EP₂ and EP₄ Receptors Regulate Aromatase Expression in Human Adipocytes and Breast Cancer Cells

EVIDENCE OF A BRCA1 AND p300 EXCHANGE

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This article has been withdrawn by the authors. The Aromatase panel in Fig. 2B was reused in Fig. 2E and in Subbaramaiah, K., et al. (2008) J. Biol. Chem. 283, 33955-33968. In Fig. 3B, lanes 2 and 3 of the 18S rRNA panel were reused in lanes 6 and 7. In Fig. 5A, the first two lanes of the 18S rRNA panel were reused in lanes 5 and 6. Additionally, a portion of the BRCA1 panel was reused in Fig. 8 as Aromatase. In Fig. 5B, the first lane of the actin panel was reused in lanes 3 and 4. The 18S rRNA panel in Fig. 5C was reused in Fig. 8. In Fig. 5E, the last lane of the BRCA1 panel was reused in the first lane of the 18S rRNA panel. Also, the 18S rRNA panel was reused in Subbaramaiah, K., et al. (2008) J. Biol. Chem. 283, 33955-33968. Portions of the BRCA1 panel in Fig. 6C were reused in Fig. 6D as BRCA1 and Aromatase and in Fig. 6F as BRCA1. In Fig. 6D, the last two lanes of the BRCA1 panel were reused in the first two lanes of the Aromatase panel. Lanes 2 and 4 of the BRCA1 panel in Fig. 8 are the same. Additionally, the last two lanes of the BRCA1 panel were reused in the first two lanes of the 18S rRNA panel.

Cytochrome P450 aromatase (aromatase), a product of the CYP19 gene, is considered an important local estrogen source. In breast cancer, aromatase activity has been linked to estrogen-dependent tumor growth and to enhanced COX-2 transcription and increased aromatase activity. Reciprocal changes in the interaction between BRCA1, p300, and the aromatase promoter I.3/II contributed to the inhibitory effects of PGE₂.

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7 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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2 The abbreviations used are: PKA, protein kinase A; CREB, cAMP-response element-binding protein; COX, cyclooxygenase; PG, prostaglandin; PGE₂, prostaglandin E₂; PR, progesterone receptor; siRNA, small interference RNA; GFP, green fluorescent protein; ChiP, chromatin immunoprecipitation; ERE, estrogen response element.
Chronic use of selective COX-2 inhibitors, prototypic inhibitors of PG synthesis, has been associated with an increased risk of cardiovascular complications (20, 21). The mechanism underlying this toxicity is not fully understood, but inhibition of COX-2 results in the loss of all downstream PGs. It has been suggested that selective COX-2 inhibitors block the production of cardioprotective PGI2 by vascular endothelium, without inhibiting COX-1-dependent platelet thromboxane A2 synthesis, supporting a pro-thrombotic mechanism (22). To improve the therapeutic index, alternate treatment strategies are being explored. One possibility is to block the actions of PGE2 rather than to inhibit its production. PGE2 exerts its effects by binding to G protein-coupled receptors that contain seven transmembrane domains. Four subtypes of PGE2 receptor (EP1, EP2, EP3, and EP4) have been cloned and defined pharmacologically (23). Different EP receptors have been implicated in regulating cell proliferation, immune function, and angiogenesis (23, 24), but little is known about the regulation of aromatase. Hence, the primary objective of this study was to identify the EP receptor(s) that mediate the induction of aromatase by PGE2 in human adipocytes and breast cancer cells. We show that PGE2 via EP2 and EP4 activates the cAMP-PKA-CREB pathway resulting in enhanced CYP19 transcription and increased aromatase activity. This was due, at least in part, to reciprocal changes in

**FIGURE 1.** EP2 is important for PGE2-mediated induction of aromatase in human adipocytes. A, cells were treated with the indicated concentration of PGE2; B–D, cells were treated with the indicated concentration of butaprost, an EP2 receptor agonist; E and F, cells were treated with vehicle, 500 nM PGE2, or 500 nM PGE2 plus the indicated concentration of AH6809, an EP2 receptor antagonist. All treatments were for 24 h. In A, B, and E, aromatase activity was determined as described under “Experimental Procedures.” Enzyme activity is expressed as femtomoles/μg of protein/min. Means ± S.D. are shown, n = 6. In A and B, *p < 0.001 versus vehicle-treated cells; E, *p < 0.01 versus PGE2-treated cells. In C, D, and F, total RNA was prepared. In C and F, 10 μg/lane RNA was subjected to Northern blotting. The blots were hybridized sequentially with the indicated probes. In D and F, aromatase mRNA was analyzed by real-time PCR as described under “Experimental Procedures.” Values were normalized to the expression levels of glyceraldehyde-3-phosphate dehydrogenase. In D and F, means ± S.D. are shown, n = 3. D, *p < 0.01 versus vehicle-treated cells and in F, *p < 0.01 versus PGE2-treated cells.
the interaction between BRCA1, p300, and the aromatase promoter I.3/I.

