Pioglitazone, a PPARγ Agonist, Suppresses CYP19 Transcription: Evidence for Involvement of 15-Hydroxyprostaglandin Dehydrogenase and BRCA1

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Abstract

Estrogen synthesis is catalyzed by cytochrome P450 aromatase, which is encoded by the CYP19 gene. In obese postmenopausal women, increased aromatase activity in white adipose tissue is believed to contribute to hormone-dependent breast cancer. Prostaglandin E2 (PGE2) stimulates the cAMP–protein kinase A (PKA) pathway leading to increased CYP19 transcription and elevated aromatase activity in inflamed white adipose tissue. 15-hydroxyprostaglandin dehydrogenase (15-PGDH) plays a major role in the catabolism of PGE2. Here, we investigated the mechanism by which pioglitazone, a ligand of the nuclear receptor PPARγ, suppressed aromatase expression. Treatment of human preadipocytes with pioglitazone suppressed Snail, a repressive transcription factor, resulting in elevated levels of 15-PGDH and reduced levels of PGE2 in the culture medium. Pioglitazone also inhibited cAMP–PKA signaling leading to reduced interaction between phosphorylated cAMP responsive element–binding protein, p300, and CYP19 I/II promoter. BRCA1, a repressor of CYP19 transcription, was induced by pioglitazone. Consistent with these in vitro findings, treatment of mice with pioglitazone activated PPARγ, induced 15-PGDH and BRCA1 while suppressing aromatase levels in the mammary gland. Collectively, these results indicate that the activation of PPARγ induces BRCA1 and suppresses the PGE2–cAMP–PKA axis leading to reduced levels of aromatase. PPARγ agonists may have a role in reducing the risk of hormone-dependent breast cancer in obese postmenopausal women. Cancer Prev Res; 5(10); 1183–94. ©2012 AACR.

Introduction

In postmenopausal women, obesity increases the risk of developing hormone receptor (HR)-positive breast cancer (1, 2). Estrogen can stimulate the development and progression of HR-positive breast cancers. Cytochrome P450 aromatase (aromatase), encoded by the CYP19 gene, catalyzes the synthesis of estrogens from androgens (3). After menopause, peripheral aromatization in adipose tissue is largely responsible for estrogen synthesis (4). The increased risk of breast cancer in obese postmenopausal women has been attributed, in part, to increased circulating levels of estrogen and enhanced estrogen receptor–dependent signaling in the breast (5). Several lines of evidence suggest that COX-derived prostaglandin E2 (PGE2) plays a significant role in stimulating CYP19 transcription resulting in increased aromatase activity. In cultured cells, PGE2 stimulates the cAMP–protein kinase A (PKA) pathway leading to increased aromatase expression (6–8). Targeted overexpression of COX-2 in the mouse mammary gland leads to increased levels of PGE2 and increased aromatase activity (9). A positive correlation has been identified between COX and aromatase levels in human breast tumor specimens (10, 11). Recently, we showed that obesity-related breast inflammation is associated with increased levels of both COX-2 and PGE2, which correlate with elevated levels of aromatase (12, 13). Finally, use of nonsteroidal anti-inflammatory agents and prototypic inhibitors of PGE2 production has been associated with reduced circulating levels of estradiol (14). Agents with anti-inflammatory activity that suppress levels of PGE2 in adipose tissue should inhibit aromatase activity.

PPARγ, a member of the nuclear receptor family of transcription factors, plays an important role in regulating glucose and lipid metabolism (15). Ligand-activated PPARγ induces adipocyte differentiation, inhibits the production...
of proinflammatory mediators, and suppresses aromatase activity (16–22). In tumor cells, PPARγ agonists suppress PGE2 levels, in part, by inducing 15-hydroxyprostaglandin dehydrogenase (15-PGDH), the key enzyme responsible for PGE2 catabolism (23, 24). Activated PPARγ can induce the transcription of tumor suppressor genes such as BRCA1 (25). Recently, BRCA1 was found to repress PGE2-mediated induction of CYP19 transcription (7, 26–28). Whether PPARγ agonist-mediated induction of BRCA1 occurs in vivo or contributes to the downregulation of aromatase is unknown.

