). The studies presented herein have been approved by Southern Health Human Research Ethics Committee B and all subjects have given informed consent. Cells were transfected using the Nucleofector electroporation apparatus (Lonza Australia) as directed by the manufacturer. Briefly, 1×10^6 cells were trypsinized, washed, and resuspended in 100 μL solution V with 2 μg DNA. For confocal imaging experiments, human adipose stromal cells were electroporated using the T-030 program on the Nucleofector and plated in 8-chamber slides and incubated overnight. For reporter assays, MCF-7 cells were transfected using program E-014, with either the PII-specific reporter constructs and CRTC2-pcDNA, LKB1-pcDNA, or pcDNA3.1+ alone as a negative control as well as 10 ng of a *Renilla* expression vector as a transfection control. Cells were plated in 24-well plates and incubated overnight. Before treatments, cells were serum starved for 24 h in phenol-red free medium containing 0.1% bovine serum albumin. After serum starvation, cells were treated with experimental agents at the concentrations indicated. Luciferase reporter assays were carried out using the Dual-Glo Luciferase Assay System (Promega) as described by the manufacturer. Experimental agents were forskolin (Sigma-Aldrich), phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich), and leptin [obtained from I.J. Clarke, Monash University, and purified as described previously (29)]. Adiponectin was prepared from stably transfected HEK293 cells expressing human adiponectin (a generous gift from Dr. J. Whitehead, University of Queensland). Cells were grown to 70% confluency in high-glucose (4,500 mg/L) DMEM supplemented with 10% (v/v) FCS, 100 μg/mL hygromycin B, 80 μg/mL gentamicin, 1 μg/mL minocycline, and 4 mmol/L L-glutamine. Cells were then incubated in serum-free medium for a further 24 h. Medium was harvested, clarified by centrifugation at 1,000 rpm for 5 min, diluted 1:2 in 50 mmol/L HEPES (pH 7.2), and loaded onto a Q-Sepharose FastFlow column (10/100; GE Healthcare Bio-Sciences) equilibrated with 50 mmol/L HEPES (pH 7.2), 50 mmol/L NaCl at 3 mL/min. Following extensive washing with equilibration buffer, adiponectin was eluted in 1.5 mL fractions using a NaCl gradient (50-1,000 mmol/L). Fractions containing adiponectin were pooled, quantitated by Western blotting (Supplementary Figure S1) using a known concentration standard (BioCore), and stored at −80°C in the presence of 10% glycerol.

Western blot analysis. Cells were washed in ice-cold PBS and lysed in ice-cold buffer (5 mmol/L HEPES, 137 mmol/L NaCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 10 mmol/L NaF, 2 mmol/L EDTA, 10 mmol/L sodium pyrophosphate, 2 mmol/L $NaVO_4$, 1% NP-40, 10% glycerol) containing protease inhibitors (Complete Mini; Roche), incubated on ice for 45 min, and centrifuged for 15 min at 14,000 × *g* before assay of supernatants for protein content by the bicinchoninic acid method (Pierce Biotechnology). Fifty micrograms of protein were diluted in sample buffer containing DTT, denatured, run on 8% polyacrylamide gels, and transferred to nitrocellulose for Western blotting. Phosphorylation of AMPK was assayed by Western blotting with antibodies to phosphopeptides based on the amino acid sequence surrounding T172 of the α subunit of human AMPK and S485/S491 of the α1/α2 AMPK subunits, respectively (Cell Signaling). The level of phosphorylation was normalized to the level of total AMPK using an antibody against the catalytic α1 and α2 subunits of AMPK (Cell Signaling). LKB1 protein levels were assessed using a specific LKB1 antibody (Cell Signaling). Proteins were visualized with an Alexa Fluor 680 goat anti-rabbit secondary antibody (Molecular Probes), and band intensities were quantified using the Odyssey infrared imaging system (LiCor Biosciences).

Reverse transcription and real-time PCR. Total RNA was extracted using the RNeasy Mini kit (Qiagen) and reverse transcription was done using AMV reverse transcription and random primers (Promega) as directed by the manufacturer. Briefly, 0.25 to 1.0 μg RNA was incubated with 0.5 μg random primers at 70°C for 5 min, and reverse transcription reaction was incubated at 37°C for 1 h. Real-time PCR amplifications were done on the Rotor-Gene (Qiagen). Quantification of human LKB1, human aromatase, and L32 transcript was done on the RotorGene using primers hLKB1-F: 5′-GCCGGGACTGACGTGTAGA-3′, hLKB1-R: 5′-CCCAAAAGGAAGGGAAAAACC-3′, hArom-F: 5′-ACCCTTCTGCGTCGTGTCA-3′, hArom-R: 5′-TCTGTGGAAATCCTGCGTCTT-3′, hL32-F: 5′-CAGGGTTCGTAGAAGATTCAAGGG-3′, and hL32-R: 5′-CTTGGAGGAAACATTGTGAGCGATC-3′. Cycling conditions were one cycle at 95°C for 5 min followed by a variable number of cycles of 95°C for 10 s, 59°C for 15 s, and 72°C for 20 s. Experimental samples were quantified by comparison with