EXPERIMENTAL PROCEDURES

Reagents—Adipocyte medium was purchased from ScienCellTM Research Laboratories (Vancouver, BC). Fetal bovine serum and TRIzol reagent were purchased from Invitrogen. PGE2, butaprost, PGE1 alcohol, and AH6809 were from Cayman Chemical Co. (Ann Arbor, MI). ONO (AE3–208) was a gift from ONO Pharmaceutical Co., Ltd. (Osaka, Japan). Rabbit polyclonal antisera for human phospho-CREB, BRCA1, p300, progesterone receptor (PR), β-actin, and control IgG were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Lowry protein assay kits, horseradish peroxidase-conjugated secondary antibody, glucose 6-phosphate, glycerol, pepstatin, leupeptin, glucose-6-phosphate dehydrogenase, and rotenone were from Sigma. A cAMP enzyme immunoassay kit was from Biomol (Plymouth Meeting, PA). PKA activity assay kits were from Calbiochem. ECL Western blotting detection reagents were from Amersham Biosciences. Nitrocellulose membranes were from Schleicher & Schuell (Keene, NH). 1β-[3H]Androstenedione and [32P]CTP were from PerkinElmer Life Sciences. Random priming kits were from Roche Molecular Biochemicals (Indianapolis, IN). pSV βgal and plasmid DNA isolation kits were from Promega Corp. (Madison, WI). Luciferase assay reagents were from Analytical Luminescence (San Diego, CA). The CYP19 cDNA was obtained from Open Biosystem Inc (Huntsville, AL). The 18S rRNA cDNA was purchased from Ambion, Inc. (Austin, TX). cDNAs for EP1–EP4 were from University of Missouri-Rolla Resource Center (Rolla, MO). siRNAs to EP1–EP4 and GFP were purchased from Dharmacon, Inc. (Lafayette, CO). Real-time PCR primers for PR were purchased from Applied Biosystems (Foster City, CA). Expression vectors for p300 and histone acetyltransferase activity mutant of p300 were from Upstate USA, Inc. (Charlottesville, VA). BRCA1 constructs were from Dr. Alvaro Monteiro (H. Lee Moffit Cancer Center, Tampa, FL) (25). The estrogen response element-luciferase construct was from Panomics (Fremont, CA).

Tissue Culture—Visceral adipocytes were obtained from ScienCellTM Research Laboratories. These primary cells were
**EP\textsubscript{2} and EP\textsubscript{4} Regulate Aromatase Expression**

**A**

\begin{center}
\begin{tabular}{ccc}

 & siRNA & siRNA & siRNA \\
EP\textsubscript{2} & GFP & EP\textsubscript{2} & EP\textsubscript{4} \\
18S rRNA & & & \\
\end{tabular}
\end{center}

**B**

\begin{center}
\begin{tabular}{ccc}

 & Aromatase & 18S rRNA \\
EP\textsubscript{4} & & \\
\end{tabular}
\end{center}

FIGURE 3. EP\textsubscript{2} and EP\textsubscript{4} receptors mediate the induction of aromatase by PGE\textsubscript{2}. In **A**, adipocytes were transfected with 2 μg of siRNAs to GFP or EP1–4 and allowed to grow for 36 h prior to analysis. In **B**, 500 nM PGE\textsubscript{2} for 24 h. In **A** and **B**, 10 μg of PGE\textsubscript{2} were added to Northern blotting (10 μg/lane). The blots were hybridized sequentially with the indicated probes. In **B**, aromatase activity was determined in cell lysates as under “Experimental Procedures.” Enzyme activity is expressed as femtomoles/μg of protein/min. Means ± S.D. are shown, n = 6. *, p < 0.01 versus PGE\textsubscript{2}-treated cells.

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grown in adipocyte medium containing 10% fetal bovine serum. In experiments requiring transfection, cells were transiently transfected using a system from Amaxa (Gaithersburg, MD). SKBR3 cells were purchased from ATCC and grown in McCoy’s 5a medium (modified) with 1.5 mM L-glutamine adjusted to contain 2.2 g/liter sodium bicarbonate, and 10% fetal bovine serum. Experimental treatments of adipocytes and SKBR3 cells were carried out under serum free conditions.