The primary objective of this study was to elucidate the mechanism by which pioglitazone, a PPARγ agonist widely used to treat type II diabetes mellitus, suppressed levels of aromatase. In addition to inducing BRCA1, we show that pioglitazone induced 15-PGDH and thereby suppressed the PGE2–cAMP—PKA—cAMP responsive element–binding protein (CREB) signal transduction pathway resulting in reduced levels of aromatase in human preadipocytes. Pioglitazone-mediated suppression of aromatase expression was due, in part, to reciprocal changes in the interaction between BRCA1, p300, and aromatase promoter I.3/I.3. Importantly, treatment of mice with pioglitazone led to similar molecular changes in the mammary gland. Collectively, these findings suggest the possibility that PPARγ agonists may alter the risk of HR-positive breast cancer, especially in the obese.

Materials and Methods

Materials

Medium to grow visceral preadipocytes was purchased from ScienCell Research Laboratories. Pioglitazone was purchased from LKT Labs, Inc. FBS was purchased from Invitrogen. Rabbit polyclonal antisera directed against human pCREB, p300, Eg1, Snail, PPARγ, BRCA1, β-actin, and control IgG were from Santa Cruz Biotechnology, Inc. Anti-human polyclonal antiserum to 15-PGDH was purchased from Novus Biologicals. 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. PGE2 EIA kits were from Cayman Chemicals. ECL Western blotting detection reagents were from Amersham Biosciences. Nitrocellulose membranes were from Schleicher & Schuell. 1β-[3H]-androstenedione, [32P]ATP, and [32P]dCTP were from PerkinElmer Life Science. pSVβgal, electrophoretic mobility shift kit, and plasmid DNA isolation kits were purchased from Promega Corp. Luciferase assay reagents were from Analytical Luminescence. The p300, Snail, BRCA1, 15-PGDH, αp2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs were obtained from Open Biosystem Inc. The 18S rRNA cDNA was purchased from Ambion, Inc. siRNAs (BRCA1, 15-PGDH, Eg1-1, PPARγ, and GFP) and RNeasy mini kits were purchased from Qiagen. ChIP assay kits were purchased from Millipore. Luciferase assay substrates A and B and cell lysis buffer were from BD Biosciences & Co. The PPRE luciferase plasmid was a gift from Dr. Ron Evans (Salk Institute for Biological Sciences, La Jolla, CA). Aromatase promoter (CYP19 1.3/I promoter) was kindly provided by Dr. S. Chen (City of Hope, Duarte, CA). Active CREB and corresponding vector constructs were from Dr. M. Montminy (Salk Institute for Biological Studies, La Jolla, CA). BRCA1 promoter constructs were provided by Dr. Sam Lee (Harvard Medical School, Boston, MA).

Tissue culture

Human visceral preadipocytes were obtained from ScienCell Research Laboratories. These primary cells were grown in preadipocyte medium containing 10% FBS. In experiments requiring transfection, cells were transiently transfected using a system from Amaxa.

Animal model

Twelve-week-old virgin FVB female mice were fed AIN-93G diets containing 0%, 0.05%, or 0.1% w/w pioglitazone ad libitum for 2 weeks before postmortem tissue harvest. Abdominal (#4) mammary glands were snap frozen in liquid nitrogen and stored at −80°C until analysis. Animal experiments were approved by the New York Blood Center Institutional Animal Care and Use Committee, and were conducted in accordance with Federal (PHS Policy on the Human Care and Use of Animals, Guide for the Use and Care of Laboratory Animals, Animal Welfare Act), state, and local laws and regulations.

