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Studies on the estrogen biosynthesis and metabolism related aromatase and CYP1B1 expression by PGE_2 and o,p'-DDT in breast cancer cells

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약 학 과

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PGE_2와 o,p′-DDT의 여성호르몬 합성 및 대사 조절 단백질 aromatase와 CYP1B1 발현 조절 연구

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이 논문을 약학 박사학위 논문으로 제출함.

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한은희의 박사학위 논문을 인준함

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List of Abbreviations

α-NF	α-Naphthoflavone
AP-1	Activator protein-1
cAMP	Cyclic adenosine monophosphate
ChIP assay	Chromatin immunoprecipitation assay
COX-2	Cyclooxygenase-2
CRE	Cyclic AMP response element
CREB	CRE binding protein
CYP1B1	Cytochrome P450 1B1
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
E2	17β-Estradiol
ELISA	Enzyme-linked immunosorbent assay
EP receptor	PGE_2 receptor
ER	Estrogen receptor
ERE	Estrogen response element
ERK1/2	Extracellular signal-related kinase1/2
FBS	Fetal bovine serum
Hsp90	Heat shock protein 90
JNK1/2	c-Jun N-terminal kinase1/2
MAP kinases	Mitogen-activated protein kinases

MXC	Methoxychlor
o,p'-DDT	o,p'-Dichlorodiphenyltrichloroethane
PCR	Polymerase chain reaction
PGE_2	Prostaglandin E_2
PKA	Protein kinase A
PMSF	Phenylmethylsulfonylfluoride
PVDF	Polyvinylidene difluoride
RSK	Ribosomal S6 kinase
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
XRE	Xenobiotic response element
3-MC	3-Methylcholanthrene
4-OHE2	4-Hydroxyestradiol

ABSTRACT

Studies on the estrogen biosynthesis and metabolism related aromatase and CYP1B1 expression by PGE_2 and o,p'-DDT in breast cancer cells

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Up-regulation of aromatase by o,p'-DDT is mediated through cyclooxygenase-2 expression in human breast cancer cells

o,p'-Dichlorodiphenyltrichloroethane (o,p'-DDT) is a DDT isomer that can induce inflammation and cancer. However, the effect of o,p'-DDT on aromatase is unclear. Therefore, this study was investigated the effect of o,p'-DDT on aromatase expression in human breast cancer cells. This study was also studied whether cyclooxygenase-2 (COX-2) was involved in o,p'-DDT-mediated aromatase expression. o,p'-DDT induced aromatase protein expression in human breast cancer

MCF-7, MDA-MB-231 and SK-BR-3 cells. In addition, o,p'-DDT enhanced aromatase gene expression and activity of enzyme and promoter in MCF-7 cells. o,p'-DDT also markedly increased the levels of COX-2 protein levels in MCF-7, MDA-MB-231 and SK-BR-3 cells. o,p'-DDT induced COX-2 mRNA expression and promoter activity in MCF-7 cells. o,p'-DDT enhanced the production of prostaglandin E_2 (PGE_2) and the gene expression of PGE_2 (EP2 and EP4) receptor. Moreover, o,p'-DDT induced cyclic AMP response element (CRE) activation, cAMP level and binding of CREB. o,p'-DDT increased the phosphorylation of PKA, Akt, ERK and JNK in their signaling pathways. Finally, o,p'-DDT-induced aromatase was inhibited by various inhibitors [COX-2 (NS-398), PKA (H-89), PI3-K/Akt (LY 294002) EP2 (AH 6809) and EP4 receptor (AH 23848)]. Taken together, these results suggest that o,p'-DDT increased aromatase and o,p'-DDT-induced aromatase was correlated with COX-2 up-regulation mediated via PKA and PI3-kinase/Akt signaling pathways in breast cancer cells.

Prostaglandin E2 induces CYP1B1 expression *via* ligand-independent activation of the ERα pathway in human breast cancer cells

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 E_2 (PGE_2), has been detected in invasive breast carcinomas. However, the interaction between PGE_2 and CYP1B1 expression in human breast cancer is unclear. Here, this study was investigated the effect of PGE_2 on CYP1B1 expression and its mechanism in breast cancer cells. PGE_2 significantly increased CYP1B1 protein and mRNA expression and dose-dependently enhanced CYP1B1 promoter activity in human breast cancer MCF-7 cells. Transient transfection with hCYP1B1 deletion promoter constructs and co-treatment with inhibitors revealed that the estrogen response element contributed to the effects of PGE_2. CYP1B1 expression was not affected by PGE_2 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 PGE_2 treatment in MCF-7 cells. However, PGE_2 rapidly induced phosphorylation of ERα at serine residues 118, 167, and 305, suggesting that PGE_2 activates ERα in a ligand-independent manner. PGE_2 also increased phosphorylation of extracellular signal-regulated kinase, Akt, and protein kinase A. Finally, a COX-2 inhibitor inhibited PGE_2-induced CYP1B1 expression, and COX-2 overexpression increased CYP1B1 expression. Our results indicate that PGE_2-

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.

I. Introduction

Up-regulation of aromatase by o,p'-DDT is mediated through cyclooxygenase-2 expression in human breast cancer cells

Breast cancer is one of the most common types of cancer among women throughout the world and it is the second leading cause of cancer deaths in women. The majority of breast tumors is hormone-dependent and expresses estrogen receptors, and their growth is regulated by potent estrogenic hormones such as estradiol. The enzyme responsible for production of estradiol is aromatase, which is a member of the cytochrome P450 family of enzymes (Brodie *et al*., 1998). Aromatase is encoded by the CYP19 gene and is regulated in a complex, tissue-specific manner (Bulun *et al*., 2003, 2004; Simpson, 2004). In breast tumors, aromatase is stimulated through cyclic adenosine monophosphate (cAMP)-mediated pathways (Zhao *et al*., 1996; Sebastian *et al*., 2002; Zhou *et al*., 2001; Bulun *et al*., 2003, 2004; Simpson, 2004). Another factor that has been shown to regulate the activity of the aromatase enzyme is the bioactive lipid prostaglandin E_2 (PGE_2). PGE_2 is formed through the activity of the cyclooxygenase-2 (COX-2) enzyme, which is the rate-limiting enzyme that catalyzes the biosynthesis of prostanoids from arachidonic acid (Hla *et al*., 1999; Simpson, 2004). Recent studies have demonstrated that COX-2 is produced in the majority of human breast tumors with invasive characteristics, regardless of their hormone receptor status (McCarthy *et al*., 2006), suggesting that COX-2 may play a more central role in mediating breast tumor development and

progression than previously believed. The observation that human breast tumors overexpress COX-2 is consistent with studies reporting that a wide variety of human tumors of epithelial origin exhibit increased expression of COX-2 mRNA and protein and produce high levels of PGE_2. Furthermore, COX-2 is upregulated in human breast tumors (Gilhooly and Rose, 1999), and an association between high levels of COX-2 gene expression and increased aromatase mRNA is reported in invasive human breast tumors (Brueggemeier *et al*., 1999; Harris *et al*., 1999; Brodie *et al*., 2001; Oliveira *et al*., 2006).

Although environmental toxicants can disrupt endocrine homeostasis with potentially disastrous consequences for wildlife, the consequences to human health is less clear and remain highly controversial. Regardless, environmental toxicants continue to be regarded as important contributing factors in the pathogenesis of estrogen-dependent diseases such as breast cancer and endometriosis (Rier and Foster, 2002; Sasco, 2003). DDT is the oldest pesticide still in use. However, its use is restricted in many countries and it is thought to act as an endocrine disruptor and to have carcinogenic effects. Due to its lipophilic nature and its slow chemical and biological rates of degradation, this molecule tends to be taken up by biological membranes and tissues. DDT and related compounds, either alone or in combination, are thought to promote human diseases (Auger *et al*., 1995). Our laboratory was the first to report that o,p'-DDT, a DDT isomer, induces COX-2 enzyme in macrophages, which subsequently increases CRE activation (Han *et al*., 2008). However, the influence of o,p'-DDT on aromatase in breast cancer and the interaction between o,p'-DDT-induced aromatase and COX-2 expression are unclear.

Therefore, the present study was investigated the effect of o,p'-DDT on aromatase in breast cancer cells. This study was demonstrated that o,p'-DDT can stimulate the expression of aromatase through COX-2-dependent mechanisms.

Prostaglandin E2 induces CYP1B1 expression *via* ligand-independent activation of the ERα pathway in human breast cancer cells

Breast cancer is the most common cancer among women and the second most common cause of cancer death in women (Bugano *et al.*, 2008). Estrogens have long been recognized as the prime risk factor for the development of breast cancer (Bugano *et al.*, 2008). In the breast, the main estrogen, 17β-estradiol (E2), is a substrate for the phase I enzyme cytochrome P450 (CYP) 1B1 and is a ligand for the estrogen receptor (ER).

CYP1B1, an extra-hepatic enzyme, is a member of the CYP1 family and is expressed constitutively in many human tissues, including breast and ovary (Muskhelishvili *et al.*, 2001; Jefcoate *et al.*, 2000; 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 (Cavalieri *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 (McFadyen *et al.*, 1999; Liehr and Ricci, 1996).

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 (Nilsson *et al.*, 2001; Zhong and Skafar, 2002; Beato and Klug,

2000). 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 (Zhong and Skafar, 2002; Klinge, 2000). 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.*, 2000); 167, by Akt (Feng *et al.*, 2001); and 305, by protein kinase A (PKA; Chen *et al.*, 1999). In addition, tyrosine 537 in the C-terminal region of ERα can be phosphorylated by Src kinase, resulting in increased transcription of target genes (Sassone-Corsi, 1998); this region is responsible for the ligand-independent transactivation functions of ERα (Shah and Rowan, 2005).

The overexpression of cyclooxygenase-2 (COX-2) has been detected in a number of solid tumors, including breast cancer (Half *et al.*, 2002; Ristimaki *et al.*, 2002). Elevated COX-2 expression in breast cancer tumors is associated with increased tumorigenic transformation (Howe *et al.*, 2005; Liu *et al.*, 2001), higher-grade tumors, and decreased overall and progression-free survival times (Ristimaki *et al.*, 2002; Surowiak *et al.*, 2005). COX, also known as prostaglandin endoperoxide synthase, is the rate-limiting enzyme in the conversion of arachidonic acid to

prostaglandin H_2 and prostaglandin E_2 (PGE_2). PGE_2 stimulates estrogen biosynthesis by increasing the expression of the aromatase (CYP19) gene in breast stromal cells (Zhao *et al.*, 1996). However, the interaction between PGE_2 and CYP1B1 expression in human breast cancer is unclear.

This study was investigated the effect of PGE_2 on CYP1B1 expression and its precise mechanism in breast cancer cells. Taken together, these results indicate that PGE_2 increases CYP1B1 expression via the ligand-independent activation of the ERα pathway in human breast cancer MCF-7 cells.

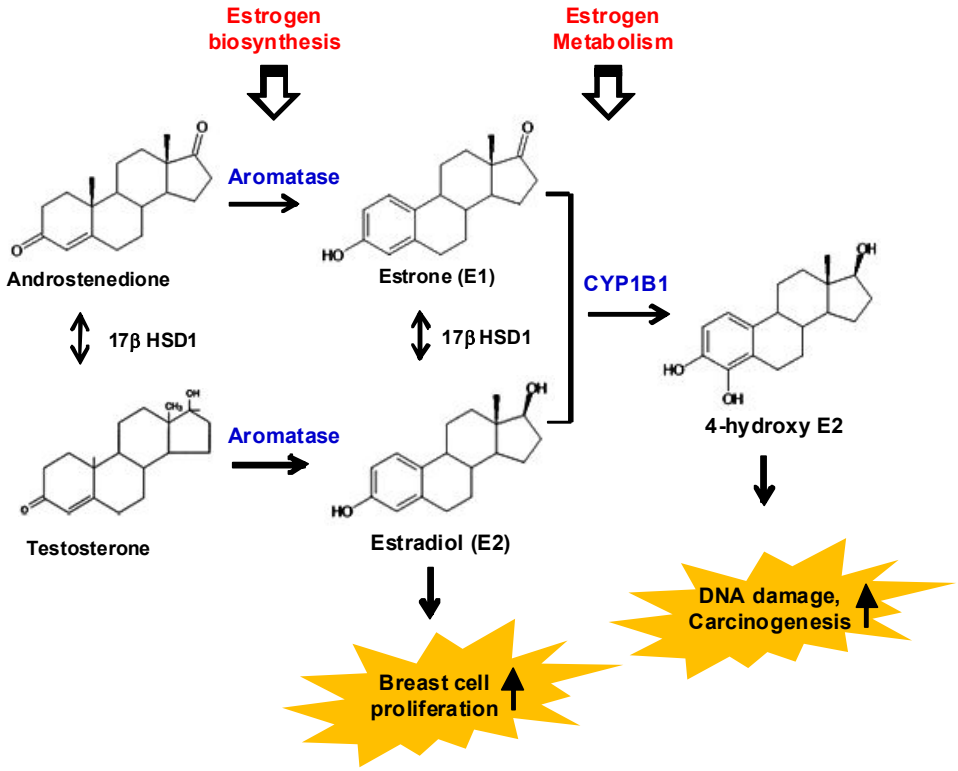


Fig. 1. The biosynthesis and metabolism of estrogen. Several genetic polymorphisms that may influence estrogen concentrations have been identified in genes involved in estrogen biosynthesis (e.g., aromatase) and estrogen metabolism (e.g., CYP1B1).

II. Materials & Methods

Up-regulation of aromatase by o,p'-DDT is mediated through cyclooxygenase-2 expression in human breast cancer cells

1. Materials

Chemicals and cell culture materials were obtained from the following sources: o,p'-DDT, methoxychlor from Supelco Co.; prostaglandin E_2 (PGE_2) immunoassay reagents and antibody against COX-2 from Cayman Co.; Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin solution from Life Technologies, Inc.; luciferase assay system and cAMP immunoassay reagents from Promega; pCMV-β-gal from Clontech; LipofectAMINE 2000 from Invitrogen, Inc.; pCRE-Luc was purchased from Stratagene; antibody against β-actin from Santa Cruz Biotechnology, Inc.; antibody against aromatase from Abcam; protein assay kit from Bio-Rad Laboratories, Inc.; primary antibodies [anti-PKA, anti-Akt/phospho-Akt (Ser473), anti-phospho-MAPK (Erkl/2) (Thr202/ Tyr204), anti-phospho-p38 MAPK (Thr180/ Tyr182), anti-phospho-SAPK/JNK (Thr183/ Tyr185), anti-aromatase, anti-CREB, anti-phospho-CREB, anti-actin and Lamin B] and secondary antibodies (HRP-linked anti-rabbit and anti-mouse IgG) from Cell Signaling Technology; ECL chemiluminescence system, polyvinylidene difluoride (PVDF) membrane and [1β-^{3}H] androst-4-ene-3,17-dione from Amersham Pharmacia Biotech.; and AH-6809,

AH-23848, H-89, NS-398 and LY 294002 from Calbiochem, Inc. Polymerase chain reaction (PCR) oligonucleotide primers were custom synthesized by Bioneer Co. (Korea). All chemicals were of the highest grade commercially available.

2. Cell culture and treatment

MCF-7, MDA-MB-231 and SK-BR-3 cells (American Type Culture Collection, Manassas, VA, USA) were grown in DMEM supplemented with 2 mM glutamine and 10% fetal bovine serum at 37°C in an atmosphere containing 5% CO_2. The o,p'-DDT was dissolved in ethanol, and stock solutions were added directly to the culture media. Control cells were treated only with solvent. The final concentration of solvent never exceeded 0.1% and did not affect the assay systems.

3. RNA preparation and mRNA expression analysis by real-time PCR

Total RNA from the treated cells was prepared with RNAiso Reagent (Takara) according to the manufacturer's protocol and stored at -80°C until use. For detection of aromatase, COX-2, EP2 and EP4 receptor, total RNA was extracted after stimulation and treatment. PCR product formation was continuously monitored during the PCR reaction using Sequence Detection System software, version 1.7 (Applied Biosystems, Foster City, CA, USA). Accumulated PCR products were detected directly by monitoring the increase of the reporter dye (SYBR). The expression levels of aromatase, COX-2, EP2 and EP4 receptor in the treated cells were compared to the expression levels in control cells at each collection time point using the comparative cycle threshold (Ct)-method (Johnson

et al., 2000). The sequences of the primers used in this study were: aromatase forward: 5'-CTC CTC ATC AAA CCA GAC ATC-3'; aromatase reverse: 5'- TAA AAT CAA CTC AGT GGC AAA G-3'; COX-2 forward: 5'-CCC TGA ACG CGT ACA CAT CA-3'; COX-2 reverse: 5'-TGT CAC TGT AGA GGG CTT TCA ATT-3'; EP2 receptor forward: 5'- CTG CTG CTG CTT CTC ATT GT-3'; EP2 receptor reverse: 5'- ATG CGG ATG AGG TTG AGA AT-3'; EP4 receptor forward: 5'- CGA CCT TCT ACA CGC TGG TAT G-3'; EP4 receptor reverse: 5'- CCG GGC TCA CCA ACA AAG T-3'; β-actin forward: 5'- TCA TCA CCA TCG GCA ACG-3', β-actin reverse: 5'- TTC CT GAT GTC CAC GTC GC-3'. The quantity of each transcript was calculated as described in the instrument manual and normalized to the amount of β-actin, a housekeeping gene.

4. PGE_2 production

MCF-7 cells were incubated with o,p'-DDT for 24 h, and the culture medium was collected. The PGE_2 released into the culture medium was measured using a specific enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. PGE_2 concentrations were determined by measuring absorbance at 415 nm.

5. Reporter gene assay

A luciferase reporter assay system (Promega) was used to determine promoter activity. Cells were transiently transfected with 0.5 mg of aromatase-Luc, human COX-2 promoter-Luc (−1432/+59), pCRE-Luc and 0.2 mg pCMV-β-gal plasmid

using the LipofectAMINE 2000 Reagent. After 4 h, the medium was replaced with basal medium. The cells were then treated with o,p'-DDT for 24 h and lysed. The luciferase and β-galactosidase activities were measured in the cellular extract as described previously (Mestre *et al*., 1997). The luciferase activity was normalized to the β-galactosidase activity and expressed relative to the activity of the control group.

6. Preparation of the nuclear fraction

o,p'-DDT-treated cells were washed with ice-cold PBS and 100 μl of lysis buffer containing 10 mM HEPES (pH 7.9), 0.5% Nonidet P-40, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonylfluoride (PMSF) were added. Cell membranes were disrupted by vortexing, and the lysates were incubated for 5 min on ice and centrifuged at 7,200 g for 5 min. Pellets containing crude nuclei were resuspended in 50 μl of extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF, and then incubated for 30 min on ice. The samples were centrifuged at 15,800 g for 30 min to obtain the supernatants containing the nuclear extracts. The nuclear extracts were stored at -80°C until used.

7. Western blotting

Cells were pretreated with inhibitors [COX-2 (NS-398), PKA (H-89), Akt (LY 294002), ERK (PD98059), JNK (SP600125), EP2 receptor (AH-6809) and EP4

receptor (AH-23848)] and then incubated with o,p'-DDT or methoxychlor. Cell lysates were resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were electroblotted onto polyvinylidene difluoride membranes. The membranes were probed with the appropriate primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibody. The blots were visualized with an ECL western blot kit according to the manufacturer's instructions.

8. Determination of aromatase activity

Aromatase activity was measured with a tritiated water release assay (Kinoshita and Chen, 2003). Cells were treated with o,p'-DDT in serum-free medium for 72 h and then treated with [1β-^{3}H] androst-4-ene-3,17-dione (100 nM) for an additional 1 h. The medium was then mixed thoroughly with 5% charcoal/0.5% dextran for 12 h and centrifuged at 10,000 × g for 30 min at 4°C to remove any residual androst-4-ene-3,17-dione. A 1-ml aliquot of the supernatant was added to a scintillation vial containing 3 ml of scintillation cocktail, and ^{3}H was measured as disintegrations per minute using a liquid scintillation counter (LS 6500, Beckman Coulter Inc., Fullerton, CA, USA).

9. cAMP determination

The 3',5'-cyclic AMP related chemiluminescent enzyme-linked immunosorbent assay (ELISA) kit (Promega, Madison, WI) was used to determine cAMP levels in o,p'-DDT-treated cell lysates, according to the manufacturer's protocol.

10. Chromatin immunoprecipitation (ChIP) Assays

Cells incubated with o,p'-DDT were cross-linked with formaldehyde at room temperature for 10 min, and the sonicated chromatin-DNA complexes were precipitated with CREB antibody or nonspecific rabbit IgG. PCR analysis was carried out using 1 μl of purified DNA, oligonucleotide primers (CRE) 5'-GCCCCCCTCCCGCTCCCATT-3' and 5'-TGGGGCTCTTCCTGGGCAGC-3', and Platinum TaqDNA polymerase. After 40 amplification cycles, PCR products were analyzed on 2 % agarose gel stained with ethidium bromide (Kim *et al*., 2008; Rao *et al*., 2008).

11. Assay for proliferative activity

Cell proliferation assays were performed using a WST-1 assay kit according to the manufacturer's instructions. Briefly, MCF-7 cells were seeded onto 96-well plates and then incubated with serum-free medium. o,p'-DDT was added to the testosterone (1 μM)-treated plates and the plates were incubated at 37°C. After 72 h, the cells were treated with 10 μl of WST-1 solution. Cell proliferation was quantified by measuring the absorbance at 550 nm using a spectrofluorometer (Varioskan, Thermo Electron Co.).