**Animals**—Generation of the mice used in this study has been described in detail previously (27). Briefly, MMTV-COX-2 transgenic mice on an FVB/N background were crossed with Ep2\textsuperscript{+/−} mice in the C57BL/6J background. The resulting MMTV-COX-2 Ep2\textsuperscript{+/−} mice in the (FVB/N and C57BL/6) background were backcrossed with FVB/N mice more than nine times. The resulting MMTV-COX-2 Ep2\textsuperscript{+/−} female mice were crossed with Ep2\textsuperscript{+/−} male mice in same strain yielding the different experimental groups. Mammary tissues were harvested, snap-frozen in liquid nitrogen and stored at −80°C until analysis.

**Northern Blotting**—Prior to RNA extraction, frozen tissue was homogenized. Total RNA was prepared from mammary tissues and cell monolayers using an RNA isolation kit from Qiagen. 10 μg of total RNA/lane were electrophoresed in a formaldehyde-containing 1% agarose gel and transferred to nylon-supported membranes. EP1–EP4, BRCA1, aromatase and 18 S rRNA probes were labeled with \([32P]\)CTP by random priming. The blots were probed as described previously (17). All experiments were repeated three times, and representative Northern blot data are shown.

**Real-time Reverse Transcription-PCR**—Total RNA was isolated using TRIzol reagent and reverse transcribed with the Superscript III First-Strand Synthesis System (Invitrogen). For total aromatase mRNA, the forward and reverse primers were 5′-CACATCCC-TCAATACCGGTCC-3′ and 5′-CAGAGATCCAGACTGGATG-3′, and the fluorescence-labeled probe was 5′-CCCTCA-TCTCCCAGGCGAGATTTCC-3′. For BRCA1, the forward and reverse primers were 5′-AAGAGGAAAGGGCTGCTTGGAA-3′, 5′-AAAAATATCGAAGGCAAGAAGCATGGATGCTTCAAGT-3′, and the fluorescence-labeled probe was 5′-CACACCCAGATGCTGCTTTCA-3′. For glyceraldehyde-3-phosphate dehydrogenase mRNA (control), the forward and reverse primers were 5′-GAAGGTTGGAAGGTTCAGGTC-3′ and 5′-GAGGATGATGATGGAGGGTGAT-3′, and the fluorescence-labeled probe was 5′-GAAGGTTGGAAGGTTCAGGCC-3′. Real-time
FIGURE 4. PGE<sub>2</sub> via EP<sub>2</sub> and EP<sub>4</sub> stimulates the cAMP →PKA →CREB pathway resulting in induction of aromatase in adipocytes. A and B, adipocytes were transfected with 2 μg of siRNAs to GFP, EP<sub>2</sub>, or EP<sub>4</sub>. Cells received 0.9 μg of siRNA to GFP, EP<sub>2</sub>, or EP<sub>4</sub> and 0.9 μg of CYP19 promoter-luciferase. All cells also received 0.2 μg of pSVβgal. In A–C, 36 h after transfection, cells received fresh medium containing vehicle (control) or 500 nM PGE<sub>2</sub>. Treatments were for 24 h. Subsequently, cellular levels of cAMP (A) and PKA activity (B) were determined. Means ± S.D. are shown, n = 6. * p < 0.01 versus PGE<sub>2</sub>-treated cells. In C, 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 PGE<sub>2</sub> plus siRNA GFP.

D, ChIP assays were performed. Top, cells were treated as indicated with vehicle (C), PGE<sub>2</sub> (500 nM), butaprost (1 μM), or PGE, alcohol (0.2 μM). Middle, cells were treated as indicated with vehicle, PGE<sub>2</sub> (500 nM), PGE<sub>2</sub> plus EP<sub>2</sub> antagonist (50 μM AH6809), or PGE<sub>2</sub> plus EP<sub>4</sub> antagonist (0.1 μM ONO AE3–208). Bottom, cells were transfected with 2 μg of siRNAs to GFP, EP<sub>2</sub>, or EP<sub>4</sub> 36 h after transfection, cells received fresh medium containing vehicle (C) or 500 nM PGE<sub>2</sub>. All treatments were for 2 h. Chromatin fragments were immunoprecipitated with antibodies against phospho-CREB, and the CYP19 1.3/II promoter was amplified by PCR (panel 1) or real-time PCR (panel 2). DNA sequencing was carried out, and the PCR product was confirmed to be the CYP19 1.3/II promoter. The CYP19 promoter was not detected when normal IgG was used or antibody was omitted from immunoprecipitation step (data not shown). For panel 2 (top, middle, and bottom), means ± S.D. are shown, n = 3. * p < 0.01.
EP₂ and EP₄ Regulate Aromatase Expression

PCR was carried out using an ABI Prism 7900 apparatus (Applied Biosystems).

