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 cAMP→protein kinase A (PKA)→CREB pathway leading to increased CYP19 transcription. The PG 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 if 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 vs. adipocytes. Aromatase levels were markedly increased in preadipocytes vs. adipocytes. This increase in aromatase was explained at least, in part, by reduced PGT levels leading to enhanced PGE2→cAMP→PKA 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.
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, at least in part, to elevated levels of circulating estradiol related to both increased adipose tissue and up-regulation of aromatase 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 E$_2$ (PGE$_2$) as an inducer of aromatase. PGE$_2$ stimulates cAMP-dependent signaling leading to enhanced CYP19 transcription (8-10). Cyclooxygenases (COX) catalyze the first step in the synthesis of PGE$_2$ from arachidonic acid. Positive correlations have been detected between COX and aromatase expression in human breast cancer specimens (11-13). In mice which express a mammary-targeted COX-2 transgene, increased PGE$_2$ and aromatase levels were observed (14). Silencing of 15-hydroxyprostaglandin dehydrogenase (15-PGDH), the key enzyme responsible for inactivating PGE$_2$ up-regulated 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 PGE$_2$. Finally, two observational studies found that 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 super family 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 demonstrated 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)→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. Fetal bovine serum (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 rotenone were from Sigma. cAMP enzyme immunoassay kit was from Biomol. PKA activity assay kits were obtained from Calbiochem. PGE$_2$ and PGE$_2$ enzyme immunoassay assay (EIA) kits were purchased from Cayman Chemicals. ECL western blotting detection reagents were from Amersham Biosciences. Nitrocellulose membranes were from Schleicher & Schuell. 1ß-[$^3$H]-androstenedione and [$^{32}$P]CTP were from Perkin-Elmer Life Science. pSVβgal and plasmid DNA isolation kits were purchased from Promega. Luciferase assay reagents were from Analytical Luminescence. The 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 I.3/II promoter-luciferase construct was kindly
provided by Dr. S. Chen (City of Hope, Duarte, CA). The estrogen response element-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 six-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 et al. (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 et al. (28). The 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 blot detection system according to the instructions of the manufacturer.

**Northern blotting**

Total RNA was prepared from cell monolayers using an RNA isolation kit from Qiagen. 10 µg of total RNA/lane were electrophoreosed 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 [32P]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 µg RNA was reversed 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 aromatase mRNA, the forward and reverse primers were-5’-CACATCCTCAATACCAGGTCC-3’ and 5’-CAGAGATCCAGACTCATG-3’; for BRCA1, the forward and reverse primers were 5’-AGCCAGCCACAGGTACAGAG-3’ and 5’-AGTAGCCAGCAGTAGAAGGAC-3’; for aP2, the forward and reverse primers were 5’-TGATGATCATGTTAGTTTGGC and TGGAACTTGTCTCCAGTGAA. Real-time PCR was performed using 2x SYBR green PCR master mix on a 7900 HT real-time PCR system (Applied Biosystems) with β-actin (forward 5’-AGAAAAATCTGGCACCACACC-3’ and reverse 5’-
AGAGCGTACAGGGATAGCA-3’) serving as an endogenous normalization control. Relative fold induction was determined using the CT (relative quantification) analysis protocol.

**Cyclic AMP levels**

Cells were plated at 5 x 10⁴/well in six-well dishes and grown to 60-70% confluence before treatment. Amounts of cAMP were measured by EIA. Production of cAMP was normalized to protein concentration.

**Protein kinase A activity**

Cells were plated at 5 x 10⁴/well in six-well dishes and grown to 60-70% confluence before treatment. PKA activity was measured according to the instructions of the manufacturer. PKA activity 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 MgCl₂, 5 mmol/L glucose-6-phosphate, 5U glucose-6-phosphate dehydrogenase, 2 µmol/L rotenone, and 12.5 nmol/L 1β-[³H] androstenedione. Following preincubation for 3 minutes, the reaction was initiated by the addition of 0.5 µmol/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 quantified by measurement of the tritiated water released from 1ß-[^3H]-androstenedione. The reaction was also performed in the presence of a specific aromatase inhibitor, as a specificity control and without NADPH as a background control. Aromatase activity was normalized to protein concentration.