Prostaglandin E2 induces CYP1B1 expression *via* ligand-independent activation of the ERα pathway in human breast cancer cells

12. Materials

Chemicals and cell culture materials were obtained from the following sources: prostaglandin E_2 (PGE_2), 3-methylcholanthrene (3-MC), and curcumin from Sigma-Aldrich; Dulbecco's Modified Eagle's Medium (DMEM), DMEM/nutrient mixture F-12 (DMEM/F12), phenol red-free DMEM, phenol red-free DMEM/F12, fetal bovine serum (FBS), fetal calf serum (FCS), and penicillin-streptomycin solution from Life Technologies, Inc.; luciferase assay system from Promega; pCMV-β-gal from Clontech; Lipofectamine™ 2000 from Invitrogen, Inc.; antibodies against actin, ERα, and ERβ from Santa Cruz Biotechnology, Inc.; antibody against CYP1B1 from Abcam; protein assay kit from Bio-Rad Laboratories, Inc.; antibody against anti-phospho-ERα (Ser305) and blocking peptide from Bethyl Laboratories, Inc.; antibodies against anti-phospho-ERα (Ser118 and 167), anti-phospho-tyrosine, anti-Akt/phospho-Akt (Ser473), anti-Src/phospho-Src (Tyr527), anti-ERK/phospho-ERKl/2 (Thr202/Tyr204), and horseradish peroxidase (HRP)-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, AH-6809, AH-23848, PD98059, and LY294002 from Calbiochem, Inc.; and ICI 182.780 from Tocris

(Fisher Bioblock Scientific). Polymerase chain reaction (PCR) oligonucleotide primers were custom synthesized by Bioneer Co. (Korea). All chemicals were of the highest grade commercially available.

13. Cell culture and treatment

Human breast cancer cell lines MCF-7 and MDA-MB-231 and the normal breast epithelial cell line MCF 10A (American Type Culture Collection, Manassas, VA, USA) were grown in DMEM (MCF-7 and MDA-MB-231) or DMEM/F12 (MCF 10A), supplemented with 2 mM glutamine and 10% FBS at 37°C in an atmosphere containing 5% CO_2. For routine culture, cells were grown in medium containing phenol red. To remove serum-derived estrogenic compounds and to avoid the estrogenic effects of phenol red, before each experiment cells were grown for 2 weeks in phenol red-free medium (DMEM or DMEM/F12) containing serum treated with 5% dextran-coated, charcoal-stripped FCS (Berthois *et al.*, 1986), and all experiments were conducted in this medium. Stock solutions of PGE_2 and each inhibitor (ICI 182.780, curcumin, CH-223191, AH-6809, AH-23848, H-89, NS-398, PP2, PD98059, and LY294002) were prepared in dimethylsulfoxide (DMSO) and added directly to the culture medium for incubation. Control cells were treated only with DMSO. The final DMSO concentration was always <0.2%.

14. Western blotting

Cells were pretreated with an inhibitor (ICI 182.780, curcumin, CH-223191, AH-6809, AH-23848, H-89, NS-398, PP2, PD98059, or LY294002) and then incubated

with PGE_2 or 3-MC. In addition, cells were transfected with a COX-2 expression vector for 24 h. Cell lysates were resolved by 10% SDS-PAGE, 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.

15. RNA preparation and mRNA analysis by Taqman PCR

Cells were pretreated with an inhibitor (ICI 182.780, curcumin, CH-223191, AH-6809, AH-23848, H-89, NS-398, PP2, PD98059, or LY294002) and treated with PGE_2 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 CT method.

16. Plasmids

The pGL3 basic vector and pERE-Luc were purchased from Promega, and the COX-2 expression vector was 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'-BglII, GAA GAT CTG CCC TAA GAA CTC CAG GCT TC; CYP1B1-3'-BglII, GAA GAT CTG GGG ACA GAG AGG AGA AGG CG; CYP1B1-5'-KpnI, GGG GTA CCG CCC TAA GAA CTC CAG GCT TC, and CYP1B1-3'-HindIII, CCC AAG CTT CTG GAG TCG CAG AAG CGC TCC. All PCR products were sequenced and confirmed to be identical to the published sequence of the CYP1B1 promoter.

17. Reporter gene assay 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 PGE_2 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.

18. Immunoprecipitation

Cells were cultured with PGE_2 for 10 min. Whole-cell lysates were prepared in 0.5% NP-40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, and 0.5% NP-40) supplemented with protease inhibitors, cellular debris was removed by centrifugation at 12,000 rpm 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 phospho-tyrosine was detected by Western blot analysis.

19. Statistical analysis

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

III. Results

Up-regulation of aromatase by o,p'-DDT is mediated through cyclooxygenase-2 expression in human breast cancer cells

1. o,p'-DDT induces aromatase mRNA expression, protein level and enzyme activity in human breast cancer cells

To examine the effect of o,p'-DDT on aromatase in breast cancer cells, this study was treated MCF-7 cells with o,p'-DDT. PGE_2 was used as a positive control in these experiments. PGE_2 is a potent stimulator of aromatase expression, and several lines of evidence suggest that PGE_2 is involved in breast cancer development and progression (Purohit *et al.*, 1995; Schrey and Patel, 1995). In our evaluation of the effect of o,p'-DDT, this study was measured the protein expression of aromatase by immunoblot assay. MCF-7 cells were treated with o,p'-DDT (0.01, 0.1 and 1 μM) or PGE_2 for 24 h. o,p'-DDT and PGE_2 each induced the aromatase protein level in a dose-dependent manner (Fig. 2A). In addition, MCF-7 cells were treated with o,p'-DDT (1 μM) for 6, 12, 24 and 48 h. Aromatase protein level was increased a peak at 24 h and declined at 48 h by o,p'-DDT (Fig. 2B). Further studies have compared that effects of o,p'-DDT on aromatase protein expression in several human breast cancer cells lines, including MDA-MB-231 and SK-BR-3 cells. o,p'-DDT induced aromatase protein level in MDA-MB-231 and SK-BR-3 cells (Fig. 3A, B). MDA-MB-231 and SK-BR-3 cells exhibited similar pattern of aromatase protein levels in

MCF-7 cells. The aromatase protein level was compared to that of a housekeeping gene such as actin. This study was next tested the effects of o,p'-DDT on aromatase gene expression in a real-time PCR assay. o,p'-DDT and PGE_2 dose-dependently increased the level of aromatase mRNA in MCF-7 cells (Fig. 4A). In addition, MCF-7 cells were treated with 1 μM o,p'-DDT for 6, 12, 24 and 48 h. Aromatase mRNA expression was increased a peak at 24 h and declined at 48 h by o,p'-DDT (Fig. 4B). The result exhibited a similar pattern to that of aromatase protein expression. The control actin gene was constitutively expressed and was unaffected by o,p'-DDT treatment. Thus, o,p'-DDT maybe regulates the transcriptional activation of aromatase in MCF-7 cells.. To study the effect of o,p'-DDT on aromatase activity, this study was used the tritiated water-release assay. Cells were treated with o,p'-DDT in serum-free medium for 72 h and then treated with [1β-^{3}H] androst-4-ene-3,17-dione (100 nM) for an additional 1 h. o,p'-DDT and PGE_2 increased the conversion of [^{3}H]androstenedione to [^{3}H]estrogen in MCF-7 cells (Fig. 5A). Further studies, this study was investigated that effects of o,p'-DDT on transcriptional activation of aromatase in MDA-MB-231 and SK-BR-3 cells. o,p'-DDT enhanced the conversion of [^{3}H]estrogen of aromatase in MDA-MB-231 and SK-BR-3 cells (Fig. 5B). PGE_2 was used as a positive control in these experiments. These results showed that MDA-MB-231 cells were sensitive more than SK-BR-3 cells. To clarify the mechanism of aromatase gene expression, this study was transfected MCF-7 cells with the aromatase-Luc reporter constructs. o,p'-DDT and PGE_2 increased the aromatase luciferase activity in MCF-7 cells (Fig. 6A). These

results demonstrate that aromatase protein and activity levels were enhanced through the induction of aromatase gene expression and promoter activity in o,p'-DDT-treated MCF-7 cells. Next, this study was investigated that effects of o,p'-DDT on and luciferase activity of aromatase in MDA-MB-231 and SK-BR-3 cells. o,p'-DDT enhanced luciferase activity of aromatase in MDA-MB-231 and SK-BR-3 cells (Fig. 6B). PGE_2 was used as a positive control in these experiments.

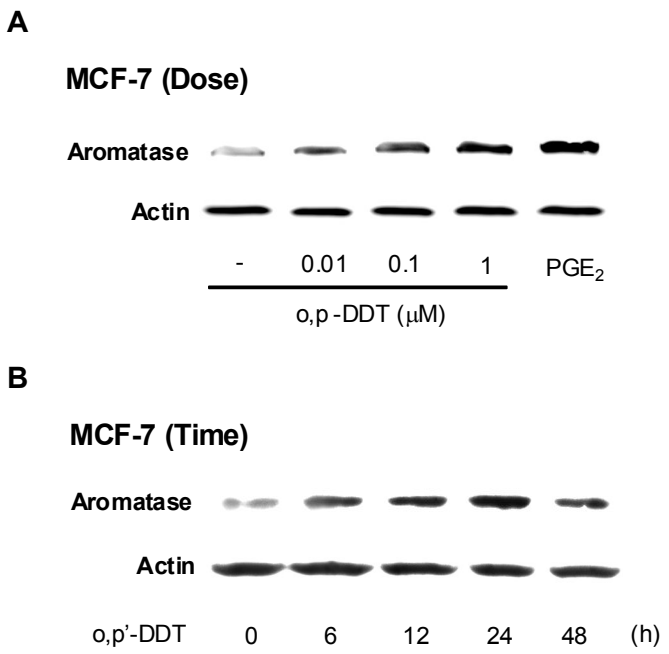


Fig. 2. Effects of o,p'-DDT on aromatase protein levels in MCF-7 cells. (A) Cells were treated with o,p'-DDT (0.01–1 μM) or PGE_2 (1 μM) for 24 h. (B) Cells were treated with o,p'-DDT (1 μM) for 6, 12, 24 and 48 h in MCF-7 cells. The membrane was probed with human aromatase-specific antibody. Each blot in this figure is representative of three independent experiments with similar results. The aromatase protein level was compared to actin protein.

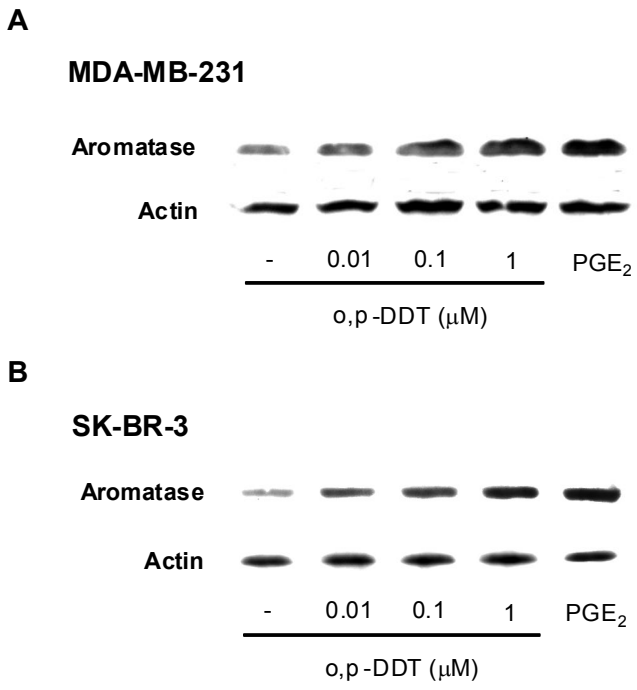


Fig. 3. Effects of o,p'-DDT on aromatase protein levels in MDA-MB-231 and SK-BR-3 cells. Cells were treated with o,p'-DDT (0.01–1 μM) or PGE_2 (1 μM) for 24 h in MDA-MB-231 (A) and SK-BR-3 cells (B). The membrane was probed with human aromatase-specific antibody. Each blot in this figure is representative of three independent experiments with similar results. The aromatase protein level was compared to actin protein.

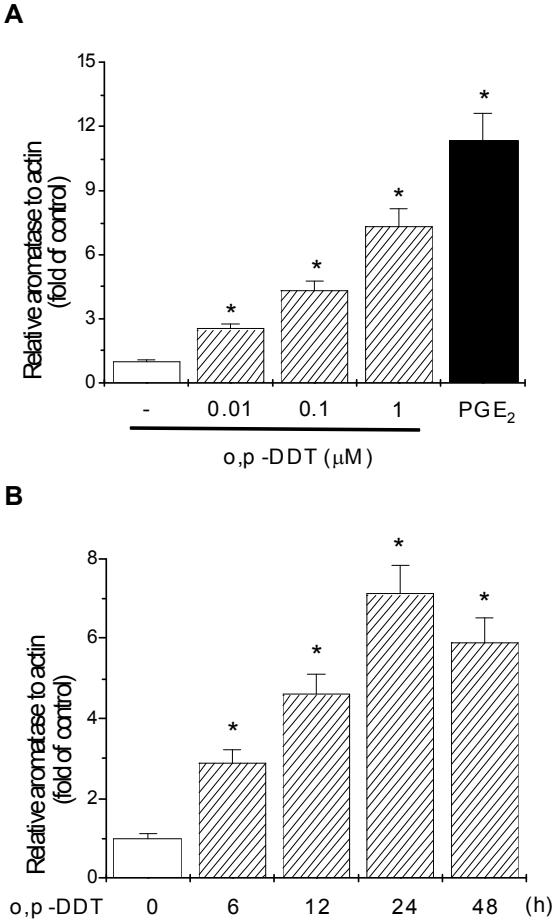


Fig. 4. Effects of o,p'-DDT on aromatase mRNA levels in MCF-7 cells. Cells were treated with o,p'-DDT (0.01–1 μM) or PGE_2 (1 μM) for 24 h (A). Cells were treated with o,p'-DDT (1 μM) for 6, 12, 24 and 48 h in MCF-7 cells (B). The cells were lysed and total RNA was prepared for analysis of aromatase gene expression. PCR amplification of the housekeeping gene, β-actin, was performed for each sample. Aromatase mRNA expression in treated cells was compared to the expression in untreated cells at each time point by real-time PCR. Each bar shows three independent experiments. * $P < 0.01$, significantly different from control as determined by analysis of variance by Newman–Keuls test.

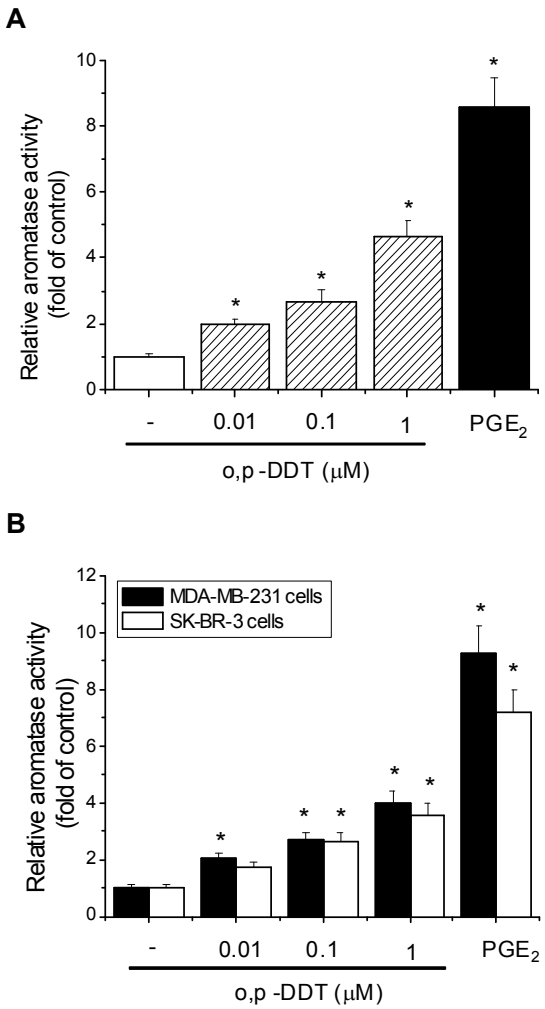


Fig. 5. Effects of o,p'-DDT on aromatase activity in human breast cancer cells. (A) After serum starvation for 24 h and then treatment with o,p'-DDT (0.01–1 μM) or PGE_2 (1 μM) for 72 h in MCF-7 cells, aromatase activity was measured. (B) Cells were treated o,p'-DDT (0.01–1 μM) or PGE_2 (1 μM) for 72 h in MDA-MB-231 and SK-BR-3 cells. Each bar shows three independent experiments. * $P < 0.01$, significantly different from control as determined by analysis of variance by Newman–Keuls test.

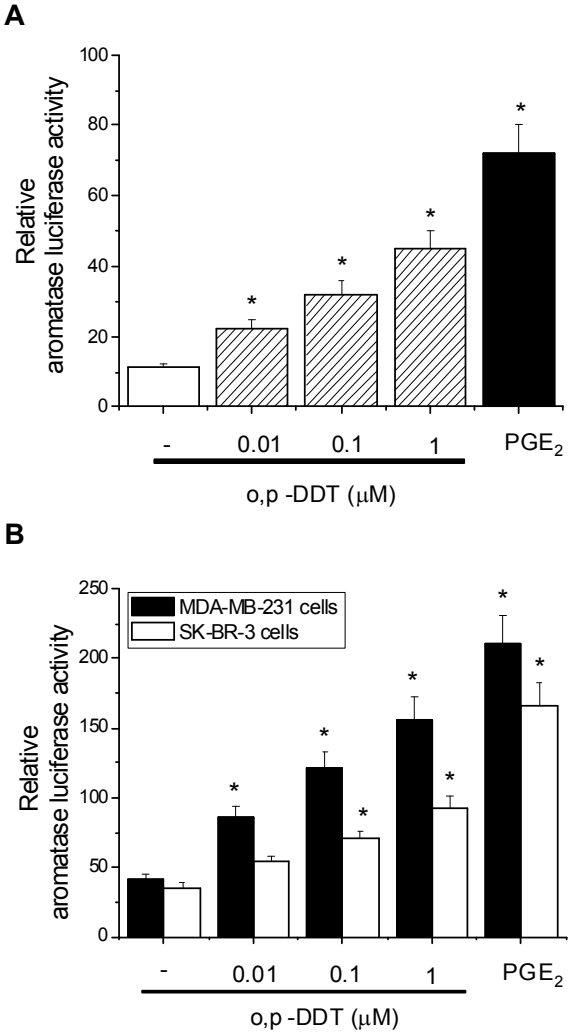


Fig. 6. Effects of o,p'-DDT on aromatase promoter activity in human breast cancer cells. (A) Cells were transfected with aromatase-Luc and then treated with o,p'-DDT (0.01–1 μM) or PGE_2 (1 μM) for 20 h in MCF-7 cells. Cells were then harvested and assayed for luciferase activity. (B) Cells were transfected with aromatase-Luc and then treated with o,p'-DDT or PGE_2 for 20 h in MDA-MB-231 and SK-BR-3 cells. Each bar shows three independent experiments. $*P < 0.01$, significantly different from control as determined by analysis of variance by Newman–Keuls test.

2. o,p'-DDT induces mRNA expression and protein level of COX-2 and PGE_2 production in human breast cancer cells

Previous studies have demonstrated that COX-2 inducers increase aromatase expression (Brueggemeier *et al*., 1999; Harris *et al*., 1999; Brodie *et al*., 2001; Oliveira *et al*., 2006). COX-2 is upregulated in many breast tumors, and one of the products of COX-2 is PGE_2, which is suggested to upregulate aromatase through cAMP signaling in breast cancer (Hwang *et al*., 1998; Gunnarsson *et al*., 2006). Our laboratory was the first to report that o,p'-DDT, a DDT isomer, induces COX-2 enzyme, which is responsible for the conversion of arachidonic acid into PGE_2, in macrophages, and COX-2 increases CRE activation (Han *et al*., 2008). To study the mechanism of o,p'-DDT induction of aromatase, this study was tested whether COX-2 protein and mRNA expression were changed in o,p'-DDT-treated MCF-7 cells. MCF-7 cells were treated with o,p'-DDT for 16 h. o,p'-DDT induced COX-2 protein level in a dose-dependent manner (Fig. 7A). Next, MCF-7 cells were treated with o,p'-DDT (1 μM) for 4, 8, 16 and 24 h. COX-2 protein level was increased a peak at 16 h and maintained at 24 h by o,p'-DDT (Fig. 7B). In addition, this study was compared that effects of o,p'-DDT on COX-2 protein expression in MDA-MB-231 and SK-BR-3 cells. o,p'-DDT dose-dependently induced COX-2 protein level in MDA-MB-231 and SK-BR-3 cells (Fig. 8A, B). MDA-MB-231 and SK-BR-3 cells exhibited similar results of COX-2 protein levels in MCF-7 cells. The COX-2 protein level was compared to that of actin. To study the effect of COX-2 mRNA expression, o,p'-DDT treated 0.01, 0.1 and 1 μM concentrations for 2 h in MCF-7

cells. o,p'-DDT induced COX-2 mRNA expression in a dose-dependent manner (Fig. 9A). Further studies, this study was investigated that effects of o,p'-DDT on transcriptional activation of COX-2 in MDA-MB-231 and SK-BR-3 cells. o,p'-DDT dose-dependently increased COX-2 gene expression in MDA-MB-231 and SK-BR-3 cells (Fig. 9B). To clarify the mechanism of COX-2 gene expression, this study was transfected MCF-7 cells with the hCOX-2-Luc reporter constructs. o,p'-DDT increased the COX-2 luciferase activity (Fig. 10A). Thus, o,p'-DDT maybe regulates the transcriptional activation of COX-2 in MCF-7 cells. In addition, this study was investigated that effects of o,p'-DDT on luciferase activity of COX-2 in MDA-MB-231 and SK-BR-3 cells. o,p'-DDT dose-dependently increased COX-2 luciferase activity in MDA-MB-231 and SK-BR-3 cells (Fig. 10B). o,p'-DDT induced the production of PGE_2, one of the major metabolites of COX-2 (Fig. 11A). To further confirm the induction of PGE_2 production by o,p'-DDT, this study was examined the effect of the COX-2 inhibitor NS-398. NS-398 inhibited o,p'-DDT-induced PGE_2 production (Fig. 11B). NS-398 also was able to suppress PGE_2 production in MCF-7 cells (Fig. 11B). A pharmacological approach was then used to identify the EP receptors that mediated this inductive effect of PGE_2. PGE_2 exerts its effects by binding to G protein-coupled receptors that contain seven transmembrane domains. Four subtypes of PGE_2 receptor (EP1, EP2, EP3, and EP4) have been cloned and defined (Sugimoto and Narumiya, 2007). Different EP receptors have been implicated in the regulation of cell proliferation, immune function, and angiogenesis (Dannenberg and Subbaramaiah, 2003; Sugimoto and Narumiya, 2007), but little is known about the regulation of aromatase. In this study,

this study was sought to identify the EP receptor(s) that mediate the induction of aromatase by o,p'-DDT in MCF-7 cells. MCF-7 cells were treated with o,p'-DDT for 24 h. o,p'-DDT increased EP2 and EP4 receptor mRNA expression in a dose-dependent manner (Fig. 12A). PGE_2 was used as a positive control. To confirm the induction of EP2 and EP4 receptors expression by o,p'-DDT, this study was observed the effect of the EP2 receptor (AH-6809) and EP4 receptor (AH-23848) inhibitors. AH-6809 reduced o,p'-DDT-induced EP2 receptor mRNA expression (Fig. 12B). AH-23848 also inhibited o,p'-DDT-induced EP4 receptor mRNA expression (Fig. 12B). EP2 and EP4 receptor mRNA expression was unaffected by AH-6809 and AH-23848 treatment in the absence of o,p'-DDT, respectively. These results indicate that EP2 and EP4 receptors are correlated with the induction of COX-2 and the production of PGE_2 in o,p'-DDT treated MCF-7 cells. To further confirm the aromatase protein levels by o,p'-DDT, this study was examined the effect of the COX-2, EP2 and EP4 receptor inhibitors. NS-398, AH-6809 and AH-23848 significantly suppressed o,p'-DDT -induced aromatase protein levels, individually (Fig. 13). NS-398 particularly inhibited o,p'-DDT -induced aromatase protein levels compared with AH-6809 and AH-23848. These results indicate that aromatase is correlated with the induction of COX-2 and the production of PGE_2 in o,p'-DDT treated MCF-7 cells.

