The Prostaglandin Transporter Regulates Adipogenesis and Aromatase Transcription

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Abstract

Cytochrome P450 aromatase, encoded by the CYP19 gene, catalyzes estrogen synthesis. In obese postmenopausal women, increased estrogen synthesis in adipose tissue has been linked to hormone-dependent breast carcinogenesis. Hence, it is important to elucidate the mechanisms that regulate CYP19 gene expression. Prostaglandin E2 (PGE2) stimulates the cyclic AMP (cAMP) → protein kinase A (PKA) → cAMP responsive element binding protein (CREB) pathway leading to increased CYP19 transcription. The prostaglandin transporter (PGT) removes PGE2 from the extracellular milieu and delivers it to the cytosol, where it is inactivated. The main objective of this study was to determine whether PGT regulates CYP19 transcription. Silencing of PGT in preadipocytes increased PGE2 levels in the extracellular medium, thereby stimulating the cAMP → PKA pathway resulting in enhanced interaction between pCREB, p300, and the CYP19 I.3/II promoter. A reciprocal decrease in the interaction between the CYP19 I.3/II promoter and BRCA1, a repressor of CYP19 transcription, was observed. Overexpressing PGT reduced extracellular PGE2 levels, suppressed the cAMP → PKA pathway, enhanced the interaction between BRCA1 and p300, and inhibited aromatase expression. We also compared the PGT → aromatase axis in preadipocytes versus adipocytes. Aromatase levels were markedly increased in preadipocytes versus adipocytes. This increase in aromatase was explained, at least in part, by reduced PGT levels leading to enhanced PGE2 signaling. In addition to regulating aromatase expression, PGT-mediated changes in extracellular PGE2 levels were a determinant of adipocyte differentiation. Collectively, these results suggest that PGT modulates adipogenesis and thereby PGE2-mediated activation of the cAMP → PKA → CREB pathway leading to altered CYP19 transcription and aromatase activity. Cancer Prev Res; 4(2); 194–206. ©2011 AACR.

Introduction

Obese postmenopausal women are at increased risk of developing hormone receptor–positive breast cancer (1). Approximately two thirds of patients with breast cancer have tumors that express estrogen receptors and require estrogen for tumor growth. After menopause, peripheral aromatization of androgen precursors in adipose tissue is largely responsible for estrogen production (2). Estrogens are synthesized from androgens in a reaction catalyzed by cytochrome P450 aromatase (aromatase), encoded by the CYP19 gene. Thus, the increased risk of hormone receptor–positive breast cancer in obese postmenopausal women is believed to be attributable, in part, to elevated levels of circulating estradiol related to both increased adipose tissue and aromatase upregulation in adipose tissue (3–6).

Given the significance of estrogen synthesis in the pathogenesis of hormone-dependent breast cancer, intense efforts have been made to elucidate the mechanisms that regulate the transcription of CYP19 (7). Several groups have carried out studies pointing to the significance of prostaglandin E2 (PGE2) as an inducer of aromatase. PGE2 stimulates cyclic AMP (cAMP)-dependent signaling leading to enhanced CYP19 transcription (8–10). COX catalyzes the first step in the synthesis of PGE2 from arachidonic acid. Positive correlations have been detected between COX and aromatase expression in human breast cancer specimens (11–13). In mice that express a mammary-targeted COX-2 transgene, increased PGE2 and aromatase levels were observed (14). Silencing of 15-hydroxyprostaglandin dehydrogenase (15-PGDH), the key enzyme responsible for inactivating PGE2, upregulated aromatase (15). Recently, the tumor suppressor BRCA1 was found to negatively regulate CYP19 expression (10, 16–18). This inhibitory effect of BRCA1 on aromatase expression was relieved by PGE2. Finally, two observational studies found that the use...
of aspirin, an inhibitor of PGE$_2$ production, was associated with a reduced risk of hormone receptor–positive breast cancer (19, 20).

Levels of PGs depend on transport in addition to synthesis and catabolism. The prostaglandin transporter (PGT) removes PGE$_2$ from the extracellular environment and thereby prevents its interaction with plasma membrane receptors (EP) for PGE$_2$ (21). This transporter is a member of the organic anion superfamily of transporting polypeptides that contain 12-transmembrane spanning domains (22). PGT is rate limiting in the delivery of PGE$_2$ to cytosolic 15-PGDH, which results in its oxidation and inactivation (23, 24). Although numerous studies have shown the significance of enzymes involved in the synthesis and catabolism of PGE$_2$ as determinants of CYP19 transcription and aromatase activity, the potential importance of PGT in regulating aromatase expression is unknown. Hence, the primary objective of this study was to evaluate whether changes in the expression of PGT modulate CYP19 transcription. Preadipocytes contain much higher levels of aromatase than mature adipocytes and were used as a model system (25). Here, we show that PGT regulates adipogenesis and thereby the PGE$_2$ → cAMP → protein kinase A (PKA) → cAMP responsive element binding protein (CREB) pathway leading, in turn, to changes in CYP19 transcription and aromatase activity. Reciprocal changes in the interaction between BRCA1, p300, and the CYP19 I.3/II promoter contribute to this effect of PGT.