PGE2 analysis

Cells were plated in 6-well dishes and grown to 60% confluence in growth medium. The amount of PGE2 released by cells was measured by enzyme immunoassay. Production of PGE2 was normalized to protein concentrations. Levels of PGs in mammary glands were quantified by liquid chromatography/tandem mass spectrometric analyses using previously described methods (12).

Western blotting

Lysates were prepared by treating cells with lysis buffer as in previous studies (12). 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 (29). SDS-PAGE was done under reducing conditions on 10% PAGE. The resolved proteins were transferred onto nitrocellulose sheets. 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 mammary tissues and 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. BRCA1 and 18S rRNA probes were labeled with [32P]dCTP by random priming. The blots were probed as described previously (12).

Quantitative real-time PCR
Total RNA was isolated using the RNeasy mini kit. One microgram of RNA was reverse transcribed using murine leukemia virus reverse transcriptase (Roche Applied Science) 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 analysis of human preadipocytes: for aromatase, the forward and reverse primers used were 5'-CACATCCCTCAATCCAGGTCC-3' and 5'-CAGAGCAGACTGCGATATG-3'; for BRCA1, the forward and reverse primers used were 5'-AGCCAGGACAGGTCAGAG-3' and 5'-AGTAGCCAGACGATGAGAGC-3'; and for 15-PGDH, the forward and reverse primers used were 5'-TCTGTGTCATCCAGTGCGATATG-3' and 5'-ATAAGATGTCCCCCTACCT-3'.

Real-time PCR was conducted using 2 x SYBR green PCR master mix on a 7900 HT real-time PCR system (Applied Biosystems) with β-actin (forward 5'-AGAAAATCTGGCACCA-3' and reverse 5'-TGTTACCAACTGGGACGACA-3') serving as an endogenous normalization control. Relative fold induction was determined using the Ct (relative quantification) analysis protocol.

To evaluate the mRNA levels in mouse mammary glands, total RNA was extracted using an RNeasy mini kit. cDNA was synthesized using oligo (dt) primers with superscript III first-strand kit as recommended by the supplier (Invitrogen). Real-time PCR was conducted with the Power SYBR green PCR kit according to the manufacturer’s instructions (Applied Biosystems). The murine primers used were as follows: for aromatase, forward and reverse primers used were 5'-CTCCAGCAAGACTACATCA-3' and 5'-ATCCGAGTCTGCTCTGAG-3'; for 15-PGDH, forward and reverse primers used were 5'-CCAAAGTGAATGATG-3' and 5'-GGGTGAGCCAGACG-3'; for BRCA1, forward and reverse primers used were 5'-CCTGCCCGAGCAGGCA-3' and 5'-CTCTGCTCGGCAGATATG-3'; for GAPDH, forward and reverse primers used were 5'-TGTGACCACCTGGGACACA-3' and 5'-GGGGTGTAGGGTCG-3'. The standard curve method was used to determine levels of each transcript per ng total RNA.

Cyclic AMP levels
Cells were plated at 5 x 10^4 cells per well in 6-well dishes and grown to 60% confluence before treatment. Amounts of cAMP were measured by enzyme immunoassay, and normalized to protein concentration.

Protein kinase A activity
Cells were plated at 5 x 10^4 cells per well in 6-well dishes and grown to 70% confluence before treatment. PKA activity was measured according to the manufacturer’s instructions and normalized to protein concentration.

Aromatase activity
To determine aromatase activity, tissues were prepared from cell lysates and tissues by differential centrifugation. Aromatase activity was quantified by measurement of the titrated water released from 1β-[3H]-androstenedione (12). The reaction was also conducted in the presence of letrozole, 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 cells per well in 6-well dishes and grown to approximately 50% confluence for each well. Cells were transfected with 2 μg of plasmid DNA using the Amaxa system. After 24 hours of incubation, the medium was replaced with basal medium. The activities of luciferase and β-galactosidase were measured in cellular extracts.