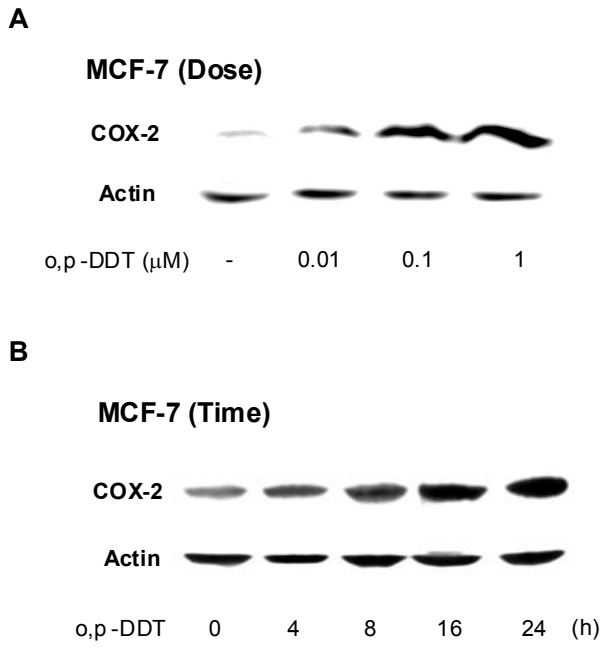


Fig. 7. Effects of o,p'-DDT on COX-2 protein levels in MCF-7 cells. (A) Cells were treated with o,p'-DDT (0.01–1 μM) for 16 h. (B) Cells were treated with o,p'-DDT (1 μM) for 4, 8, 16 and 24 h in MCF-7 cells. The blot was probed with COX-2-specific antibody, and the bands were visualized with ECL reagents. Each blot in this figure is representative of three independent experiments with similar results. The COX-2 protein level was compared to actin protein.

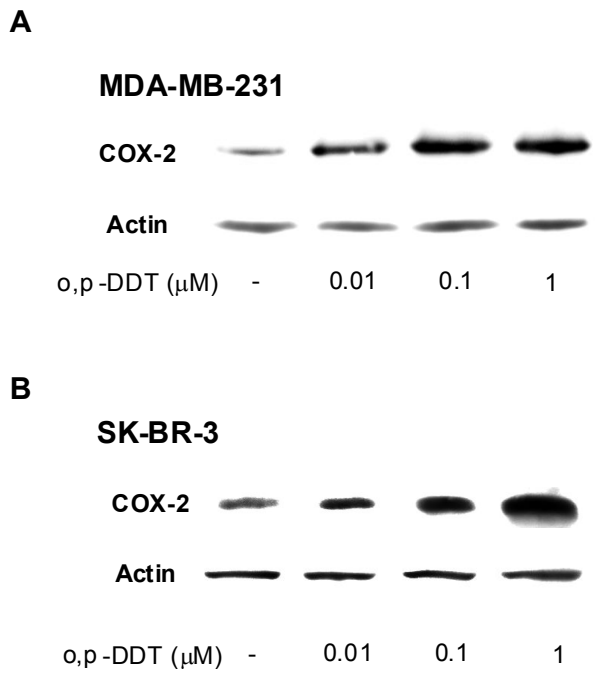


Fig. 8. Effects of o,p'-DDT on COX-2 protein levels in MDA-MB-231 and SK-BR-3 cells. Cells were treated with o,p'-DDT (0.01–1 μM) for 16 h in MDA-MB-231 (A) and SK-BR-3 (B) cells. Each blot in this figure is representative of three independent experiments with similar results. The COX-2 protein level was compared to actin protein.

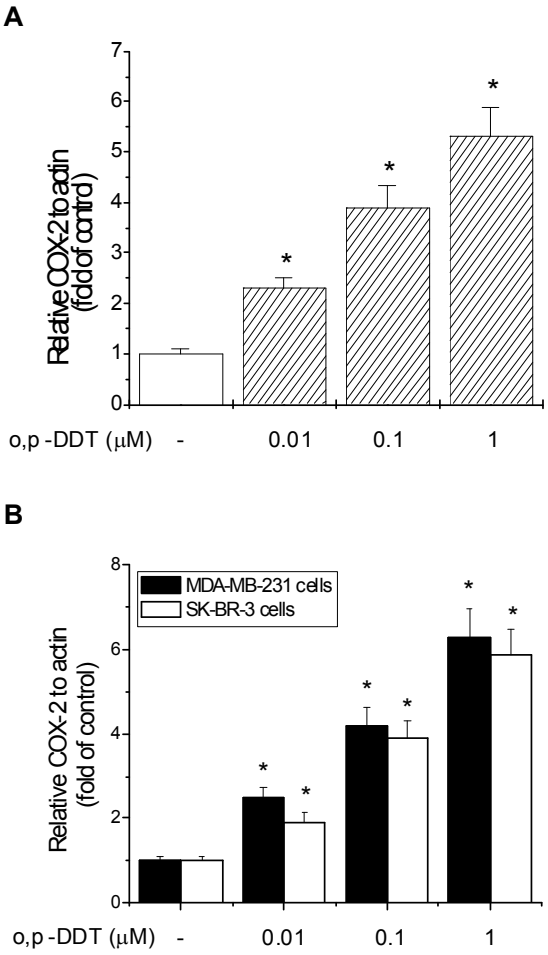


Fig. 9. Effects of o,p'-DDT on COX-2 gene expression in human breast cancer cells. (A) Cells were treated with o,p'-DDT (0.01–1 μM) for 2 h in MCF-7 cells (A), MDA-MB-231, SK-BR-3 cells (B). The cells were lysed and total RNA was prepared for analysis of COX-2 gene expression. COX-2 mRNA expression in treated cells was compared to the expression in untreated cells at each time point by real-time PCR. Each bar shows three independent experiments. * $P < 0.01$, significantly different from control as determined by analysis of variance by Newman–Keuls test.

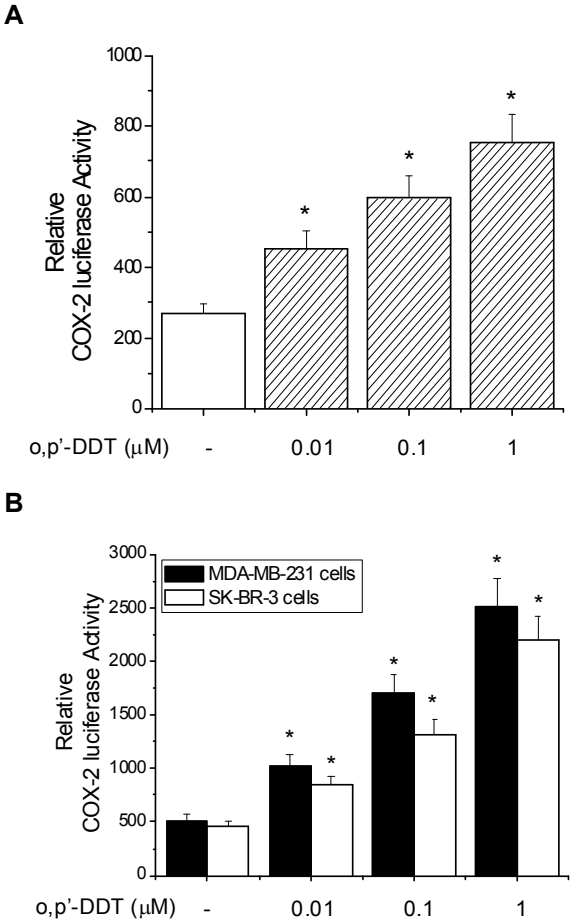


Fig. 10. Effects of o,p'-DDT on hCOX-2-Luc promoter activity in human breast cancer cells. Cells were transfected with hCOX-2-Luc and then treated with o,p'-DDT (0.01–1 μM) for 20 h in MCF-7 cells (A), MDA-MB-231, SK-BR-3 cells (B). Cells were then harvested and assayed for luciferase activity. Each bar shows three independent experiments. *$P < 0.01$, significantly different from control as determined by analysis of variance by Newman–Keuls test.

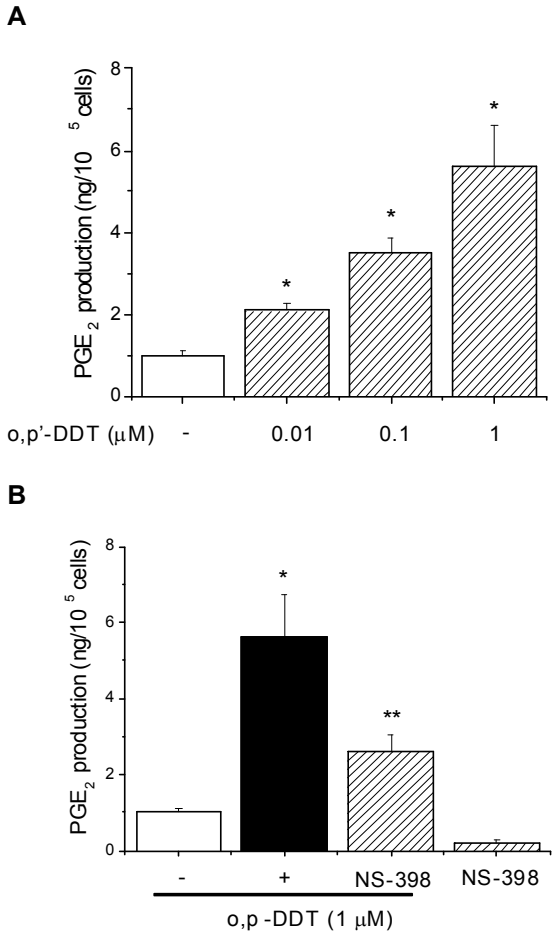


Fig. 11. Effects of o,p'-DDT on PGE_2 production and EP receptor expression in MCF-7 cells. (A) Effects of o,p'-DDT on PGE_2 production in MCF-7 cells. Cells were treated with o,p'-DDT (0.01–1 μM) for 24 h. (B) Effects of COX-2 inhibitor (NS-398) on o,p'-DDT-mediated PGE_2 production. Cells were pretreated with NS-398, a COX-2-specific inhibitor, for 30 min and then treated with o,p'-DDT (0.01–1 μM) for 24 h. The medium was collected and assayed for PGE_2 production. PGE_2 levels were determined by enzyme immunoassay. Each bar shows three independent experiments. * $P < 0.01$, significantly different from control as determined by analysis of variance by Newman–Keuls test.

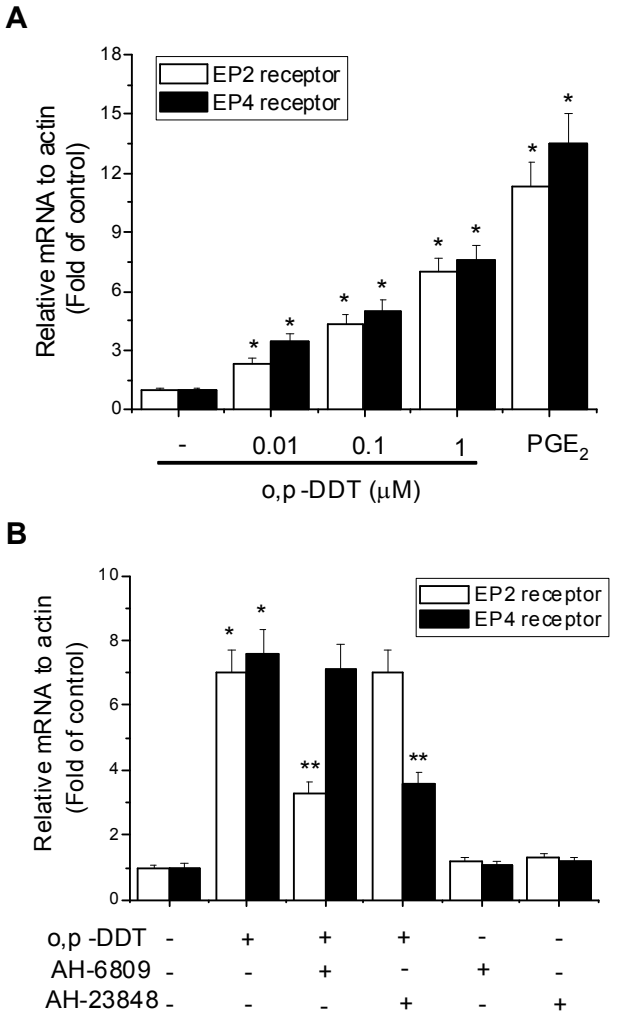


Fig. 12. Effects of o,p'-DDT on EP2 and EP4 receptor gene expression in MCF-7 cells. (A) Cells were treated with o,p'-DDT (0.01–1 μM) or PGE_2 (1 μM) for 24 h. (B) Effects of EP2 and EP4 receptor inhibitors on o,p'-DDT-induced these mRNA levels. Cells were pretreated with EP2 receptor inhibitor (AH-6809, 10 μM) and EP4 receptor inhibitor (AH-23848, 30 μM) for 30 min and then treated with o,p'-DDT (1 μM) for 24 h. PCR amplification of the housekeeping gene, β-actin, was

performed for each sample. These mRNA expressions in treated cells were compared to the expression in untreated cells at each time point by real-time PCR. Each bar shows three independent experiments. * P and ** $P < 0.01$, significantly different from control and o,p'-DDT, respectively, as determined by analysis of variance by Newman–Keuls test.

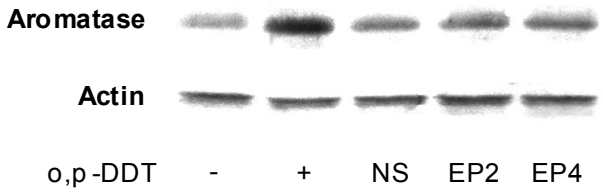


Fig. 13. Effects of various inhibitors [COX-2 (NS-398), EP2 receptor (AH-6809) and EP4 receptor (AH-23848)] on o,p'-DDT-induced aromatase protein levels in MCF-7 cells. Cells were pretreated with inhibitors (NS-398, 100 μM; AH-6809, 10 μM and AH-23848, 30 μM) for 30 min and then treated with o,p'-DDT for 24 h. The membrane was probed with human aromatase-specific antibody, and the bands were visualized with ECL reagents. Each blot in this figure is representative of three independent experiments with similar results. The aromatase protein level was compared to actin.

3. CRE activation and PKA are important for o,p'-DDT-mediated induction of aromatase in human breast cancer cells

Aromatase mRNA is regulated by multiple promoters such as CREB binding to CRE (Means *et al*., 1991; Young and McPhaul, 1998). CRE can regulate the transcription of the COX-2 and aromatase genes in breast cancer (Utsumi *et al*., 1996; Young and McPhaul, 1998), so this study was examined whether o,p'-DDT-induced aromatase was mediated by the activation of CRE. The CRE-Luc reporter activity was remarkably increased in o,p'-DDT treated MCF-7 cells (Fig. 14). Moreover, cAMP was induced in o,p'-DDT-treated MCF-7 cells (Fig. 15). This study subsequently performed ChIP assays using an antibody against CREB. The results of the ChIP assay further confirmed that o,p'-DDT directly bound to endogenous CRE and CREB. o,p'-DDT induced binding to endogenous CREB and CRE in a concentration-dependent manner (Fig. 16). To clarify the induction of CRE reporter activity, this study was tested the changes in the levels of nuclear CREB and CREB phosphorylation. o,p'-DDT significantly increased the levels of nuclear CREB protein in a dose-dependent manner (Fig. 17A). In addition, o,p'-DDT induced activation of the serine 133-phosphorylated CREB (Fig. 17B). However, total CREB protein expression was not changed by o,p'-DDT (Fig. 17C). Protein kinase A (PKA) stimulates cAMP activation and then activates the CREB pathway (Zhao *et al*., 1996; Cai *et al*., 2007). This study show that o,p'-DDT activates the cAMP→CREB pathway via the EP2 receptor, resulting in enhanced aromatase transcription and activity. PKA is a common signaling effector activated by EP2 receptor (Subbaramaiah *et al*., 2006). Therefore, this study was investigated

the effects of o,p'-DDT on PKA phospholyration in MCF-7 cells. MCF-7 cells were treated with o,p'-DDT for 10 min, and stimulation with o,p'-DDT increased PKA phosphorylation (Fig. 18). Therefore, o,p'-DDT treatment increased PKA/CREB phosphorylation and CREB nuclear translocation in MCF-7 cells.

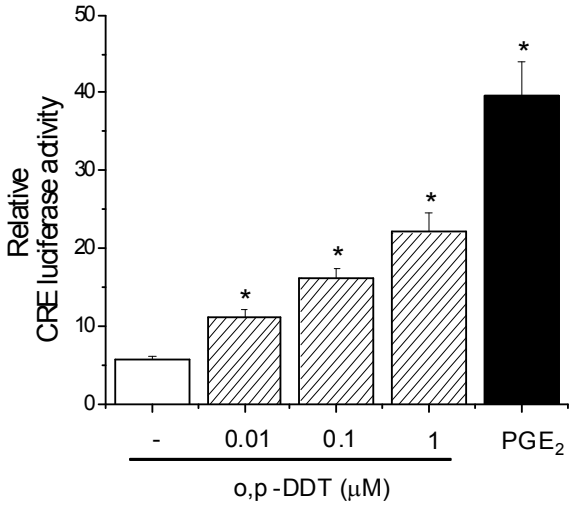


Fig. 14. Effects of o,p'-DDT on CRE-Luc promoter activity in MCF-7 cells. Cells were transfected with CRE-Luc and then treated with o,p'-DDT (0.01–1 μM) or PGE2 (1 μM) for 20 h. Cells were then harvested and assayed for luciferase activity. Each bar shows three independent experiments. *$P < 0.01$, significantly different from control as determined by analysis of variance by Newman–Keuls test.

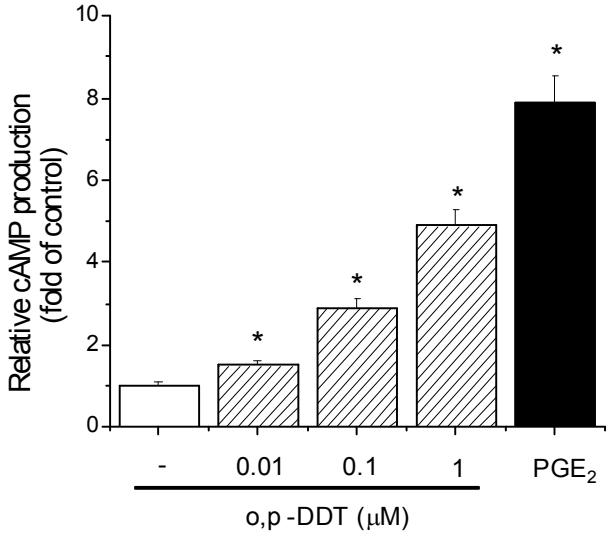


Fig. 15. Effects of o,p'-DDT on cAMP production in MCF-7 cells. Cells were treated with o,p'-DDT (0.01–1 μM) or PGE_2 (1 μM) for 18 h. cAMP levels in cell lysates were determined using ELISA kit. Each bar shows three independent experiments. *$P < 0.01$, significantly different from control as determined by analysis of variance by Newman–Keuls test.

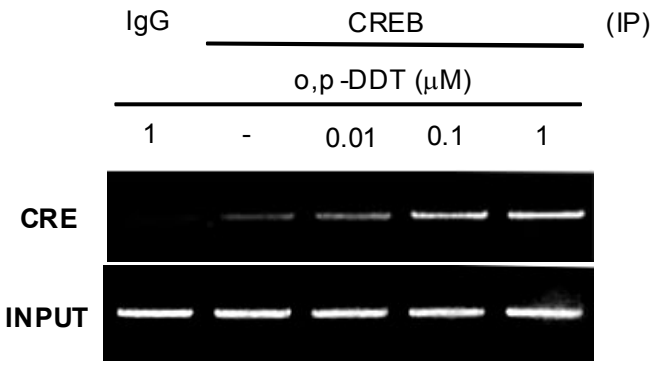


Fig. 16. The cells were treated with o,p'-DDT for 24 h in MCF-7 cells. Chromatin immunoprecipitation using the anti-CREB antibody was performed on chromatin extracted from o,p'-DDT-stimulated cells, and the specific CRE regions were amplified by PCR. The PCR amplification products were electrophoresed in 2% agarose gel and stained with ethidium bromide. One of three representative experiments is shown.

