Effect of Genistein and 2,3,7,8-tetrachlorodibenzo-*para*-TCDD On Aromatase Activity

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ABSTRACT

Aromatase or CYP19 is the rate limiting enzyme in converting androgen to estrogen. Estrogen is important in the prevention of the cardiovascular disease. Previous researches have shown that genistein, a dietary phytoestrogen, could increase LDL receptor and apolipoprotein A-1 expression, but the mechanism is unclear. This study investigated the relationship between genistein and estrogen synthesis in a human hepatic cell line. On the other hand, the female hormone is critical in the initiation and development of breast cancer. Environmental toxicants are thought to play a role in several estrogen-dependent diseases including breast cancer. Therefore, the other objective of this study was to determine the effect of TCDD on aromatase expression and activity in human breast cancer cell lines in culture.

In this study, 0.1μM to 10μM genistein could induce aromatase activity in a dose dependent manner in ERα-transfected HepG2 cells. The induction of aromatase activity was consistent with the up-regulations in aromatase protein expression, mRNA expression and promoter I.1 activity. From the promoter deletion analysis, we found that the sequences between -212 to -190 and -300 to -260 bp upstream of exon I.1 might be responsible for the activity induced by genistein. Within these regions of promoter I.1, several AP-1 sites can be located. Genistein also increased the AP-1 transactivation activity, suggesting that AP-1 might be involved in the induced aromatase activity in ERα-transfected HepG2 cells. Besides, inhibition of p38 pathway could partly abolish the genistein-induced aromatase activity. The possibility that genistein promotes the aromatase activity through p38 kinase activation and ER phosphorylation needs further investigation.

Tritiated water release assay had shown that TCDD could increase aromatase activity in MCF-7, but not the other breast cancer cells. 1ηM to 10ηM TCDD were found to increase the aromatase activity in MCF-7 cells, but not in human CYP19 Supersomes®. These results indicated that the enzyme induction was not the result of a direct stimulation on the aromatase enzyme complex. TCDD could up-regulate the aromatase protein and mRNA expression in MCF-7 cells. However, it could not induce promoter I.1, I.3/II activity and AP-1 transactivation activity. We also

investigated the role of ER, MAP kinase and protein kinase pathways by adding the corresponding inhibitors. The results showed that ER, ERK, JNK, PKA and PKC might be involved in the regulation process.

Instead of regulation through typical transcriptional control, data in the present study suggested that TCDD could increase the half-life of CYP19 mRNA. Such induction can be abolished by ERK inhibitor. However, the mechanism through which TCDD mediated aromatase mRNA degradation was not fully understood. The aromatase activity, protein and mRNA expression in MCF-7 cells expressing ERK were higher than that of the control cells. On the other hand, TCDD could increase the active ERK protein in MCF-7 in a dose-dependent manner, and ERK inhibitor could block TCDD-induced aromatase. Combining these observations, the aromatase activity could be mediated by ERK.

In the present study, we demonstrated that genistein could significantly increase the aromatase activity in the ERα-transfected HepG2 cells. Genistein might increase apoA1 and LDLR by way of estrogen synthesis. This study provided a new insight for the gene-regulatory mechanism of genistein, or other estrogen-like compounds. Besides, we also demonstrated that TCDD could enhance the aromatase activity through a post-transcriptional stabilization of aromatase mRNA in MCF-7 cells. Such process could be mediated by ERK. In conclusion, genistein and TCDD could increase the aromatase activity through different mechanisms.

摘要

芳香化酶是作爲催化類固醇向雌激素的轉化的關鍵限速酶。能引發及發展乳腺癌。雌激素能有效預防心血管疾病。研究發現食品中的植物雌激素金雀異黃酮能有效增加低密度脂蛋白受體及載脂蛋白 A1 的表達，但其潛在機理尚未完全清楚。本項研究主要探討在人類肝細胞中金雀異黃酮和雌激素的關係。另一方面，環境毒物一直被視爲在一些依賴雌激素的疾病發病機制中擔當在重要的角色， 其中包括乳腺癌。本項研究並就二惡英在人類乳腺癌細胞中對芳香化酶的表達及活性的作用進行了研究。