Chromatin immunoprecipitation assay
Chromatin immunoprecipitation (ChIP) assays were conducted with a kit according to the manufacturer’s instructions. A total of 2 x 10^6 human preadipocytes 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 to 1,000 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. Ten microliters of each sample was used as a template for PCR amplification. CYP19 oligonucleotide sequences for PCR primers were forward, 5'-AACCTGTCTGATGAACTCACAA-3' and reverse, 5'-TCAGACATTTAGCACACTG-3'. This primer set encompasses the CYP19 promoter I.3/II segment from nucleotide −302 to −38. 15-PGDH oligonucleotide sequences for PCR primers were forward, 5'-TCTGGCTCGGACCGACG-3' and reverse, 5'-AACCACAGCTGTCACCC-3'. This primer set encompasses the 15-PGDH promoter sequence from nucleotide −366 to −155, which contains an E-box site where Snail is known to bind (30). For BRCA1, primers were purchased from Superarray Bioscience Corp. These primers encompass the pPRE site (−241 to −229 bp). PCR was conducted 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 promoters for CYP19, 15-PGDH, and BRCA1 was confirmed for real-time PCR analysis, ChIP-qPCR assay kits from Superarray Bioscience Corp. were used. Real-time PCR was conducted as described above.

Electrophoretic mobility shift assay
Nuclear extracts were prepared from mouse mammary glands using an EMSA kit from Promega. For binding
studies, oligonucleotides containing PPRE sites were obtained from Active Motif. The complementary oligonucleotides were annealed in 20 mmol/L Tris (pH 7.6), 50 mmol/L NaCl, 10 mmol/L MgCl₂, and 1 mmol/L dithiothreitol. The annealed oligonucleotide was phosphorylated at the 5' end with [γ-³²P]ATP and T4 polynucleotide kinase. The binding reaction was conducted by incubating 5 µg of nuclear protein in 20 mmol/L HEPES (pH 7.9), 10% glycerol, 300 µg of bovine serum albumin, and 1 µg of poly (dl-dC) in a final volume of 10 µL for 10 minutes at 25°C. The labeled oligonucleotides were added to the reaction mixture and allowed to incubate for an additional 20 minutes at 25°C. The samples were electrophoresed on a 4% non-denaturing PAGE. The gel was then dried and subjected to autoradiography at −80°C.

Statistics

For data generated from in vitro experiments, comparisons between groups were made by Student t test. For data generated in the animal experiment, the biomarker levels across experimental groups were compared using the non-parametric Kruskal–Wallis test. Treatment versus control comparisons were carried out using the Wilcoxon rank-sum test. P values were adjusted for multiple comparisons using the conservative Bonferroni method. A difference between groups with P < 0.05 was considered statistically significant.

Results

Pioglitazone inhibits expression of aromatase

To determine whether pioglitazone could stimulate PPAR-mediated transcription in primary human visceral preadipocytes, cells were transiently transfected with a PPAR response element cloned upstream of luciferase. Treatment with pioglitazone caused a dose-dependent increase in promoter activity (Fig. 1A) concomitant with dose-dependent suppression of aromatase activity, mRNA levels, and promoter activity (Fig. 1B–D). To confirm that these effects of pioglitazone were mediated by PPARγ, we silenced PPARγ. Silencing of PPARγ blocked pioglitazone-mediated suppression of aromatase promoter activity (Fig. 1E).

