CYP1B1 expression was not affected by PGE2 in estrogen receptor
a ligand-independent manner. PGE2 also increased phosphorylation
expression. Our results indicate that PGE2-induced CYP1B1
expression, and COX-2 overexpression increased CYP1B1
in breast cancer cells.

ER
expression is mediated by ligand-independent activation of the
a (ER)
pathway as a result of the activation of ERK, Akt, and PKA

Transient transfection with human CYP1B1 (hCYP1B1) deletion
promoter constructs and cotreatment with inhibitors revealed that
the estrogen response element contributed to the effects of PGE2
CYP1B1 expression and its mechanism in breast cancer cells. PGE2 significantly increased CYP1B1 protein and
messenger RNA expression and dose dependently enhanced CYP1B1 promoter activity in human breast cancer MCF-7 cells. Transient transfection with human CYP1B1 (hCYP1B1) deletion promoter constructs and cotreatment with inhibitors revealed that the estrogen response element contributed to the effects of PGE2-CYP1B1 expression was not affected by PGE2 in estrogen receptor
(ER) α-negative MDA-MB-231 breast cancer cells or in ERα/
β-negative MCF-10A normal breast cells, and protein expression of
ERα and ERβ was not affected by PGE2 treatment in MCF-7 cells.
However, PGE2 rapidly induced phosphorylation of ERα at serine
residues 118, 167, and 305, suggesting that PGE2 activates ERα in
a ligand-independent manner. PGE2 also increased phosphorylation of extracellular signal-regulated kinase (ERK), Akt, and protein
kinase A (PKA). Finally, a COX-2 inhibitor inhibited PGE2-induced
CYP1B1 expression, and COX-2 overexpression increased CYP1B1
expression. Our results indicate that PGE2-induced CYP1B1
expression is mediated by ligand-independent activation of the
ERα pathway as a result of the activation of ERK, Akt, and PKA
in breast cancer cells.

Key Words: prostaglandin E2; CYP1B1; ERα; cyclooxygenase-2.

Breast cancer is a major cause of death worldwide. Human
cytochrome P450 (CYP) 1B1 is a key enzyme in the metabolism of
17β-estradiol, and CYP1B1-metabolized 4-hydroxyestradiol is a
marker for breast cancer. Furthermore, overexpression of
cyclooxygenase-2 (COX-2), which produces prostaglandin E2
(PGE2), has been detected in invasive breast carcinomas. However, the interaction between PGE2 and CYP1B1 expression
in human breast cancer is unclear. Here, we investigated the effect of PGE2 on CYP1B1 expression and its mechanism in breast
cancer cells. PGE2 significantly increased CYP1B1 protein and
messenger RNA expression and dose dependently enhanced CYP1B1 promoter activity in human breast cancer MCF-7 cells. Transient transfection with human CYP1B1 (hCYP1B1) deletion
promoter constructs and cotreatment with inhibitors revealed that
the estrogen response element contributed to the effects of PGE2-CYP1B1 expression was not affected by PGE2 in estrogen receptor
(ER) α-negative MDA-MB-231 breast cancer cells or in ERα/
β-negative MCF-10A normal breast cells, and protein expression of
ERα and ERβ was not affected by PGE2 treatment in MCF-7 cells.
However, PGE2 rapidly induced phosphorylation of ERα at serine
residues 118, 167, and 305, suggesting that PGE2 activates ERα in
a ligand-independent manner. PGE2 also increased phosphorylation of extracellular signal-regulated kinase (ERK), Akt, and protein
kinase A (PKA). Finally, a COX-2 inhibitor inhibited PGE2-induced
CYP1B1 expression, and COX-2 overexpression increased CYP1B1
expression. Our results indicate that PGE2-induced CYP1B1
expression is mediated by ligand-independent activation of the
ERα pathway as a result of the activation of ERK, Akt, and PKA
in breast cancer cells.

Key Words: prostaglandin E2; CYP1B1; ERα; cyclooxygenase-2.

Breast cancer is a major cause of death worldwide. Human
cytochrome P450 (CYP) 1B1 is a key enzyme in the metabolism of
17β-estradiol, and CYP1B1-metabolized 4-hydroxyestradiol is a
marker for breast cancer. Furthermore, overexpression of
cyclooxygenase-2 (COX-2), which produces prostaglandin E2
(PGE2), has been detected in invasive breast carcinomas. However, the interaction between PGE2 and CYP1B1 expression
in human breast cancer is unclear. Here, we investigated the effect of PGE2 on CYP1B1 expression and its mechanism in breast
cancer cells. PGE2 significantly increased CYP1B1 protein and
messenger RNA expression and dose dependently enhanced CYP1B1 promoter activity in human breast cancer MCF-7 cells. Transient transfection with human CYP1B1 (hCYP1B1) deletion
promoter constructs and cotreatment with inhibitors revealed that
the estrogen response element contributed to the effects of PGE2-CYP1B1 expression was not affected by PGE2 in estrogen receptor
(ER) α-negative MDA-MB-231 breast cancer cells or in ERα/
β-negative MCF-10A normal breast cells, and protein expression of
ERα and ERβ was not affected by PGE2 treatment in MCF-7 cells.
However, PGE2 rapidly induced phosphorylation of ERα at serine
residues 118, 167, and 305, suggesting that PGE2 activates ERα in
a ligand-independent manner. PGE2 also increased phosphorylation of extracellular signal-regulated kinase (ERK), Akt, and protein
kinase A (PKA). Finally, a COX-2 inhibitor inhibited PGE2-induced
CYP1B1 expression, and COX-2 overexpression increased CYP1B1
expression. Our results indicate that PGE2-induced CYP1B1
expression is mediated by ligand-independent activation of the
ERα pathway as a result of the activation of ERK, Akt, and PKA
in breast cancer cells.