Transient Transfections—Cells were seeded at a density of 5 × 10⁴ per well in six-well dishes and grown to 70% confluence before treatment. PKA activity was determined according to the instructions of the manufacturer. PKA activity was normalized to protein concentration.

PKA Activity—Cells were plated at a density of 10⁴/well in six-well dishes and grown to 60% confluence before treatment. Amounts of cAMP were measured by enzyme immunoassay. Production of cAMP was normalized to protein concentration.

Measurements of cAMP Levels—Cells were plated at a density of 5 × 10⁴/well in six-well dishes and grown to 70% confluence before treatment. Amounts of cAMP were measured by enzyme immunoassay. Production of cAMP was normalized to protein concentration.

Aromatase Assays—To determine aromatase activity, microsomes were prepared from cells or primary tissues by differential centrifugation using established methods (17). Briefly, tissue was sliced into small pieces and then ground in liquid nitrogen before being suspended in buffer (20 mM Tris/HCl (pH 7.5), 1 mM EDTA, 10% glycerol, 5 μM pepstatin and 5 μM leupeptin). The tissue lysate was then centrifuged for 20 min at 800 × g, and the pellet containing the nuclear fraction was discarded. The supernatant was subjected to ultracentrifugation (1 h at 100,000 × g) to separate microsomes from cytosol. To determine aromatase activity, microsomal protein was added to a 0.5-mL reaction mixture containing 50 mM Tris/HCl (pH 7.5), 5 mM MgCl₂, 5 mM glucose 6-phosphate, 5 units of glucose-6-phosphate dehydrogenase, 2 μM rotenone, and 1β-[³H]androstenedione (200 pm for microsomes for tissue, 12.5 pm for microsomes from cells). Following preincubation for 3 min, the reaction was initiated by the addition of 0.5 μM NADPH and allowed to run for up to several hours at 37 °C. Adding 3 mL of ice-cold chloroform, and applying vigorous shaking and brief centrifugation terminated the reaction. The resulting aqueous layer was further extracted with 3 mL of chloroform and treated with 0.5 mL of 5% activated charcoal/0.5% dextran. Following centrifugation of the mixture, the radioac-

Western Blotting—Lysozymes were prepared by treating cells with lysis buffer (150 mM NaCl, 100 mM Tris (pH 8.0), 1% Tween 20, 50 mM diethyldithiocarbamate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, and 10 μg/ml leupeptin). Lysozymes were sonicated for 20 s on ice and centrifuged at 10,000 × g for 10 min to sediment the particulate material. The protein concentration of the supernatant was measured by the method of Lowry et al. (28). SDS-PAGE was done under reducing conditions on 10% polyacrylamide gels as described by Laemmli (29). The resolved proteins were transferred onto nitrocellulose sheets as detailed by Towbin et al. (30). 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.

Chromatin Immunoprecipitation Assay—ChIP assay was performed with a kit (Upstate) according to the manufacturer’s instructions. 3 × 10⁶ cells were crosslinked in a 1% formaldehyde solution for 10 min at 37 °C. Cells were then lysed in 200 μl of SDS buffer and sonicated to generate 200- to 1000-bp DNA fragments. After centrifugation, the cleared supernatant was counted. Aromatase activity was determined by measurement of the tritiated water released from [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.

Figure 5. PGE₂ via EP₂ and EP₄ stimulates aromatase and suppresses levels of BRCA1 and β-actin. Adipocytes were treated with the indicated agonists or vehicle (C). Cells were treated with vehicle (C), 500 nM PGE₂ and 500 nM PGE₄ plus an EP₂ antagonist (D), or 500 nM PGE₂ plus an EP₄ agonist (E). 36 h after transfection, cell lysates (100 μg of protein/lane) were prepared and 10 μg/lane were hybridized sequentially with probes for BRCA1 and β-actin, respectively.

Data are means ± S.E. of four to six experiments. *p < 0.05; **p < 0.01; ***p < 0.001 versus vehicle control (A), vehicle + PGE₂ (B), vehicle + PGE₄ (C).
EP<sub>2</sub> and EP<sub>4</sub> Regulate Aromatase Expression

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**FIGURE 6.** PGE<sub>2</sub> via EP<sub>2</sub> and EP<sub>4</sub> induces aromatase activity and expression in human breast cancer cells. A, SKBR3 cells were treated with vehicle, 500 nM PGE2, or 500 nM PGE2 plus 0.1 μM butaprost, an EP2 agonist. B–D, cells were treated with the indicated concentration of PGE2, butaprost, or PGE1 alcohol, an EP4 agonist. In A, aromatase activity was determined as under “Experimental Procedures.” Enzyme activity is expressed as femtomoles/H11021. S.D. are shown, *p* < 0.05. E–F, cells were treated with vehicle, 500 nM PGE2, or 500 nM PGE2 plus the indicated concentration of butaprost, an EP2 agonist, or PGE1 alcohol, an EP4 agonist. In E, the indicated concentrations of butaprost were for 24 h. In C–F, total RNA was prepared and 10 μg/lane was subjected to Northern blotting. The blots were hybridized sequentially with the indicated probes.