Pioglitazone-mediated suppression of PGE₂ levels contributes to the downregulation of aromatase

Because PGE₂ is a known inducer of aromatase, we next determined whether exogenous PGE₂ induced aromatase in preadipocytes. Treatment with PGE₂ caused a dose-dependent increase in aromatase activity (Fig. 1F). We then investigated whether pioglitazone modulated the amount of PGE₂ released into the cell culture medium. Pioglitazone inhibited PGE₂ levels (Fig. 1G) over the same concentration range that it downregulated aromatase expression and activity in preadipocytes (Fig. 1B and C). This finding suggested that pioglitazone-mediated inhibition of aromatase expression was due at least, in part, to its ability to reduce PGE₂ levels in the medium. PGE₂ can stimulate the cAMP—PKA pathway leading to increased aromatase expression. Consistent with the reduction of PGE₂ levels in the culture medium of pioglitazone-treated preadipocytes, cellular levels of cAMP and PKA activity were reduced (Fig. 1H and I).

Pioglitazone-mediated inhibition of PGE₂ levels is associated with induction of 15-PGDH

Next, we investigated whether pioglitazone suppressed the amount of PGE₂ released into the cell culture media through an effect on 15-PGDH, the enzyme responsible for PGE₂ catabolism. As shown in Fig. 2A, pioglitazone induced 15-PGDH levels, an effect that could be responsible for both reduced levels of PGE₂ in the medium and inhibition of aromatase expression. This possibility was tested using small interfering RNA to suppress levels of 15-PGDH. Notably, the ability of pioglitazone to suppress PGE₂ levels in the medium and inhibit aromatase activity in the preadipocytes was attenuated by silencing of 15-PGDH (Fig. 2B and C).

Egr-1 has been reported to induce Snail, a repressive transcription factor that inhibits 15-PGDH gene expression (30). Hence, we next determined if pioglitazone suppressed levels of Egr-1 and Snail leading to induction of 15-PGDH. As shown in Fig. 2D and E, pioglitazone caused dose-dependent suppression of both Egr-1 and Snail at concentrations that inhibited PGE₂ and aromatase levels. Experiments were also carried out to confirm that the down-regulation of Egr-1 contributed to suppression of Snail levels. In fact, silencing of Egr-1 (Fig. 2F) led to reduced levels of Snail (Fig. 2G). Importantly, silencing of Egr-1 also led to increased levels of 15-PGDH (Fig. 2H). Previously, Snail was reported to bind to a region of the 15-PGDH promoter that contains E-boxes (31). ChIP assays were carried out using a primer set that included the 15-PGDH promoter segment containing E-boxes. Consistent with its ability to suppress levels of Egr-1 and thereby Snail, treatment with pioglitazone inhibited the binding of Snail to the 15-PGDH promoter (Fig. 2I). Given the importance of 15-PGDH in the catabolism of PGE₂, we next evaluated whether Snail was important for the pioglitazone-mediated decrease in PGE₂ concentration in the medium. Overexpressing Snail blocked pioglitazone-mediated induction of 15-PGDH (Fig. 2J top) and reversed its inhibitory effects on PGE₂ levels in the medium (Fig. 2J). Finally, overexpressing Snail reversed the inhibitory effects of pioglitazone on aromatase promoter activity (Fig. 2K).

The tumor suppressor BRCA1 is induced by pioglitazone and plays a role in suppressing aromatase levels

Earlier results suggest that BRCA1 can repress aromatase transcription and be induced by PPARγ ligands (7, 25–28). Hence, we investigated if pioglitazone induced BRCA1. Here, we show that pioglitazone caused dose-dependent induction of BRCA1 mRNA and promoter activity (Figs. 3A and B). ChIP assays were conducted and showed the recruitment of PPARγ to the BRCA1 promoter in response to treatment with pioglitazone (Fig. 3C). The inductive effect of pioglitazone on BRCA1 promoter activity was abrogated when a construct containing a mutant PPRE was used (Fig. 3D).
The effects of pioglitazone on components of the transcriptional machinery that influence PGE2-mediated induction of aromatase