Key Words: prostaglandin E2; CYP1B1; ERα; cyclooxygenase-2.

Breast cancer is a major cause of death worldwide. Human
cytochrome P450 (CYP) 1B1 is a key enzyme in the metabolism of
17β-estradiol, and CYP1B1-metabolized 4-hydroxyestradiol is a
marker for breast cancer. Furthermore, overexpression of
cyclooxygenase-2 (COX-2), which produces prostaglandin E2
(PGE2), has been detected in invasive breast carcinomas. However, the interaction between PGE2 and CYP1B1 expression
in human breast cancer is unclear. Here, we investigated the effect of PGE2 on CYP1B1 expression and its mechanism in breast
cancer cells. PGE2 significantly increased CYP1B1 protein and
messenger RNA expression and dose dependently enhanced CYP1B1 promoter activity in human breast cancer MCF-7 cells. Transient transfection with human CYP1B1 (hCYP1B1) deletion
promoter constructs and cotreatment with inhibitors revealed that
the estrogen response element contributed to the effects of PGE2-CYP1B1 expression was not affected by PGE2 in estrogen receptor
(ER) α-negative MDA-MB-231 breast cancer cells or in ERα/
β-negative MCF-10A normal breast cells, and protein expression of
ERα and ERβ was not affected by PGE2 treatment in MCF-7 cells.
However, PGE2 rapidly induced phosphorylation of ERα at serine
residues 118, 167, and 305, suggesting that PGE2 activates ERα in
a ligand-independent manner. PGE2 also increased phosphorylation of extracellular signal-regulated kinase (ERK), Akt, and protein
kinase A (PKA). Finally, a COX-2 inhibitor inhibited PGE2-induced
CYP1B1 expression, and COX-2 overexpression increased CYP1B1
expression. Our results indicate that PGE2-induced CYP1B1
expression is mediated by ligand-independent activation of the
ERα pathway as a result of the activation of ERK, Akt, and PKA
in breast cancer cells.

Key Words: prostaglandin E2; CYP1B1; ERα; cyclooxygenase-2.

Breast cancer is a major cause of death worldwide. Human
cytochrome P450 (CYP) 1B1 is a key enzyme in the metabolism of
17β-estradiol (E2), a substrate for the phase I enzyme

cytochrome P450 (CYP) 1B1 and a ligand for the estrogen
receptor (ER).

CYP1B1, an extrahepatic enzyme, is a member of the CYP1
family and is expressed constitutively in many human tissues,
including breast and ovary (Jefcoate et al., 2000; Muskhelishvili
et al., 2001; Shimada et al., 1996; Sutter et al., 1994). CYP1B1 can oxidize the catechol estrogens to the chemically
reactive semiquinone and quinine intermediates, which can
form DNA adducts to initiate breast, prostate, and other types of
cancers (Cavaleri et al., 2006). Increased E2 4-hydroxylase
activity has been detected in human breast cancer compared
with normal breast tissue, and increased expression of CYP1B1
protein has been demonstrated in several types of human
cancers, including breast and ovary (Liehr and Ricci, 1996;
McFadyen et al., 1999).

The ER is a ligand-dependent transcription factor involved in
normal growth and differentiation of mammary tissue. Ligand
binding to both isoforms, ERα and ERβ, is regulated by both
heat shock protein 90 (Hsp90) association and nuclear
receptor phosphorylation (Beato and Klug, 2000; Nilsson
et al., 2001; Zhong and Skafar, 2002). Hsp90 interacts with the
ligand-binding domain of ER in the absence of ligand and
dissociates upon ligand binding, leading to a tight association
of ERα with the nuclear compartment (Klinge, 2000; Zhong
and Skafar, 2002). In the classical ligand-dependent activation
of ER, estrogen binding increases ER phosphorylation at
specific sites that facilitate ER dimerization and direct the
interaction with estrogen response elements (EREs) in the
promoters of estrogen target genes (Loven et al., 2001; Nilsson
et al., 2001). Furthermore, ERα can be transcriptionally
activated in the absence of estrogen, a process referred to as
ligand-independent activation. Three serine residues in the N-
terminal region of ERα can be phosphorylated: 118, by
extracellular signal-regulated kinase (ERK; Kushner
et al., 1997); 167, by Akt (Feng et al., 2001); and 305, by protein

© The Author 2010. Published by Oxford University Press on behalf of the Society of Toxicology. All rights reserved.
For permissions, please email: journals.permissions@oxfordjournals.org
Serum-derived estrogenic compounds and to avoid the estrogenic effects of routine culture, cells were grown in medium containing phenol red. To remove Invitrogen, Inc.; antibodies against actin, ERα and blocking peptide from Bethyl Laboratories, Inc.; antibodies against anti-phospho-ERα (Ser118 and 167), anti-phosphotyrosine, anti-Akt/phospho-Akt (Ser473), anti- Src/phospho-Src (Tyr527), anti-ERK/phospho-ERK1/2 (Thr202/Tyr204), and horseradish peroxidase–linked anti-rabbit and anti-mouse IgG from Cell Signaling Technology; enhanced chemiluminescence (ECL) system and polyvinylidene difluoride (PVDF) membrane from Amersham Pharmacia Biotech; CH-223191, H-89, NS-398, PP2, PD98059, and LY294002) and was always < 0.2%. Western blotting. Cells were pretreated with an inhibitor (ICI 182.780, curcumin, CH-223191, H-8609, CH-23848, H-89, NS-398, PP2, PD98059, or LY294002) and then incubated with PGE2 or 3-MC. In addition, cells were transfected with a COX-2 expression vector for 24 h. Cell lysates were resolved by 10% SDS-polyacrylamide gel electrophoresis followed by electroblotting onto a PVDF membrane. The membrane was probed with the appropriate primary antibody followed by incubation with secondary antibody. The immunoreactive bands were visualized using an ECL kit, according to the manufacturer’s instructions.