本項研究發現在轉染雌激素受體 α 的 HepG2 細胞中， 0.1 至 10μM 的金雀異黃酮以劑量遞增效應來誘導芳香化酶活性。 同樣地， 芳香化酶的表達、 mRNA 及啓動子 P.I.1 的轉錄活性均被誘導。 應答金雀異黃酮誘導作用的反應應答組件位於外顯子 I.1 上游 -212 至 -190 以及 -300 至 -260 之間。 若干 AP-1 位置被發現在這些應答區內。 由於金雀異黃酮能增誘 AP-1 轉錄活性， 轉錄因數 AP-1 應參與了金雀異黃酮誘導芳香化酶活性中的機制。另外， p38 MAP Kinase 抑制劑能抑制金雀異黃酮所引發的芳香化酶活性，但不能抑壓其引發的啓動子轉錄活動。 鑒此，p38 MAP Kinase 的調控作用機制應進行更仔細的研究。

氚水釋放檢測證實二惡英只能刺激 MCF-7，但不能刺激其他乳腺細胞中的芳香化酶活性。在 MCF-7 細胞中， 0.1 至 10ηM 二惡英能增加芳香化酶活性， 但在人類芳香化酶超載體中則不能。 這結果指出二惡英不大可能直接刺激芳香化酶的酵素合成物來誘發芳香化酶活性。 另一方面， 在 MCF-7 細胞中二惡英能提升芳香化酶蛋白質及 mRNA 的表達。 不過，二惡英不能刺激啓動子 P.I.1、I.3/II 及 AP-1 的轉錄活性。 因此， 此機制不大可能透過典型的轉錄調控機制。雌激素受體拮抗劑 ICI 182 780 的附加能阻止二惡英對芳香化酶的誘導作用。 這表明雌激素受體 α 在這機制中扮演一定的角色。 我們用相應的抑制劑研究促絲裂原活化蛋白激酶 (MAPK)和蛋白激酶的相關信號通路的作用機制。結果顯示細胞外信號調節激酶 (ERK)、 氨基末端激酶 (JNK)、 蛋白激酶 A 和蛋白激酶 C 可能參與其調控機制。

以目前的資料顯示， 二惡英能增加芳香化酶的 mRNA 的半衰期。細胞外信號調節激酶拮抗劑的附加能阻止二惡英增加芳香化酶的 mRNA 的半衰期的作用。然而，二惡英怎樣影響芳香化酶 mRNA 的半衰期仍未被完全理解。在一個穩定表達細胞外信號調節激酶基因的 MCF-7 細胞內， 其芳香化酶的活性、蛋白質的表達及 mRNA 的表達均較控制細胞爲高。 另一方面， 在 MCF-7 細胞內， 二惡英可以以劑量遞增效應來誘導細胞外信號調節激酶的蛋白質表達。二惡英所誘發的芳香化酶活性同時能被細胞外信號調節激酶抑制劑所抑制。 綜觀以上的研究， 芳香化酶活性可能是被細胞外信號調節激酶所調控。

本項研究發現在轉染雌激素受體 α 的 HepG2 細胞中， 金雀異黃酮誘導芳香化酶。 金雀異黃酮可能誘導雌激素的表達， 從而增加低密度脂蛋白受體及載脂蛋白 A1 的表達。 是此研究爲金雀異黃酮及其它類似雌激素作用機制提供一個新的途徑。另外，在 MCF-7 細胞中，二惡英能透過細胞外信號調節激酶來增加增加芳香化酶的 mRNA 的半衰期，從而刺激芳香化酶活性。

LIST OF ABBREVIATIONS

AF-1	**Activation Function-1**
AF-2	**Activation Function-2**
AhR	**Aryl Hydrocarbon Receptor**
AP-1	**Activator Protein 1**
ApoA1	**Apolipoprotein A-1**
ARNT	**AhR nuclear translocator protein**
Bp	**Base pair**
BSA	**Bovine serum albumin**
cDNA	**Complementary DNA**
CDS	**Coding sequence**
CREB	**cAMP response element binding protein**
CT	**Threshold cycle**
CVD	**Cardiovascular diseases**
CYP	**Cytochrome P450**
DMSO	**Dimethyl sulfoside**
DNA	**Deoxynucleic acid**
dNTP	**Deoxyribonucleotide triphosphate**
E_2	**17ß-Estradiol**
E.coli	**Escherichea Coli**
ER	**Estrogen Receptor**
ERE	**Estrogen Response Element**
ERK	**Extracellular Signal Regulated Kinase**
FBE	**Fetal bovine serum**
HDL	**High Density Lipoprotein**
JNK	**c-Jun N-terminal Kinase**
kDa	**Kilo-base**
LB	**Luria-Bertani medium**
LCAT	**Lecithin:cholesterol acyltransferase**
LDL	**Low Density Lipoprotein**
LDLR	**Low Density Lipoprotein Receptor**
MAPK	**Mitogen-Activated Protein Kinase**
MAPKK	**MAPK Kinase**