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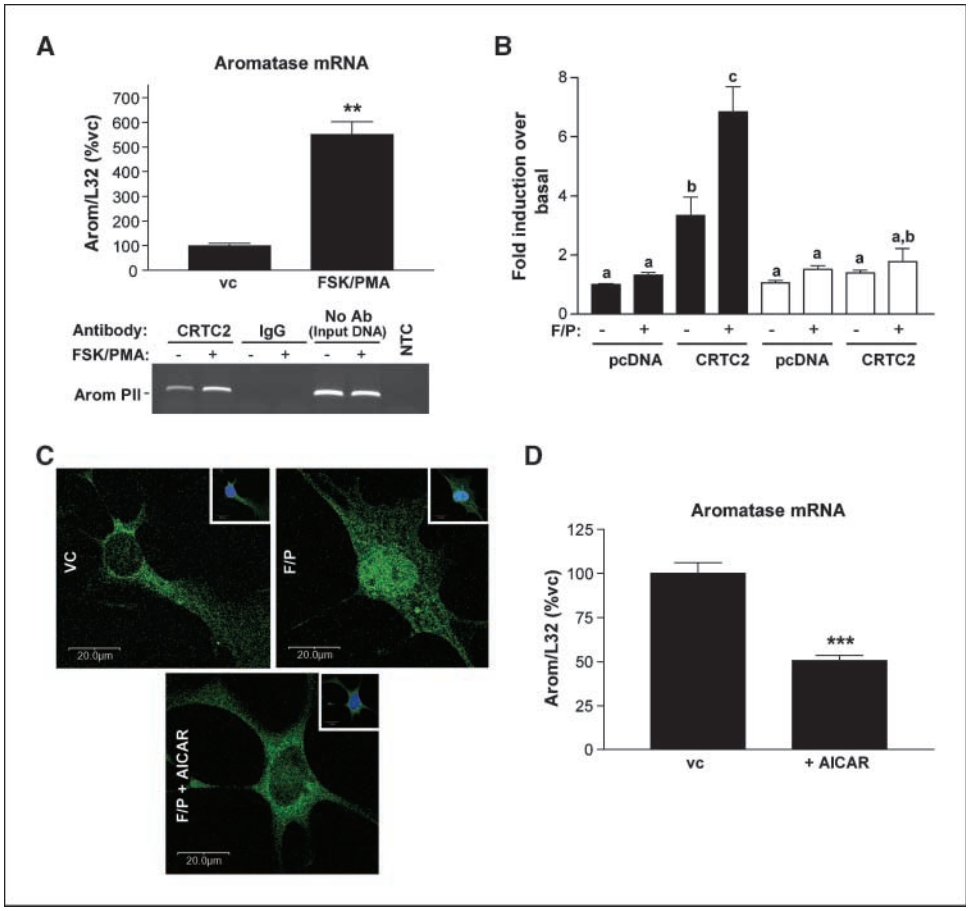

Figure 1. Role of CRTC2 in aromatase PII activation. *A, top,* forskolin/PMA induced a significant increase in aromatase transcript; *bottom,* interaction of CRTC2 with aromatase PII was shown by chromatin immunoprecipitation and was shown to be stimulated by forskolin/PMA (n = 3). *B,* forskolin/PMA induced a significant increase in aromatase PII activity in CRTC2-transfected cells. Mutation of the proximal CRE abolished the CRTC2-mediated activation of PII (*white columns*). *C, top left,* basally, CRTC2 is mainly located in the cytoplasm (merged 4′,6-diamidino-2-phenylindole nuclear stain and CRTC2-GFP in *top right, inset*); *top right,* forskolin/PMA stimulated the translocation of CRTC2 to the nucleus. Stimulation of AMPK activity in forskolin/PMA-treated preadipocyte cells with AICAR prevented the nuclear translocation of CRTC2 (*C, bottom*) and resulted in a significant decrease in aromatase (*D*). *Arom,* aromatase; *vc,* vehicle control; *Ab,* antibody; *NTC,* no template control. Unless otherwise specified, n = 6.

standards of known concentrations. All samples were normalized to L32 transcript levels.