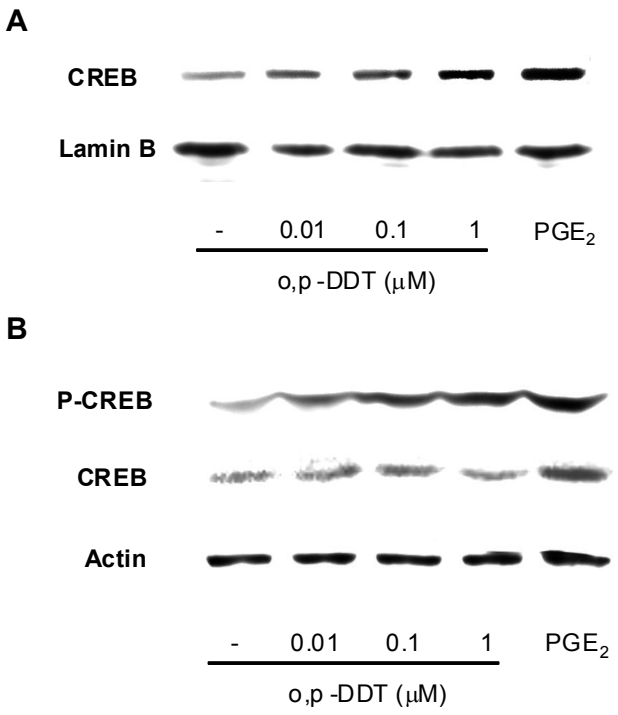


Fig. 17. Effects of o,p'-DDT on CREB activation in MCF-7 cells. Cells were treated with o,p'-DDT (0.01–1 μM) or PGE_2 (1 μM) for 3 h (A) or 30 min (B). The membranes were probed with CREB and phosphorylated CREB-specific antibodies, and the bands were visualized with ECL reagents. Each blot in this figure is representative of three independent experiments with similar results. These protein levels are shown as the ratio of CREB/lamin B and p-CREB/actin protein levels.

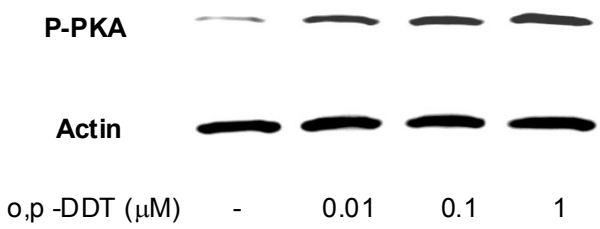


Fig. 18. Effects of o,p'-DDT on phosphorylation of PKA in MCF-7 cells. Cells were treated with o,p'-DDT (0.01–1 μM) for 10 min. The membrane was probed with phosphorylated PKA-specific antibody, and the bands were visualized with ECL reagents. Each blot in this figure is representative of three independent experiments with similar results. These protein levels are shown as the ratio of p-PKA/actin protein levels.

4. PI3-kinase/Akt are involved in the induction of aromatase by o,p'-DDT in human breast cancer cells

Stimulation of the EP4 receptor induces phosphatidylinositol 3-kinase (PI3-kinase)-dependent activation of Akt (Fujino *et al*., 2003). PI3-kinase/Akt contributes to the activation of three MAPKs (ERK, JNK, and p38), resulting in up-regulation of aromatase (Brodie *et al*., 2006). Cells were treated with o,p'-DDT for 5 min and 20 min, respectively. o,p'-DDT enhanced the phosphorylation of Akt, ERK and JNK (Fig. 19, 20A). o,p'-DDT treatment did not affect the levels of phosphorylated p38. The phosphorylated MAPK protein level was compared to that of the housekeeping gene, actin. In addition, this study was investigated the activation of the MAPKs by o,p'-DDT using specific inhibitors. Treatment with the MAPK signaling inhibitors PD98059 (ERK) and SP600125 (JNK) significantly reduced the o,p'-DDT-induced phosphorylation of ERK and JNK (Fig. 20B, C). At the concentrations used, PD98059 and SP600125 inhibited the activities of phosphorylated ERK and JNK, respectively, without producing any cytotoxic effects. The phosphorylated ERK and JNK protein levels were compared to those of the housekeeping gene, actin. In addition, Akt (LY294002), ERK and JNK inhibitors inhibited o,p'-DDT induced nucleus CREB level (Fig. 21). These results suggest that activation of Akt, ERK and JNK signaling pathways regulates CREB activation in o,p'-DDT-treated MCF-7 cells. Furthermore, PKA (H-89), PI3-kinase/Akt (LY 294002), ERK and JNK inhibitors significantly suppressed o,p'-DDT-induced aromatase protein levels, respectively (Fig. 22). Therefore, activation of PKA, Akt, ERK and JNK signaling pathways regulates aromatase up-regulation in o,p'-DDT-treated cells.

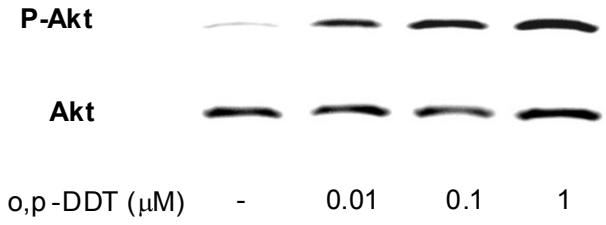


Fig. 19. Effects of o,p'-DDT on phosphorylation of Akt in MCF-7 cells. Cells were treated with o,p'-DDT (0.01–1 μM) for 5 min. The membrane was probed with phosphorylated Akt-specific antibody, and the bands were visualized with ECL reagents. Each blot in this figure is representative of three independent experiments with similar results. These protein levels are shown as the ratio of p-Akt/Akt protein levels.

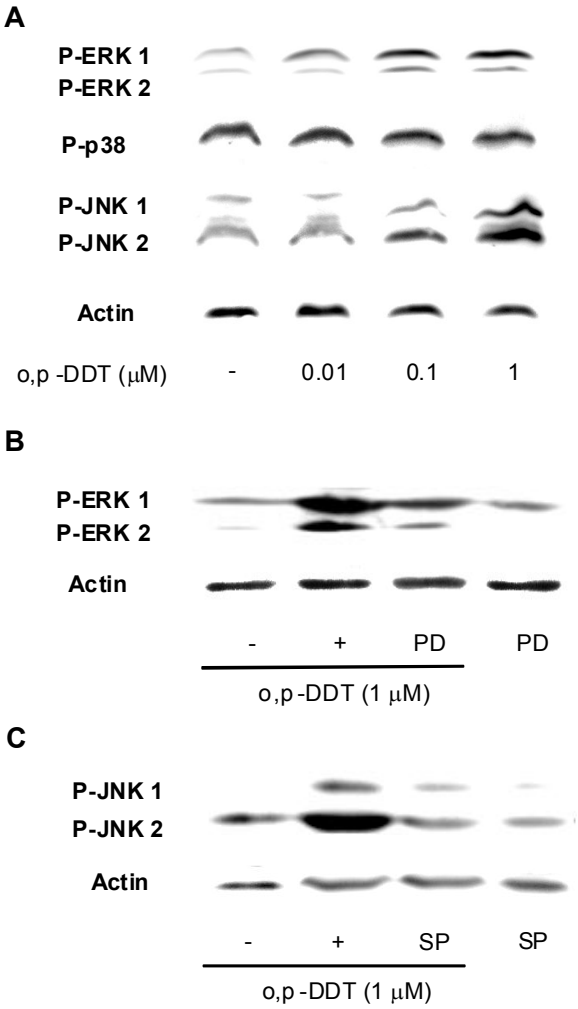


Fig. 20. Effects of o,p'-DDT on phosphorylation of MAPK in MCF-7 cells. (A) Cells were treated with o,p'-DDT (0.01–1 μM) for 20 min. The membranes were probed with phosphorylated ERK, p38, and JNK-specific antibodies, and the bands were visualized with ECL reagents. Each blot in this figure is representative of three independent experiments with similar results. These protein levels are shown as the ratio of target protein/actin protein levels. (B, C) Cells were pretreated with PD (PD98059; 10 μM) and SP (SP600125; 10 μM) for 30 min and then o,p'-DDT (20 min)

was added. Each blot in this figure is representative of three independent experiments with similar results. These protein levels are shown as the ratio of target protein/actin protein levels.

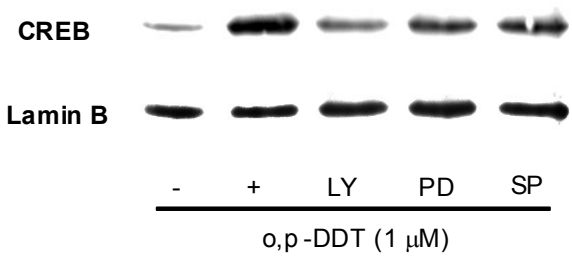


Fig. 21. Effects of inhibitors [Akt, ERK and JNK] on o.p'-DDT induced CREB activation in MCF-7 cells. Cells were pretreated with LY (LY294002; 10 μM), PD (PD98059; 10 μM) and SP (SP600125; 10 μM) for 30 min and then o,p'-DDT (3 h) was added. The membranes were probed with CREB-specific antibodies, and the bands were visualized with ECL reagents. Each blot in this figure is representative of three independent experiments with similar results. These protein levels are shown as the ratio of CREB/lamin B protein levels.

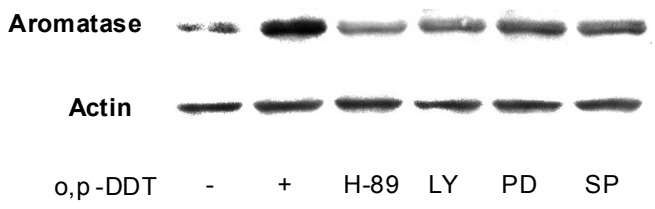


Fig. 22. Effects of various inhibitors [PKA (H-89), Akt (LY 294002), ERK (PD98059), JNK (SP600125)] on o,p'-DDT-induced aromatase protein levels in MCF-7 cells. Cells were pretreated with inhibitors (H-89, 10 μM; LY 294002 (LY), 5 μM; PD98059 (PD), 10 μM; SP600125 (SP), 10 μM) for 30 min and then treated with o,p'-DDT for 24 h. The membrane was probed with human aromatase-specific antibody, and the bands were visualized with ECL reagents. Each blot in this figure is representative of three independent experiments with similar results. The aromatase protein level was compared to actin.

5. Role of COX-2 and related signaling pathway in o,p'-DDT induction of aromatase in human breast cancer cells

To determine whether o,p'-DDT-induced aromatase was correlated with a COX-2-dependent and PKA/PI3-kinase/Akt signaling pathway, this study was examined the effect of various inhibitors [COX-2 (NS-398), PKA (H-89), PI3-kinase/Akt (LY 294002) EP2 (AH-6809) and EP4 receptor (AH-23848)]. NS-398, H-89, LY 294002, AH-6809 and AH-23848 reduced the conversion of [^{3}H]androstenedione to [^{3}H]estrogen in o,p'-DDT-stimulated MCF-7 cells (Fig. 23). In addition, these inhibitors significantly decreased aromatase expression in o,p'-DDT-stimulated MCF-7 cells (Fig. 24). The control, actin, was constitutively expressed and was unaffected by o,p'-DDT treatment. Morevoer, these inhibitors reduced aromatase luciferase activity in o,p'-DDT-treated MCF-7 cells (Fig. 25A). Therefore, o,p'-DDT-induced aromatase maybe correlated with COX-2 level and PKA/Akt/MAP kinase upstream signaling pathways. However, various inhibitors [COX-2 (NS-398), PKA (H-89), PI3-kinase/Akt (LY 294002) EP2 (AH- 6809) and EP4 receptor (AH-23848)] effect was very similar results and this study did not find specificity. Further studies were investigated combined inhibitors effect using a several inhibitors. NS-398 + H-89 was completely blocked o,p'-DDT-induced aromatase luciferase activity (Fig. 25B). NS-398 + LY 294002, H-89 + LY 294002 and AH-6809 + AH-23848 were also inhibited o,p'-DDT-induced aromatase luciferase

activity although short ability compared of NS-398 + H-89 (Fig. 25B). Therefore, COX-2 and upstream signal pathway maybe co-working and assist mechanism. Finally, this study was investigated the effect of o,p'-DDT on MCF-7 cell proliferation because aromatase-induced estrogen increases breast cancer cell proliferation. In breast tissues, estrogens are produced mainly through aromatization of adrenal androgen precursors (androstenedione and testosterone) (Simpson *et al.*, 1999). Proliferation of MCF-7 cells incubated for 72 h with serum-free medium in the presence of 1 μM testosterone was induced by 1 μM o,p'-DDT (Fig. 26). To further confirm the induction of cell proliferation by o,p'-DDT, this study was examined the effects of the COX-2 inhibitor NS-398 and the aromatase inhibitor α-naphthoflavone (α-NF). NS-398 and α-NF inhibited o,p'-DDT-induced cell proliferation (Fig. 26). Cell proliferation in the absence of o,p'-DDT was unaffected by NS-398 and α-NF treatment. Proliferation of MCF-7 cells incubated for 72 h with serum-free medium in the presence of 1 μM testosterone was induced by 0.01 and 0.1 μM o,p'-DDT (data not shown). These increased proliferation was shown that o,p'-DDT maybe induced estrogen-dependent diseases including breast cancer and endometriosis at least in vitro system.

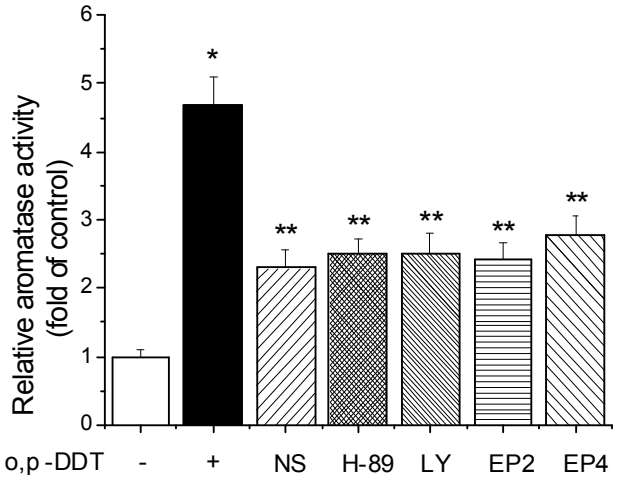


Fig. 23. Effects of inhibitors [COX-2 (NS-398), PKA (H-89), Akt (LY 294002), EP2 receptor (AH-6809) and EP4 receptor (AH-23848)] on o,p'-DDT-induced aromatase activity in MCF-7 cells. Cells were pretreated with inhibitors [NS-398 (NS), 100 μM; H-89, 10 μM; LY 294002 (LY), 5 μM; AH-6809 (EP2), 10 μM and AH-23848 (EP4), 30 μM] for 30 min and then treated with o,p'-DDT for 72 h in serum-free medium. Aromatase activity was then measured. Each bar shows three independent experiments. * P and ** $P < 0.01$, significantly different from control and o,p'-DDT, respectively, as determined by analysis of variance by Newman–Keuls test.

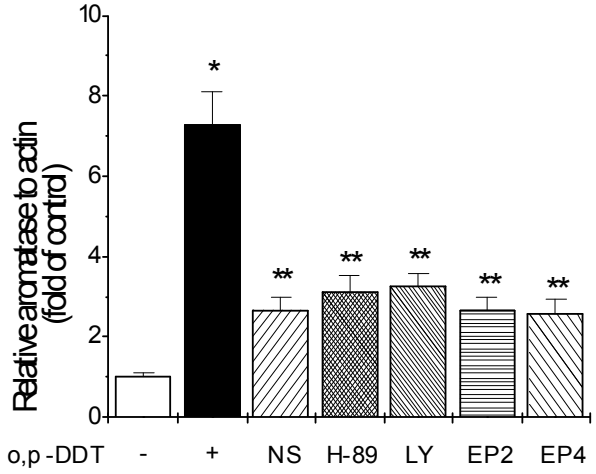


Fig. 24. Effects of inhibitors [COX-2 (NS-398), PKA (H-89), Akt (LY 294002), EP2 receptor (AH-6809) and EP4 receptor (AH-23848)] on o,p'-DDT-induced aromatase mRNA levels in MCF-7 cells. Cells were pretreated with these inhibitors [NS-398 (NS), 100 μM; H-89, 10 μM; LY 294002 (LY), 5 μM; AH-6809 (EP2), 10 μM and AH-23848 (EP4), 30 μM] for 30 min and then treated with o,p'-DDT for 24 h. The cells were lysed and total RNA was prepared for analysis of aromatase gene expression. PCR amplification of the housekeeping gene, β-actin, was performed for each sample. Aromatase mRNA expression in treated cells was compared to the expression in untreated cells at each time point by real-time PCR. Each bar shows three independent experiments. * P and ** $P < 0.01$, significantly different from control and o,p'-DDT, respectively, as determined by analysis of variance by Newman–Keuls test.

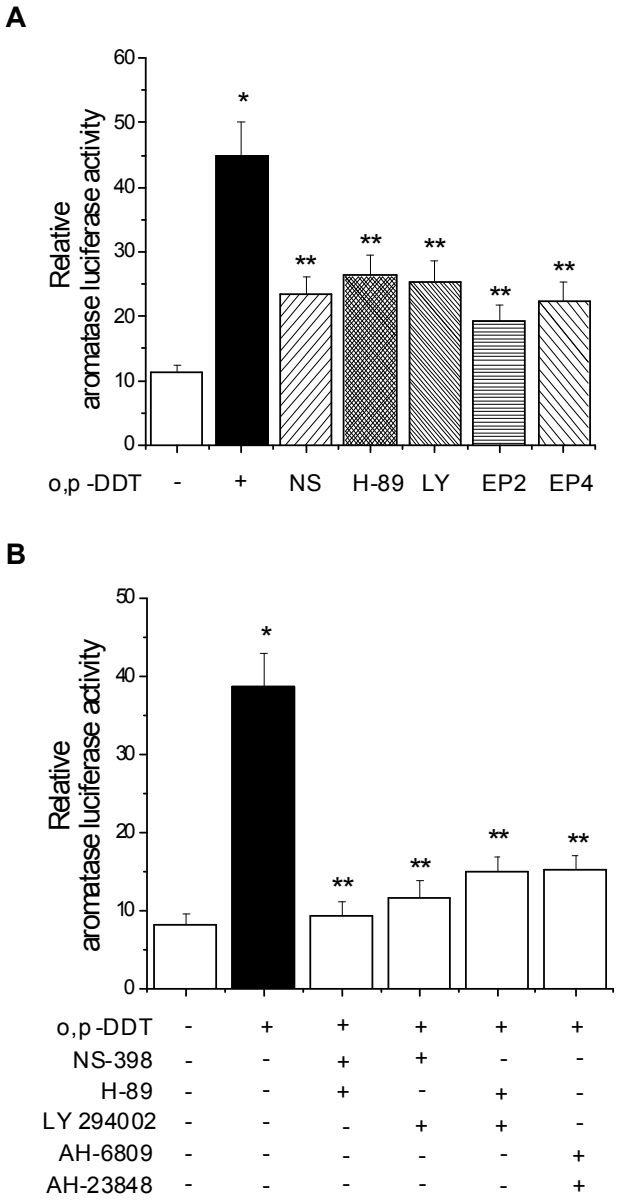


Fig. 25. Effects of inhibitors [COX-2 (NS-398), PKA (H-89), Akt (LY 294002), EP2 receptor (AH-6809) and EP4 receptor (AH-23848)] on o,p'-DDT-induced aromatase promoter activity in MCF-7 cells. (A) Cells were transfected with aromatase-Luc

vector for 4 h. Cells were pretreated with these combined inhibitors [NS-398 (NS), 100 μM; H-89, 10 μM; LY 294002 (LY), 5 μM; AH-6809 (EP2), 10 μM and AH-23848 (EP4), 30 μM] for 30 min and then treated with o,p'-DDT (0.01–1 μM) for 20 h. (B) Cells were pretreated NS-398 + H-89, NS-398 + LY 294002, H-89 + LY 294002 and AH-6809 + AH-23848 for 30 min and then treated with o,p'-DDT (1 μM) for 20 h. Cells were then harvested and assayed for luciferase activity. Each bar shows three independent experiments. * P and ** $P < 0.01$, significantly different from control and o,p'-DDT, respectively, as determined by analysis of variance by Newman–Keuls test.

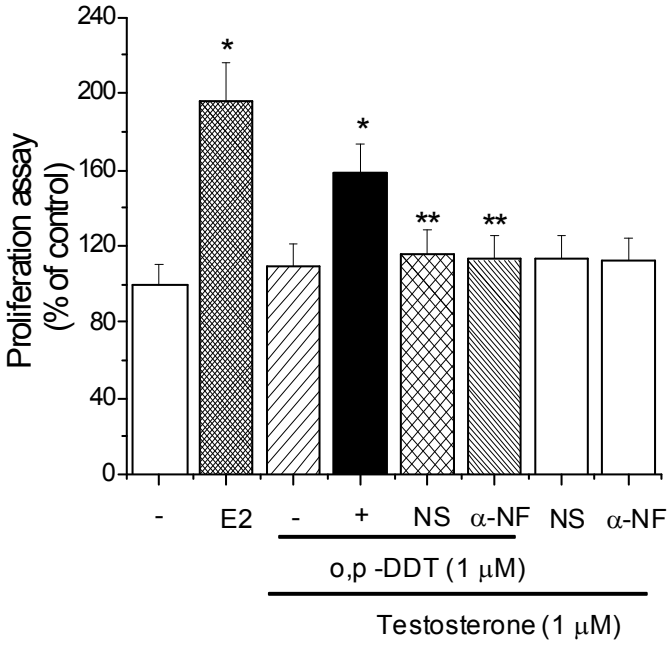


Fig. 26. Effects of o,p'-DDT on breast cancer cell proliferation. Cells were pretreated with these inhibitors (NS-398; 100 μM and aromatase inhibitor α-NF; 10 μM) for 30 min in the presence of testosterone (1 μM). After that cells were treated with o,p'-DDT (1 μM) for 72 h. Cell proliferation was assessed using WST-1 assays. Each bar shows three independent experiments. * P and ** $P < 0.01$, significantly different from control and o,p'-DDT, respectively, as determined by analysis of variance by Newman–Keuls test.