MAPKKK	**MAPKK Kinase**
mRNA	**Messenger ribonucleic acid**
NF-κB	**Nuclear Factor-κB**
PAH	**Polycyclic Aromatase Hydrocarbons**
PBS	**Phosphate buffered saline**
PCR	**Polymerase chain reaction**
PKA	**Protein Kinase A**
PKC	**Protein Kinase C**
RNA	**Ribonucleic acid**
RNase	**Ribonuclease**
rpm	**Revolutions per minute**
RPMI 1640 Medium	**Rosewell Park Memorial Institute tissue culture medium 1640**
RT-PCR	**Reverse transcription polymerase chain reaction**
SDS	**Sodium Dodecyl Sulfate**
TAE	**Tris-acetate-EDTA**
TCDD	**2,3,7,8-tetrachlorodibenzo-para-TCDD**
Tris	**Trizma base**
XRE	**Xenobiotic Response Element**

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CHAPTER 1

GENERAL INTRODUCTION

1.1 AROMATASE

Aromatase is a cytochrome P450 superfamily enzyme, which is critical in the conversion of androgen into estrogen, i.e. testosterone (C19) and androstenedione (C19) to estradiol and estrone (C18) respectively. Estone can be further processed to estradiol by 17β-hydroxysteroid dehydrogenase type 1 as shown in Figure1.1 (Simpson *et al.,* 1994a; Labrie *et al*, 2000).

Aromatase is expressed only in gonads and brain in most vertebrates. However, primates also express aromatase in placenta, bone and adipose tissue (Lephart & Simpson, 1991; Simapson *et al*, 1994b). The principal estrogen source of the premenopausal non-pregnant woman is synthesized in the ovary. Other sites of estrogen biosynthesis are present throughout the body beyond menopause. The estrogen synthesized within these extragonadal sites is probably biologically active only at local tissue level (Labrie *et al.,* 1997). These sites include osteoblasts (Bruch *et al.,* 1992), aortic smooth muscle cells (Sasano *et al.,* 1999), mesenchymal cells of the skin and adipose tissue (Simpson *et al.,* 1997), chondrocytes in bone (Bayard *et al.,* 1995); amygdala and the medial basal hyptothalamus in brain (Naftolin *et al.,* 1975). After menopause, adipose tissue becomes the main source of estrogen (Siiteri and MacDonald 1973; Simpson *et al.,* 1997). For the male, estrogen play an important role in the lipoprotein synthesis, maintenance of bone mineralization and spermatogenesis (Tsai-Morris *et al.,* 1985; Nitta *et al.,* 1993; Morishima *et al.,* 1995, Carani *et al.,* 1997; Smith *et al.,* 1994; Bagatell *et al.,* 1994). The level of estrogens is far higher within the reproductive tract then in the general blood compartment (Hess 2000). It has been estimated that testes account for 15% of circulating estrogen (Hemsell *et al.,* 1974). In the testis, aromatase is mainly localized in Leydig cells (Carreau *et al.,* 1999). Mutation of aromatase gene causes failure of epiphysical fusion, osteopenia and delayed bone age in male (Morishima *et al.,* 1995). Male mice with a null mutation in the aromatase gene also exhibit alterations in bone histomorphometry (Oz *et al.,* 2000).

Estrogen plays an important role in the initiation and development of breast cancer. It can stimulate cancer cell growth by triggering estrogen receptor-mediated signal transduction, resulting in increased DNA synthesis and cell proliferation (Feigelson and Henderson, 1996). Estradiol would interact with plasma membrane estrogen receptor (ER), change the regulation of cell cycle and Bcl-2 family protein expression (Dickson, 1987; Watson *et al.,* 1999; Leung & Wang, 1999). Estrogen metabolites formed by cytochrome P450s may also play a role in the initiation of cancer. CYP1A1 and CYP1B1 hydroxylate 17ß-estradiol (E_2) at the C-2 and C-4 positions respectively to form 2-hydroxyestradiol (2-OHE_2) and 4-hydroxyestradiol (4-OHE_2). These hydroxylated metabolites can further be oxidized to quinones, which are putative tumor initiators (Yager, 2000; Zhu *et al.,* 1998; Cavalien *et al.,* 1997; Cavalien *et al.,* 2002 and Rogan *et al.,* 2003). Some metabolites retain estrogenic activity and generate mutagenic free radicals and cause DNA damage (Zhu & Connery, 1998). The DNA damaging-effects of estrogen have been observed in rat mammary tissues and MCF-7 cells (Zhang *et al.,* 2001; Yared *et al., 2*002).