Materials and Methods

Materials

Medium to grow visceral preadipocytes was purchased from ScienCell Research Laboratories. Differentiating medium was obtained from ZenBio. FBS was purchased from Invitrogen. Rabbit polyclonal antisera for human phospho-CREB (pCREB), CREB, p300, BRCA1, β-actin, and control IgG were from Santa Cruz Biotechnology. Lowry protein assay kits, horseradish peroxidase–conjugated secondary antibody, glucose-6-phosphate, glycerol, pepstatin, leupeptin, glucose-6-phosphate dehydrogenase, and rRNA probes were labeled with [$^{32}$P]CTP by random priming. The resolved proteins were transferred onto nitrocellulose membrane was then incubated with primary antisera. Secondary antibody to IgG conjugated to horseradish peroxidase was used. The blot was probed with the ECL Western blotting detection reagents from Amersham Biosciences. Nitrocellulose membranes were purchased from Schleicher & Schuell. 1β-[$^{3}H$]androstenedione and [$^{32}$P]CTP were from Perkin-Elmer Life Science. pSVgal and plasmid DNA isolation kits were purchased from Promega. Luciferase assay reagents were from Analytical Luminescence. The PGT, BRCA1, aromatase, progesterone receptor (PR), aP2, and β-actin cDNAs were obtained from Open Biosystems. The 18S rRNA cDNA was purchased from Ambion. siRNAs [PGT, green fluorescent protein (GFP)] and RNeasy mini kits were purchased from Qiagen. Adipogenesis and chromatin immunoprecipitation (ChIP) assay kits were purchased from Millipore. MuLV reverse transcriptase, RNase inhibitor, oligo-(dT)$_{16}$, and SYBR green PCR master mix were obtained from Applied Biosystems. Luciferase assay substrates and cell lysis buffer were from BD Biosciences. The CYP19 L.3/II promoter-luciferase construct was kindly provided by Dr. S. Chen (City of Hope, Duarte, CA). The estrogen response element (ERE)-luciferase construct was from Panomics.

Cell culture and Oil Red O staining

Human visceral preadipocytes were obtained from ScienCell Research Laboratories. These primary cells were grown in preadipocyte medium containing 10% FBS. Preadipocytes were grown in differentiation medium for 48 hours to produce mature adipocytes. Oil Red O staining of lipid vesicles was carried out and quantified to confirm the differentiation state of adipocytes. As detailed by the manufacturer (Millipore), the absorbance of extracted Oil Red O was measured in a plate reader at 490 nm.

PGE$_2$ production

Cells were plated in 6-well dishes and grown to 60% confluence in growth medium. The amount of PGE$_2$ in cell culture medium was measured by EIA. Levels of PGE$_2$ were normalized to protein concentrations.

Western blotting

Lysates were prepared by treating cells with lysis buffer [150 mmol/L NaCl, 100 mmol/L Tris (pH 8.0), 1% Tween 20, 50 mmol/L diethyldithiocarbamate, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 10 µg/mL aprotinin, 10 µg/mL trypsin inhibitor, and 10 µg/mL leupeptin]. Lysates were sonicated for 20 seconds on ice and centrifuged at 10,000 × g for 10 minutes to sediment the particulate material. The protein concentration of the supernatant was measured by the method of Lowry and colleagues (26). SDS-PAGE was performed under reducing conditions according to the procedure of Laemmli (27). The resolved proteins were transferred onto nitrocellulose sheets as detailed by Towbin and colleagues (28). The nitrocellulose membrane was then incubated with primary antisera. Secondary antibody to IgG conjugated to horse-radish peroxidase was used. The blot was probed with the ECL Western blot detection system according to the instructions of the manufacturer.

Northern blotting

Total RNA was isolated from cell monolayers, using an RNA isolation kit from Qiagen. Ten micrograms of total RNA per lane was electrophoresed in a formaldehyde-containing 1% agarose gel and transferred to nylon-supported membranes. Aromatase, PGT, BRCA1, PR, aP2, β-actin, and 18S rRNA probes were labeled with [$^{32}$P]CTP by random priming. The blots were probed as described previously (14).