6. Methoxychlor induces aromatase and COX-2 protein expression in human breast cancer cells

To discover whether o,p'-DDT-induced aromatase was correlated with a COX-2, this study was investigated the effect of another endocrine-disrupting chemicals, for example methoxychlor. Insecticide methoxychlor (MXC, 1,1,1-trichloro-2,2-bis(p-methoxy phenyl)ethane) is an endocrine-disrupting chemicals that is widely used and was developed to replace DDT. Although the more apparent toxic signs during acute exposure to MXC on the immune system, few studies suggested that chronic exposure alters sexual steroidal hormone homeostasis (Richter-Reichhelm *et al.*, 2002; You *et al.*, 2002; Golub *et al.*, 2003, 2004; Laviola *et al.*, 2005). Perinatal exposure to MXC can result in alterations in the size of the adult prostate and increase the incidence of inflammation, such as prostatitis (Stoker *et al.*, 1999). However, the influence of MXC on COX-2 and aromatase protein is unknown. MCF-7 cells were treated with MXC (1, 5 and 10 μM) or PGE_2 for 24 h. MXC dose and time dependently induced COX-2 protein level in MCF-7 cells (Fig. 27). In addition, MXC dose-dependently enhanced COX-2 protein expression in MDA-MB-231 and SK-BR-3 cells (Fig. 28). Moreover, MXC concentration and time dependently increased aromatase protein level in MCF-7 cells (Fig. 29). MXC induced aromatase protein level in MDA-MB-231 and SK-BR-3 cells (Fig. 30). The COX-2 and aromatase protein level was compared to that of a housekeeping gene such as actin. Therefore, MXC exhibited similar mechanism of o,p'-DDT in human breast cancer cells. These results demonstrate that endocrine-disrupting chemicals,

including o,p’-DDT and MXC, were enhanced aromatase and COX-2 protein level and may induced-aromatase was correlated with COX-2 up-regulation.

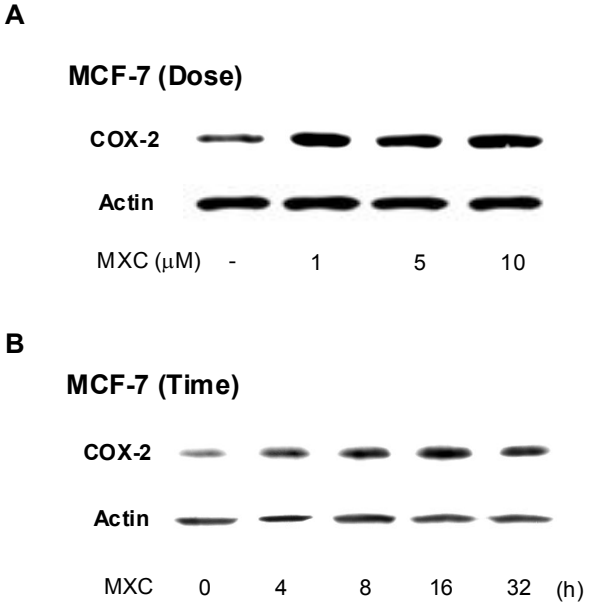


Fig. 27. Effects of MXC on COX-2 protein levels in MCF-7 cells. Cells were treated with MXC (1–10 μM) for 16 h. Another blot was treated with MXC (10 μM) for 4, 8, 16 and 24 h in MCF-7 cells. The membrane was probed with human COX-2-specific antibody. Each blot in this figure is representative of three independent experiments with similar results. The COX-2 protein level was compared to actin protein.

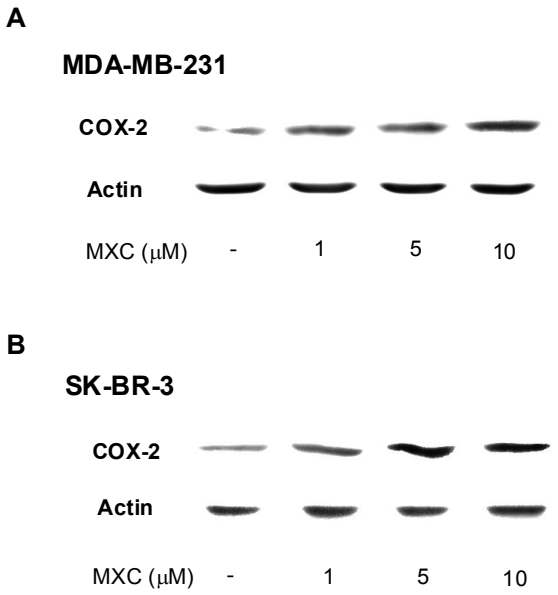


Fig. 28. Effects of MXC on COX-2 protein levels in MDA-MB-231 and SK-BR-3 cells. Cells were treated with MXC (1–10 μM) for 16 h. Each blot in this figure is representative of three independent experiments with similar results. The COX-2 protein level was compared to actin protein.

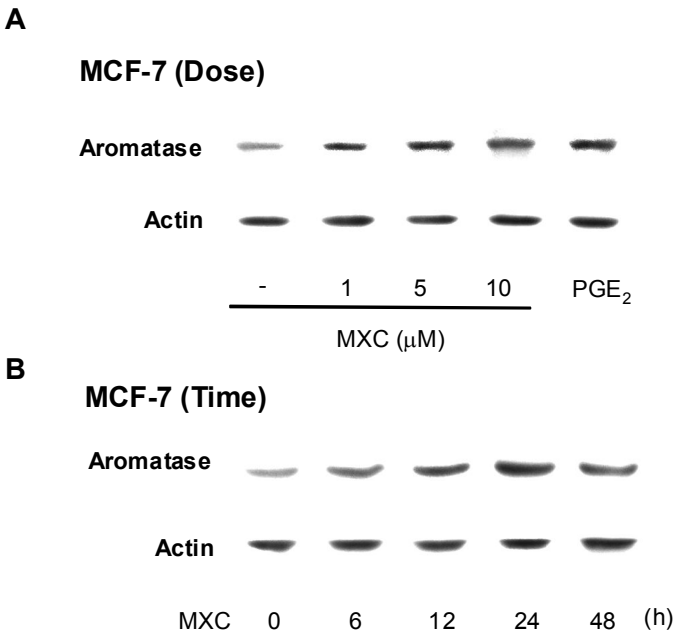


Fig. 29. Effects of MXC on aromatase protein expression in MCF-7 cells. Cells were treated with MXC (1–10 μM) or PGE_2 (1 μM) for 24 h. Another membrane was treated with MXC (10 μM) for 6, 12, 24 and 48 h in MCF-7 cells. Each blot in this figure is representative of three independent experiments with similar results. The aromatase protein expression was compared to actin protein.

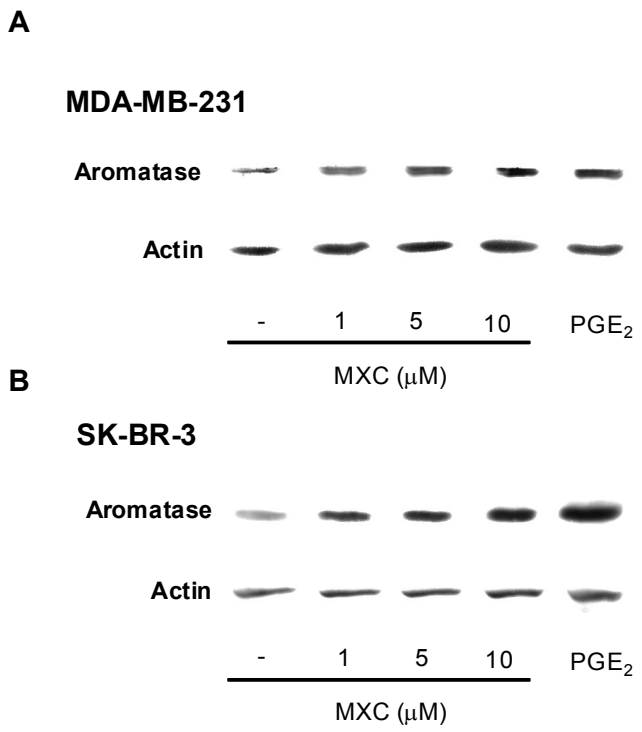


Fig. 30. Effects of MXC on aromatase protein expression in MDA-MB-231 and SK-BR-3 cells. Cells were treated with MXC (1–10 μM) or PGE_2 (1 μM) for 24 h. Each blot in this figure is representative of three independent experiments with similar results. The aromatase protein level was compared to actin protein.

Prostaglandin E2 induces CYP1B1 expression *via* ligand-independent activation of the ERα pathway in human breast cancer cells

7. PGE_2 induces CYP1B1 protein and mRNA expression and CYP1B1 promoter activity in MCF-7 cells

To examine the effect of PGE_2 on CYP1B1 expression in breast cancer cells, this study was treated MCF-7 cells with various concentrations of PGE_2 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 PGE_2 treatment. As a positive control, 3-MC was used instead of PGE_2, because it leads to the activation of both ER and aryl hydrocarbon receptor (AhR) target genes (Swedenborg *et al.*, 2008) such as CYP1B1. PGE_2 dose-dependently induced CYP1B1 protein expression in MCF-7 cells (Fig. 1A). Treatment with 1 μM 3-MC also increased CYP1B1 protein expression. When MCF-7 cells were treated with PGE_2 (10 μM) for 6, 12, 24, and 48 h, the CYP1B1 protein level peaked at 24 h and had declined by 48 h (Fig. 1B). This study was also tested the effect of PGE_2 on CYP1B1 gene expression using a PCR assay. Both PGE_2 and 3-MC increased the level of CYP1B1 mRNA in a dose-dependent manner and with a pattern similar to that of CYP1B1 protein expression (Fig. 1C). Thus, PGE_2 may regulate the transcriptional activation of CYP1B1 in MCF-7 cells.

To investigate the mechanism by which PGE_2 regulates CYP1B1 gene expression, this study was transfected MCF-7 cells with the CYP1B1-Luc reporter construct.

Both PGE_2 and 3-MC induced luciferase activity in MCF-7 cells (Fig. 1D). These results demonstrate that PGE_2 induced CYP1B1 gene expression and enhanced the CYP1B1 protein level by inducing CYP1B1 promoter activity in MCF-7 cells.

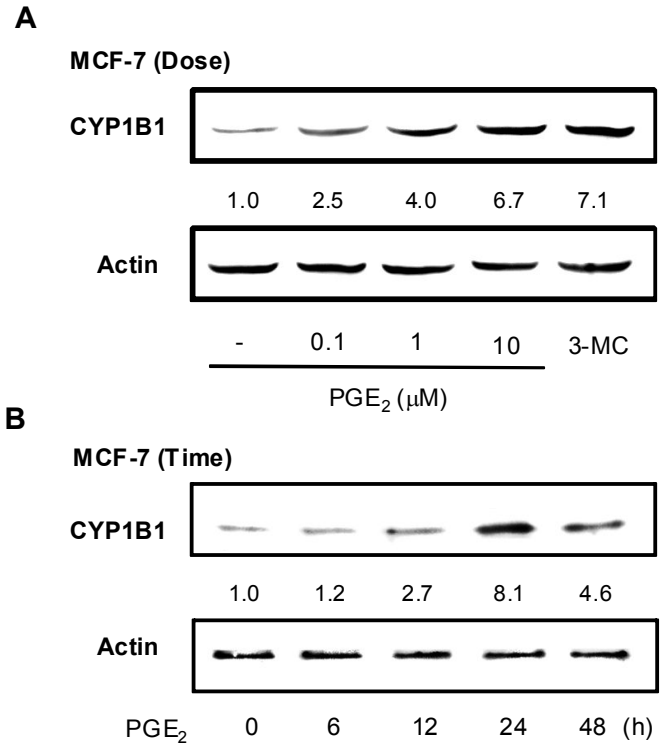


Fig. 31. Effect of PGE_2 on CYP1B1 protein levels in MCF-7 cells. Cells were treated with PGE_2 (0.1–10 μM) or 3-MC (1 μM) for 24 h (A) or cells cultured with 10 μM of PGE_2 for 6, 12, 24 and 48 h (B). The membrane was probed with human CYP1B1-specific antibody. Each blot in this figure is representative of three independent experiments with similar results. The CYP1B1 protein level was compared to actin protein. The densitometry data presented below the bands are "fold change" compared with control after normalization with respective loading control value.

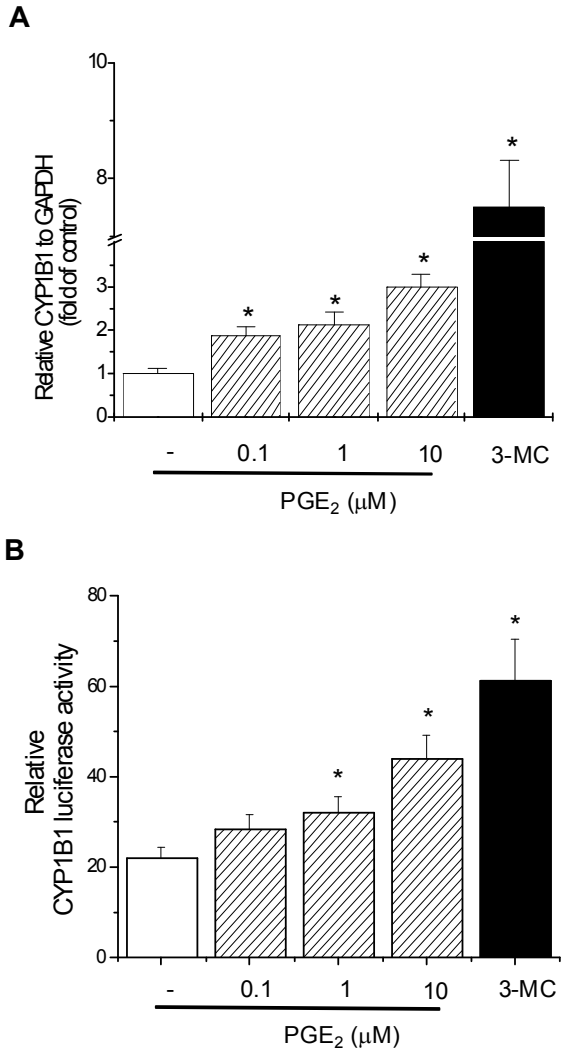


Fig. 32. Effect of PGE_2 on CYP1B1 mRNA expression and promoter activity in MCF-7 cells. (A) Effect of PGE_2 on CYP1B1 mRNA expression. Cells were incubated with PGE_2 (0.1–10 μM) or 3-MC (1 μM) for 24 h. Cells were lysed and total RNA was prepared for analysis of CYP1B1 gene expression. PCR amplification of the housekeeping gene, GAPDH, was performed for each sample. CYP1B1 mRNA expression in treated cells was compared to the expression in

untreated cells at each time point by real-time PCR. * $P < 0.01$, significantly different from control as determined by Newman–Keuls test. (B) Effects of PGE_2 on CYP1B1 promoter activity. Cells were transfected with CYP1B1-Luc and then cultured with PGE_2 (0.1–10 μM) or 3-MC (1 μM) for 24 h. Cells were then harvested and assayed for luciferase activity. *$P < 0.01$, significantly different from control as determined by Newman–Keuls test.

8. EP receptor inhibitors inhibit PGE_2-induced CYP1B1 expression and promoter activity in MCF-7 cells

PGE_2 is the ligand for four EP receptor subtypes, termed EP1 through EP4 (Breyer and Breyer, 2000), and the binding of PGE_2 to EP2 and EP4 signals an increase in intracellular cyclic AMP. This study was examined the role of EP receptors in PGE_2-induced CYP1B1 expression using inhibitors of EP2 (AH-6809) and EP4 (AH-23848). Both inhibitors reduced PGE_2-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 PGE_2-induced CYP1B1 mRNA expression and CYP1B1 promoter activity (Fig. 2B, C). Neither AH-6809 nor AH-23848 alone affected CYP1B1 protein or mRNA expression or luciferase activity. Thus, PGE_2-induced CYP1B1 expression and promoter activity were decreased in the presence of EP receptor inhibitors.

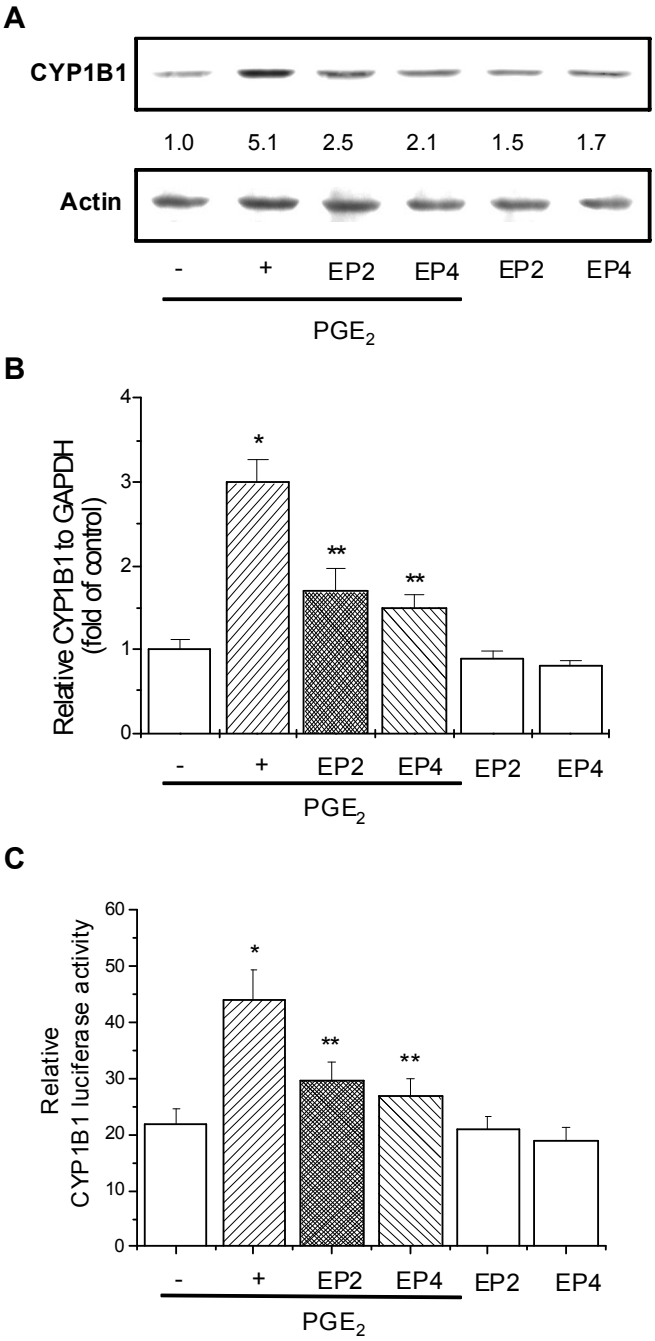


Fig. 33. Effects of PGE_2 on EP receptors in MCF-7 cells Effects of PGE_2 on EP

receptors in MCF-7 cells. (A) Effects of EP receptors on PGE_2-induced CYP1B1 protein expression. Cells were pretreated antagonist of EP2 receptor (AH-6809; 10 μM) and EP4 receptor (AH-23848; 30 μM) for 30 min and then incubated with PGE_2 (10 μM) for 24 h. The membrane was probed with CYP1B1-specific antibody, and the bands were visualized with ECL reagents. Each blot is representative of three independent experiments with similar results. The CYP1B1 protein level was compared to actin protein. The densitometry data presented below the bands are "fold change" compared with control after normalization with respective loading control value. (B) Effects of EP receptors on PGE_2-induced CYP1B1 gene expression. Cells were pretreated antagonist of AH-6809 (10 μM) and AH-23848 (30 μM) for 30 min and then cultured with PGE_2 (10 μM) for 24 h. The gene expression level was expressed as a ratio to the expression of GAPDH. CYP1B1 mRNA expression in treated cells was compared to the expression in untreated cells at each time point by real-time PCR. Graphic results shown are representative of three independent experiments. * P and ** $P < 0.01$, significantly different from control and PGE_2, respectively, as determined by Newman–Keuls test. (C) Effects of EP receptors on PGE_2-induced CYP1B1 luciferase activity. Cells were transfected with CYP1B1-Luc and then pretreated antagonist AH-6809 (10 μM) and AH-23848 (30 μM) in the presence or absence of PGE_2 (10 μM) for another 24 h. Cells were collected and lysed for luciferase activity. * P and ** $P < 0.01$, significantly different from control and PGE_2, respectively, as determined by Newman–Keuls test.

9. PGE_2 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 estrogen response element (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 PGE_2, this study was transfected cells with the −910/+25 hCYP1B1 deletion construct, which contains all of the transcription factor binding sites, or the −91/+25 hCYP1B1 deletion construct, which has only the ERE binding site. PGE_2 induced luciferase activity in a dose-dependent manner in cells transfected with either of the hCYP1B1 deletion constructs (Fig. 3B). In addition, PGE_2 enhanced the reporter activity of the ERE-Luc vector in a dose-dependent manner (Fig. 3C). Next, this study was 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 PGE_2-induced CYP1B1 protein expression, whereas CH-223191 and curcumin did not (Fig. 3D). ICI 182.780 also inhibited PGE_2-induced CYP1B1 mRNA expression and promoter reporter activity, but CH-223191 and curcumin had no effects (Fig. 3E, F). Thus, PGE_2-induced CYP1B1 expression occurs via promoter activation at the ERE binding site, suggesting that the estrogen receptor may be particularly important for understanding the role of PGE_2 in CYP1B1 regulation.