RNA preparation and messenger RNA analysis by TaqMan PCR. Cells were pretreated with an inhibitor (ICI 182.780, curcumin, CH-223191, H-8609, CH-23848, H-89, NS-398, PP2, PD98059, or LY294002) and treated with PGE2 or 3-MC for 24 h. Total RNA from the treated cells was prepared using RNAiso reagent (Takara), according to the manufacturer’s protocol. TaqMan probes and primers for CYP1B1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were Assay-on-Demand gene expression products (Applied Biosystems). TaqMan PCR was performed with a Prism 7000 sequence detection system (Applied Biosystems), according to the manufacturer’s instructions. The primers and probe for CYP1B1 were from a human GAPDH control reagent kit (Applied Biosystems). Data were analyzed using the comparative cycle of threshold method.

Plasmids. The pGL3-basic vector and pERE-Luc were purchased from Promega, and the COX-2 expression vector was a gift from Dr Soo Jung Lim (Sejong University, South Korea). The human CYP1B1-Luc vector (−1635 to +1588) was a gift from Dr Robert Barouki (Coumoul et al., 2001). Human CYP1B1-Luc deletion plasmids were constructed to test for promoter activity using a luciferase reporter assay system. Two DNA fragments, −910 to +25 and −91 to +25, containing CYP1B1 promoter regions were obtained by PCR using the following primers (5′ → 3′): CYP1B1-5′/β-gII, GAA GAT CTG CCC TAA GAA CTC CAG GCT TC; CYP1B1-3′/βII, GAA GAT CTG GGG ACA GAG AGG AGA AGG CG; CYP1B1-5′/Kpln, GGG GTA CGCC CCC TAA GAA A CTC CAG GCT TC; and CYP1B1-3′/HidinIII, CCC AAG CTG GAG TGG CAG CGG CCC TCC. All PCR products were sequenced and confirmed to be identical to the published sequence of the CYP1B1 promoter.

Luciferase and β-galactosidase assays. Using Lipofectamine 2000 reagent, cells were transiently transfected with 1 μg of hCYP1B1-Luc, one of the hCYP1B1-Luc deletion plasmids containing promoter regions (−910/−25 or −91/−25), or pERE-Luc and 0.5 μg of pCMV-β-gal plasmid. After 4 h, the mixture was replaced with basal medium. The cells were treated with an inhibitor (ICI 182.780, H-89, NS-398, PP2, PD98059, or LY294002) and/or PGE2 or 3-MC for 24 h and then lysed. The luciferase and β-galactosidase activities were measured in cellular extracts as described previously (Mestre et al., 1997). The luciferase activity was normalized to the β-galactosidase activity and is expressed relative to the activity of the control group.

Immunoprecipitation. Cells were cultured with PGE2 for 10 min. Whole-cell lysates were prepared in 0.5% Nonidet P-40 (NP-40) lysis buffer (50mM Tris-HCl, 150mM NaCl, 5mM EDTA, and 0.5% NP-40) supplemented with protease inhibitors, cellular debris was removed by centrifugation at 12,000 revolutions per minute for 10 min, and the supernatant was subjected to immunoprecipitation. First, anti-ERα antibody was incubated with protein A agarose beads, and the beads were washed. Then total cellular extract (2 mg) was diluted with PBS and incubated with the washed beads for 18 h at 4°C.
with rotation. The beads were washed twice with PBS, and the samples were boiled for 5 min. Immunoprecipitated phosphotyrosine was detected by Western blot analysis.

**Statistical analysis.** All experiments were repeated at least three times. One-way ANOVA was used to determine the significance of differences between treatment groups. The Newman-Keuls test was used for multigroup comparisons. Statistical significance was accepted for p values < 0.01.

**RESULTS**

**PGE₂ Induces CYP1B1 Protein and Messenger RNA Expression and CYP1B1 Promoter Activity in MCF-7 Cells**

To examine the effect of PGE₂ on CYP1B1 expression in breast cancer cells, we treated MCF-7 cells with various concentrations of PGE₂ for 24 h, and the protein expression of CYP1B1 was analyzed by immunoblotting followed by normalization to the actin protein level, which was unaffected by PGE₂ treatment. As a positive control, 3-MC was used instead of PGE₂ because it leads to the activation of both ER and aryl hydrocarbon receptor target genes (Swedenborg et al., 2008) such as CYP1B1. PGE₂ dose dependently induced CYP1B1 protein expression in MCF-7 cells (Fig. 1A). The concentrations of 0.1, 1, and 10μM of PGE₂ increased 2.5-, 4-, and 6.7-fold from control, respectively. Treatment with 1μM 3-MC also increased CYP1B1 protein expression to 7.1-fold of control. When MCF-7 cells were treated with PGE₂ (10μM) for 6, 12, 24, and 48 h, the CYP1B1 protein level peaked at 24 h and had declined by 48 h (Fig. 1B). Fold protein expression increased concomitant with PGE₂ dose with a maximum of 8.1-fold at 10μM. We also tested the effect of PGE₂ on CYP1B1 gene expression using a PCR assay. Both PGE₂ and 3-MC increased the level of CYP1B1 messenger RNA (mRNA) in a dose-dependent manner and with a pattern similar to that of CYP1B1 protein expression (Fig. 1C). The concentrations of 0.1, 1, and 10μM of PGE₂ increased 3.2-, 5.6-, and 8.2-fold from control, respectively. Thus, PGE₂ may regulate the transcriptional activation of CYP1B1 in MCF-7 cells. To investigate the mechanism by which PGE₂ regulates CYP1B1 gene expression, we transfected MCF-7 cells with the CYP1B1-Luc reporter construct. Both PGE₂ and 3-MC