Real-time PCR

Total RNA was isolated using the RNeasy mini kit. One microgram of RNA was reversed transcribed using murine
leukemia virus reverse transcriptase and oligo-(dT)\textsubscript{16} primer. The resulting cDNA was then used for amplification. The volume of the PCR was 20 \muL and contained 5 \muL of cDNA with the following primers: for aromatase mRNA, the forward and reverse primers were 5’-CACATCTCAA-TACCAGCTCC-3’ and 5’-CAGAGATCCAGACTCG CATTG-3’; for BRCA1, the forward and reverse primers were 5’-AGCCAGCCACAGGTACAGAG-3’ and 5’-AGTAGCCACAGCATGAGAAGGAC-3’; for aP2, the forward and reverse primers were 5’-TGGATGATCATGTTAGGTTTGGC and TGG AAACCTTTCTCCAGTGA. Real-time PCR was performed using 2x SYBR green PCR master mix on a 7900 HT real-time PCR system (Applied Biosystems) with 2\beta-actin (forward, 5’-AGAAAATCTGCGACCACAACC-3’; reverse, 5’-AGAGCCTACAGGATAGCA-3’) serving as an endogenous normalization control. Relative fold induction was determined using the C\textsubscript{T} (relative quantification) analysis protocol.

**cAMP levels**

Cells were plated at 5 \times 10^4 per well in 6-well dishes and grown to 60% to 70% confluence before treatment. Amounts of cAMP were measured by EIA. Production of cAMP was normalized to protein concentration.

**PKA activity**

Cells were plated at 5 \times 10^4 per well in 6-well dishes and grown to 60% to 70% confluence before treatment. PKA activity was measured according to the instructions of the manufacturer and was normalized to protein concentration.

**Aromatase activity**

To determine aromatase activity, microsomes were prepared from cell lysates by differential centrifugation using established methods (14). To determine aromatase activity, microsomal protein was added to a 0.5 mL reaction mixture containing 50 mmol/L Tris-HCl (pH 7.5), 5 mmol/L MgCl\textsubscript{2}, 5 mmol/L glucose-6-phosphate, 5 U glucose-6-phosphate dehydrogenase, 2 mmol/L rotenone, and 12.5 mmol/L 1\beta-[\textsuperscript{3}H]androstenedione. Following preincubation for 3 minutes, the reaction was initiated by the addition of 0.5 mmol/L NADPH and allowed to run for up to several hours at 37°C. Adding 3 mL ice-cold chloroform and applying vigorous shaking and brief centrifugation terminated the reaction. The resulting aqueous layer was further extracted with 3 mL chloroform and treated with 0.5 mL 5% activated charcoal/0.5% dextran. Following centrifugation of the mixture, the radioactivity in the supernatant was counted. Aromatase activity was measured according to the instructions of the manufacturer and was normalized to protein concentration.

**Transient transfections**

Cells were seeded at a density of 5 \times 10^4 per well in 6-well dishes and grown to approximately 50% confluence. For each well, 2 \muL of plasmid DNA were introduced into cells, using the Amaxa system. After 24 hours of incubation, the medium was replaced with basal medium. The activities of luciferase and b-galactosidase were measured in cellular extract. Preadipocytes overexpressing PGT or control vector were obtained by transfecting PGT expression vector or control vector, using the Amaxa system. Following transfection, cells that could grow in puromycin (10 \mug/mL) were selected and used.

**ChiP assay**

ChiP assay was performed with a kit according to the manufacturer’s instructions. A total 2 \times 10^6 cells were cross-linked in a 1% formaldehyde solution for 10 minutes at 37°C. Cells were then lysed in 200 \muL of SDS buffer and sonicated to generate 200- to 1,000-bp DNA fragments. After centrifugation, the cleared supernatant was diluted 10-fold with ChiP buffer and incubated with 1.5 \mug of the indicated antibody at 4°C. Immune complexes were precipitated, washed, and eluted as recommended. DNA-protein cross-links were reversed by heating at 65°C for 4 hours, and the DNA fragments were purified and dissolved in 50 \muL of water. Ten microliters of each sample was used as a template for PCR amplification. CYP19 oligonucleotide sequences for PCR primers were as follows: forward, 5’-AACCTGCTGATGAAATCTGACGAGGATAC-3’; reverse, 5’-TGACAGATTACGCTGAC-3’. This primer set encompasses the CYP19 I.3/II promoter segment from nucleotide –302 to –38. PCR was performed at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds for 30 cycles. The PCR products generated from the ChiP template were sequenced, and the identity of the CYP19 promoter was confirmed. For real-time PCR analysis, ChiP-qPCR assay kits from Superarray Bioscience Corp. were used. Real-time PCR was performed as described previously.

**Statistics**

Comparisons between groups were made by Student’s \textit{t} test. A difference between groups of \textit{P} < 0.05 was considered significant. All experiments were carried out a minimum of 3 times. Representative data are shown.