**Statistics**—Comparisons between groups were made by Student’s *t* test. A difference between groups of *p* < 0.05 was considered significant.

**RESULTS**

**EP<sub>2</sub> and EP<sub>4</sub> Are Important for PGE<sub>2</sub>-mediated Induction of Aromatase Expression**—Initially, we showed that PGE<sub>2</sub> caused dose-dependent induction of aromatase activity in human adipocytes (Fig. 1A). A pharmacological approach was then used to identify the EP receptors that mediated this inductive effect of PGE<sub>2</sub>. Similar to PGE<sub>2</sub>, butaprost, an EP2 agonist, caused a dose-dependent increase in aromatase activity and expression (Fig. 1, B–D). Next we evaluated AH6809, a nonselective EP<sub>2</sub> receptor antagonist. This compound blocked PGE<sub>2</sub>-mediated induction of aromatase activity and expression (Fig. 1, E and F). Because both EP<sub>2</sub> and EP<sub>4</sub> signal via the cAMP→PKA→CREB pathway, we next evaluated the role of EP<sub>2</sub> in mediating the induction of aromatase. Treatment with PGE<sub>1</sub> alcohol, an EP<sub>4</sub> agonist, caused dose-dependent induction of aromatase activity and expression (Fig. 2, A–C). ONO AE3–208, an EP<sub>4</sub> antagonist, suppressed PGE<sub>2</sub>-mediated induction of aromatase activity and expression (Fig. 2, D and E). The inductive effects of the EP<sub>2</sub> receptor agonist (butaprost) were blocked by the EP<sub>2</sub> receptor antagonist (AH6809) but not by the EP<sub>4</sub> receptor antagonist (ONO AE3–208) (Fig. 2F). Similarly, the EP<sub>4</sub> antagonist blocked the inductive effects of PGE<sub>1</sub> alcohol but not butaprost.

To complement this pharmacological approach, a genetic strategy was employed. As shown in Fig. 3A, siRNAs to EP<sub>2</sub> and EP<sub>4</sub> suppressed levels of EP<sub>2</sub> and EP<sub>4</sub>, respectively. Consistent with the pharmacological findings, silencing of EP<sub>2</sub> or EP<sub>4</sub> blocked PGE<sub>2</sub>-mediated induction of aromatase expression (Fig. 3B) and activity (Fig. 3B). Although we were not able to completely knock out these EP receptors (Fig. 3), we did observe an effect on the inductive effects of PGE<sub>2</sub> on aromatase expression and activity (Fig. 3). Taken together, these data indicate that EP<sub>2</sub> and EP<sub>4</sub> mediate the inductive effects of PGE<sub>2</sub> on aromatase expression in human adipocytes.

**Signal Transduction Pathway by which EP<sub>2</sub> and EP<sub>4</sub> Regulate CYP19 Transcription**—As mentioned above, both EP<sub>2</sub> and EP<sub>4</sub> can signal via the cAMP→PKA→CREB pathway. Accordingly, we investigated whether this pathway was involved in PGE<sub>2</sub>-mediated induction of aromatase. The induction of cAMP levels and PKA activity by PGE<sub>2</sub> was suppressed by siRNAs to either EP<sub>2</sub> or EP<sub>4</sub> (Fig. 4, A and B). Moreover, siRNA to EP<sub>2</sub> or EP<sub>4</sub> blocked PGE<sub>2</sub>-mediated activation of the CYP19 promoter (Fig. 4C). ChIP assays were performed to evaluate the role of EP<sub>2</sub> and EP<sub>4</sub> in modulating the binding of p-CREB to the CYP19 promoter. Treatment with PGE<sub>2</sub>, an EP<sub>2</sub> agonist (butaprost) or an EP<sub>4</sub> agonist (PGE<sub>1</sub> alcohol) stimulated the association between p-CREB and the CYP19 promoter (Fig. 4D, top panel). This interaction was blocked by pharmacological antagonists to EP<sub>2</sub> (AH6809) or EP<sub>4</sub> (ONO AE3–208) (Fig. 4D, middle panel) or siRNAs to these receptors (Fig. 4D, bottom panel).