**FIG. 1.** Effect of PGE₂ on CYP1B1 expression in MCF-7 breast cancer cells. (A, B) Effect of PGE₂ on CYP1B1 protein expression. Cells were treated with PGE₂ (0.1–10μM) or 3-MC (1μM) for 24 h (A) or cultured with 10μM PGE₂ for 6, 12, 24, and 48 h (B). The CYP1B1 protein level was analyzed on immunoblots of cell lysates probed with anti-hCYP1B1 antibody. The CYP1B1 protein level was normalized to that of actin. Each blot is representative of three independent experiments. The densitometry data presented below the bands are “fold change” compared with control after normalization with respective loading control value. (C) Effect of PGE₂ on CYP1B1 mRNA expression. Cells were incubated with PGE₂ (0.1–10μM) or 3-MC (1μM) for 24 h. Cells were lysed, and total RNA was prepared for PCR analysis of CYP1B1 mRNA expression relative to GAPDH expression. CYP1B1 mRNA expression was compared between treated and untreated cells at each time point. *p < 0.01, significantly different from control, by the Newman-Keuls test. (D) Effect of PGE₂ on CYP1B1 promoter activity. Cells transfected with CYP1B1-Luc were cultured with PGE₂ (0.1–10μM) or 3-MC (1μM) for 24 h, harvested, and assayed for luciferase activity. *p < 0.01, significantly different from control, by the Newman-Keuls test.
induced luciferase activity in MCF-7 cells (Fig. 1D). This result exhibited PGE2-induced luciferase activity rates about 2.5-fold higher than the control value. These results demonstrate that PGE2 induced CYP1B1 gene expression and enhanced the CYP1B1 protein level by inducing CYP1B1 promoter activity in MCF-7 cells.

**EP Receptor Inhibitors Inhibit PGE2-Induced CYP1B1 Expression and Promoter Activity in MCF-7 Cells**

PGE2 is the ligand for four PGE2 receptor (EP) receptor subtypes, termed EP1 through EP4 (Breyer and Breyer, 2000), and the binding of PGE2 to EP2 and EP4 signals an increase in intracellular cyclic 3′-5′-cyclic adenosine monophosphate. We examined the role of EP receptors in PGE2-induced CYP1B1 expression using inhibitors of EP2 (AH-6809) and EP4 (AH-23848). Both inhibitors reduced PGE2-induced CYP1B1 protein expression (Fig. 2A). The results suggested a greater sensitivity to the EP4 receptor inhibitor than the EP2 receptor inhibitor. AH-6809 and AH-23848 also reduced PGE2-induced CYP1B1 mRNA expression and CYP1B1 promoter activity (Figs. 2B and 2C). Neither AH-6809 nor AH-23848 alone affected CYP1B1 protein or mRNA expression or luciferase activity. Thus, PGE2-induced CYP1B1 expression and promoter activity were decreased in the presence of EP receptor inhibitors.

**PGE2 Induces CYP1B1 Expression via Activation of the ERE-Binding Site**

In the hCYP1B1 promoter (Fig. 3A), xenobiotic response element (XRE), Sp-1, activator protein 1 (AP-1), and ERE transcription factor–binding sites are important for regulating the transcription of CYP1B1 (Sissung et al., 2006; Tsuchiya et al., 2004). To identify the region of the hCYP1B1 promoter that mediates the inductive effects of PGE2, we transfected cells with the −910/+25 hCYP1B1 deletion construct, which contains all the transcription factor–binding sites, or the −91/+25 hCYP1B1 deletion construct, which has only the ERE-binding site. PGE2 induced luciferase activity in a dose-dependent manner in cells transfected with either of the hCYP1B1 deletion constructs (Fig. 3B). Luciferase activity was increased when treated with PGE2 with a maximum of 2.7- and 3.8-fold at 10μM in the −910/+25 and −91/+25 hCYP1B1 deletion constructs transfected cells. In addition, PGE2 enhanced the reporter activity of the ERE-Luc vector in a dose-dependent manner (Fig. 3C). The concentrations of 0.1, 1, and 10μM of PGE2 increased 1.9-, 2.7-, and 3.7-fold from control, respectively. Next, we used the inhibitors ICI 182.780, CH-223191, and curcumin to inhibit transcription factor activation at ERE, XRE, and AP-1 sites, respectively. The ERE-binding site activation inhibitor, ICI 182.780, blocked PGE2-induced CYP1B1 protein expression, whereas CH-223191 and curcumin did not (Fig. 3D). ICI 182.780 also inhibited PGE2-induced CYP1B1 mRNA expression and promoter reporter activity, but CH-223191 and curcumin had no effects (Figs. 3E and 3F). Thus, PGE2-induced CYP1B1 expression occurs via promoter activation at the ERE-binding site, suggesting that the ER may be particularly important for understanding the role of PGE2 in CYP1B1 regulation.