**Results**

**PGT regulates aromatase expression in human preadipocytes**

Initially, we determined the effect of silencing PGT on levels of PGE\textsubscript{2} in the cell culture medium. As shown in Figure 1A, silencing of PGT led to approximately a 1-fold increase in PGE\textsubscript{2} levels (Fig. 1A). This increase in PGE\textsubscript{2} was accompanied by a corresponding increase in both aromatase expression and activity (Fig. 1B and C). To further investigate the significance of PGT as a determinant of aromatase expression, it was overexpressed in preadipocytes. Overexpression of PGT led to reduced levels of PGE\textsubscript{2} in the cell culture medium, decreased aromatase expression, and reduced aromatase activity (Fig. 1D–F).
Figure 1. PGT regulates aromatase expression. A–C, human preadipocytes were transfected with 2 μg of siRNAs to GFP (control) or PGT and allowed to grow for 36 hours prior to analysis. A, levels of PGE2 in the cell culture medium were determined by enzyme immunoassay. Means ± SD are shown; n = 6. *, P < 0.01 compared with control. Inset, Northern blotting was done using 10 μg total RNA per lane; the blot was probed for PGT and β-actin. B, total RNA was prepared from cells and levels of aromatase mRNA were determined by real-time PCR. Values were normalized to the expression levels of β-actin. Means ± SD are shown; n = 6. *, P < 0.01 compared with control. Inset, Northern blot analysis was done on 10 μg total RNA per lane and the blot was probed for aromatase and β-actin. C, aromatase activity was determined using microsomes prepared from cell lysates as in "Materials and Methods." Enzyme activity is expressed as fmol/μg protein/min. Means ± SD are shown; n = 6. *, P < 0.01 compared with control. Inset, Northern blot analysis was done using 10 μg of RNA per lane; the blot was probed for PGT and β-actin. D–F, PGT or control vector were overexpressed in human preadipocytes. D, levels of PGE2 in the cell culture medium were determined by enzyme immunoassay. Means ± SD are shown; n = 6. *, P < 0.001 compared with control. Inset, Northern blot analysis was done using 10 μg of RNA per lane; the blot was probed for PGT and β-actin. E, total RNA was prepared from cells, and levels of aromatase mRNA were determined by real-time PCR. Values were normalized to the expression levels of β-actin. Means ± SD are shown; n = 6. *, P < 0.01 compared with control. Inset, Northern blot analysis was done using 10 μg of RNA per lane; the blot was probed for aromatase and β-actin. F, aromatase activity was determined using microsomes prepared from cell lysates as in "Materials and Methods." Enzyme activity is expressed as fmol/μg protein/min. Means ± SD are shown; n = 6. *, P < 0.01 compared with control.
Signal transduction pathway by which PGT regulates CYP19 transcription

PGT is known to stimulate the cAMP → PKA → CREB pathway leading to induction of CYP19 gene expression and increased aromatase activity (10). Hence, we next evaluated the importance of PGT as a modulator of this signal transduction pathway. Silencing PGT increased levels of cAMP and PKA activity (Fig. 2A and B). Conversely, overexpressing PGT led to a significant reduction in cAMP levels and PKA activity (Fig. 2C and D). We next evaluated whether changes in PGT expression modulated aromatase promoter activity. As shown in Figure 2E, silencing PGT stimulated aromatase promoter activity. In contrast, overexpressing PGT inhibited aromatase promoter activity (Fig. 2F). ChIP assays were performed to evaluate whether changes in the expression of PGT modulated the binding of pCREB to the CYP19 promoter. Silencing PGT stimulated the phosphorylation of CREB and the recruitment of pCREB to the CYP19 promoter (Fig. 2G); overexpression of PGT inhibited the phosphorylation of pCREB and its recruitment to the CYP19 promoter (Fig. 2H).

Recently, PGE2 was found to suppress levels of BRCA1, which contributed, in turn, to enhanced CYP19 transcription and increased aromatase activity (10, 17). Therefore, we attempted to determine whether modulating PGT levels would impact on BRCA1 levels. Silencing PGT suppressed BRCA1 levels (Fig. 3A), whereas overexpressing PGT induced BRCA1 (Fig. 3B). ChIP assays were carried out to explore the effects of PGT on the interaction between BRCA1 and the CYP19 promoter. Silencing PGT suppressed the interaction between BRCA1 and the CYP19 promoter (Fig. 3C), whereas overexpressing PGT stimulated the interaction between BRCA1 and the CYP19 promoter (Fig. 3D). Because p300 is important for pCREB-dependent activation of aromatase transcription, the interaction between the CYP19 promoter and p300 was also investigated. Silencing PGT increased the interaction between p300 and the CYP19 promoter (Fig. 3E). In contrast, overexpressing PGT reduced the recruitment of p300 to the CYP19 promoter (Fig. 3F). To further understand the role of PGT in regulating the transcription of aromatase, we also investigated the interactions between BRCA1, p300, and pCREB under basal conditions and following silencing or overexpression of PGT. In control cells, immunoprecipitation experiments suggested that p300 and BRCA1 were in a complex (Fig. 3G and H). Following silencing of PGT, p300 and pCREB were in the complex but BRCA1 was not found (Fig. 3G). In contrast, overexpression of PGT stimulated the interaction between p300 and BRCA1 but pCREB was not found in the complex (Fig. 3H).