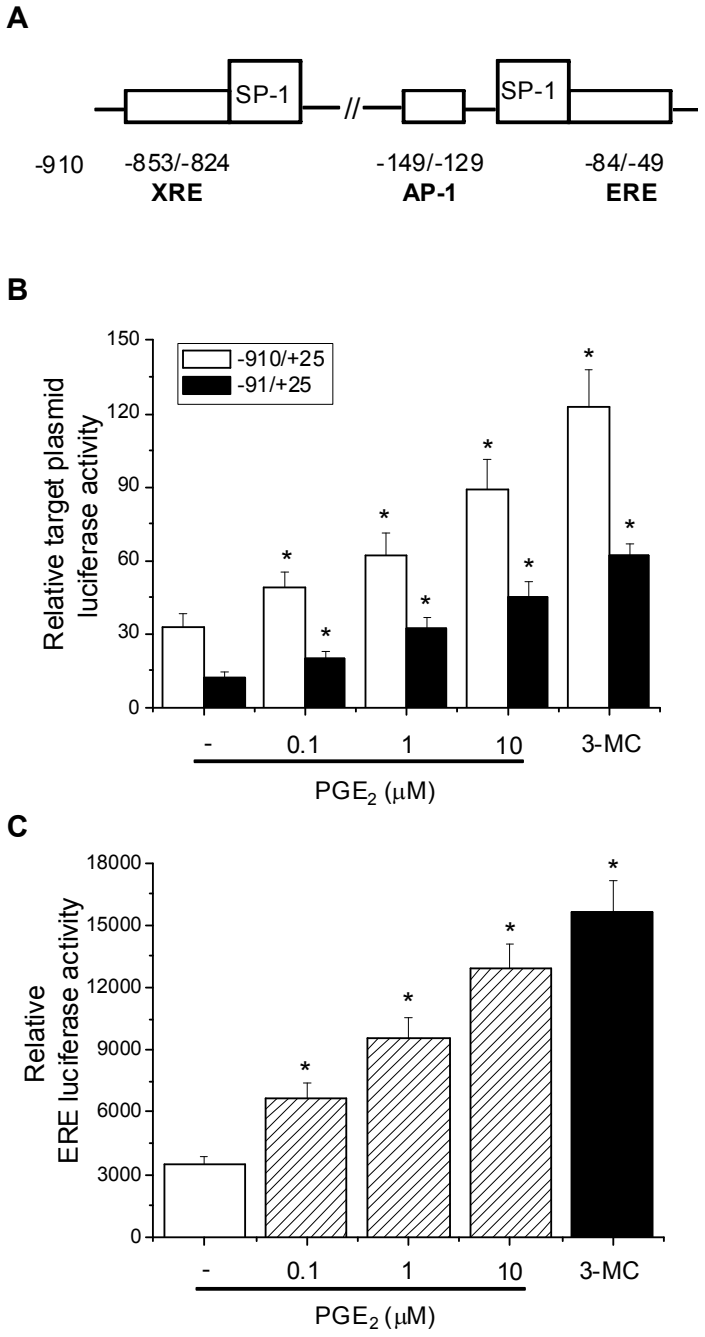

A
SP-1
//
SP-1
-910
-853/-824
XRE
-149/-129
AP-1
-84/-49
ERE
B
-910/+25
-91/+25
Relative target plasmid luciferase activity
150
120
90
60
30
0
-
0.1
1
10
3-MC
PGE2 (μM)
C
Relative ERE luciferase activity
18000
15000
12000
9000
6000
3000
0
-
0.1
1
10
3-MC
PGE2 (μM)

Fig. 34. Effects of PGE_2 on CYP1B1 related transcriptional factors activity in MCF-7 cells (A) Schematic diagram of the hCYP1B1 promoter. (B) Effects of PGE_2 on CYP1B1 related transcriptional factors activity. Cells were transfected with 1 μg of a series of hCYP1B1 promoter deletion constructs –910/+25 (white bars), –91/+25 (black bars) and 0.5 μg of pCMV-β-gal for 4 h after that treated with PGE_2 (0.1–10 μM) or 3-MC (1 μM) for 24 h. (C) Effects of PGE_2 on ERE luciferase activity. Cells were transfected with 1 μg of a series of ERE-Luc vector and pCMV-β-gal and then cultured with PGE_2 (0.1–10 μM) or 3-MC (1 μM). Cells were harvested and lysed for luciferase activity. The luciferase activity was normalized to the β-galactosidase activity. This result shown is representative of three independent experiments. $*P < 0.01$, significantly different from control as determined by Newman–Keuls test.

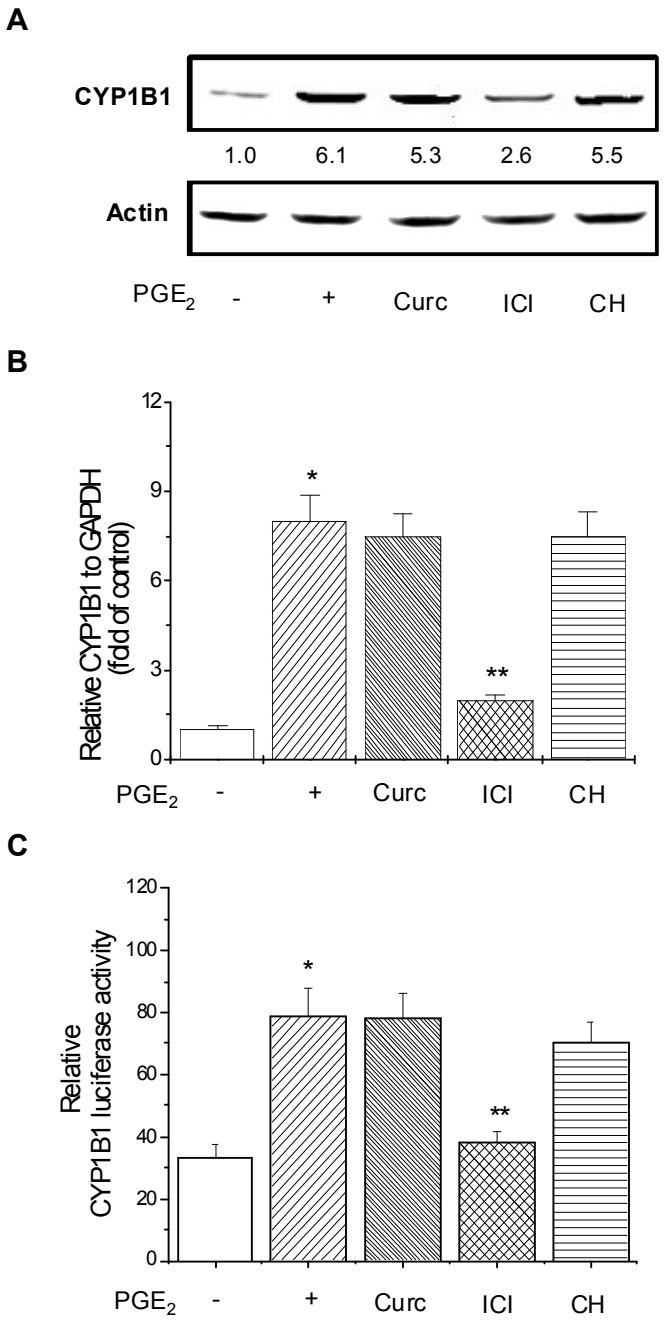


Fig. 35. Effects of transcriptional factors (ERE, AP-1 and XRE) on PGE_2-regulated

CYP1B1 protein level (A) Effects of transcriptional factors (ERE, AP-1 and XRE) on PGE_2-regulated CYP1B1 protein level. Cells were pretreated with inhibitors of ERE (ICI 182.780; 100 nM), AP-1 (curcumin; 20 μM) and AhR (CH-223191; 10 μM) and then incubated with PGE_2 (10 μM) for 24 h. The blot was probed with CYP1B1-specific antibody, and the bands were visualized with ECL reagents. Each membrane is representative of three independent experiments. The CYP1B1 protein level was compared to actin protein. The densitometry data presented below the bands are "fold change" compared with control after normalization with respective loading control value. (B) Effects of transcriptional factors (ERE, AP-1 and XRE) on PGE_2-regulated CYP1B1 mRNA expression. Cells were pretreated antagonist of ICI 182.780 (100 nM), curcumin (20 μM) and CH-223191 (10 μM) for 30 min and then cultured with PGE_2 (10 μM) for 24 h. The gene expression level was expressed as a ratio to the expression of GAPDH. Graphic result shown is representative of three independent experiments. * P and ** $P < 0.01$, significantly different from control and PGE_2, respectively, as determined by Newman–Keuls test. (C) Effects of transcriptional factors (ERE, AP-1 and XRE) on PGE_2-regulated CYP1B1 promoter activity. Cells were transfected with CYP1B1-Luc and then pretreated ICI 182.780 (100 nM), curcumin (20 μM) and CH-223191 (10 μM) for 30 min in the presence or absence of PGE_2 (10 μM) for another 24 h. Cells were lysed for luciferase activity and normalized to the β-galactosidase activity. This result shown is representative of three independent experiments. * P and ** $P < 0.01$, significantly different from control and PGE_2, respectively, as determined by Newman–Keuls test.

10. Role of ERα in PGE_2-induced CYP1B1 expression in human breast cancer cells

The two major estrogen receptors, ERα and ERβ, differ in some ligand specificities and physiological functions (Enmark and Gustafsson, 1999); however, the natural hormone 17β-estradiol (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 PGE_2-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–). PGE_2 treatment induced CYP1B1 protein expression in MCF-7 cells in a dose- and time-dependent manner (Fig. 1A, B); however, the CYP1B1 protein level was not affected by PGE_2 in MDA-MB-231 or MCF-10A cells (Fig. 4A, B). Treatment with 3-MC increased the CYP1B1 protein level in all three cell lines (Fig. 1A, B; Fig. 4A, B), because it leads to promoter activation at both the ERE and XRE binding sites (Swedenborg *et al.*, 2008). Furthermore, CYP1B1 mRNA expression and CYP1B1 promoter activity were not increased in PGE_2-treated MDA-MB-231 or MCF-10A cells, although 3-MC alone enhanced both CYP1B1 mRNA expression and promoter activity in these cells (Fig. 4C, D). As MDA-MB-231 cells express ERβ but not ERα, these results indicate that PGE_2 regulates CYP1B1 through an ERα-related mechanism. To further confirm this, this study was examined the effect of PGE_2 on the protein expression of ERα and ERβ in MCF-7 cells. Treatment of MCF-7 cells with 0.1, 1,

and 10 μM PGE_2 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 10 μM of PGE_2 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 PGE_2-induced CYP1B1 expression and PGE_2 regulates CYP1B1 through an ERα-related mechanism in breast cancer cells.

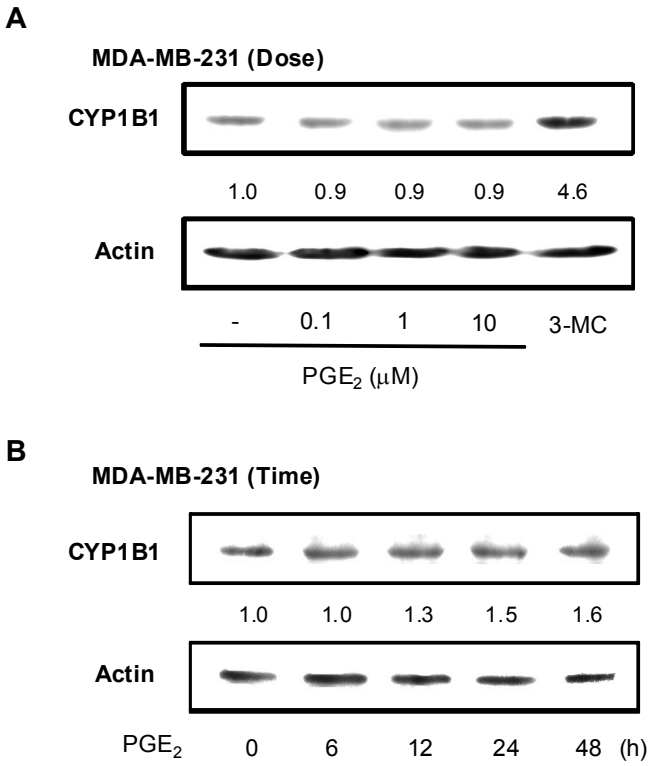


Fig. 36. Effects of PGE_2 on CYP1B1 protein level in MDA-MB-231 cells. (A) PGE_2 (0.1–10 μM) or 3-MC (1 μM) incubated with for 24 h in MDA-MB-231 cells. (B) Another blot was treated with 10 μM of PGE_2 for 6, 12, 24 and 48 h in MDA-MB-231 cells. The blot was probed with human CYP1B1-specific antibody. Each membrane is representative of three independent experiments with similar results. The CYP1B1 protein level was compared to actin protein.

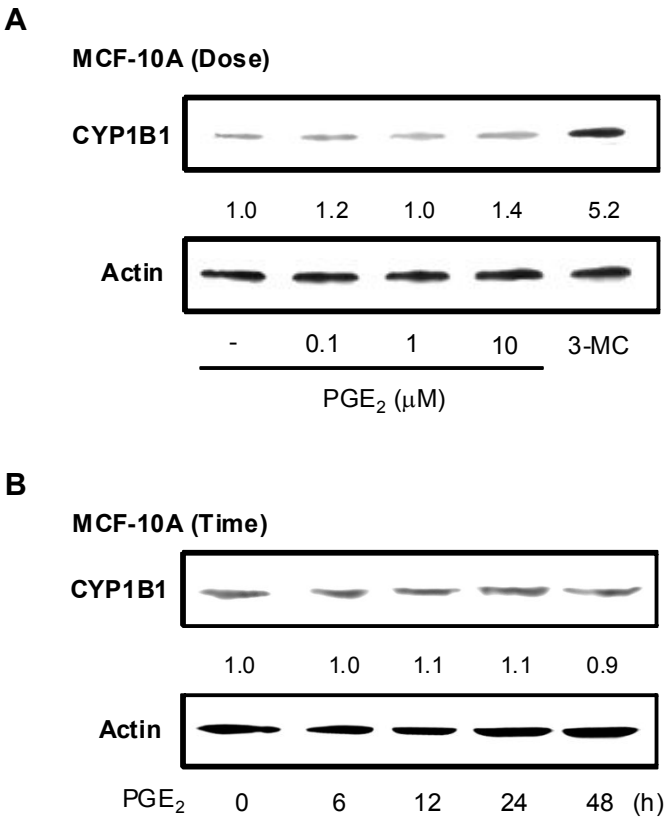


Fig. 37. Effects of PGE_2 on CYP1B1 protein level in MCF-10A cells. (A) PGE_2 (0.1–10 μM) or 3-MC (1 μM) incubated with for 24 h in MCF-10A cells. (B) Another blot was treated with 10 μM of PGE_2 for 6, 12, 24 and 48 h in MCF-10A cells. The blot was probed with human CYP1B1-specific antibody. Each membrane is representative of three independent experiments with similar results. The CYP1B1 protein level was compared to actin protein.

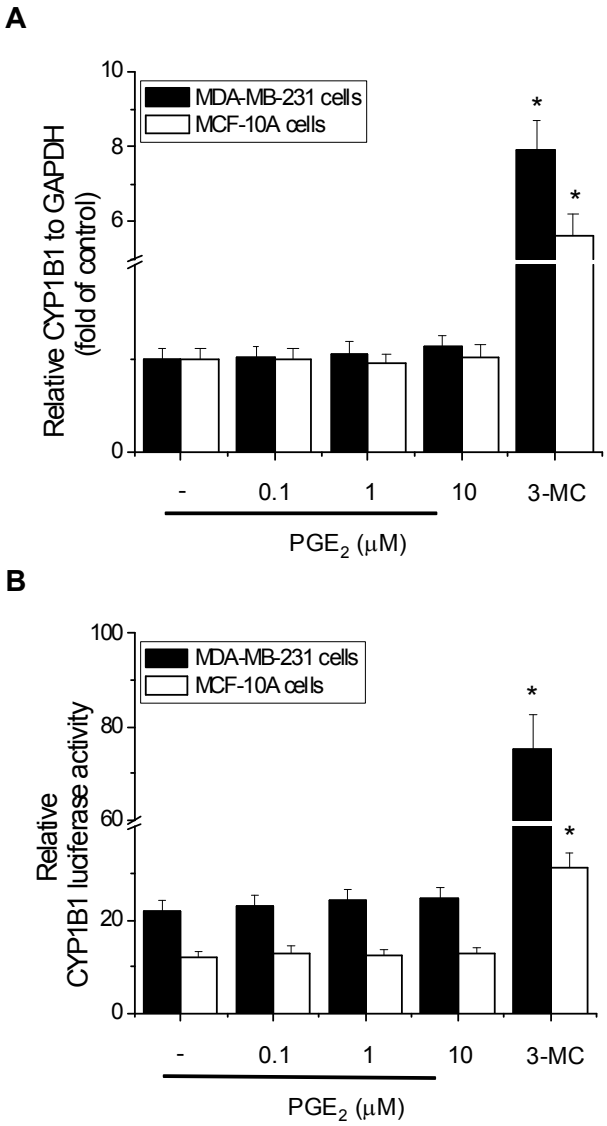


Fig. 38. Effects of PGE_2 on CYP1B1 mRNA expression and luciferase activity in MDA-MB-231 and MCF-10A cells. (A) Effects of PGE_2 on CYP1B1 mRNA expression in MDA-MB-231 and MCF-10A cells. PGE_2 (0.1–10 μM) or 3-MC (1 μM) treated with for 24 h in MDA-MB-231 (black bars) and MCF-10A (white

bars) cells. The gene expression level was expressed as a ratio to the expression of GAPDH. Results shown are representative of three independent experiments. *P < 0.01, significantly different from control as determined by Newman–Keuls test. (B) Effects of PGE_2 on CYP1B1 luciferase activity in MDA-MB-231 and MCF-10A cells. Cells were transfected with CYP1B1-Luc and then 0.1–10 μM of PGE_2 or 3-MC cultured with for 24 h in MDA-MB-231 (black bars) and MCF-10A (white bars) cells. Cells were lysed for luciferase activity and normalized to the β-galactosidase activity. Graphic results shown are representative of three independent experiments. *P < 0.01, significantly different from control as determined by Newman–Keuls test.

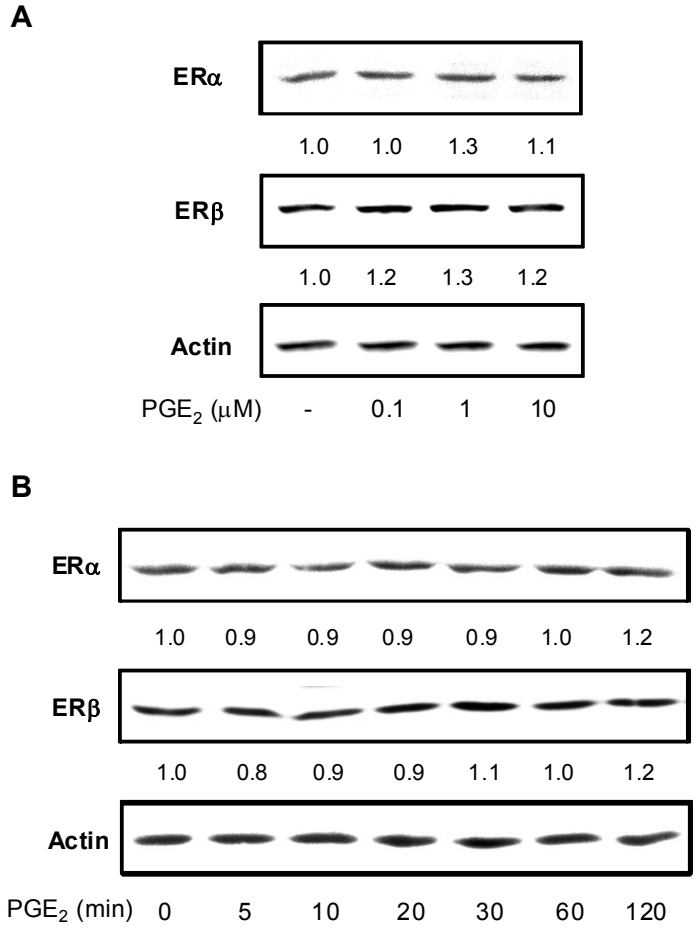


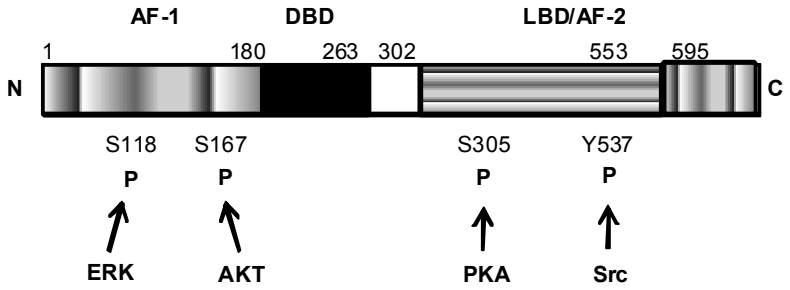
Fig. 39. Effects of PGE_2 on ERα and ERβ protein level in MCF-7 cells. Cells were incubated with 0.1–10 μM of PGE_2 for 10 min (A) or cultured with 10 μM of PGE_2 for 5, 10, 20, 30, 60 and 120 min (B). The membrane was probed with ERα and ERβ-specific antibodies and the bands were visualized with ECL reagents. Each blot is representative of three independent experiments with similar results. The ERα and ERβ protein level were compared to actin protein. The densitometry data presented below the bands are "fold change" compared with control after normalization with respective loading control value.