Recently, PGE<sub>2</sub> was found to suppress levels of BRCA1 resulting in enhanced CYP19 expression (19). Here we attempted to identify the EP receptors that are responsible for these effects. First, we confirmed that PGE<sub>2</sub>-mediated suppression of BRCA1 mRNA and protein levels occurred over a concentration range that also induced aromatase (Fig. 5, A and B, and supplemental Fig. S1A). Similar to PGE<sub>2</sub>, both the EP<sub>2</sub> and EP<sub>4</sub> receptor agonists suppressed BRCA1 levels and induced
aromatase (Fig. 5, C and D; and supplemental Fig. S1, B and C). Consistent with these findings, the effects of PGE₂ on BRCA1 and aromatase were blocked by antagonists to EP₂ and EP₄ (Fig. 5E and supplemental Fig. S1D) or silencing of these receptors (Fig. 5F and supplemental Fig. S1E).

In breast cancer, increased aromatase activity occurs in tumor cells in addition to stromal cells (2). Hence, we also evaluated whether EP₂ and EP₄ were important for regulating aromatase expression and activity in a human breast cancer cell line (SKBR3 cells). Similar to the findings in adipocytes, treat-
ment with PGE$_2$, an EP$_2$ receptor agonist (butaprost) or an EP$_4$ receptor agonist (PGE$_4$ alcohol) caused dose-dependent induction of aromatase activity and expression (Fig. 6, A, C and D, and supplemental Fig. S2, A and B). Both the EP$_2$ (AH6809) and EP$_4$ (ONO AE3–208) receptor antagonists suppressed PGE$_2$-mediated induction of aromatase activity and expression (Fig. 6, B, E and F, and supplemental Fig. S2, C and D). Importantly, reciprocal changes in amounts of BRCA1 occurred (Fig. 6, C–F, and supplemental Fig. S2).

To further investigate the role of BRCA1 in repressing CYP19 transcription, transient transfections experiments were carried out. The induction of CYP19 promoter activity by PGE$_2$ or agonists of EP$_2$ and EP$_4$ was suppressed by overexpressing wild-type BRCA1 but not a mutant form of BRCA1 that does not bind to DNA (Fig. 7A). ChIP assays were performed to further explore the interaction between BRCA1 and the CYP19 promoter. Treatment with PGE$_2$ or agonists of EP$_2$ and EP$_4$ suppressed the interaction between BRCA1 and the CYP19 promoter (Fig. 7B). Because p300 is important for CREB-dependent activation of gene expression, the interaction between p300 and the CYP19 promoter was also investigated. Treatment with PGE$_2$ or agonists of EP$_2$ and EP$_4$ increased the interaction between p300 and the CYP19 promoter (Fig. 7B). Hence, PGE$_2$ via EP$_2$ or EP$_4$ reduced the interaction between BRCA1 and the CYP19 promoter but caused a reciprocal increase in interaction between p300 and the CYP19 promoter. To further evaluate the role of p300, transient transfections experiments were carried out. The induction of CYP19 promoter activity by agonists of EP$_2$ and EP$_4$ was suppressed by overexpressing a histone acetyltransferase mutant form of p300, which we used to understand the transcriptional activity of BRCA1. To investigate the interaction between BRCA1 and p-CREB under basal conditions and following treatment with PGE$_2$, p-CREB was in the complex, but BRCA1 was not found. Following treatment with PGE$_2$, p-CREB and BRCA1 were in the complex, but BRCA1 was not found (Fig. 7D).

In an effort to determine the relevance of these findings, an MMTV-COX-2 transgenic mouse model was employed. Previously, we utilized this model and demonstrated that overexpression of COX-2 led to both increased levels of intramammary PGE$_2$ and aromatase activity (17). This was confirmed in the current study; overexpression of COX-2 in the mammary gland also led to increased aromatase activity and expression (Fig. 8). To evaluate whether the EP$_2$ receptor contributed to PGE$_2$-mediated induction of aromatase activity, MMTV-COX-2 EP$_2$–/– mice were generated. Remarkably, when the EP$_2$ receptor was absent, the COX-2-mediated increase in aromatase activity and expression was abrogated (Fig. 8). Consistent with our in vitro findings, levels of BRCA1 were also reduced in the mammary glands of MMTV-COX-2 transgenic mice, an effect that was reversed by knocking out EP$_2$ (Fig. 8).