Because aromatase activity can be rate limiting for the synthesis of estradiol, we also evaluated the role of PGT as a determinant of estrogen-dependent gene expression. The PR, an estrogen target gene, is positively regulated by an ERE (29, 30). As shown in Supplementary Figure 1, silencing PGT stimulated ERE-luciferase activity and induced PR (Supplementary Fig. S1A and B). Overexpression of PGT suppressed ERE-luciferase activity (Supplementary Fig. S1C) and reduced levels of PR (Supplementary Fig. S1D). Collectively, these results indicate that PGT modulates PGE2-mediated activation of the cAMP → PKA → CREB pathway and thereby regulates aromatase transcription and estrogen-dependent gene expression in preadipocytes.

PGT regulates adipogenesis resulting in changes in aromatase expression

As mentioned earlier, aromatase expression is known to be markedly higher in preadipocytes than in adipocytes (25). On the basis of our finding that PGT regulates aromatase expression, we next investigated whether the difference in aromatase levels in preadipocytes versus adipocytes could be explained at least, in part, by differences in PGT levels. Differentiating medium was used to convert human preadipocytes to adipocytes. aP2 is a marker of differentiated adipocytes. Higher levels of aP2 were found in adipocytes than preadipocytes, confirming the differentiation state of the cells (Fig. 4A, inset). Levels of PGT mRNA were markedly increased in adipocytes compared with preadipocytes (Fig. 4A). Consistent with this finding, levels of PGE2 were approximately 4-fold lower in the cell culture medium of adipocytes than in preadipocytes (Fig. 4B). Given the evidence that PGE2 regulates the cAMP → PKA → CREB signal transduction pathway leading to changes in aromatase transcription, we next evaluated the impact of differentiation on this axis. Reduced levels of cAMP, PKA activity, pCREB, and aromatase were detected in adipocytes versus preadipocytes (Fig. 4C–G). Levels of PR, an estrogen-regulated gene, were also reduced in adipocytes (Fig. 4H). On the basis of our findings in preadipocytes that were engineered to overexpress PGT, we also investigated whether the transcription machinery that controls aromatase expression was altered in adipocytes versus preadipocytes. Here, we show reduced binding of pCREB, increased BRCA1 expression and binding, and decreased recruitment of p300 to the CYP19 1.3/II promoter in adipocytes versus preadipocytes (Fig. 5A–D). The interaction between p300 and BRCA1 was increased, with a reciprocal decrease in the interaction between p300 and pCREB in adipocytes versus preadipocytes (Fig. 5E). Taken together, these results suggest that the higher levels of PGT in adipocytes contribute to the reduced expression of aromatase in these cells compared with preadipocytes.