11. PGE_2 induces phosphorylation of ERα at serines 118, 167, and 305 in MCF-7 cells

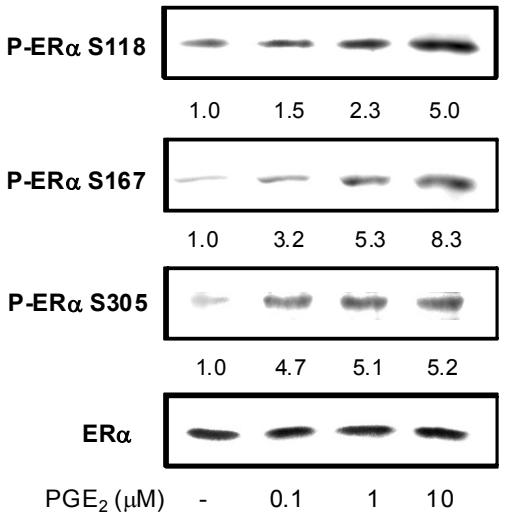
ERα can be activated through genomic (transcriptional regulation) and nongenomic mechanisms (activation of signaling cascades, including phosphorylation) (Katzenellenbogen and Katzenellenbogen, 2000), although ERα phosphorylation by various cellular kinases is considered to be the most important regulator 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 (Kushner *et al.*, 2000; Feng *et al.*, 2001; Chen *et al.*, 1999), 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 PGE_2 activates ERα by phosphorylation, this study was determined the extent of ERα phosphorylation by comparing the amounts of phosphorylated and non-phosphorylated ERα on immunoblots of lysates from cells treated with PGE_2. When MCF-7 cells were incubated with 0.1, 1, and 10 μM PGE_2 for 10 min, PGE_2 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 PGE_2 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, PGE_2 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 PGE_2 treatment (Fig. 5D). These results demonstrate that PGE_2 stimulates the phosphorylation of ERα at serines 118, 167, and 305 in MCF-7 cells prior to the induction of CYP1B1 expression.

A


AF-1
DBD
LBD/AF-2
1
180
263
302
553
595
N
C
S118
S167
S305
Y537
P
P
P
P
ERK
AKT
PKA
Src

B


P-ERα S118
1.0
1.5
2.3
5.0
P-ERα S167
1.0
3.2
5.3
8.3
P-ERα S305
1.0
4.7
5.1
5.2
ERα
PGE2 (μM)
-
0.1
1
10

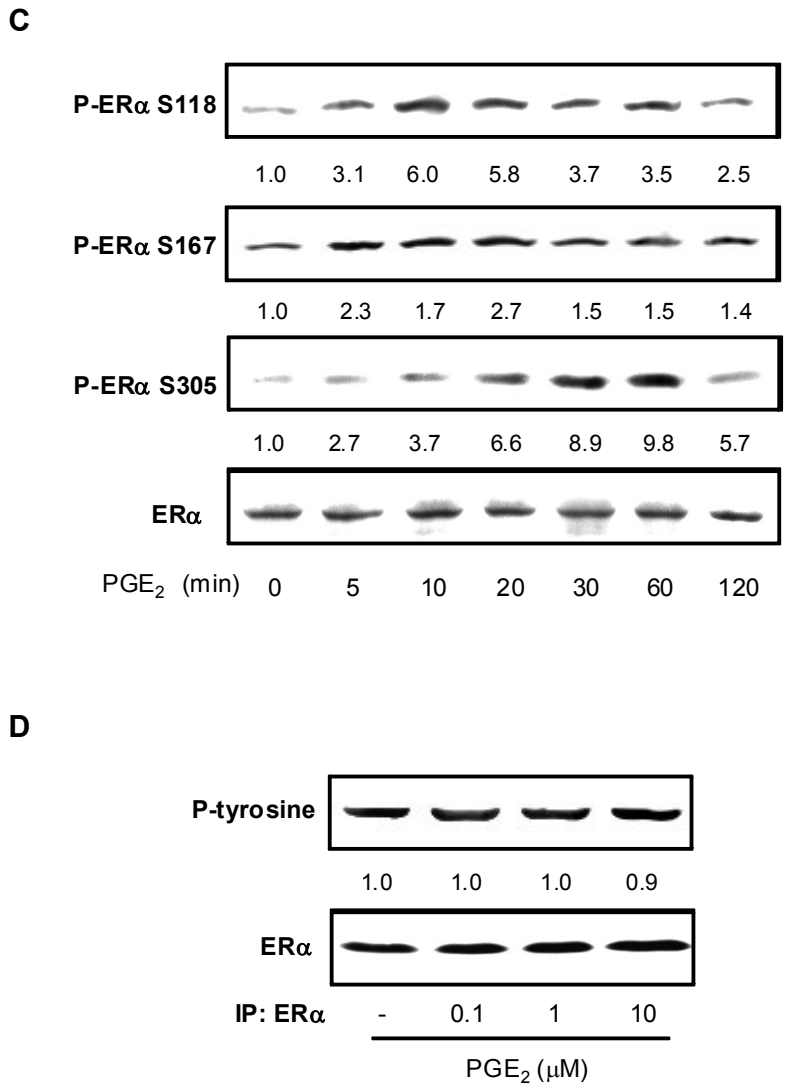


Fig. 40. Effects of PGE_2 on ERα phosphorylation in MCF-7 cells. (A) A schematic presentation of ERα phosphorylation sites. (B, C) Effects of PGE_2 on phosphorylated-ERα at serine residues in MCF-7 cells. Cells were incubated with 0.1–10 μM of PGE_2

for 10 min (B) or cultured with 10 μM of PGE_2 for 5, 10, 20, 30, 60 and 120 min (C). The membrane was probed with phospho-ERα S118, S167 and S305-specific antibodies and the bands were visualized with ECL reagents. Each blot is representative of three independent experiments with similar results. The ERα and ERβ protein level were compared to actin protein. (D) Effects of PGE_2 on phosphorylated-ERα at tyrosine residue in MCF-7 cells. Cells were incubated with 0.1–10 μM of PGE_2 for 10 min and harvested after that total cellular extract was prepared. ERα antibody was incubated first with Protein A agarose beads for 2 h and the beads were washed twice with PBS and then incubated with the total cellular extraction at 4 °C for 18 h with rotation. Beads were washed three times with PBS/0.1% Triton X-100, and ERα was eluted by boiling 5 min in SDS loading buffer. Phospho-tyrosine was detected by Western blot and compared to ERα protein. The densitometry data presented below the bands are "fold change" compared with control after normalization with respective loading control value.

12. PGE_2 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 PGE_2-induced ERα activation, this study was examined the activation of these kinases in MCF-7 cells. Cells were cultured with 10 μM PGE_2 for 5, 10, and 20 min, and the levels of phosphorylated and non-phosphorylated kinase on immunoblots of cell lysates were compared, after normalization to actin expression. The level of phosphorylated ERK increased with PGE_2 treatment, peaking at 10 min and declining by 20 min (Fig. 6A), and the phosphorylation of Akt exhibited a similar pattern (Fig. 6B). PGE_2 also induced the phosphorylation of PKA in a time-dependent manner (Fig. 6C). However, the phosphorylation of Src did not change with PGE_2 treatment (Fig. 6D). The results suggest that PGE_2-regulated ERα activation is mediated by the phosphorylation of ERK, Akt, and PKA. To further examine the upstream signaling pathway related to PGE_2-induced CYP1B1 expression, this study was 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 PGE_2-induced CYP1B1 protein expression, but the Src inhibitor, PP2, did not (Fig. 6E). This study was next examined the gene expression and reporter activity of PGE_2-induced CYP1B1 in the presence of these inhibitors. Similarly, PGE_2-induced CYP1B1 mRNA expression

and reporter activity were inhibited by PD98059, LY294002, and H-89, but not by PP2 (Fig. 6F, G). Therefore, PGE_2-induced CYP1B1 expression and ligand-independent ERα activation result from the activation of ERK, Akt, and PKA.

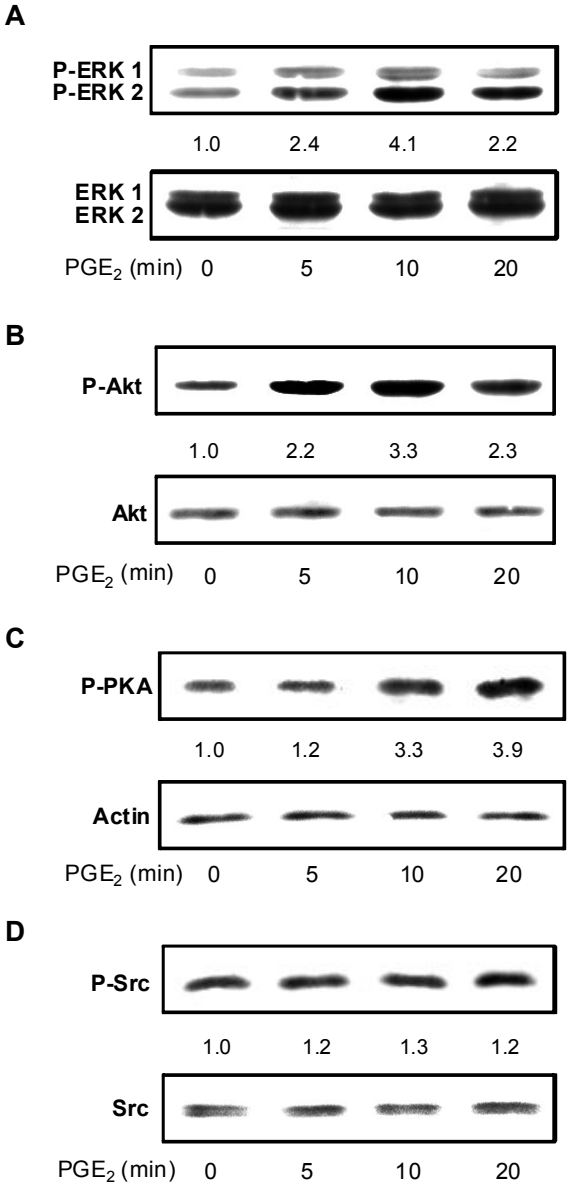


Fig. 41. Effects of PGE_2 on phosphorylation of ERK, Akt, PKA and Src in MCF-7 cells. Cells were incubated with 10 μM of PGE_2 for 5, 10 and 20 min. The membrane was probed with phospho-ERK (A), Akt (B), PKA (C) and Src (D)-specific antibodies and these were compared to total ERK, Akt, actin and Sre

protein, respectively. Each blot is representative of three independent experiments with similar results. The CYP1B1 protein level was compared to actin protein. The densitometry data presented below the bands are “fold change” compared with control after normalization with respective loading control value.

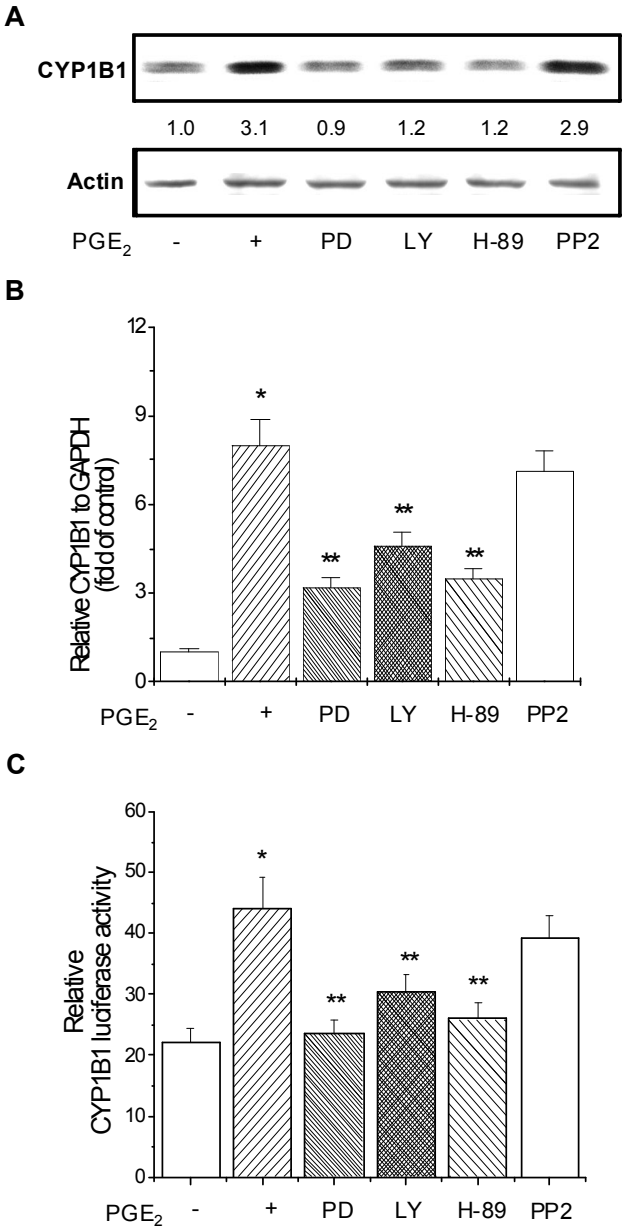


Fig. 42. Effects of ERK, Akt, PKA and Src on PGE_2-induced CYP1B1 in MCF-7 cells. (A) Effects of phosphorylation of ERK, Akt, PKA and Src on PGE_2-regulated

CYP1B1 protein expression. Cells were pretreated with ERK [PD98059 (PD; 10 μM)], Akt [LY294002 (LY; 5 μM)], PKA (H-89; 10 μM) and Src (PP2; 20 μM) and then incubated with 10 μM of PGE_2 for 24 h. The blot was probed with human CYP1B1-specific antibody. Each blot is representative of three independent experiments. The CYP1B1 protein level was compared to actin protein. The densitometry data presented below the bands are "fold change" compared with control after normalization with respective loading control value. (B) Effects of phosphorylation of ERK, Akt, PKA and Src on PGE_2-regulated CYP1B1 gene expression. Cells were pretreated inhibitors of PD98059 (PD; 10 μM), LY294002 (LY; 5 μM), H-89 (10 μM) and PP2 (20 μM) for 30 min and then treated with 10 μM of PGE_2 for 24 h. The gene expression level was expressed as a ratio to the expression of GAPDH. Result shown is representative of three independent experiments. * P and ** $P < 0.01$, significantly different from control and PGE_2, respectively, as determined by Newman–Keuls test. (C) Effects of phosphorylation of ERK, Akt, PKA and Src on PGE_2-regulated CYP1B1 promoter activity. Cells were transfected with CYP1B1-Luc and then pretreated inhibitors after that incubated 10 μM of PGE_2 in 24 h. Cells were harvested and lysed for luciferase activity and normalized to the β-galactosidase activity. This result shown is representative of three independent experiments. * P and ** $P < 0.01$, significantly different from control and PGE_2, respectively, as determined by Newman–Keuls test.

13. 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 PGE_2 and COX-2 mediated induction of PGE_2 maybe regulates CYP1B1 expression. To investigate the relationship between COX-2 and CYP1B1, this study was examined the effects of a COX-2 inhibitor, NS-398, on PGE_2-induced CYP1B1 expression. NS-398 significantly reduced PGE_2-induced CYP1B1 protein expression (Fig. 7A) as well as PGE_2-induced CYP1B1 mRNA expression and reporter activity in MCF-7 cells (Fig. 7B, C). 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, this study was 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, this study was investigated its effect on CYP1B1 mRNA expression and promoter activity. MCF-7 cells co-transfected with hCYP1B1-Luc vector and the COX-2 expression vector had increased CYP1B1 mRNA expression and luciferase activity (Fig. 7E, F). These results demonstrate that COX-2 may induce CYP1B1 expression in human breast cancer.

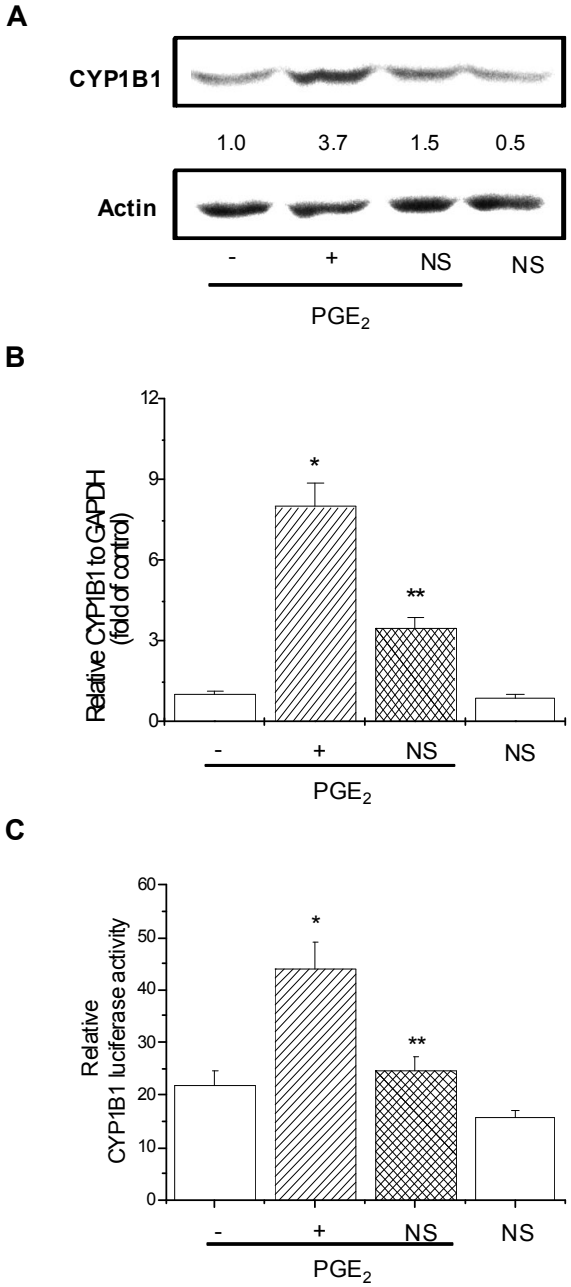


Fig. 43. Effects of COX-2 inhibitor on PGE_2-induced CYP1B1 expression in MCF-

7 cells. (A) Effects of COX-2 on PGE_2-regulated CYP1B1 protein level. Cells were pretreated COX-2 inhibitor [NS-398 (NS; 100 μM)] for 30 min and then incubated with 10 μM of PGE_2 for 24 h. The membrane was probed with human CYP1B1-specific antibody and each blot is representative of three independent experiments. The CYP1B1 protein level was compared to actin protein. The densitometry data presented below the bands are "fold change" compared with control after normalization with respective loading control value. (B) Effects of COX-2 on PGE_2-regulated CYP1B1 mRNA level. Cells were pretreated NS-398 (NS; 100 μM) and then treated with 10 μM of PGE_2 for 24 h. The gene expression level was expressed as a ratio to the expression of GAPDH and results shown are representative of three independent experiments. * P and ** $P < 0.01$, significantly different from control and PGE_2, respectively, as determined by Newman–Keuls test. (C) Effects of COX-2 on PGE_2-regulated CYP1B1 luciferase activity. Cells were transfected with hCYP1B1-Luc construct for 4 h and then treated with NS-398 (NS; 100 μM) and PGE_2 (10 μM) for 24 h. Cells were lysed for luciferase activity and normalized to the β-galactosidase activity. This result shown is representative of three independent experiments. * P and ** $P < 0.01$, significantly different from control and PGE_2, respectively, as determined by Newman–Keuls test.

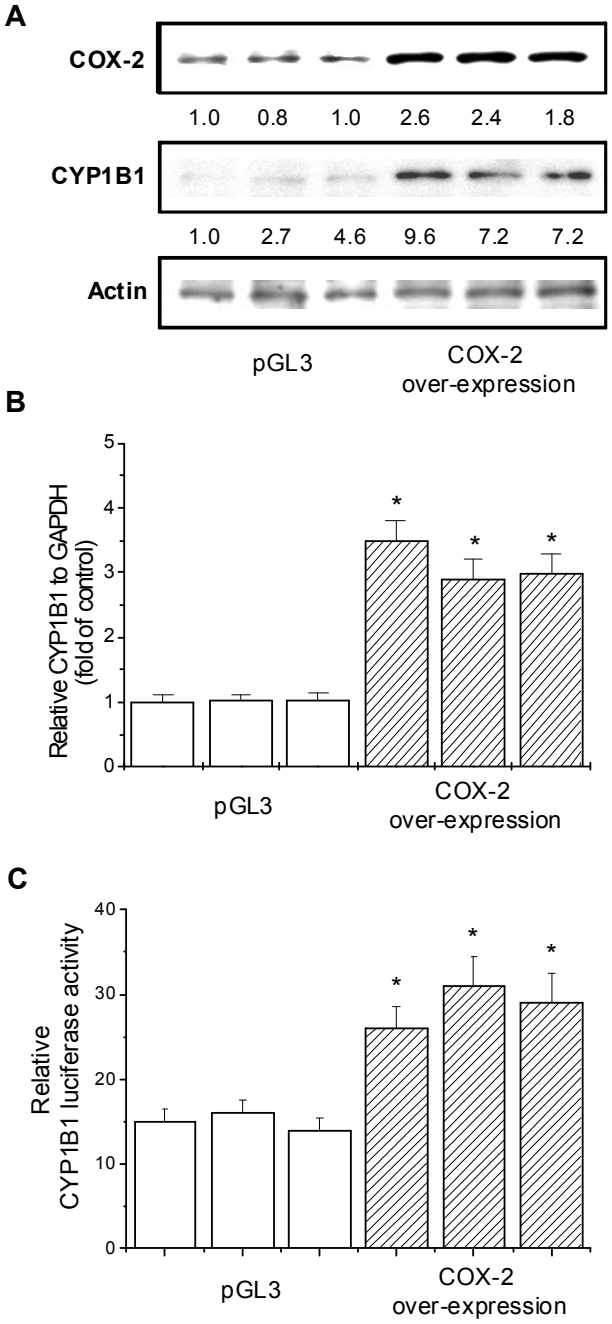

A
COX-2
1.0 0.8 1.0 2.6 2.4 1.8
CYP1B1
1.0 2.7 4.6 9.6 7.2 7.2
Actin
pGL3
COX-2 over-expression
B
Relative CYP1B1 to GAPDH (fold of control)
0
1
2
3
4
5
*
*
*
pGL3
COX-2 over-expression
C
Relative CYP1B1 luciferase activity
0
10
20
30
40
*
*
*
pGL3
COX-2 over-expression

Fig. 44. Effects of COX-2 over-expression on PGE_2-regulated CYP1B1 level in MCF-7 cells. (A) Effects of COX-2 over-expression on CYP1B1 protein level. Cells were transfected with pGL3-basic vector or COX-2 expression vector for 24 h and then harvested and lysed for Western blot. The membrane was probed with human COX-2 and CYP1B1-specific antibodies and each blot is representative of three independent experiments. The COX-2 and CYP1B1 protein level was compared to actin protein. The densitometry data presented below the bands are "fold change" compared with control after normalization with respective loading control value. (B, C) Effects of COX-2 over-expression on CYP1B1 mRNA expression and luciferase activity. Cells were transfected with pGL3-basic vector or COX-2 expression vector for 24 h. The gene expression level was expressed as a ratio to the expression of GAPDH (B). Cells were harvested and lysed for luciferase activity and normalized to the β-galactosidase activity (C). Results shown are representative of three independent experiments. $*P < 0.01$, significantly different from control as determined by Newman–Keuls test.