Chromatin immunoprecipitation. Chromatin immunoprecipitation was done to examine aromatase PII binding on cells treated with experimental agents for 6 h. Sample preparation was done using adaptations of the protocols of Alberts and colleagues (30) and Braunstein and colleagues (31). Briefly, serum-starved cells were grown to 50% confluency and treated for 6 h at 37°C for study of binding of transcriptional regulators to the aromatase PII. Cells were then cross-linked using 1% formaldehyde for 5 min at room temperature and collected in PBS containing protease inhibitors. Cells were lysed [1% SDS, 10 mmol/L EDTA, and 50 mmol/L Tris (pH 8.0) plus protease inhibitors] and sonicated at 20% max power 7 for 30 s pulses using a Sonics sonifier. After sonication, one tenth of the total sample was removed for input. Chromatin immunoprecipitation was done using the ChIP-IT express kit (Australian Biosearch) as directed by the manufacturer. Briefly, 5 μg DNA was immunoprecipitated overnight at 4°C with 5.0 μg antibody (CRTC2 and IgG; Biolab). Protein/DNA complexes were eluted from the beads and treated with proteinase K solution at 37°C for 1 h. Real-time PCR was done on the purified DNA as described above using primers flanking the CREs of *CYP19A1* PII (PII-ChIP-F: 5′-TTTCCAC-ACTACCGTTGGCCG-3′ and PII-ChIP-R: 5′-GGCAATCTTCTTCCCTTGAA-GC-3′). Images presented are representative of three separate experiments.

Fluorescence imaging. After transfection, cells were serum starved for 24 h and treated for a further 24 h with experimental agents. Slides were covered by coverglasses using the fluorescence mounting medium (Dako Australia) and 4′,6-diamidino-2-phenylindole nuclear stain. Fluorescence was visualized and captured using the FluoView FV500 confocal laser scanning microscope (Olympus Imaging Australia) at ×60 magnification. Images are representative of the majority of cells examined for that treatment.

Statistical analyses. All experiments were done at least three times. All data are reported as mean ± SE. Statistical analyses for experiments comparing two groups were done by two-tailed Student's t test. For experiments where the effect of treatment was compared with control (Fig. 3*A*), statistical analysis was done using one-way ANOVA followed by Dunnett's multiple comparison test. Asterisks indicate statistically significant differences: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. For experiments where more than two groups were compared, statistical analyses were done using one-way ANOVA followed by Tukey's multiple comparison test. Results that were statistically significant were labeled with different letters. GraphPad Prism version 3.00 was used.

Results

Role of CRTC2 in aromatase PII activation. The role of CRTC2 in PII-driven aromatase expression was examined in primary human adipose stromal cells treated with 25 μmol/L forskolin and 4 nmol/L PMA to mimic the effects of PGE_2. Treatment resulted in a significant 5.5-fold increase in aromatase mRNA (Fig. 1*A, top*), consistent with previously published results (32). The endogenous interaction of CRTC2 with PII was shown by chromatin immunoprecipitation and was shown to be stimulated by 74.6 ± 26.05% (mean ± SE; n = 3) in the presence of forskolin/PMA (Fig. 1*A, bottom*). To determine whether CRTC2 is involved in PII activation, MCF-7 cells were cotransfected with CRTC2 and activity of a luciferase reporter construct containing 516 bp immediately upstream of the transcription start site of PII was examined. Treatment of CRTC2-transfected cells with forskolin/PMA caused a significant increase in PII activity over untreated cells (Fig. 1*B, black columns*). Moreover, mutation of the proximal CRE completely abolished the CRTC2-mediated activation of PII (Fig. 1*B,*

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white columns) compared with the effect observed with cotransfection of CRTC2 with wild-type PII and forskolin/PMA treatment (*black columns*). Because CRTC2 activity is largely mediated by its subcellular localization, a mammalian expression vector encoding a CRTC2/GFP fusion protein was generated and transfected into human adipose stromal cells. Under resting conditions, CRTC2 is mainly located in the cytoplasm (Fig. 1*C, top left*; representative of all cells examined; $n = 6$) and treatment with forskolin/PMA resulted in the translocation of CRTC2 to the nucleus (Fig. 1*C, top right*; representative of all cells examined; $n = 6$). Furthermore, stimulation of AMPK activity in these forskolin/PMA-treated cells with 500 μmol/L AICAR (an AMP analogue) resulted in a significant decrease in aromatase expression (Fig. 1*D*). Interestingly, the forskolin/PMA-mediated translocation of CRTC2 to the nucleus was prevented by treatment with AICAR (Fig. 1*C, bottom*; representative of all cells examined; $n = 6$). These data show that stimulation of the protein kinase A and C pathways in human adipose stromal cells results in nuclear translocation of CRTC2 and that this is accompanied by an increase in CRTC2-dependent activation of aromatase PII.