PGE2 suppresses adipocyte differentiation, the process by which preadipocytes become mature adipocytes (31, 32). It is possible, therefore, that PGT regulates PGE2 levels in the extracellular milieu and thereby adipogenesis and aromatase levels. Our finding that levels of PGT are reduced in preadipocytes versus adipocytes with a reciprocal increase in PGE2 levels in the cell culture medium of preadipocytes (Fig. 4A and B) is consistent with this possibility. The conversion of preadipocytes to adipocytes is associated with increased triglyceride levels in addition to elevated aP2 levels. To interrogate the potential importance of PGT in regulating adipogenesis, siRNA was used. Silencing of PGT in preadipocytes markedly inhibited the
Figure 2. PGT modulates the cAMP → PKA → aromatase pathway in preadipocytes. A and B, preadipocytes were transfected with 2 μg of siRNAs to GFP (control) or PGT and allowed to grow for 36 hours prior to analysis. C and D, control vector or PGT were overexpressed. A and C, cellular levels of cAMP were determined. B and D, PKA activity was determined. Means ± SD are shown; n = 6. *, P < 0.01 compared with control. E, preadipocytes were transfected with 0.9 μg of CYP19 promoter-luciferase and 0.9 μg of control siRNA or PGT siRNA. All cells also received 0.2 μg of pSVβgal. Thirty-six hours after transfection, aromatase promoter activity was measured. F, preadipocytes that overexpressed PGT or control vector were transiently transfected with 1.8 μg of CYP19 promoter-luciferase and 0.2 μg of pSVβgal. Following transfection, cells were harvested and aromatase promoter activity was measured. E and F, luciferase activity was measured in cell lysates, and the activities represent data that have been normalized to β-galactosidase activity. Means ± SD are shown; n = 6. *, P < 0.01 compared with control. G and H, ChIP assays were performed. G, cells were transfected with 2 μg of siRNAs to GFP (control) or PGT and allowed to grow for 36 hours prior to analysis. H, control vector or PGT were overexpressed in preadipocytes and then subjected to analysis. G and H, chromatin fragments were immunoprecipitated with antibodies against pCREB and the CYP19 1.3/II promoter was amplified by real-time PCR. DNA sequencing was carried out, and the PCR product was confirmed to be the CYP19 1.3/II promoter, which was not detected when normal IgG was used or antibody was omitted from immunoprecipitation step (data not shown). Means ± SD are shown; n = 3. *, P < 0.01. Insets (G and H), Western blotting was performed using 100 μg of protein per lane and the blots were probed with antibodies to pCREB and CREB.
ability of adipocyte differentiating medium to stimulate triglyceride accumulation and induce aP2 expression (Fig. 6A and B). Differentiation of adipocytes also led to reduced amounts of PGE2 in the cell culture medium, an effect that was blocked by silencing of PGT (Fig. 6C). In this context, it was important to carry out additional experiments to further evaluate the importance of extracellular PGE2 in regulating adipocyte differentiation. Treatment of preadipocytes with exogenous PGE2 suppressed the ability of differentiating medium to stimulate triglyceride accumulation and induce aP2 expression (Fig. 6A and B). Differentiation of adipocytes also led to reduced amounts of PGE2 in the cell culture medium, an effect that was blocked by silencing of PGT (Fig. 6C).
PGT Regulates Adipogenesis and Aromatase Transcription

Figure 4. Differentiation of preadipocytes induces PGT, leading to reduced aromatase expression. A, total RNA was prepared from cells and levels of PGT mRNA were determined by real-time PCR. Values were normalized to the expression levels of β-actin. Inset, Northern blotting was done using 10 μg of total RNA per lane; the blot was probed for αP2 and β-actin. PA, preadipocytes; A, adipocytes. B, levels of PGE2 in the cell culture medium were determined by enzyme immunoassay. C, cellular levels of cAMP were determined. D, PKA activity was determined. E, Western blotting was performed using 100 μg of protein per lane, and the blot was probed with antibodies to pCREB and CREB. F, Northern blot analysis was done using 10 μg of RNA per lane; the blot was probed for aromatase and β-actin. G, aromatase activity was determined using microsomes prepared from cell lysates as in “Materials and Methods.” Enzyme activity is expressed as fmol/μg protein/min. H, Northern blotting was performed using 10 μg of total RNA per lane. The blot was probed for the PR and 18S rRNA. A–D and G, means ± SD are shown; n = 6. *, P < 0.01 compared with preadipocytes.

Trygliceride accumulation and αP2 expression, hallmarks of adipogenesis (Fig. 6D and E). Thus, either silencing PGT or treatment of preadipocytes with exogenous PGE2 suppressed the ability of differentiating medium to stimulate adipogenesis. Treatment with PGE2 also suppressed the increase in PGT levels associated with adipocyte differentiation (Fig. 6F). Taken together, our data suggest that PGT is a critical determinant of extracellular levels of PGE2 and thereby adipogenesis and aromatase expression.
Obese postmenopausal women are at increased risk of developing hormone receptor–positive breast cancer (33, 34). This increased risk has been attributed, in part, to elevated levels of circulating and tissue estrogen related to both increased adipose tissue mass and enhanced aromatase expression (3–6). Previous studies indicate that PGE2 can stimulate transcription resulting in elevated aromatase levels (8–10, 14, 35, 36). The induction of aromatase by PGE2 seems to be mediated, in part, by suppression of BRCA1, a repressor of CYP19 transcription (10, 17). Given the link between estrogen synthesis and the development and progression of hormone receptor–positive breast cancer (37, 38), we have attempted to further elucidate the mechanisms that control the activation of CYP19 transcription by PGE2.