**ERα Did Not Affect PGE2-Induced CYP1B1 Expression in Human Breast Cancer Cells**

The two major ERs, ERα and ERβ, differ in some ligand specificities and physiological functions (Enmark and Gustafsson, 1999); however, the natural hormone E2 binds as a ligand with equal affinity to ERα and ERβ, after which the E2–ERα/β complex translocates into the nucleus, where it regulates gene transcription via the interaction between the activated ER and ERE. To investigate the role of ER in PGE2-regulated CYP1B1 expression, cell lines exhibiting different ER status were used: MCF-7 (ERα+/ERβ+), MDA-MB-231 (ERα−/ERβ+), and MCF-10A (ERα−/ERβ−). PGE2 treatment induced CYP1B1 protein expression in MCF-7 cells in a dose- and time-dependent manner (Figs. 1A and 1B); however, the CYP1B1 protein level was not affected by PGE2 in MDA-MB-231 or MCF-10A cells (Figs. 4A and 4B). Treatment with 3-MC increased the CYP1B1 protein level in all three cell lines (Figs. 1A and 1B and 4A and 4B) because it leads to promoter activation at both the ERE- and the XRE-binding sites (Swedenborg et al., 2008). Furthermore, CYP1B1 mRNA expression and CYP1B1 promoter activity were not increased in PGE2-treated MDA-MB-231 or MCF-10A cells, although 3-MC alone enhanced both CYP1B1 mRNA expression and promoter activity in these cells (Figs. 4C and 4D). As MDA-MB-231 cells express ERβ but not ERα, these results indicate that PGE2 regulates CYP1B1 through an ERα-related mechanism. To further confirm this, we examined the effect of PGE2 on the protein expression of ERα and ERβ in MCF-7 cells. Treatment of MCF-7 cells with 0.1, 1, and 10μM PGE2 for 10 min did not produce a change in the ERα or ERβ protein level (Fig. 4E). In addition, when MCF-7 cells were treated with 10μM of PGE2 for 5, 10, 20, 30, 60, and 120 min, no change in the ERα or ERβ protein level was observed (Fig. 4F). Therefore, ERα may be important for PGE2-induced CYP1B1 expression, and PGE2 regulates CYP1B1 through an ERα-related mechanism in breast cancer cells.

**PGE2 Induces Phosphorylation of ERα at Serines 118, 167, and 305 in MCF-7 Cells**

ERα can be activated through genomic (transcriptional regulation) and nongenomic mechanisms (activation of signaling cascades, including phosphorylation) (Katzenellenbogen and Katzenellenbogen, 2000), although ERα phosphorylation by various cellular kinases is considered to be the most important regulator of ER activity (Likhite et al., 2006). Figure 5A shows the ER phosphorylation sites (Likhite et al., 2006);
serines 118, 167, and 305 in the N-terminal region are phosphorylated by ERK, Akt, and PKA, respectively (Chen et al., 1999; Feng et al., 2001; Kushner et al., 2000), and Tyr537 in the C-terminal region is phosphorylated by Src kinase (Sassone-Corsi, 1998). These phosphorylation sites are responsible for the ligand-independent transactivation functions of ERα (Shah and Rowan, 2005). To investigate whether PGE2 activates ERα by phosphorylation, we determined the extent of ERα phosphorylation by comparing the amounts of phosphorylated and nonphosphorylated ERα on immunoblots of lysates from cells treated with PGE2. When MCF-7 cells were incubated with 0.1, 1, and 10 μM PGE2 for 10 min, PGE2 induced the phosphorylation of ERα at serines 118, 167, and 305 in a concentration-dependent manner (Fig. 5B). When MCF-7 cells were treated with 10 μM PGE2 for 5, 10, 20, 30, 60, and 120 min, phosphorylation at serines 118, 167, and 305 of ERα showed a time-dependent increase, peaking at 10, 5, and 30 min, respectively (Fig. 5C). Thus, PGE2 rapidly induced a ligand-independent ERα activation pathway in MCF-7 cells. The phosphorylation of ERα at Tyr537 was determined in a similar manner, and the degree of Tyr537 phosphorylation did not change with PGE2 treatment (Fig. 5D). These results demonstrate that PGE2 stimulates the phosphorylation of ERα at serines 118, 167, and 305 in MCF-7 cells prior to the induction of CYP1B1 expression.

PGE2 Induces CYP1B1 Expression through Phosphorylation of PKA, ERK, and Akt Signaling Pathways in MCF-7 Cells

The nongenomic actions of estrogen are most notably associated with the activation of Src, ERK, PKA, and Akt, all of which phosphorylate ERα (Lannigan, 2003), and a number of factors activate these kinases (Ellis, 2004; Johnston, 2005). To evaluate the upstream signal for PGE2-induced ERα activation, we examined the activation of these kinases in MCF-7 cells. Cells were cultured with 10 μM PGE2 for 5, 10, and 20 min, and the levels of phosphorylated and

FIG. 2. Role of EP receptors in PGE2-induced CYP1B1 expression in MCF-7 cells. (A) Role of EP receptors in PGE2-induced CYP1B1 protein expression. Cells were pretreated with an inhibitor of EP2 receptor (AH-6809; 10 μM) or EP4 receptor (AH-23848; 30 μM) for 30 min and then incubated with