Role of LKB1 in regulating CRTC2 activity and regulation of the LKB1/AMPK pathway in human adipose stromal cells. The LKB1 protein has been shown to inhibit CRTC2 activity via the intermediate phosphorylation of AMPK. Cotransfection of the PII reporter construct and CRTC2 with LKB1 prevented the forskolin/PMA-mediated activation of aromatase PII (Fig. 2*A*). Cells were also cotransfected with the CRTC2/GFP-vector and LKB1 or small interfering RNA against LKB1, synchronized by starvation, and treated with forskolin/PMA. Interestingly, the forskolin/PMA-mediated translocation of CRTC2 to the nucleus (Fig. 1*C*) was prevented by cotransfection with LKB1 (Fig. 2*B, left*; representative of all transfected cells examined; $n = 6$) and knockdown of LKB1 using siRNA was sufficient to cause nuclear localization of CRTC2 (Fig. 2*B, right*; representative of all cells examined; $n = 6$). LKB1 expression and activity was also examined in primary human adipose stromal cells after forskolin/PMA treatment. Treatment resulted in a significant decrease in LKB1 transcript and protein expression (Fig. 2*C, top* and *bottom*, respectively) and this was associated with a significant decrease in phosphorylation of AMPK at T172 (Fig. 2*D, top right*). Interestingly, total AMPK protein expression also significantly decreased in response to forskolin/PMA (Fig. 2*D, top left*), indicating that the net effect of the treatment is greater than that measured by phosphorylation alone. Furthermore, phosphorylation of the α subunits at S485, which is inhibitory of AMPK activity, significantly increased with forskolin/PMA treatment (Fig. 2*D, bottom*). These data show that inhibition of the LKB1/AMPK pathway by factors that stimulate the protein kinase A and C pathways contributes to the CRTC2-dependent PII-driven stimulation of aromatase expression in human adipose stromal cells.

Leptin induces aromatase expression via CRTC2 and inhibition of LKB1 in human adipose stromal cells. The treatment of primary human adipose stromal cells with 0.5 μg/mL leptin resulted in a significant increase in aromatase transcript expression compared with the control treatment (Fig. 3*A, top*). To determine the involvement of CRTC2 in the leptin-mediated stimulation of

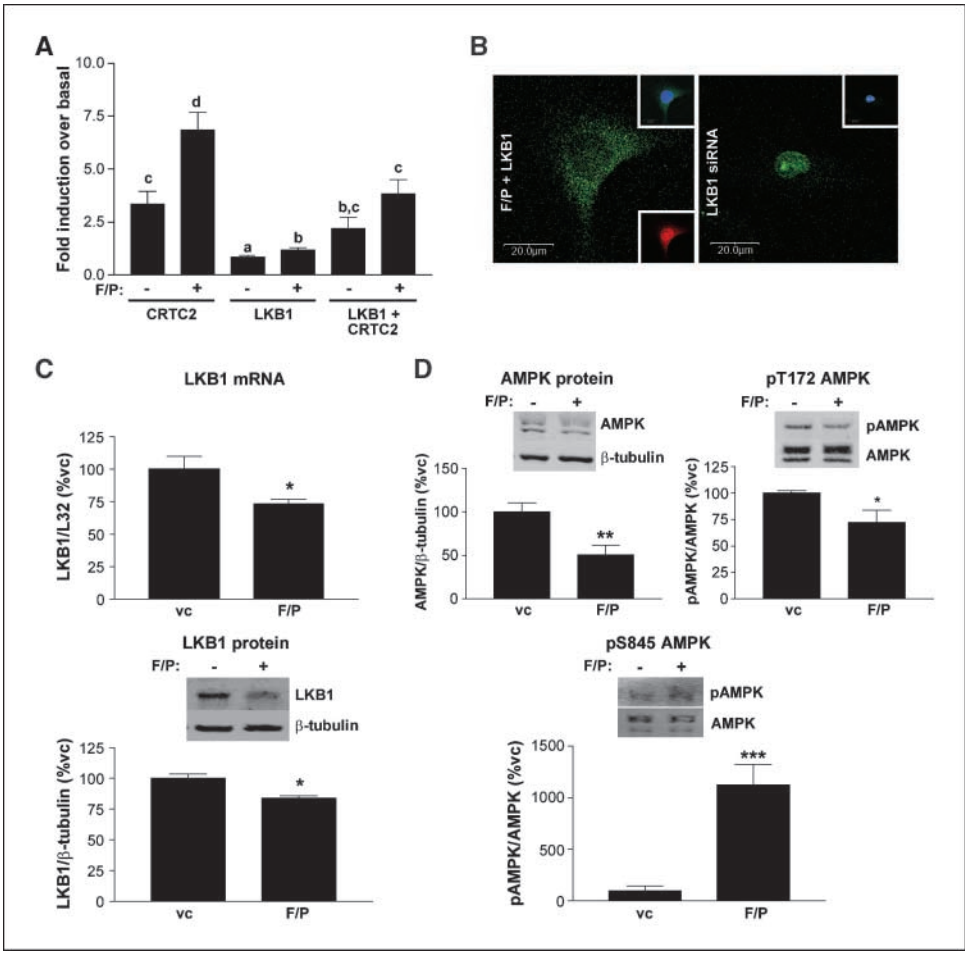

Figure 2. Effect of LKB1 on CRTC2 and regulation of LKB1 and AMPK in human adipose stromal cells. Cotransfection of LKB1 with CRTC2 prevented the forskolin/PMA-dependent activation of aromatase PII using a reporter assay (*A*) and prevented the forskolin/PMA-mediated translocation of CRTC2 to the nucleus (*B, left, inset*, with red fluorescence, immunocytochemistry of LKB1-transfected cell; *top right, inset,* merged signal of CRTC2-GFP with 4′,6-diamidino-2-phenylindole nuclear stain). *B, right,* knockdown of LKB1 resulted in the nuclear localization of CRTC2. Forskolin/PMA resulted in a decrease in LKB1 mRNA (*C, top*; $n = 3$), protein (*C, bottom*), and activity as measured by AMPK phosphorylation of T172 (*D, top right*). Forskolin/PMA treatment was also associated with a decrease in total AMPK protein (*D, top left*) and a significant increase in phosphorylation of S485 of AMPK (*D, bottom*). *F/P,* forskolin/PMA. Unless otherwise specified, $n = 6$.