Because aromatase activity can be rate-limiting for the synthesis of estradiol, we also evaluated the role of EP$_2$ and EP$_4$ as determinants of estrogen-dependent gene expression. The progesterone receptor, an estrogen target gene, is positively regulated by an estrogen response element (31, 32). As shown in Fig. 9, treatment with PGE$_2$ stimulated ERE-luciferase activity and induced the PR, effects that were suppressed by silencing of either EP$_2$ or EP$_4$.

**DISCUSSION**

Previous studies indicate that COX-derived PGE$_2$ can activate CYP19 transcription leading to enhanced aromatase activity and possibly an increased risk of breast cancer (9, 10, 17, 18, 33). Recent evidence suggests that the induction of aromatase by PGE$_2$ is mediated, in part, by suppression of BRCA1, a repressor of CYP19 transcription (19). Given the established link between estrogen biosynthesis and the development and progression of hormone receptor-positive breast cancer, we have attempted to further elucidate the signaling pathway that mediates the activation of CYP19 transcription by PGE$_2$. Several lines of evidence indicate that the EP$_2$ and EP$_4$ receptors are involved in mediating this inductive effect. First, agonists of EP$_2$ and EP$_4$ mimicked PGE$_2$ in inducing aromatase expression. Moreover, EP$_2$ and EP$_4$ receptor antagonists blocked PGE$_2$-mediated induction of aromatase. Consistent with these pharmacological findings, silencing of EP$_2$ or EP$_4$ suppressed PGE$_2$-mediated induction of aromatase expression and activity. Although both EP$_2$ and EP$_4$ play a role in mediating the induction of aromatase, we note that inhibiting either receptor abro-
EP\textsubscript{2} and EP\textsubscript{4} Regulate Aromatase Expression

A

![Graph showing PR and β-actin expression levels with siRNA treatment](Image)

B

![Graph showing ERE luciferase activity with siRNA treatment](Image)

**Figure 9.** PGE\textsubscript{2} via EP\textsubscript{2} and EP\textsubscript{4} induce aromatase expression in adipocytes. A, adipocytes were first transfected with 0.9 g of siRNAs to GFP, EP\textsubscript{2}, or EP\textsubscript{4}. Thirty-six hours after transfection, cells were treated with vehicle (C) or 500 nM PGE\textsubscript{2} for another 24 h. ERE luciferase activity represents data that have been normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase. Means ± S.D. are shown, n = 3, *p < 0.01 versus PGE\textsubscript{2}-treated cells. B, a construct for a ERE luciferase, 0.2 μg of p5Vβgal, and 0.9 μg of siRNAs to EP\textsubscript{2} or vehicle (control) or 500 nM PGE\textsubscript{2} were transfected to the adipocytes. The luciferase activity represents data that have been normalized to β-galactosidase activity. Means ± S.D. are shown, n = 6, *p < 0.01 compared with PGE\textsubscript{2} treatment.

gated the stimulatory effects of PGE\textsubscript{2}. This finding suggests that EP\textsubscript{2} and EP\textsubscript{4} are functionally interdependent. There is growing evidence that G protein-coupled receptors can heterodimerize and that this physical interaction can affect the function of either receptor (34, 35). Recently, functionally important heterodimerization of the thromboxane and prostacyclin receptors (34) and the EP\textsubscript{1} and β\textsubscript{2}-adrenergic receptors (35) was observed. Studies are underway to determine whether the current findings can be explained by heterodimerization of the EP\textsubscript{2} and EP\textsubscript{4} receptors.

Because activation of EP\textsubscript{2} or EP\textsubscript{4} induced aromatase activity, we also investigated the role of these receptors in regulating estrogen-dependent gene expression. Silencing of EP\textsubscript{2} or EP\textsubscript{4} suppressed PGE\textsubscript{2}-mediated induction of the progesterone receptor, a prototypic estrogen-response gene. The significance of these in vitro findings is supported by evidence that the increase in aromatase expression and activity in the mammary glands of COX-2 transgenic mice was abrogated by knocking out EP\textsubscript{2}. Because the survival of EP\textsubscript{4}-deficient mice is poor, the role of EP\textsubscript{4} as a determinant of aromatase expression was not assessed in vivo. A previous study suggested that EP\textsubscript{1} was also a determinant of aromatase expression in adipose stromal cells (36). Although it’s possible that EP\textsubscript{1} signaling contributes to aromatase expression in this cell type, the earlier study did not utilize a genetic approach to confirm the results suggested by EP receptor agonists and antagonists. Our study was carried out in visceral adipocytes and a breast cancer cell line, because both cell types are believed to be relevant sources of estrogen in breast carcinogenesis. Whether our findings for EP\textsubscript{2} and EP\textsubscript{4} extend to other conditions, e.g. uterine leiomyomata, in which PGE\textsubscript{2}-mediated induction of aromatase appears to be important should be determined (38).