**Transient transfections**

Cells were seeded at a density of 5 x 10^4 per well in six-well dishes and grown to approximately 50% confluence. For each well, 2 µg 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 ß-galactosidase were measured in cellular extract. Preadipocytes overexpressing PGT or control vector were made by transfecting PGT expression vector or control vector using the Amaxa system. Following transfection, cells that were able to grow in puromycin (10 µg/mL) were selected and used.

**Chromatin immunoprecipitation assay**

ChiP assay was performed with a kit according to the manufacturer's instructions. 2 x 10^6 cells were cross-linked in a 1% formaldehyde solution for 10 minutes at 37°C. Cells were then lysed in 200 µL of SDS buffer and sonicated to generate 200-1000-bp DNA fragments. After centrifugation, the cleared supernatant was diluted 10-fold with ChIP buffer and incubated with 1.5 µg 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 µL of water. 10 µL of each sample were used as a template for PCR amplification. CYP19 oligonucleotide sequences for PCR primers were forward, 5’-AACCTGCTGATGAAGTCACAA-3’ and reverse, 5’-TCAGACATTTAGGCAAGACT-3’. This primer set encompasses the CYP19 promoter I.3/II
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 above.

Statistics

Comparisons between groups were made by Student’s \( t \) test. A difference between groups of \( p<0.05 \) was considered significant. All experiments were carried out a minimum of three 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\(_2\) in the cell culture medium. As shown in Fig. 1A, silencing of PGT led to approximately a one-fold increase in PGE\(_2\) levels (Fig. 1A). This increase in PGE\(_2\) was accompanied by a corresponding increase in both aromatase expression and activity (Fig. 1, B and C). To further investigate the significance of PGT as a determinant of aromatase expression, PGT was overexpressed in preadipocytes. Overexpression of PGT led to reduced levels of PGE\(_2\) in the cell culture medium, decreased aromatase expression and reduced aromatase activity (Fig. 1, D-F).

Signal transduction pathway by which PGT regulates CYP19 transcription

PGE\(_2\) 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. 2, A and B). Conversely, over expressing PGT led to a significant reduction in cAMP levels and PKA activity (Fig. 2, C and D). We next evaluated whether changes in PGT expression modulated aromatase promoter activity. As shown in Fig. 2E, silencing PGT stimulated aromatase promoter activity. In contrast, over expressing 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); over expression 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 over expressing 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 over expressing 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, over expressing 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 over expression of PGT. In control cells, immunoprecipitation experiments suggested that p300 and BRCA1 were in a complex (Fig. 3, G and H). Following silencing of PGT, p300 and pCREB were in the complex.
but BRCA1 was not found (Fig. 3G). In contrast, over expression 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 estrogen response element (ERE) (29, 30). As shown in Supplementary Fig. 1, silencing PGT stimulated ERE-luciferase activity and induced PR (Fig. S1, A and B). Over expression of PGT suppressed ERE-luciferase activity (Fig. S1C) and reduced levels of PR (Fig. S1D). Collectively, the above results indicate that PGT modulates PGE$_2$-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 above, aromatase expression is known to be markedly higher in preadipocytes compared to adipocytes (25). Based on our finding that PGT regulates aromatase expression, we next investigated if the difference in aromatase levels in preadipocytes vs. 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 PGE$_2$ were approximately 4-fold lower in the cell culture medium of adipocytes compared with preadipocytes (Fig. 4B). Given the evidence that PGE$_2$ 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 vs.
preadipocytes (Fig. 4, C-G). Levels of PR, an estrogen regulated gene, were also reduced in adipocytes (Fig. 4H). Based on 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 vs. preadipocytes. Here we demonstrate reduced binding of pCREB, increased BRCA1 expression and binding and decreased recruitment of p300 to the CYP19 1.3/II promoter in adipocytes vs. preadipocytes (Fig. 5, A-D). The interaction between p300 and BRCA1 was increased with a reciprocal decrease in the interaction between p300 and pCREB in adipocytes vs. 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.