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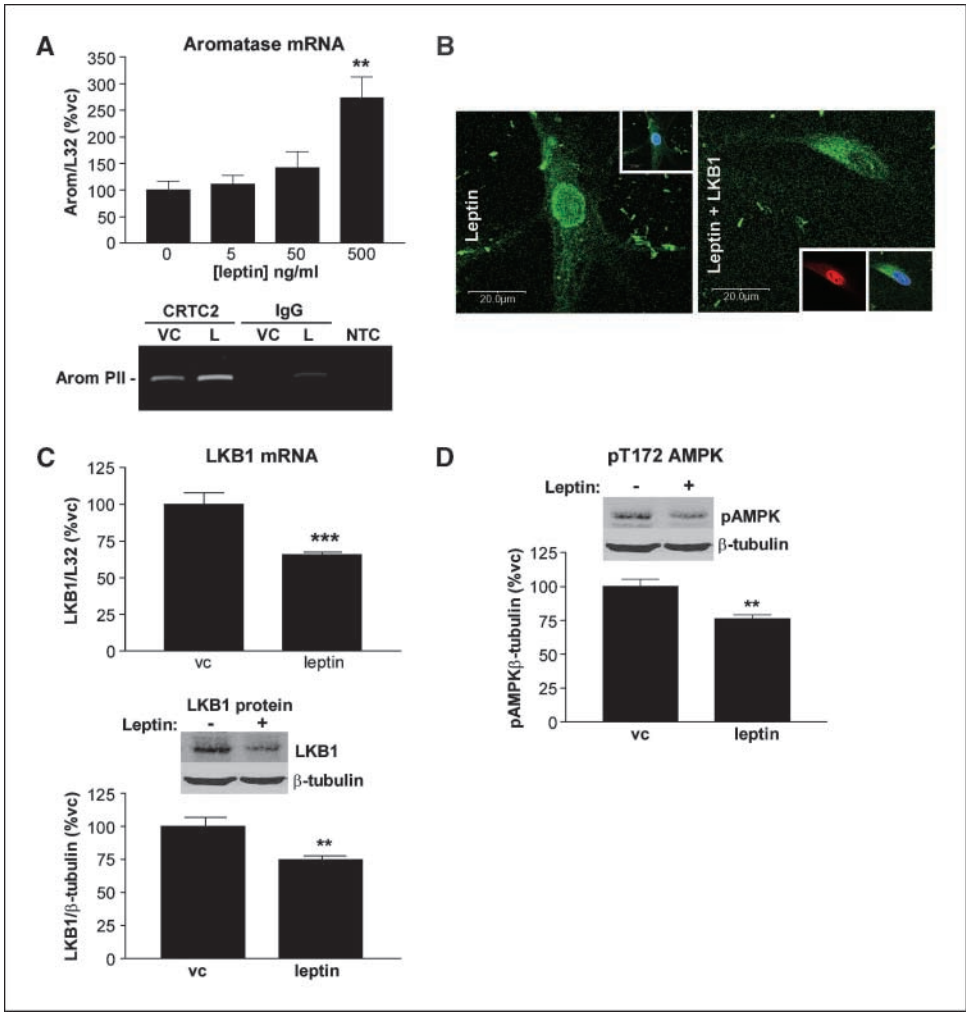

Figure 3. Leptin induces aromatase expression via CRTC2 and inhibition of LKB1 in human adipose stromal cells. *A, top,* leptin induced a significant increase in aromatase transcript expression at a concentration of 500 ng/mL (n = 3); *bottom,* chromatin immunoprecipitation analysis revealed that binding of CRTC2 to aromatase PII was increased with 500 ng/mL leptin treatment (n = 3) and this was associated with the pronounced nuclear translocation of CRTC2 (*B, left*). The leptin-dependent change in subcellular localization of CRTC2 was prevented when cells where cotransfected with LKB1 (*B, right*). *Inset*, with red fluorescence, immunocytochemistry of LKB1-transfected cell. Leptin resulted in a decrease of LKB1 transcript (*C, top*; n = 3) and protein (*C, bottom*) expression in human adipose stromal cells and caused a decrease in AMPK phosphorylation at T172 (*D*). *L,* leptin. Unless otherwise specified, n = 6.