Extracellular PGE2 exerts its actions via cell surface G protein–coupled receptors (EP receptors) that activate a number of signaling cascades (39). PGE2 via EP2 and EP4 activates the cAMP → PKA → CREB pathway leading to enhanced CYP19 transcription and increased aromatase activity (10). Extracellular PGE2 can be transported via PGT into the cell, where it is inactivated by cytosolic 15-PGDH (24). Here, we show that changes in the levels of PGT modulate extracellular levels of PGE2, which lead, in turn, to changes in CYP19 transcription and aromatase activity. These findings highlight the importance of prostaglandin transport into the cell as a determinant of local PGE2 levels and aromatase expression. Because changes in the levels of PGT led to changes in aromatase activity, we...
Figure 6. PGT regulates adipogenesis. A–C, the bar labeled Vehicle represents human preadipocytes treated with preadipocyte medium containing vehicle for 48 hours; the bar labeled Diff. Med. represents preadipocytes treated with differentiating medium for 48 hours; bars labeled Diff. Med. + Control siRNA or Diff. Med. + PGT siRNA represent preadipocytes that were transfected with 2 μg of siRNAs to GFP (Control) or PGT before receiving differentiating medium for 48 hours. A, top, total RNA was prepared from cells. The Northern blot was probed for PGT and β-actin. Bottom, cells were stained with Oil Red O and dye was extracted. Absorbance of extracted dye was measured at 490 nm. Means ± SD are shown; n = 6. *P < 0.01 compared with cells treated with control siRNA. B, total RNA was prepared from cells and levels of aP2 mRNA were determined by real-time PCR. Values were normalized to the expression levels of β-actin. Means ± SD are shown; n = 6. *P < 0.01 compared with control. C, levels of PGE2 in the cell culture medium were determined by enzyme immunoassay. Means ± SD are shown; n = 6. *P < 0.01 compared with cells treated with control siRNA. D–F, bars labeled Vehicle represent cells that received preadipocyte medium containing vehicle for 48 hours; bars labeled Diff. Med. represent cells that received differentiating medium for 48 hours; bars labeled Diff. Med. + PGE2 represent cells that received differentiating medium containing either 250 nmol/L or 500 nmol/L PGE2 for 48 hours. D, cells were stained with Oil Red O and dye was extracted. Absorbance of extracted dye was measured at 490 nm. Means ± SD are shown; n = 6. *P < 0.01 compared with cells treated with differentiating medium alone. E, total RNA was prepared from cells and levels of aP2 mRNA were determined by real-time PCR. Values were normalized to the expression levels of β-actin. Means ± SD are shown; n = 6. *P < 0.01 compared with cells treated with differentiating medium. F, total RNA was prepared from cells. The Northern blot was probed for PGT and β-actin.
also investigated the role of PGT as a determinant of estrogen-dependent gene expression. Silencing PGT increased extracellular levels of PGE₂, stimulated aromatase activity, and induced the expression of the PR, a prototypic estrogen response gene. Consistent with these findings, overexpressing PGT led to reduced extracellular levels of PGE₂, decreased aromatase activity, and downregulation of PR.

Because PGE₂ activates the cAMP → PKA → CREB pathway leading to enhanced CYP19 transcription, we investigated whether changes in the levels of PGT affected this signal transduction pathway. Consistent with the change in extracellular PGE₂ levels, silencing PGT induced cAMP levels and PKA activity; overexpressing PGT led to reduced cAMP levels and PKA activity. Furthermore, silencing PGT stimulated the binding of pCREB to the CYP19 I.3/II promoter whereas binding of pCREB to the CYP19 I.3/II promoter was suppressed when PGT was overexpressed. The tumor suppressor BRCA1 plays a significant role in repressing aromatase expression (10, 16–18, 40). BRCA1 binds directly to the CYP19 I.3/II promoter region and suppresses transcription (17). Agents including PGE₂ that stimulate cAMP signaling suppress BRCA1 levels resulting in enhanced CYP19 transcription (17, 41). We extend upon these findings and show that PGT is a determinant of BRCA1 levels. Silencing PGT suppressed levels of BRCA1 and caused a decrease in its interaction with the CYP19 I.3/II promoter. In contrast, overexpression of PGT resulted in both an increase in BRCA1 levels and an increase in its interaction with the CYP19 I.3/II promoter. To our knowledge, this is the first time that changes in the expression of any type of transporter have been linked to altered expression of BRCA1. Our data also support the notion that changes in the expression of BRCA1 in the stroma could be very important for the development of hormone-dependent cancers.

The coactivator CBP/p300, which possesses histone acetyltransferase activity (42), is also known to be important for PGE₂-mediated activation of CYP19 transcription (10). Previously, PGE₂ was found to stimulate the recruitment of p300 to the CYP19 I.3/II promoter. In addition, the interaction between p300 and pCREB was enhanced by treatment with PGE₂. In the current study, we present evidence that silencing PGT stimulated the recruitment of p300 to the CYP19 I.3/II promoter. Importantly, the interaction between p300 and pCREB was enhanced whereas that between p300 and BRCA1 was reduced by silencing PGT. In contrast, overexpressing PGT inhibited the recruitment of p300 to the CYP19 I.3/II promoter and stimulated the interaction between p300 and BRCA1.