IV. Discussion

Up-regulation of aromatase by o,p'-DDT is mediated through cyclooxygenase-2 expression in human breast cancer cells

The objective of this study was to determine the effect of o,p'-DDT on aromatase in human breast cancer cells. This study show that o,p'-DDT stimulates aromatase protein and gene expression and that o,p'-DDT-induced aromatase correlates with COX-2 up-regulation in human breast cancer cells. This study also suggests that this effect of o,p'-DDT is mediated via activation of the CREB, PKA and Akt/ERK/JNK signaling pathway.

This study was first examined the effects of o,p'-DDT on the regulation of aromatase in human breast cancer MCF-7 cells. o,p'-DDT treatment increased aromatase protein, activity, gene expression and transcriptional activity in MCF-7 cells, thus providing a mechanism by which o,p'-DDT can increase the availability of estrogen in breast cancer. In addition, o,p'-DDT induced aromatase protein level, enzyme activity and luciferase activity in MDA-MB-231 and SK-BR-3 cells. Therefore, o,p'-DDT may enhanced aromatase expression in breast. In a previous paper, this study was reported that o,p'-DDT-induced COX-2 enzyme increased intracellular cAMP and estrogen production in macrophages (Han *et al*., 2008). The significant association between COX-2 and aromatase is in line with earlier studies (Brueggemeier *et al*., 1999; Brodie *et al*., 2006). This study was checked the effects

of o,p'-DDT on the regulation of COX-2 in MCF-7 cells. o,p'-DDT induced COX-2 protein, gene expression and transcriptional activity and PGE_2 production in MCF-7 cells. o,p'-DDT also induced COX-2 protein level, gene expression and luciferase activity in MDA-MB-231 and SK-BR-3 cells. Based on these results, o,p'-DDT-induced aromatase correlated with induction of COX-2 expression and PGE_2 production in MCF-7 cells. Previous studies indicate that COX-derived PGE_2 can activate aromatase and possibly increase the risk of breast cancer (Zhao *et al*., 1996; Terry *et al*., 2004; DuBois, 2004; Subbaramaiah *et al*., 2006).

PGE_2 (as a result of COX-2 activity) exerts its effects by binding to G protein-coupled receptors that contain seven transmembrane domains. The subtypes of PGE_2 receptor (EP1, EP2, EP3, and EP4) have been defined (Sugimoto and Narumiya, 2007). Different EP receptors have been implicated in the regulation of cell proliferation, immune function, and angiogenesis (Dannenberg and Subbaramaiah, 2003; Sugimoto and Narumiya, 2007), but little is known about the regulation of aromatase. This study show that o,p'-DDT induced EP2 and EP4 receptor gene expression in MCF-7 cells. In addition, PGE_2, a known stimulant of cAMP in breast cancer cells, is suggested to be important in the switching of aromatase promoters (Bulun and Simpson, 2008). The expression of aromatase is also known to be regulated by cAMP responsive element (CRE) in cancer tissues (Simpson, 2003). o,p'-DDT induced CRE transcriptional activity, increasing the levels of cAMP, nuclear CREB and CREB phosphorylation. These results suggest that o,p'-DDT activates the cAMP and CREB pathway via EP2 receptor, resulting in enhanced aromatase transcription and increased aromatase activity. Stimulation

of cAMP induces PKA activation and then activates CREB pathway (Zhao *et al*., 1996; Cai *et al*., 2007). In addition, stimulation of the EP4 receptor induces PI3-kinase-dependent activation of Akt (Fujino *et al*., 2003). This study was found that PKA and Akt were involved in the up-regulation of aromatase by o,p'-DDT in MCF-7 cells. Moreover, PI3-kinase/Akt contributes to activation of three MAPKs (ERK, JNK, and p38), resulting in up-regulation of aromatase (Brodie *et al*., 2006). This study was also found that ERK and JNK were involved in the up-regulation of aromatase by o,p'-DDT in MCF-7 cells. Finally, to determine whether o,p'-DDT-induced aromatase correlated with a COX-2-dependent pathway and PKA/PI3-kinase/Akt activation, this study was examined the effects of various inhibitors [COX-2 (NS-398), PKA (H-89), PI3-kinase/Akt (LY 294002), ERK (PD 98059), JNK (SP 600125), EP2 (AH-6809) and EP4 receptor (AH-23848)]. These inhibitors significantly reduced o,p'-DDT-induced aromatase protein expression in MCF-7 cells. Therefore, o,p'-DDT-induced aromatase correlated with COX-2 up-regulation mediated by the PKA and PI3-kinase/Akt signaling pathways in MCF-7 cells (Fig. 8). Moreover, MXC is used to protect crops, ornamentals, livestock, and pets against fleas, mosquitoes, cockroaches, and other insects. It has been used to some degree as a replacement for DDT as it is metabolized faster and does not lead to bioaccumulation. Methoxychlor, other environmental toxicants, was also induced aromatase and COX-2. Therefore, environmental toxicants may induce aromatase and that aromatase correlates with COX-2 up-regulation.

Endocrine-disrupting chemicals are widely distributed in the environment and are derived from many human activities. Chemical compounds that mimic the effects

of estrogen, including o,p'-DDT, are largely considered endocrine disruptors. Endocrine disruptors are thought to affect human health by influencing fetal development (Iguchi, 1998) and increasing the rate of breast cancer (Davis *et al*., 1993) and other endocrinological diseases (Mosconi *et al*., 2002). Environmental toxicants such as o,p'-DDT may affect aromatase in several estrogen-dependent diseases including breast cancer and endometriosis. The aromatase enzyme is unique to the pathway of estrogen biosynthesis and converts androgen precursors into estrogens, which are major stimulatory factors for breast cancer proliferation (Bulun and Simpson, 2008). Our data suggest that o,p'-DDT can increase aromatase and thus increase breast estrogen levels, which could have implications for estrogen-dependent functions in target tissues. Our results suggest that environmental toxicants, such as o,p'-DDT and MXC, can increase aromatase and thus increase breast estrogen levels, which could have implications for estrogen-dependent functions in target tissues. In addition, DDT was used for malaria control in some African countries and would exceed the World Health Organization (WHO)/Food and Agricultural Organization (FAO) acceptable daily intakes for DDT, 20 mg/kg (Kinyamu et al, 1998). Mothers residing in these parts of south Africa had mean breast milk DDT levels (15.83 mg/kg in milk fat) that were statistically significantly higher than those of mothers residing in parts of the study area where DDT was not used (0.69 mg/kg in milk fat) (Nweke OC and Sanders, 2009). Among women who were = 14 years of age by 1945 (when DDT was first introduced for use by the general public), those with blood concentrations in the highest tertile were five times more likely to develop breast cancer than those with

blood levels in the lowest tertile (Eskenazi et al., 2009). Our study suggests that o,p'-DDT induced aromatase was risk of breast cancer among women who were irrespective of age.

In summary, our study demonstrates that o,p'-DDT induces aromatase and that o,p'-DDT-induced aromatase correlates with COX-2 up-regulation mediated by the PKA and PI3-kinase/Akt signaling pathways in breast cancer cells.

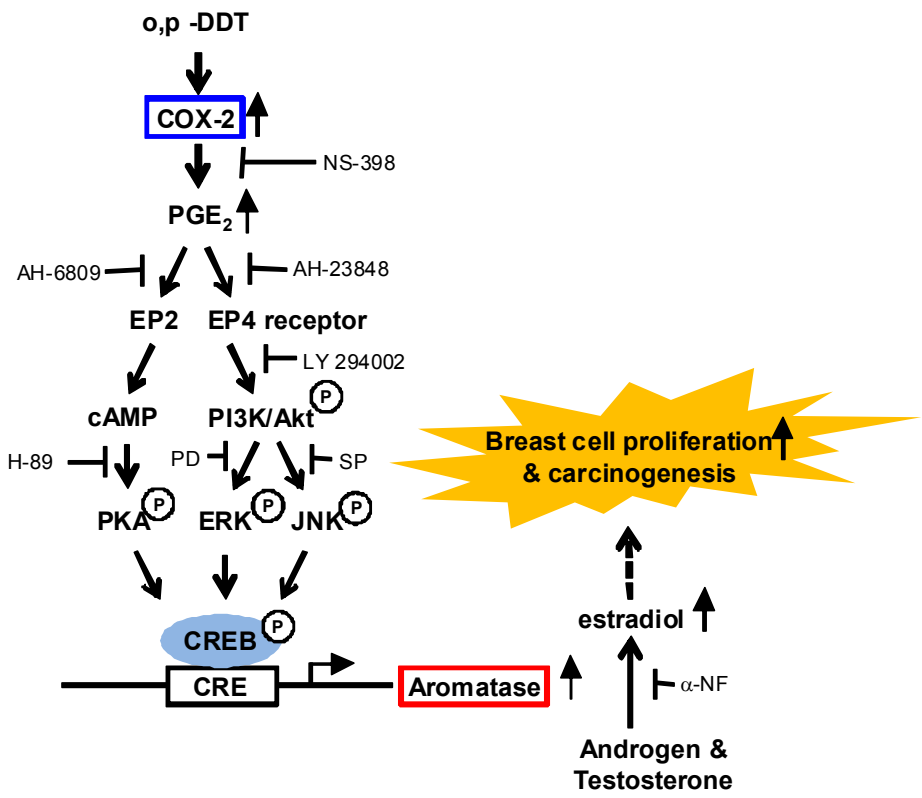


Fig. 45. A scheme of the aromatase induction pathway by o,p'-DDT in MCF-7 cells. o,p'-DDT induced COX-2 and increased PGE_2 production. o,p'-DDT-induced PGE_2 activated the EP2 and 4 receptors. Activated-EP2 and 4 receptors lead to cAMP and phosphorylation of other proteins such as PKA, PI3-kinase/Akt, ERK, and JNK. These initial interactions led to CREB phosphorylation and then increased aromatase levels. Thus, o,p'-DDT-induced COX-2 significantly induced aromatase expression through signaling pathways, such as PKA, PI3-kinase/Akt, ERK, JNK and CRE activation. Various inhibitors can inhibit the action of o,p'-DDT-induced aromatase expression. Finally, o,p'-DDT-induced aromatase can increase estradiol production, which participates in breast cancer. Abbreviations: aromatase (α-NF), COX-2 (NS-398; NS), PKA (H-89), PI3-kinase/Akt (LY 294002; LY), ERK (PD98059; PD) and JNK (SP600125; SP)

Prostaglandin E2 induces CYP1B1 expression *via* ligand-independent activation of the ERα pathway in human breast cancer cells

PGE_2 is the predominant pro-tumorigenic prostanoid, and this study was investigated the effect of PGE_2 on CYP1B1 expression in breast cancer cells. PGE_2 treatment was shown to significantly increase CYP1B1 protein and mRNA expression and promoter activity in MCF-7 breast cancer cells. The actions of PGE_2 depend on the activation of one or more of the four PGE_2 receptors (EP1−EP4) expressed by target cells (Timoshenko *et al.*, 2006), and this study was demonstrated that PGE_2-induced CYP1B1 expression is mediated through the EP2 and EP4 receptors. However, we don't know yet whether PGE_2 stimulates CYP1B1 expression by activation of EP1 and EP3 receptor signal pathway. Recent studies have been 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 GC-box and ERE half site (Petz *et al.,* 2000; Saville *et al.,* 2000). Next, using deletion promoter constructs of hCYP1B1 and co-treatment with inhibitors of AP-1, XRE, and ERE binding site activation, this study was showed that the ERE transcription factor binding site of the CYP1B1 promoter contributes to CYP1B1 regulation by PGE_2. Furthermore, PGE_2 was shown to rapidly induce the phosphorylation of ERα 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 PGE_2-induced CYP1B1

expression, and COX-2 overexpression induced CYP1B1 expression. Taken together, our results demonstrate that PGE_2-induced CYP1B1 expression is mediated by PGE_2-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 PGE_2, has been found at high levels in tumor cells (Schrey and Patel, 1995) and is synthesized in several human breast cancer cell lines. PGE_2 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 (Clemons and Goss, 2001; Eliassen *et al.*, 2006; Tamimi *et al.*, 2007; Yager and Davidson, 2006; Clamp *et al.*, 2002).

Several lines of evidence suggest that CYP1B1 plays a role in carcinogenesis. CYP1B1 is commonly overexpressed in human malignancies (Murray *et al.*, 1997; Gibson *et al.*, 2003; Lin *et al.*, 2003). CYP1B1 readily metabolizes 17β-estradiol via its primary hydroxylase activity at C-4 (Spink *et al.*, 1998), 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

(Cavalieri *et al.*, 2006; Cavalieri *et al.*, 1997; Zhang *et al.*, 2007). Thus, 4-hydroxyestradiol is a unique carcinogen 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_2 significantly induces CYP1B1 expression through ligand-independent ERα activation via the ERK, Akt, and PKA signaling pathways in human breast cancer cells. PGE_2-induced CYP1B1 metabolizes estradiol to 4-hydroxyestradiol, which is associated with the promotion of breast cancer.

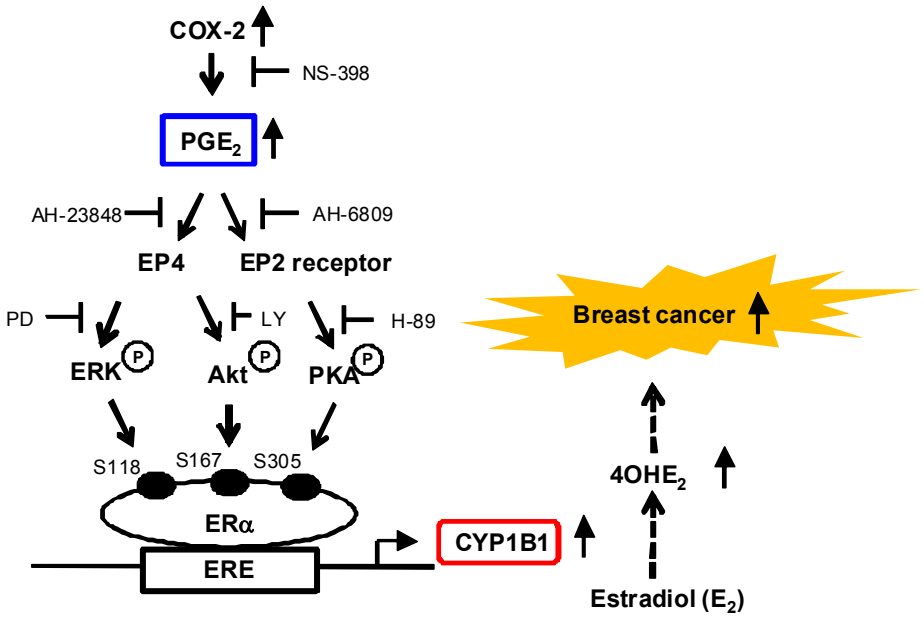


Fig. 46. A scheme of the CYP1B1 induction pathway by PGE_2 in MCF-7 cells. COX-2-induced PGE_2 activated the EP2 and 4 receptors. Activated-EP2 and 4 receptors lead to phosphorylation of other proteins such as ERK, Akt and PKA. These initial interactions led to ERα phosphorylation at serine 118, 167 and 305 residues and then increased CYP1B1 levels. Thus, COX-2-induced PGE_2 significantly induced CYP1B1 expression through signaling pathways, such as ERK, Akt and PKA activation. Various inhibitors [COX-2 (NS-398; NS), EP2 receptor (AH-6809), EP4 receptor (AH-23848), ERK (PD98059; PD), Akt (LY 294002; LY) and PKA (H-89)] can inhibit the action of PGE_2-induced CYP1B1 expression. Finally, PGE_2 induces the CYP1B1 expression and PGE_2-induced CYP1B1 was correlates with ligand-independent ERα activation mechanisms in human breast cancer cells. PGE_2-induced CYP1B1 can metabolize estradiol to 4-hydroxyestradiol, which participates in breast cancer.

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[국문 초록]

PGE_2와 o,p′-DDT의 여성호르몬 합성 및 대사 조절 단백질 aromatase와 CYP1B1 발현 조절 연구

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본 연구에서는 염증 반응을 유도하는 유기염소계 살충제 o,p'-DDT (o,p'-dichlorodiphenyltrichloroethan)와 염증 반응 최종산물인 PGE_2 (prostaglandin E_2)의 유방암 유발에 관여하는 estrogen의 합성 및 대사 조절 단백질 발현에 미치는 영향과 작용기전을 조사하였다.

Estrogen 합성 조절 단백질 aromatase에 대한 o,p'-DDT의 영향을 조사하기 위하여 인간 유방암세포주인 MCF-7 세포주에 o,p'-DDT를 처리하였을 때 aromatase 단백질과 유전자 발현, 전사활성 및 효소 활성도가 증가하였다. PGE_2는 aromatase 발현을 유발하는 대표적인 물질로 염증반응 매개 단백질인 COX-2 (cyclooxygenase-2)의 최종

산물이다. o,p'-DDT에 의해 COX-2 단백질과 유전자 발현이 증가하였으며, PGE_2 생성량이 증가하였다. 또한 MCF-7 세포주 외에 인간 유방암 세포주 MDA-MB-231과 SK-BR-3 세포에서도 o,p'-DDT에 의해 aromatase와 COX-2 단백질 및 유전자 발현이 증가하였다. o,p'-DDT의 PGE_2 신호전달에 관여하는 EP2, 4 receptor 발현이 증가하였으며, 이들의 억제제를 처리하여 이를 재확인하였다. o,p'-DDT는 aromatase 발현에 관여하는 CRE (cAMP response element)의 전사활성과 cAMP 생성량을 증가시켰으며, CREB (CRE binding protein) 단백질의 세포질에서 핵으로의 translocation을 유발하였다. cAMP 하위 신호전달체계인 PKA와 EP receptor 활성에 관여하는 PI3K/Akt 및 MAPKs ERK, JNK 등의 신호전달 단백질이 활성화되었다. Testosterone을 전처리한 MCF-7 세포에서 o,p'-DDT에 의해 aromatase 발현이 증가되고, estradiol 생성이 유발되어 세포 생장율이 증가되었다. 또한 o,p'-DDT 대체 내분비계 장애물질인 methoxychlor에 의해 COX-2와 aromatase 단백질 발현이 증가하였으며, COX-2의 증가가 aromatase 발현을 조절한다는 것을 재확인하였다.

Estrogen 대사 조절 단백질 CYP1B1에 대한 PGE_2의 영향을 조사하기 위하여 인간 유방암세포주인 MCF-7 세포주에 PGE_2를 처리하였을 때 CYP1B1 단백질과 유전자 발현, 전사활성이 증가하였다. PGE_2 신호전달에 관여하는 EP2와 EP4 receptor에 의해 CYP1B1 발현이 유도되었다. ERE (estrogen response element), XRE (xenobiotic response element), AP-1

(activator protein-1)등이 CYP1B1 발현을 조절하는 대표적인 promoter로 PGE_2 처리에 의한 ERE에 활성화로 CYP1B1 발현이 유발 되었다. ERα가 결핍된 인간 유방암 세포주인 MDA-MB-231과 ERα와 β가 결핍된 MCF-10A 세포에서 PGE_2에 의해 CYP1B1 단백질과 유전자 발현 및 전사활성의 변화가 없었다. 또한 MCF-7 세포에 PGE_2를 처리하였을 때 ERα와 β의 단백질 발현도 변화가 없었다. PGE_2에 의해 ERα의 serine 118, 167, 305의 인산화가 증가하였다. 또한 PGE_2에 의해 PKA, Akt 및 ERK의 단백질이 활성화되었다. COX-2 inhibitor를 처리했을 때 PGE_2에 의해 증가된 CYP1B1 발현이 감소하였다. 마지막으로 COX-2를 과발현시켰을 때 CYP1B1의 단백질, 유전자 발현 및 전사활성이 증가하였다. 따라서 염증반응이 증가되었을 때 estradiol을 4-hyroxyestradiol로 대사시켜 유전독성을 유발하는 CYP1B1의 발현이 증가되었으며, 염증반응 유발 시 유방암 발병에 영향을 줄 수 있는 가능성을 확인하였다.

저작물 이용 허락서

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논문 제목	한글: PGE_2와 o,p′-DDT의 여성호르몬의 합성 및 대사 조절 단백질 aromatase와 CYP1B1 발현 조절 연구				
	영문: Studies on the estrogen biosynthesis and metabolism related aromatase and CYP1B1 expression by PGE_2 and o.p'-DDT in breast cancer cells				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함.
2. 위의 목적을 위하여 필요한 범위 내에서의 편집과 형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포・전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물 이용의 허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음.
7. 소속 대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송・출력을 허락함.

동의 여부 : 동의 ( ) 반대 (○)

2010년 2 월 일

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