aromatase expression, chromatin immunoprecipitation analysis was done and revealed that CRTC2 binding to aromatase PII was increased by 129.9 ± 75.21% (mean ± SE; n = 3) with leptin treatment (Fig. 3*A, bottom*). CRTC2 localization was followed by transfecting human breast adipose stromal cells with a mammalian expression vector encoding a CRTC2/GFP fusion protein and treated with 0.5 μg/mL leptin. Figure 3*B* (*left*) shows that leptin treatment resulted in the pronounced nuclear translocation of CRTC2 (representative of all cells examined; n = 6) compared with the vehicle control (Fig. 1*C*). This leptin-dependent change in subcellular localization of CRTC2 was prevented when cells were cotransfected with a LKB1 expression vector (Fig. 3*B, right*; representative of all transfected cells examined; n = 6). Importantly, treatment of human adipose stromal cells with 0.5 μg/mL leptin resulted in a decrease in LKB1 transcript and protein expression (Fig. 3*C, top* and *bottom*, respectively) and resulted in reduced AMPK phosphorylation at T172 (Fig. 3*D*), consistent with previous studies in the hypothalamus (32).

Adiponectin down-regulates stimulated aromatase expression in human adipose stromal cells via CRTC2. In contrast to leptin, adiponectin secretion is negatively correlated with obesity and has been shown to activate AMPK in liver and skeletal muscle cells (17). Interestingly, adiponectin significantly reduced the forskolin/PMA-dependent up-regulation of aromatase transcript expression in human adipose stromal cells (Fig. 4*A, top*). Consistent with this, adiponectin also reduced the forskolin/PMA-dependent interaction of CRTC2 and aromatase PII by 55.2 ± 12.03% (mean ± SE; n = 3) at concentrations of 1.0 μg/mL as shown by chromatin immunoprecipitation (Fig. 4*A, bottom*). Moreover, the subcellular localization of CRTC2 was followed by confocal microscopy and revealed that the forskolin/PMA-induced translocation of CRTC2 to the nucleus (Fig. 1*C*) was prevented by adiponectin treatment (Fig. 4*B*; representative of all cells examined; n = 6). The effect of adiponectin on LKB1 expression and AMPK phosphorylation was also examined using primary human breast adipose stromal cells. In contrast to the results with leptin, LKB1 transcript and protein levels were increased with 1.0 μg/mL adiponectin treatment (Fig. 4*C, top* and *bottom*, respectively), with a concomitant increase in AMPK phosphorylation at T172 (Fig. 4*D*).

Discussion

In the present work, we have sought to establish whether there is a relationship between adipokine concentrations and the activity of the LKB1/AMPK pathway, as well as to identify CRTC2 as a regulator of aromatase expression, in the human breast. Specifically, we have sought to determine if the adipokines leptin and adiponectin serve as upstream regulators, because this would provide a cellular and molecular link between obesity and breast cancer risk.

With increasing obesity and aging, the risk of breast cancer increases and several studies have highlighted the importance of

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local estrogen production in adipose tissue (2, 33, 34). In particular, in the postmenopausal woman, it is this extragonadal local source of estrogen via the action of the aromatase enzyme within the breast that mainly contributes to breast cancer development and progression. In normal breast adipose tissue, aromatase activity and expression are low; however, aromatase expression is elevated 3- to 4-fold within breast adipose tissue bearing a tumor due to increased activation of the aromatase PII (7, 21, 35). In this study, we have observed that activation of the LKB1/AMPK pathway results in inhibition of expression of aromatase via PII in human adipose stromal cells. Moreover, stimulation of the protein kinase A and C pathways, which activates PII (as is the case with PGE_2 action), results in a decrease in LKB1 expression and activity, an increase in phosphorylation of AMPK at the inhibitory S485 site, and the concomitant nuclear translocation of CRTC2 into the nucleus. Moreover, this translocation coincides with an increase in CRTC2 binding to aromatase PII as well as an increase in PII activity dependent on the proximal CRE. Considering the already established role for CREB in activating aromatase PII (36, 37) and that recent evidence has suggested that activation of the majority of CREB target genes requires the coactivation of CREB by CRTCs (38), our results provide a substantial advance in understanding the pathways involved in aromatase regulation in the breast. Furthermore, CRTC2 has been shown to regulate peroxisome proliferator-activated receptor-γ coactivator-1α (39), which also appears to be involved in aromatase expression as a coactivator of liver receptor homologue-1 (24). Interestingly, previous reports using pancreatic islet cells have also identified cyclic AMP as a mediator of CRTC2 nuclear translocation (40).