PGE$_2$ suppresses adipocyte differentiation, the process by which preadipocytes become mature adipocytes (31, 32). It's possible, therefore, that PGT regulates PGE$_2$ levels in the extracellular milieu and thereby adipogenesis and aromatase levels. Our finding that levels of PGT are reduced in preadipocytes vs. adipocytes with a reciprocal increase in PGE$_2$ levels in the cell culture medium of preadipocytes (Fig. 4, A 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, small interfering RNA was used. Silencing of PGT in preadipocytes markedly inhibited the ability of adipocyte differentiating medium to stimulate triglyceride accumulation and induce aP2 expression (Fig. 6, A and B). Differentiation of adipocytes also led to reduced amounts of PGE$_2$ 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 PGE$_2$ in regulating adipocyte differentiation. Treatment of preadipocytes with exogenous PGE$_2$ suppressed the ability of differentiating medium to stimulate triglyceride accumulation and aP2 expression, hallmarks of adipogenesis (Fig. 6, D
and E). Thus, either silencing PGT or treatment of preadipocytes with exogenous PGE$_2$ suppressed the ability of differentiating medium to stimulate adipogenesis. Treatment with PGE$_2$ 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 PGE$_2$ and thereby adipogenesis and aromatase expression.

**Discussion**

Obese postmenopausal women are at increased risk of developing hormone receptor-positive breast cancer (33, 34). This increased risk has been attributed, at least 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 PGE$_2$ can stimulate CYP19 transcription resulting in elevated aromatase levels (8-10, 14, 35, 36). The induction of aromatase by PGE$_2$ appears 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 PGE$_2$.

Extracellular PGE$_2$ exerts its actions via cell surface G protein-coupled receptors (EP receptors) that activate a number of signaling cascades (39). PGE$_2$ via EP$_2$ and EP$_4$ activates the cAMP→PKA→CREB pathway leading to enhanced CYP19 transcription and increased aromatase activity (10). Extracellular PGE$_2$ can be transported via PGT into the cell, where it is inactivated by cytosolic 15-PGDH (24). Here we demonstrate that changes in the levels of PGT modulate extracellular levels of PGE$_2$ which lead, in turn, to changes in CYP19 transcription and aromatase activity. These findings highlight the importance of PG transport into the cell as a determinant of local PGE$_2$ levels and aromatase expression. Because changes in the levels of PGT led to changes in aromatase activity, we also investigated the role of PGT as a
determinant of estrogen-dependent gene expression. Silencing PGT increased extracellular levels of PGE$_2$, stimulated aromatase activity and induced the expression of the PR, a prototypic estrogen-response gene. Consistent with these findings, over expressing PGT led to reduced extracellular levels of PGE$_2$, decreased aromatase activity and down regulation of PR.