Changes in the interaction among pCREB, BRCA1, p300, and the aromatase promoter I.3/II contribute to the inducive effects of PGE2 (7). We conducted ChIP assays to explore the interaction of pCREB, BRCA1, and p300 with the aromatase promoter I.3/II following treatment with pioglitazone (Fig. 4A). Pioglitazone stimulated interaction with BRCA1 but caused a decrease in the interactions of pCREB and p300 with the aromatase promoter. To further evaluate the importance of these pioglitazone-induced changes in regulating aromatase expression, transient transfections were carried out. The suppressive effect of pioglitazone on aromatase promoter activity was attenuated by overexpressing activated CREB or p300 (Fig. 4B). Silencing of BRCA1 also reversed the suppressive effect of pioglitazone on aromatase promoter activity (Fig. 4C). To further understand the transcriptional regulation of aromatase by pioglitazone, we investigated the interactions between BRCA1, p300, and pCREB under basal conditions and following treatment with pioglitazone. In untreated cells, immunoprecipitation experiments suggested that BRCA1, pCREB, and p300 were in a complex (Fig. 4D). Following treatment with pioglitazone, p300, BRCA1, and PPARγ were in the complex but pCREB was not found (Fig. 4D).
Figure 2. Pioglitazone-mediated downregulation of Egr-1 and Snail induces 15-PGDH. A, cells were treated with indicated concentrations of pioglitazone for 12 hours. Total RNA was prepared and 15-PGDH mRNA was quantified by real-time PCR. Values were normalized to the levels of β-actin. In the inset, Western blotting was conducted and the blot was probed with antibodies to 15-PGDH and β-actin. B and C, cells were transfected with 2 μg of control siRNA (GFP) or 15-PGDH siRNA as indicated. 24 hours after transfection, cells were treated with vehicle or 10 μmol/L pioglitazone for an additional 24 hours. B, levels of PGE2...
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Immunoblotting was conducted to determine levels of each of these proteins (Fig. 4E). Treatment with pioglitazone induced levels of BRCA1 while suppressing levels of pCREB. In contrast, amounts of PPARγ and p300 seemed to be unaffected.

Pioglitazone induces 15-PGDH and BRCA1 while suppressing aromatase levels in murine mammary gland in vivo

We next attempted to translate our in vitro findings. Mice were fed control diet or control diet supplemented with...
0.05% or 0.1% pioglitazone for 2 weeks before being sacrificed. Treatment with pioglitazone suppressed aromatase activity and mRNA levels while inducing 15-PGDH expression (Fig. 5A–C). Intramammary levels of PGE2 were suppressed ($P = 0.08$), whereas levels of 13,14-dihydro-15-keto-PGE$_2$, a catabolic product of PGE$_2$, were increased ($P < 0.05$) following treatment with pioglitazone (Fig. 5D and E). Consistent with our in vitro findings, intramammary
BRCA1 mRNA levels were also increased by treatment with pioglitazone (Fig. 5F). Further experiments were carried out to confirm that these effects of pioglitazone were likely to be mediated by PPARγ. We showed that feeding either 0.05% or 0.1% pioglitazone induced aP2, a prototypic PPARγ target gene (Fig. 6A). In addition, electrophoretic mobility shift assay (EMSA) analyses of nuclear proteins isolated from the mammary gland showed that feeding either dose of pioglitazone increased binding to a PPRE site (Fig. 6B) and supershift analysis identified PPARγ in the binding complex.

**Discussion**

PPARγ agonists are known to suppress aromatase expression and liver receptor homologue-1 has been implicated as a mediator of this effect (17–22). Here, we identify additional components of this inhibitory mechanism. Pioglitazone-mediated induction of both 15-PGDH and BRCA1 contributed to reduced aromatase expression. Although PPARγ agonists have been reported to induce 15-PGDH (23), the key enzyme for PGE2 catabolism, our findings are the first to connect this effect with the downregulation of aromatase. The fact that our in vitro results were mirrored by