Moreover, this study is the first to characterize the action of adiponectin to inhibit, and leptin to stimulate, aromatase expression in human breast adipose stromal cells and to relate this to the regulation of the LKB1/AMPK pathway and CRTC2 translocation to and from the nucleus. Although it is well established that adipokines play an important role in AMPK activity (reviewed in refs. 41, 42), it has never been shown that leptin and adiponectin alter LKB1 expression. Exploring links between adipokines and aromatase expression in the context of breast cancer has been the focus of a

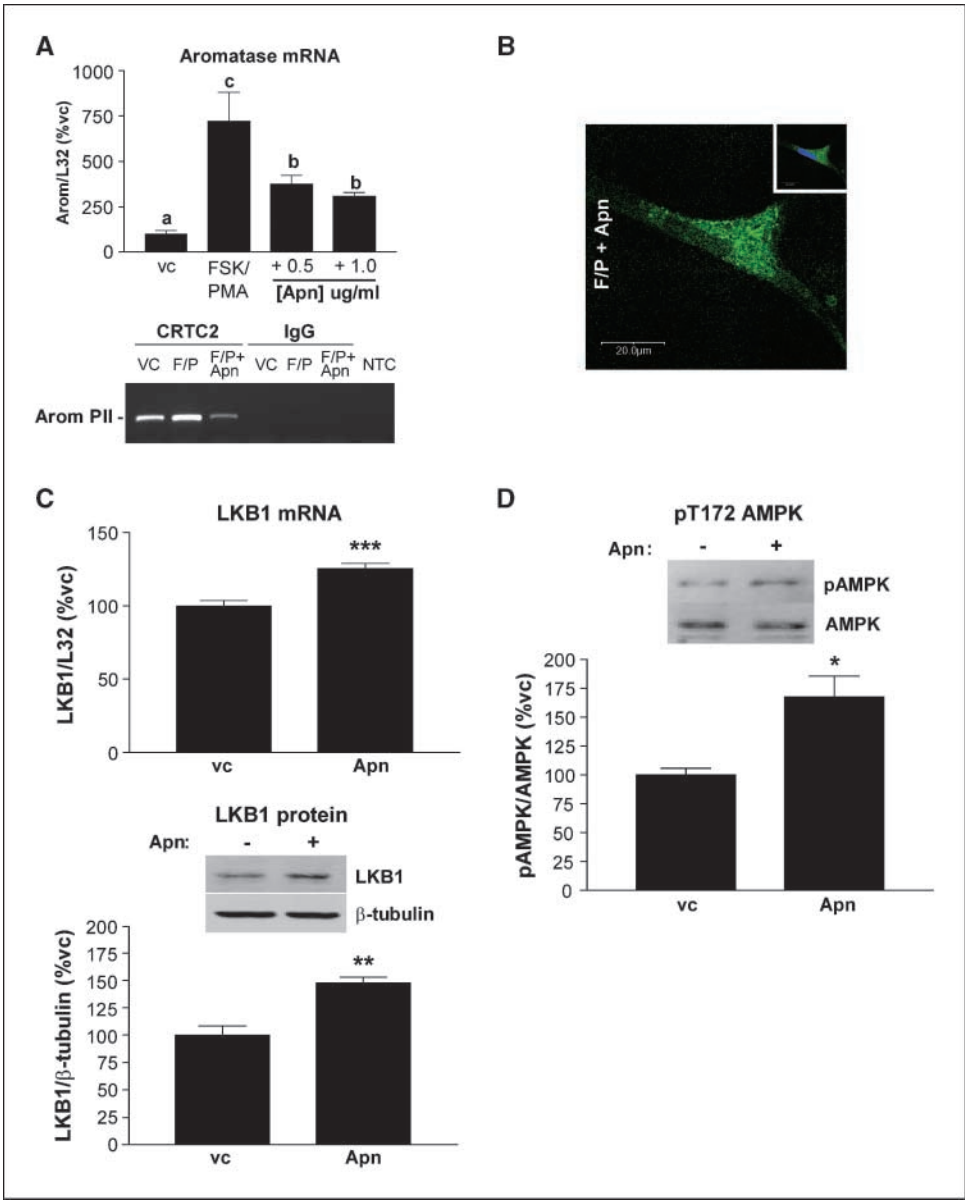

Figure 4. Adiponectin down-regulates stimulated aromatase expression in human adipose stromal cells via CRTC2. *A, top,* adiponectin significantly reduced the forskolin/PMA-dependent up-regulation of aromatase transcript expression in human adipose stromal cells ($n = 3$); *bottom,* chromatin immunoprecipitation analysis revealed that 1.0 μg/mL adiponectin reduces the forskolin/PMA-dependent interaction of CRTC2 and aromatase PII ($n = 3$). *B,* adiponectin prevented the forskolin/PMA-induced translocation of CRTC2 to the nucleus. LKB1 transcript (*C, top*; $n = 3$) and protein (*C, bottom*) expression is significantly up-regulated by adiponectin in human adipose stromal cells and is associated with a significant increase in AMPK phosphorylation at T172 (*D*). *Apn,* adiponectin. Unless otherwise specified, $n = 6$.