Because PGE$_2$ 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 PGE$_2$ levels, silencing PGT induced cAMP levels and PKA activity; over expressing 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 over expressed. The tumor suppressor BRCA1 plays a significant role in repressing aromatase expression (10, 16-18, 40). BRCA1 binds directly to the CYP19 1.3/II promoter region and suppresses transcription (17). Agents including PGE$_2$ 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, over expression of PGT resulted in both increased BRCA1 levels and an increase in its interaction with the CYP19 I.3/II promoter. To our knowledge, this is 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 PGE$_2$-mediated activation of CYP19 transcription (10). Previously, PGE$_2$ was found to stimulate the recruitment of p300 to the CYP19 I.3/II promoter. Additionally, the interaction between p300 and pCREB was enhanced by treatment with PGE$_2$. In the current
study, we present evidence that silencing PGT stimulated the recruitment of p300 to the \textit{CYP19} \textit{I.3/II} promoter. Importantly, the interaction between p300 and pCREB was enhanced whereas the interaction between p300 and BRCA1 was reduced by silencing PGT. In contrast, over expressing PGT inhibited the recruitment of p300 to the \textit{CYP19} \textit{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). PGE$_2$ can suppress adipogenesis, the process by which preadipocytes become mature adipocytes (32). Importantly, we found that levels of PGT were reduced in preadipocytes vs. adipocytes with a reciprocal increase in PGE$_2$ 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 PGE$_2$ in the cell culture medium and inhibition of adipocyte differentiation. The significance of this effect of PGT on PGE$_2$ levels was highlighted by our finding that treatment of preadipocytes with exogenous PGE$_2$ also inhibited adipocyte differentiation. Finally, we explored the possibility that reduced levels of PGT in preadipocytes vs. adipocytes could account for the significantly increased levels of aromatase in preadipocytes. The reduction in PGT levels in preadipocytes vs. adipocytes led to enhanced PGE$_2$$\rightarrow$ cAMP$\rightarrow$ PKA signaling. Increased binding of pCREB, reduced binding of BRCA1 and increased recruitment of p300 to the \textit{CYP19} \textit{I.3/II} promoter were found in preadipocytes vs. adipocytes. These findings strongly suggest that the differentiation of preadipocytes to adipocytes leads to a change in the PGT$\rightarrow$\textit{CYP19} axis resulting in changes in aromatase levels. Notably, ligands of PPAR$_\gamma$ stimulate adipocyte differentiation (43) and suppress aromatase expression (44). Based on 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 PGE\textsubscript{2} synthesis and catabolism as determinants of aromatase expression (10-15). This study is the first to suggest that PG 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 PG levels and estrogen synthesis in the local microenvironment, which could be important for normal menstrual function. Treatment with LPS down regulates PGT in the lung and liver (46), which could lead to increased aromatase levels and estrogen synthesis. This possibility should be explored since estrogen antagonizes some of the pro-inflammatory effects of LPS (47). Levels of PGT are reduced in a variety of tumors including lung cancer (48). Future studies are warranted to determine if 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 if 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 PGE\textsubscript{2} from the extracellular milieu to the cytoplasm regulates adipocyte differentiation and the cAMP→PKA→CREB pathway leading to changes in the interaction between pCREB, BRCA1, p300 and the CYP19 I.3/I.2 promoter (Fig. 7). Agents that induce PGT should enhance PGE\textsubscript{2} 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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Legends to Figures

Fig. 1. PGT regulates aromatase expression. In 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 PGE$_2$ in the cell culture medium were determined by enzyme immunoassay. Means
± S.D. are shown, n=6. *, p < 0.01 compared with control. Inset, northern blotting was done using 10 µg total RNA/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 ± S.D. are shown, n = 6. *, p < 0.01 compared with control. Inset, northern blot analysis was done on 10 µg total RNA/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/minute. Means ± S.D. are shown, n=6. *, p<0.01 compared with control. In 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 ± S.D. are shown, n=6. *, p < 0.001 compared with control. Inset, northern blot analysis was done using 10 µg of RNA/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 ± S.D. are shown, n = 6. *, p < 0.01 compared with control. Inset, northern blot analysis was done using 10 µg of RNA/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/minute. Means ± S.D. are shown, n=6. *, P<0.01 compared with control.

**Fig. 2.** PGT modulates the cAMP →PKA →aromatase pathway in preadipocytes. In 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. In 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 ± S.D. 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. In E and F, luciferase activity was measured in cell lysates, and the activities represent data that have been normalized to β-galactosidase activity. Means ± S.D. 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 I.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. The CYP19 I.3/II promoter was not detected when normal IgG was used or antibody was omitted from immunoprecipitation step (data not shown). Means ± S.D. are shown; n=3. *p <0.01. In the insets (G and H), western blotting was performed using 100 μg of protein/lane and the blots were probed with antibodies to pCREB and CREB.

**Fig. 3.** PGT modulates the interactions of p300 with BRCA1 and pCREB at the aromatase promoter. In A, C, E and G, preadipocytes were transfected with 2 μg of siRNAs to GFP (control) or PGT. In B, D, F and H, control vector or PGT expression vector were overexpressed; cells were then subjected to analysis. In A and B, total RNA was prepared from cells and levels of BRCA1 mRNA were determined by real-time PCR. Values were normalized to the expression levels of β-actin. Means ± S.D. are shown, n = 6. *, p < 0.01 compared with control. In the insets, northern blotting was done on 10 μg RNA/lane and the blot was probed for BRCA1 and β-actin. In C-F, ChIP assays were performed. Chromatin fragments were
immunoprecipitated with antibodies against BRCA1 (C, D) or p300 (E, F) and the CYP19 I.3/II promoter was amplified by real-time PCR. DNA sequencing was carried out, and the PCR product was confirmed to be the CYP19 I.3/II promoter. The CYP19 I.3/II promoter was not detected when normal IgG was used or antibody was omitted from immunoprecipitation step (data not shown). Means ± S.D. are shown; n=3. *p <0.01. G and H, 500 µg of cell lysate were subjected to immunoprecipitation with p300 antiserum and western blotting was performed for p300, BRCA1 and pCREB as indicated. These proteins were not immunoprecipitated with control IgG. Input is p300.