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**Figure 5.** Pioglitazone induces 15-PGDH and BRCA1, and suppresses aromatase levels in mouse mammary gland. Mice were fed control diet or control diet supplemented with 0.05% or 0.1% (w/w) pioglitazone for 2 weeks before being sacrificed. Levels of aromatase activity (A), aromatase mRNA (B), 15-PGDH mRNA (C), PGE2 (D) 13,14-dihydro-15-keto PGE2 (E), and BRCA1 mRNA (F) were measured in mammary glands. A, aromatase activity is expressed as femtomoles/μg protein/h. Total RNA was prepared and aromatase (B), 15-PGDH (C), and BRCA1 (F) mRNA levels were quantified by real-time PCR. D and E, PGE2 and 13,14-dihydro-15-keto-PGE2 levels were measured in mammary glands. The biomarker levels under each experimental condition (n=10) were summarized in terms of mean ± SD. *P < 0.1; †, P < 0.05; ‡, P < 0.01; ††, P < 0.001 versus mice that received control diet.
the findings in the mammary glands of mice treated with pioglitazone underscores the potential relevance of the work.

The mechanism by which PPARγ agonists induce 15-PGDH seems to differ between non–small cell lung cancer cells and adipose stromal cells (23). In tumor cells, pioglitazone induced 15-PGDH by a PPARγ-independent mechanism. In contrast, the effects of pioglitazone on 15-PGDH depended on the presence of PPARγ in preadipocytes. As a part of our effort to understand the mechanism by which pioglitazone induced 15-PGDH, we focused on Snail. Snail is a transcription factor that binds to the E-box in the 15-PGDH promoter and represses transcription (30, 31). We showed that the same concentration range of pioglitazone that inhibited the binding of Snail to the 15-PGDH promoter induced 15-PGDH. Moreover, overexpressing Snail attenuated the ability of pioglitazone to induce 15-PGDH, suppress PGE2 release, and decrease aromatase promoter activity. These results suggest that pioglitazone-mediated downregulation of Snail plays a key role in inducing 15-PGDH. We also provide evidence that pioglitazone-mediated down regulation of Egr-1 led to decreased Snail expression resulting in a reciprocal increase in 15-PGDH levels. PPARγ agonist–mediated suppression of Egr-1 has been reported previously (32, 33). Consistent with these findings, treatment of mice with pioglitazone induced 15-PGDH, augmented the catabolism of PGE2, and suppressed aromatase expression and activity in the mammary gland.

PGE2 via the EP2 and EP4 receptors can activate cAMP signaling leading to enhanced CYP19 transcription and increased aromatase activity (7). Because pioglitazone-mediated induction of 15-PGDH led to reduced extracellular levels of PGE2, we investigated its effects on the cAMP→PKA→pCREB pathway. Pioglitazone suppressed levels of PGE2, cAMP, and PKA activity while reducing the binding of pCREB to the CYP19 promoter. Forced expression of active CREB or its coactivator p300 relieved pioglitazone-mediated suppression of aromatase expression. Together, these data support our conclusion that pioglitazone suppresses aromatase expression, at least in part, by inhibiting the cAMP→PKA→pCREB pathway.

CBP/p300 is important for CREB-dependent activation of gene expression. Previously, we showed that the interaction between p300 and the CYP19 promoter was important for PGE2-mediated activation of the CYP19 I.3/II promoter (7). Overexpressing a p300 mutant that lacked histone acetyltransferase activity suppressed activation of the CYP19 I.3/II promoter by PGE2. Ligands of nuclear receptors stimulate an interaction between their receptors and CBP/p300 (34) reducing the availability of relatively low concentrations of CBP/p300.