EP\textsubscript{2} and EP\textsubscript{4} can signal through the cAMP→PKA→CREB pathway. We investigated whether this signaling pathway was responsible for PGE\textsubscript{2}-mediated activation of CYP19 transcription. The induction of cAMP levels and PKA activity by PGE\textsubscript{2} was suppressed by silencing of EP\textsubscript{2} or EP\textsubscript{4}. Furthermore, the PGE\textsubscript{2}-mediated increase in binding of p-CREB to the CYP19 I.3/II promoter was suppressed by EP\textsubscript{2} and EP\textsubscript{4} antagonists or silencing of these receptors. Although CYP19 transcription is regulated by CREB (11), little is known about the potential involvement of the coactivator CBP/p300 in regulating aromatase expression (39). We present evidence that PGE\textsubscript{2} via EP\textsubscript{2} and EP\textsubscript{4} stimulated the interaction between p300 and the CYP19 I.3/II promoter. The interaction between p300 and pCREB was also enhanced by treatment with PGE\textsubscript{2}. The interaction between p300 and the CYP19 I.3/II promoter was functionally important, because overexpression of a p300 mutant that lacked histone acetyltransferase activity suppressed activation of the CYP19 I.3/II promoter by PGE\textsubscript{2}. Our observation that p300 is important in the regulation of CYP19 transcription provides mechanistic insights that may help to explain previous findings. More specifically, ligands of nuclear receptors, e.g. retinoids and PPAR-γ ligands, have been reported to inhibit aromatase expression (40, 41). Ligands can stimulate an interaction between nuclear receptors and CBP/p300 to interact with transcription factors and enhance gene expression. Hence, the current results provide the basis for future experiments to determine whether...
squelching of CBP/p300 explains the ability of ligands of nuclear receptors to suppress the expression of aromatase.

The tumor suppressor BRCA1 plays a significant role in repressing aromatase expression (19, 44, 45). BRCA1 binds directly to the CYP19 L3/II promoter region and suppresses transcription (19). Previously, PGE₂ and other agents that stimulate cAMP signaling were found to suppress amounts of BRCA1 resulting in enhanced CYP19 transcript levels (19, 44). We extend upon these findings and show that PGE₂-mediated suppression of BRCA1 is mediated by EP₂ and EP₄. In fact, EP₂ and EP₄ agonists mimicked PGE₂ in suppressing levels of BRCA1 while inducing aromatase. Conversely, antagonists of EP₂ and EP₄ or silencing of these receptors blocked PGE₂-mediated suppression of BRCA1. Additionally, PGE₂ via EP₂ and EP₄ caused a decrease in the interaction between BRCA1 and the CYP19 L3/II promoter. The reduced interaction between repressor and promoter was functionally important because overexpression of wild-type but not mutant BRCA1 abrogated PGE₂-mediated activation of the aromatase promoter. Consistently with these in vitro findings, overexpression of COX-2 in the murine mammary gland, a known inducer of intramammary PGE₂ (17), led to reduced levels of BRCA1 in association with increased aromatase expression; this effect was reversed by knocking out EP₂. The mechanism by which PGE₂ inhibits the expression of BRCA1 is likely to be complex. One recent study that utilized a human ovarian granulosa cell line demonstrates that overexpression of wild-type BRCA1 resulted in enhanced proteasome degradation of BRCA1 after a cAMP stimulus. Possibly, PGE₂ via EP₂ and EP₄ acts, in part, to inhibit degradation of BRCA1 protein, additional levels of repression are required.

Regardless of the mechanism, the EP₂ and EP₄ agonists demonstrated potential implications of BRCA1 suppression of aromatase expression, BRCA1 expression in response to important cellular processes, including cell growth and death, DNA repair, chromatin remodeling, histone deacetylase activity, and spindle formation (47). BRCA1 is also involved in transcriptional regulation through interactions with a number of transcription factors (48). Hence, the reduction in amounts of BRCA1 mediated by activation of EP₂ or EP₄ is likely to affect multiple mechanisms that reduce the threshold for carcinogenesis. Taken together, PGE₂ via EP₂ and EP₄ activates the CAMP→PKA→CREB pathway resulting in reciprocal changes in the interaction between BRCA1, p300, and the aromatase promoter L3/II, which contributes, in turn, to enhanced CYP19 transcription and increased aromatase activity. Inhibitors of this pathway, including antagonists of EP₂ and EP₄, may reduce the risk of breast cancer.

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WITHDRAWN
EP$_2$ and EP$_4$ Regulate Aromatase Expression

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