Previously, levels of aromatase were reported to be significantly higher in preadipocytes than in adipocytes, a finding that we confirmed (25). PGE₂ can suppress adipogenesis, the process by which preadipocytes become mature adipocytes (32). Importantly, we found that levels of PGT were reduced in preadipocytes versus adipocytes with a reciprocal increase in PGE₂ levels in the cell culture medium. Collectively, these findings suggested the possibility that PGT may regulate adipogenesis leading, in turn, to changes in aromatase expression. To this end, we showed that silencing of PGT led to increased levels of PGE₂ in the cell culture medium and inhibition of adipocyte differentiation. The significance of this effect of PGT on PGE₂ levels was highlighted by our finding that the treatment of preadipocytes with exogenous PGE₂ also inhibited adipocyte differentiation. Finally, we explored the possibility that reduced levels of PGT in preadipocytes versus adipocytes could account for the significantly increased levels of aromatase in preadipocytes. The reduction in PGT levels in preadipocytes versus adipocytes led to an increased PGE₂ → cAMP → PKA signaling. Increased binding of pCREB, reduced binding of BRCA1, and increased recruitment of p300 to the CYP19 I.3/II promoter were found in preadipocytes versus adipocytes. These findings strongly suggest that the differentiation of preadipocytes to adipocytes leads to a change in the PGT → CYP19 axis, resulting in changes in aromatase levels. Notably, ligands of PPARγ stimulate adipocyte differentiation (43) and suppress aromatase expression (44). On the basis of these findings, we suggest that PGT may regulate adipogenesis and play a role in the regulation of aromatase expression. Therefore, further studies are needed to elucidate the role of PGT in regulating adipogenesis and aromatase expression.

Figure 7. Signal transduction pathway by which PGT regulates aromatase expression. PGT, a transporter containing 12-transmembrane spanning domains, mediates the rate-limiting step in PGE₂ signal termination. It carries PGE₂ from the extracellular environment across the plasma membrane to the cytoplasm where 15-PGDH oxidizes it to 15-keto PGE₂. Changes in amounts of PGT alter extracellular levels of PGE₂, which exerts its effects by binding to EP receptors, G protein–coupled receptors. PGE₂ via EP₂ and EP₄ activates the cAMP → PKA pathway resulting in enhanced interaction between pCREB, p300, and aromatase promoter I.3/II. PGE₂ also causes a decrease in amounts of BRCA1, a repressor of aromatase transcription, and reduced interaction between BRCA1 and the aromatase promoter I.3/II. The increased interaction between pCREB and p300 and the reduced interaction between BRCA1 and p300 contribute to enhanced aromatase transcription and increased aromatase activity. Levels of PGT are reduced in preadipocytes versus adipocytes, resulting in increased extracellular levels of PGE₂. This increase leads, in turn, to activation of the cAMP → PKA → CREB pathway resulting in enhanced aromatase transcription.
of our results, it will be worthwhile to determine whether these agents induce PGT and thereby suppress aromatase levels.

Previous studies have focused on the importance of enzymes involved in PGE₂ synthesis and catabolism as determinants of aromatase expression (10–15). This study is the first to suggest that prostaglandin transport is a determinant of CYP19 transcription and aromatase expression. Notably, the current findings could be important for understanding both normal physiology and disease. For example, PGT expression is modulated in epithelial and stromal cells of the human endometrium during the menstrual cycle (45). Our results suggest that changes in PGT levels will affect both prostaglandin levels and estrogen synthesis in the local microenvironment, which could be important for normal menstrual function. Treatment with lipopolysaccharide (LPS) downregulates PGT in both the lung and the liver (46), which could lead to increased aromatase levels and estrogen synthesis. This possibility should be explored, as estrogen antagonizes some of the proinflammatory effects of LPS (47). Levels of PGT are reduced in a variety of tumors including lung cancer (48). Future studies are warranted to determine whether reduced levels of PGT contribute to the elevated levels of aromatase found in some tumors (49). Given the importance of changes in expression of PGT, an influx transporter, on aromatase expression, it will also be worthwhile to determine whether multidrug resistance-associated protein 4, an efflux transporter, plays a role in regulating aromatase expression. Taken together, our data provide evidence that PGT-mediated delivery of PGE₂ from the extracellular milieu to the cytoplasm regulates adipoocyte differentiation and the cAMP → PKA → CREB pathway leading to changes in the interaction between pCREB, BRCA1, p500, and the CYP19 1.3/II promoter (Fig. 7). Agents that induce PGT should enhance PGE₂ uptake and catabolism, suppress aromatase activity, and thereby reduce the risk of hormone receptor–positive breast cancer, perhaps especially so among obese postmenopausal women.

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.

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The Prostaglandin Transporter Regulates Adipogenesis and Aromatase Transcription

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