PGE2 (10 μM) for 24 h. The CYP1B1 protein level was analyzed on immunoblots of cell lysates probed with anti-hCYP1B1 antibody. The CYP1B1 protein level was normalized to that of actin. Each blot is representative of three independent experiments. The densitometry data presented below the bands are “fold change” compared with control after normalization with respective loading control value. (B) Role of EP receptors in PGE2-induced CYP1B1 mRNA expression. Cells were pretreated with the inhibitor AH-6809 (10 μM) or AH-23848 (30 μM) for 30 min and then incubated with PGE2 (10 μM) for 24 h. Cells were lysed, and total RNA was prepared for PCR analysis of CYP1B1 mRNA expression relative to the expression of GAPDH. CYP1B1 mRNA expression was compared between treated and untreated cells at each time point. Data shown are representative of three independent experiments. *p and **p < 0.01, significantly different from control and PGE2, respectively, by the Newman-Keuls test. (C) Role of EP receptors in PGE2-induced CYP1B1 promoter activity. Cells were transfected with CYP1B1-Luc, pretreated with inhibitor AH-6809 (10 μM) or AH-23848 (30 μM), and incubated in the presence or absence of PGE2 (10 μM) for 24 h. Cells were harvested and assayed for luciferase activity. *p and **p < 0.01, significantly different from control and PGE2, respectively, by the Newman-Keuls test.
FIG. 3. Roles of transcriptional factor–binding sites in PGE2-induced CYP1B1 expression in MCF-7 cells. (A) Schematic of the hCYP1B1 promoter. (B) Effect of PGE2 on the activity of CYP1B1 promoter regions. Cells were transfected with 1 μg hCYP1B1 promoter deletion construct −910/+25 (white bars) or −91/+25 (black bars) and 0.5 μg of pCMV-β-gal for 4 h and then treated with PGE2 (0.1–10 μM) or 3-MC (1 μM) for 24 h. Cells were harvested and assayed for luciferase activity, which was normalized to β-galactosidase activity. (C) Effect of PGE2 on ERE activity. Cells were transfected with 1 μg of ERE-Luc vector and pCMV-β-gal and then cultured with PGE2 (0.1–10 μM) or 3-MC (1 μM). Cells were harvested and assayed for luciferase activity, which was normalized to β-galactosidase activity. Results shown are representative of three independent experiments. *p < 0.01, significantly different from control, by the Newman-Keuls test. (D) Roles of transcriptional factor–binding sites (ERE, AP-1, and XRE) in PGE2-induced CYP1B1 protein expression. Cells were pretreated with an inhibitor of ERE (ICI 182.780; 100 nM), AP-1 (curcumin; 20 μM), or aryl hydrocarbon receptor (CH-223191; 10 μM) and then incubated with PGE2 (10 μM) for 24 h. The CYP1B1 protein level was analyzed on immunoblots of cell lysates probed with anti-hCYP1B1 antibody. The CYP1B1 protein level was normalized to that of actin. Each blot is representative of three independent experiments. The densitometry data presented below the bands are “fold change” compared with control after normalization with respective loading control value. *p < 0.01, significantly different from control and PGE2, respectively, by the Newman-Keuls test. (E) Roles of transcriptional factor–binding sites (ERE, AP-1, and XRE) in PGE2-induced CYP1B1 mRNA expression. Cells were pretreated with the inhibitor ICI 182.780 (100 nM), curcumin (20 μM), or CH-223191 (10 μM) for 30 min and then cultured with PGE2 (10 μM) for 24 h. Cells were lysed, and total RNA was prepared for PCR analysis of CYP1B1 mRNA expression relative to the expression of GAPDH. Data shown are representative of three independent experiments. *p and **p < 0.01, significantly different from control and PGE2, respectively, by the Newman-Keuls test. (F) Roles of transcriptional factor–binding sites (ERE, AP-1, and XRE) in PGE2-induced CYP1B1 promoter activity. Cells were transfected with CYP1B1-Luc, pretreated with ICI 182.780 (100 nM), curcumin (20 μM), or CH-223191 (10 μM) for 30 min, and incubated in the presence or absence of PGE2 (10 μM) for 24 h. Cells were harvested and assayed for luciferase activity, which was normalized to β-galactosidase activity. Results shown are representative of three independent experiments. *p and **p < 0.01, significantly different from control and PGE2, respectively, by the Newman-Keuls test.
FIG. 4. Effect of PGE2 on ERα and ERβ in human breast cancer cells. (A, B) Effect of PGE2 on CYP1B1 protein expression in MDA-MB-231 and MCF-10A cells. MDA-MB-231 (A) and MCF-10A (B) cells were treated with PGE2 (0.1–10 μM) or 3-MC (1 μM) for 24 h or with 10 μM PGE2 for 6, 12, 24, and 48 h. Cells were lysed and immunoblotted. The blot was probed with anti-actin antibody, for normalization, and with anti-hCYP1B1 antibody. Each membrane is representative of three independent experiments. The densitometry data presented below the bands are “fold change” compared with control after normalization with respective loading control value. (C) Effect of PGE2 on CYP1B1 mRNA expression in MDA-MB-231 and MCF-10A cells. MDA-MB-231 (black bars) or MCF-10A cells (white bars) were treated with PGE2 (0.1–10 μM) or 3-MC (1 μM) for 24 h. Cells were lysed, and total RNA was prepared for PCR analysis of CYP1B1 mRNA expression relative to the expression of GAPDH. Results shown are representative of three independent experiments. *p < 0.01, significantly different from control, by the Newman-Keuls test. (D) Effect of PGE2 on CYP1B1 promoter activity in MDA-MB-231 and MCF-10A cells. MDA-MB-231 (black bars) or MCF-10A cells (white bars) were transfected with CYP1B1-Luc and cultured with 0.1–10 μM PGE2 or 3-MC for 24 h. Cells were harvested and assayed for luciferase activity, which was normalized to β-galactosidase activity. Data shown are representative of three independent experiments. *p < 0.01, significantly different from control, by the Newman-Keuls test. (E, F) Effect of PGE2 on ERα and ERβ protein levels in MCF-7 cells. Cells were incubated with 0.1–10 μM PGE2 for 10 min (E) or with 10 μM PGE2 for 5, 10, 20, 30, 60, and 120 min (F). Cells were lysed and immunoblotted. The blots were probed with anti-actin antibody, for normalization, and with anti-ERα and anti-ERβ antibodies. Each blot is representative of three independent experiments. The densitometry data presented below the bands are fold change compared with control after normalization with respective loading control value.
nonphosphorylated kinase on immunoblots of cell lysates were compared after normalization to actin expression. The level of phosphorylated ERK increased with PGE2 treatment, peaking at 10 min and declining by 20 min (Fig. 6A), and the phosphorylation of Akt exhibited a similar pattern (Fig. 6B). PGE2 also induced the phosphorylation of PKA in a time-dependent manner (Fig. 6C). However, the phosphorylation of Src did not change with PGE2 treatment (Fig. 6D). The results suggest that PGE2-regulated ERα activation is mediated by the phosphorylation of ERK, Akt, and PKA.