Figure 6. Pioglitazone activates PPARγ and induces aP2 in murine mammary gland. Mice were fed control diet or control diet supplemented with 0.05% or 0.1% (w/w) pioglitazone for 2 weeks prior to being sacrificed. A, Northern blotting was conducted on total RNA (10 μg/lane) and the blots were probed for aP2 and 18S rRNA. B, electrophoretic mobility shift assays were conducted using nuclear protein isolated from the mammary glands. 5 μg of nuclear protein from individual mammary glands were incubated with a 32P-labeled oligonucleotide containing a PPRE canonical site. Left, (control diet), C represents purified PPARγ protein and served as a positive control. Right, (0.1% w/w pioglitazone), lane 6 represents nuclear protein incubated with a 32P-labeled PPRE containing oligonucleotide and a 100-fold excess of unlabeled oligonucleotide; lanes 7 and 8, nuclear protein incubated with a 32P-labeled oligonucleotide and IgG (lane 7) or antibody to PPARγ (lane 8). The protein–DNA complex that formed was separated on a 4% PAGE. The arrow shows a supershift for PPARγ.
CBP/p300 that are able to interact with transcription factors such as CREB and enhance gene expression. Hence, in the current study, we determined whether such squelching of CBP/p300 contributed to the ability of pioglitazone to suppress the expression of aromatase. Immunoprecipitation experiments indicated that pioglitazone enhanced the interaction between PPARY and p300 while reducing the interaction between pCREB and p300. Moreover, overexpressing p300 partially reversed the suppressive effects of pioglitazone on aromatase promoter activity. To our knowledge, these findings represent the first evidence that a PPARY ligand inhibits aromatase expression via a squelching mechanism.

BRCA1 plays a significant role in suppressing aromatase expression (7, 26–28). In fact, the levels of aromatase are increased in the breast tissue of women with germline BRCA1 mutations (35). PPARY agonists have been reported to induce BRCA1 (25). In this study, treatment with pioglitazone increased levels of BRCA1 in both preadipocytes and in the mouse mammary gland. ChIP assays indicated that treatment with pioglitazone increased PPARY binding to the BRCA1 promoter. Pioglitazone also enhanced the binding of BRCA1 to the aromatase promoter. Silencing BRCA1 relieved pioglitazone-mediated suppression of aromatase promoter activity. Taken together, these data provide strong evidence that induction of BRCA1 contributes to pioglitazone-mediated suppression of aromatase expression. Reciprocal occupancy of aromatase promoter I.3/II by BRCA1 and p300 is critical for this effect of pioglitazone. In addition to inhibiting aromatase expression, BRCA1 is involved in several important cellular processes, including DNA damage control, DNA repair, chromatin remodeling, and mitotic spindle formation (36). If haploinsufficiency plays a role in the pathogenesis of breast cancer in carriers of mutant BRCA1, one can posit that a PPARY agonist could delay the onset of disease via increasing expression of the wild-type BRCA1 allele.

Obesity-related breast inflammation is associated with activation of the PGE2→cAMP→PKA pathway resulting in elevated aromatase expression (12, 13). On the basis of the current findings, PPARY agonists may have a role in reducing the risk of HR-positive breast cancer in obese postmenopausal women. However, it is unlikely that this idea will be tested in the near term because of a recent report that use of pioglitazone was associated with an increased risk of bladder cancer in a cohort of diabetic patients (37). Novel synthetic compounds with a unique mode of binding to PPARY that lack some of the serious side effects of traditional thiazolidinediones (e.g., pioglitazone) are in development (38). Future studies to evaluate the effects of these new agents on aromatase expression are warranted.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
8. Zhao Y, Agarwal VR, Mendelson CR, Simpson ER. Estrogen biosynthesis proximal to a breast tumor is stimulated by PGE2 via cyclic AMP, leading to activation of promoter II of the CYP19 (aromatase) gene. Endocrinology 1996;137:5739–42.
11. Brueggemeier RW, Quinn AL, Parrett ML, Joarder FS, Harris RE, Robertson FM. Correlation of aromatase and cyclooxygenase gene
Pioglitazone, a PPARγ Agonist, Suppresses CYP19 Transcription: Evidence for Involvement of 15-Hydroxyprostaglandin Dehydrogenase and BRCA1

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