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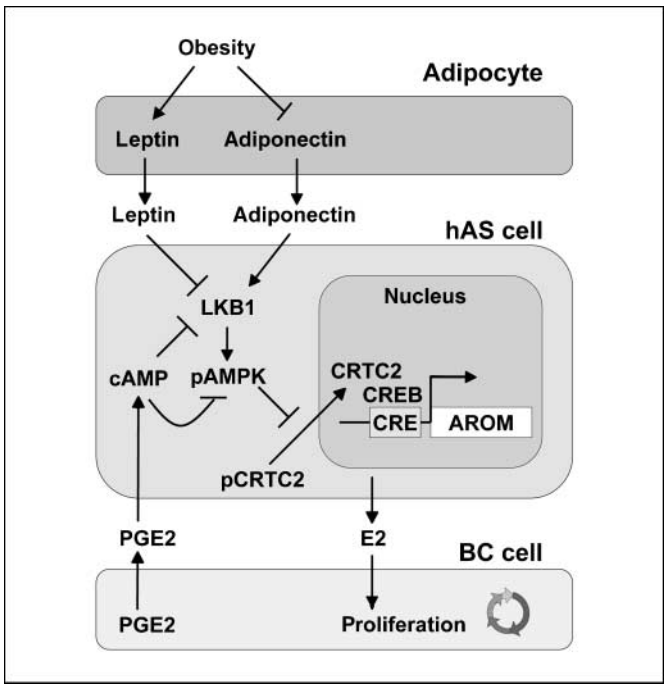

Figure 5. Model of the regulation of CRTC2 as a link between obesity and breast cancer. Leptin secretion from adipocytes, which is associated with obesity, down-regulates LKB1 expression in human adipose stromal cells, leading to a decrease in AMPK phosphorylation, translocation of CRTC2 to the nucleus, and an increase in aromatase expression. Adiponectin has the opposite effect to leptin. Estradiol (*E2*) leads to an increase in breast cancer cell proliferation. The tumor-derived factor PGE_2 causes the down-regulation of LKB1 and AMPK in human adipose stromal cells resulting in a positive feedback loop leading to a further increase in aromatase expression and further breast cancer proliferation; however, adiponectin can counteract this and prevent the CRTC2-mediated induction of aromatase.

few publications (12, 13, 43). Catalano and colleagues described an increase in aromatase expression with leptin via activator protein-1 in MCF-7 cells, whereas leptin had no effect on aromatase expression in any of the breast cancer cell lines studied by Sulkowska and colleagues. One other publication describes adiponectin as a negative regulator of aromatase transcript expression in porcine granulosa cells but does not provide evidence as to the signaling pathways involved (44). Nonetheless, it is aromatase expression within the breast adipose that has been correlated with an increase in breast cancer cell proliferation. We believe that these findings offer, in part, an explanation for the well-established epidemiologic observation that obesity is a risk factor for breast cancer.

Based on these considerations, we propose the following model (Fig. 5): Increasing obesity is associated with increased leptin and decreased adiponectin formation. This change in adipokine secretion would have the net effect of stimulating aromatase expression in adjacent stromal cells via inhibition of the LKB1/AMPK pathway and consequent nuclear translocation of CRTC2 where it can coactivate CREB. Whether the changes in LKB1 expression, which we report here, are adequate to solely account for the changes in nuclear translocation of CRTC2 remains to be investigated. The increase in aromatase expression will lead to an increase in the local concentrations of estrogen in subcutaneous adipose and in breast adipose in particular. This will result in a stimulation of proliferation of breast cancer cells that are in the process of developing. Indeed, it has been shown that tumors are most likely to be found in regions of the breast that have the highest aromatase expression and activity (5, 45). Once a tumor is established, it will produce PGE_2 and probably other factors that stimulate aromatase expression via PII. The PGE_2 will also inhibit LKB1 and AMPK expression, as we have described, as well as inhibit AMPK activity by phosphorylation at the inhibitory site α1 S485, thus potentially further enhancing aromatase expression. The net effect of all of these reactions is to optimize conditions within the breast, which maximize the stimulation of aromatase expression and hence estrogen formation and breast cancer proliferation.

Taken together, we believe that these observations provide a comprehensive explanation for the observed effects of obesity to increase breast cancer risk. They also point to strategies of therapeutic intervention to inhibit aromatase expression within the breast and breast cancer proliferation. Targets for such intervention could be at multiple sites such as subunits of LKB1 and AMPK, and also liver receptor homologue-1, which we are currently investigating as a potential breast-specific therapeutic target. Currently phase III aromatase inhibitors are proven superior to tamoxifen as endocrine therapy for breast cancer; however, because these compounds inhibit the catalytic activity of aromatase, they inhibit the enzyme in all body sites, leading to contraindications such as bone loss, arthralgia, and possibly cognitive defects. Breast-specific inhibition of aromatase expression in the postmenopausal woman via these targets is possible because PII is not used in bone and brain, and the ovaries cease to make estrogens at the time of menopause. Finally, because most obese individuals find it difficult or impossible to permanently reduce weight via diet and exercise, such therapeutic intervention may offer the best hope, at least in the short term, for preventing the obesity pandemic developing into a breast cancer epidemic.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Subcellular Localization of Cyclic AMP-Responsive Element Binding Protein-Regulated Transcription Coactivator 2 Provides a Link between Obesity and Breast Cancer in Postmenopausal Women

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