To further examine the upstream signaling pathway related to PGE2-induced CYP1B1 expression, we evaluated the effect of inhibitors of ERK, Akt, PKA, and Src (PD98059, LY294002, H-89, and PP2, respectively) on CYP1B1 expression. PD98059, LY294002, and H-89 blocked PGE2-induced CYP1B1 protein expression, but the Src inhibitor, PP2, did not (Fig. 6E). We next examined the gene expression and reporter activity of PGE2-induced CYP1B1 in the presence of these inhibitors. Similarly, PGE2-induced CYP1B1 mRNA expression and reporter activity were inhibited by PD98059, LY294002, and H-89 but not by PP2 (Figs. 6F and 6G). Therefore, PGE2-induced CYP1B1 expression and ligand-independent ERα activation result from the activation of ERK, Akt, and PKA.

Role of COX-2 in CYP1B1 Expression in MCF-7 Cells

COX-2 is upregulated in many breast tumors, and estrogen is recognized as a risk factor for the development of breast cancer (Bugano et al., 2008). COX-2 is the rate-limiting enzyme in the conversion of arachidonic acid to PGE2, and COX-2–mediated induction of PGE2 may regulate CYP1B1 expression. To
FIG. 6. Effect of PGE2 on the phosphorylation of ERK, Akt, PKA, and Src in MCF-7 cells. (A–D) Effect of PGE2 on the levels of phosphorylated ERK, Akt, PKA, and Src proteins in MCF-7 cells. Cells were incubated with 10 μM PGE2 for 5, 10, and 20 min. Cells were lysed and immunoblotted. The blot was probed with anti-actin antibody for normalization and with (A) anti-ERK and anti-phospho-ERK antibodies; (B) anti-Akt and anti-phospho-Akt antibodies; (C) anti-PKA and anti-phospho-PKA antibodies; and (D) anti-Src and anti-phospho-Src antibodies. The normalized signals from each nonphosphorylated and phosphorylated kinase were compared. Each blot is representative of three independent experiments. The densitometry data presented below the bands are “fold change” compared with control after normalization with respective loading control value. (E) Effect of phosphorylation of ERK, Akt, PKA, and Src on PGE2-induced CYP1B1 protein expression. Cells were pretreated with an inhibitor of ERK (PD98059 [PD; 10 μM]), Akt (LY294002 [LY; 5 μM]), PKA (H-89; 10 μM), or Src (PP2; 20 μM) and then incubated with 10 μM PGE2 for 24 h. Cells were lysed and immunoblotted. The blot was probed with anti-actin antibody, for normalization, and with anti-hCYP1B1 antibody. Each blot is representative of three independent experiments. The densitometry data presented below the bands are fold change compared with control after normalization with respective loading control value. (F) Effect of phosphorylation of ERK, Akt, PKA, and Src on PGE2-induced CYP1B1 mRNA expression. Cells were pretreated with the inhibitor PD98059 (PD; 10 μM), LY294002 (LY; 5 μM), H-89 (10 μM), or PP2 (20 μM) for 30 min and then treated with 10 μM PGE2 for 24 h. Cells were lysed, and total RNA was prepared for PCR analysis of CYP1B1 mRNA expression relative to the expression of GAPDH. Results shown are representative of three independent experiments. *p and **p < 0.01, significantly different from control and PGE2, respectively, by the Newman-Keuls test. (G) Effect of phosphorylation of ERK, Akt, PKA, and Src on PGE2-induced CYP1B1 promoter activity. Cells were transfected with CYP1B1-Luc, pretreated with inhibitors, and then incubated with 10 μM PGE2 for 24 h. Cells were harvested and assayed for luciferase activity, which was normalized to the β-galactosidase activity. Results shown are representative of three independent experiments. *p and **p < 0.01, significantly different from control and PGE2, respectively, by the Newman-Keuls test.
FIG. 7. Effect of COX-2 on CYP1B1 expression in MCF-7 cells. (A) Effect of COX-2 on PGE2-induced CYP1B1 protein expression. Cells were pretreated with a COX-2 inhibitor (NS-398 [NS; 100 μM]) for 30 min and then incubated with 10 μM PGE2 for 24 h. Cells were lysed and immunoblotted. The blot was probed with anti-actin antibody, for normalization, and with anti-hCYP1B1 antibody. Each blot is representative of three independent experiments. The densitometry data presented below the bands are “fold change” compared with control after normalization with respective loading control value. (B) Effect of COX-2 on PGE2-induced CYP1B1 mRNA expression. Cells were pretreated with NS-398 (NS; 100 μM) and then treated with 10 μM PGE2 for 24 h. Cells were lysed, and total RNA was prepared for PCR analysis of CYP1B1 mRNA expression relative to the expression of GAPDH. Results shown are representative of three independent experiments. *p < 0.05, significantly different from control and PGE2, respectively, by the Newman-Keuls test. (C) Effect of COX-2 on PGE2-induced CYP1B1 promoter activity. Cells were transfected with the hCYP1B1-Luc construct for 4 h and then treated with NS-398 (NS; 100 μM) and PGE2 (10 μM) for 24 h. Cells were harvested and assayed for luciferase activity, which was normalized to the β-galactosidase activity. Results shown are representative of three independent experiments. *p < 0.05, significantly different from control and PGE2, respectively, by the Newman-Keuls test. (D) Effect of COX-2 overexpression on CYP1B1 protein expression. Cells were transfected with pGL3-basic vector or COX-2 expression vector for 24 h. Cells were lysed and immunoblotted. The blot was probed with anti-actin antibody, for normalization, and with anti-hCOX-2 and anti-hCYP1B1 antibodies. Each blot is representative of three independent experiments. The densitometry data presented below the bands are “fold change” compared with control after normalization with respective loading control value. (E) Effect of COX-2 overexpression on CYP1B1 mRNA expression. Cells were transfected with pGL3-basic vector or COX-2 expression vector for 24 h. Cells were lysed, and total RNA was prepared for PCR analysis of CYP1B1 mRNA expression relative to the expression of GAPDH. (F) Effect of COX-2 overexpression on CYP1B1 promoter activity. Cells were transfected with pGL3-basic vector or COX-2 expression vector and the hCYP1B1-Luc construct for 24 h. Cells were harvested and assayed for luciferase activity, which was normalized to the β-galactosidase activity. Results shown are representative of three independent experiments. *p < 0.01, significantly different from control, by the Newman-Keuls test.
investigate the relationship between COX-2 and CYP1B1, we examined the effects of a COX-2 inhibitor, NS-398, on PGE2-induced CYP1B1 expression. NS-398 significantly reduced PGE2-induced CYP1B1 protein expression (Fig. 7A) as well as PGE2-induced CYP1B1 mRNA expression and reporter activity in MCF-7 cells (Figs. 7B and 7C). Furthermore, NS-398 alone suppressed CYP1B1 protein and mRNA expression and reporter activity in MCF-7 cells. Taken together, these results suggest that CYP1B1 expression may correlate with COX-2 protein expression in MCF-7 cells. To examine the direct effect of COX-2 on CYP1B1 expression in breast cancer cells, we used a COX-2 expression vector, which increased the level of COX-2 protein in MCF-7 cells. Compared with cells transfected with pGL3-basic vector, the COX-2 overexpressing cells displayed elevated CYP1B1 protein levels (Fig. 7D). To further confirm the induction of CYP1B1 expression by COX-2, we investigated its effect on CYP1B1 mRNA expression and promoter activity. MCF-7 cells cotransfected with hCYP1B1-Luc vector, and the COX-2 expression vector had increased CYP1B1 mRNA expression and luciferase activity (Figs. 7E and 7F). These results demonstrate that COX-2 may induce CYP1B1 expression in human breast cancer.