**Fig. 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/lane; the blot was probed for aP2 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/lane and the blot was probed with antibodies to pCREB and CREB. F, northern blot analysis was done using 10 µg of RNA/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/minute. H, northern blotting was performed using 10 µg of total RNA/lane. The blot was probed for the progesterone receptor (PR) and 18S rRNA. In panels A-D and G, means ± S.D. are shown, n = 6. *, p< 0.01 compared with preadipocytes.

**Fig. 5. Differentiation of preadipocytes to adipocytes modulates the composition of the transcription complex at the aromatase promoter.** A, chromatin fragments were

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immunoprecipitated with antibodies against pCREB and the CYP19 I.3/II promoter was amplified by real-time PCR. B, total RNA was prepared from cells and levels of BRCA1 mRNA were determined by real-time PCR. Values were normalized to the expression levels of β-actin. Means ± S.D. are shown, n = 6. *, p < 0.01. C, ChIP assays were performed. Chromatin fragments were immunoprecipitated with antibodies against BRCA1 and the CYP19 I.3/II promoter was amplified by real-time PCR. D, ChIP assays were performed. Chromatin fragments were immunoprecipitated with antibodies against p300 and the CYP19 I.3/II promoter was amplified by real-time PCR. In A, C and D, DNA sequencing was carried out, and the PCR product was confirmed to be the CYP19 1.3/II promoter. The CYP19 I.3/II promoter was not detected when normal IgG was used or antibody was omitted from immunoprecipitation step (data not shown). Means ± S.D. are shown; n=3. *p < 0.01 compared with preadipocytes. E, 500 µg of cell lysate were subjected to immunoprecipitation with p300 antiserum and western blotting was performed for p300, BRCA1 and pCREB as indicated. These proteins were not immunoprecipitated with control IgG. Input is p300.

**Fig. 6. PGT regulates adipogenesis.** In 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 prior to receiving differentiating medium for 48 hours. A (top panel), total RNA was prepared from cells. The northern blot was probed for PGT and β-actin. Bottom panel, cells were stained with Oil Red O and dye was extracted. Absorbance of extracted dye was measured at 490 nm. Means ± S.D. 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 ± S.D. 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 ± S.D. are shown, n=6. *, p < 0.01 compared with cells treated with control siRNA. In 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 ± S.D. 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 ± S.D. are shown, n = 6. *, p < 0.01 compared to cells treated with differentiating medium. F, total RNA was prepared from cells. The northern blot was probed for PGT and β-actin.

Fig. 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. PGE₂ 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 vs. 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.

Supplementary Fig. 1. PGT regulates estrogen receptor-dependent gene expression in preadipocytes. A, cells were transfected with 0.9 μg of ERE-luciferase and either 0.9 μg of siRNA to GFP (Control) or 0.9 μg of siRNA to PGT. Cells also received 0.2 μg of pSVβgal. B, cells were transfected with 2 μg of siRNAs to GFP (Control) or PGT. C, preadipocytes that overexpressed control vector or PGT were transiently transfected with 1.8 μg of ERE-luciferase and 0.2 μg pSVβgal. D, cells overexpressing control vector or PGT expression vector were utilized. In A and C, ERE luciferase activity represents data that have been normalized to β-galactosidase activity. Means ± S.D. are shown, n = 6. *, p < 0.01 compared with control. In B and D, northern blotting was performed using 10 μg of total RNA/lane. The blots were probed for the progesterone receptor (PR) and 18S rRNA.

References


Fig. 1
Fig. 2
Fig. 3
Fig. 5
Fig. 6
The Prostaglandin Transporter Regulates Adipogenesis and
Aromatase Transcription

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