DISCUSSION

PGE2 is the predominant protumorigenic prostanoid, and we investigated the effect of PGE2 on CYP1B1 expression in breast cancer cells. PGE2 treatment was shown to significantly increase CYP1B1 protein and mRNA expression and promoter activity in MCF-7 breast cancer cells. The actions of PGE2 depend on the activation of one or more of the four PGE2 receptors (EP1–EP4) expressed by target cells (Timoshenko et al., 2006), and we demonstrated that PGE2-induced CYP1B1 expression is mediated through the EP2 and EP4 receptors. However, we do not know yet whether PGE2 stimulates CYP1B1 expression by activation of EP1 and EP3 receptor signal pathway. Recent studies have reported that estrogen induced CYP1B1 expression by ERE activation (Tsuchiya et al., 2004). In addition, some estrogen-regulated genes are indirectly regulated by the cooperation of Sp1 and ER within a guanin- and cytosin-rich regulation element (GC-box) and ERE half site (Pett and Nardulli, 2000; Saville et al., 2000). Next, using deletion promoter constructs of hCYP1B1 and cotreatment with inhibitors of AP-1-, XRE-, and ERE-binding site activation, we showed that the ERE transcription factor–binding site of the CYP1B1 promoter contributes to CYP1B1 regulation by PGE2. Furthermore, PGE2 was shown to rapidly induce the phosphorylation of ERs at serine residues 118, 167, and 305 and to increase the phosphorylation of ERK, Akt, and PKA. Finally, a COX-2 inhibitor (NS-398) was shown to reduce PGE2-induced CYP1B1 expression, and COX-2 overexpression induced CYP1B1 expression. Taken together, our results demonstrate that PGE2-induced CYP1B1 expression is mediated by PGE2-induced ligand-independent activation of the ERα pathway via the ERK, Akt, and PKA signaling pathways (Fig. 8).

COX-2 is upregulated rapidly by growth factors and cytokines and contributes to inflammation, which is one of the first responses of the immune system to infection or irritation. The main product of COX-2, namely PGE2, has been found at high levels in tumor cells (Schrey and Patel, 1995) and is synthesized in several human breast cancer cell lines. PGE2 expression has been shown to correlate with the expression of aromatase, an estrogen synthetase, in human breast cancer tissue (Michael et al., 1997). Estrogens have long been associated with breast cancer, and it is thought that a woman’s cumulative exposure to estrogen is an important determinant of her risk for the disease (Clamp et al., 2002; Clemons and Goss, 2001; Eliassen et al., 2006; Tamimi et al., 2007; Yager and Davidson, 2006).

Several lines of evidence suggest that CYP1B1 plays a role in carcinogenesis. CYP1B1 is commonly overexpressed in human malignancies (Gibson et al., 2003; Lin et al., 2003; Murray et al., 1997). CYP1B1 readily metabolizes E2 via its primary hydroxylase activity at C-4 (Spink et al., 2001), and 4-hydroxyestradiol has been shown to be carcinogenic in animal models (Spivack et al., 2001). Additionally, 4-hydroxyestradiol generates free radicals from reductive-oxidative cycling with the corresponding semiquinone and quinone forms, which cause cellular damage and stimulate cell proliferation and gene expression via the ER (Cavaleri et al., 1997, 2006; Zhang et al., 2007). Thus, 4-hydroxyestradiol is a unique carcinogen.

![Schematic of the CYP1B1 induction pathway by PGE2 in breast cancer cells](https://academic.oup.com/toxsci/article-abstract/114/2/204/1673301)
that affects both tumor initiation and promotion (Gaikwad et al., 2008; Yager and Davidson, 2006; Yue et al., 2003). The present study indicates that COX-2–synthesized PGE\textsubscript{2} significantly induces CYP1B1 expression through ligand-independent ER\textsubscript{x} activation via the ERK, Akt, and PKA signaling pathways in human breast cancer cells. PGE\textsubscript{2}-induced CYP1B1 metabolizes estradiol to 4-hydroxyestradiol, which is associated with the promotion of breast cancer.

FUNDING

National Institute of Toxicological Research, KFDA (08152KFDA424) and Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by Ministry of Education, Science and Technology (2009-0093815), Republic of Korea.

ACKNOWLEDGMENTS

Conflict of Interest: None declared.

REFERENCES