Previously, genistein and estradiol were shown to increase LDLR and ApoA1 expression in HepG2 cells (Yuen, 2005; Lamon-Fava *et al.*, 1999). As aromatase is a critical enzyme to convert androgen into estrogen, this study investigated the effect of genistein on aromatase activity and its underlying mechanism in the hepatic cells HepG2. On the other hand, environmental toxicants, including TCDD, are thought to play a role in breast cancer (Mocarelli and Pocchiari, 1988). The possibility that TCDD possesses estrogenic effect should be considered. Due to the functional role of aromatase, it may play a role in the TCDD-induced breast cancer. In this study, we investigated the effect and the underlying mechanism of genistein and TCDD on aromatase activity.

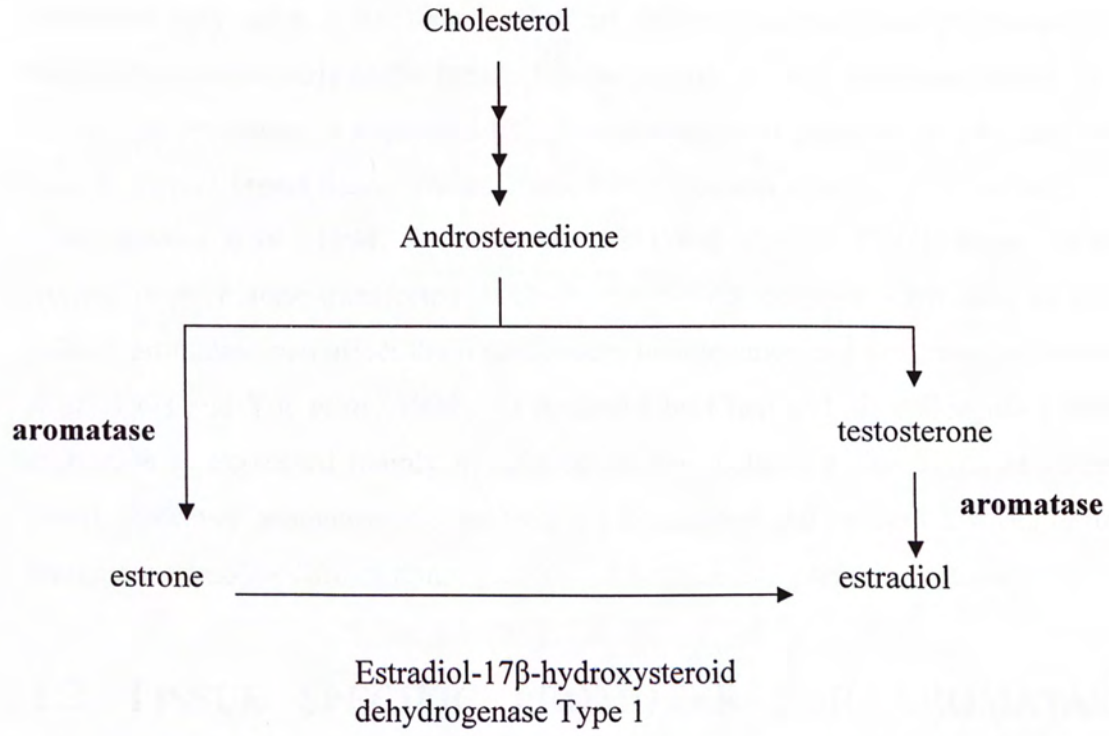

Figure 1.1 Scheme illustrates aromatase act as the final rate-limiting enzyme in the conversion of estrogen

As aromatase is responsible for the synthesis of estrogens, abnormal expression of aromatase may have a significant effect in the development and progression of malignancy, particularly in the breast (Simpson *et al.*, 1994a). Previous studies have shown that aromatase is expressed in breast cancer tissue probably at a higher level than in normal breast tissue (James *et al.*, 1987; Esteban *et al.*, 1992; Santen *et al.*, 1994; Sasano *et al.*, 1994; Bulum *et al.*, 1993 and Harada, 1997). Based on the studies in aromatase-transfected MCF-7 and T-47D cells *in vitro* and *in vivo*, cellular aromatase can affect the breast cancer maintenance and progression (Santner *et al.*, 1993 and Yue *et al.*, 1994). As reviewed by Chen and his colleagues (1999), aromatase is expressed mainly in adipose stromal cells and fibroblasts in normal breast. However, aromatase is expressed in both stromal and cancer cells, suggesting aromatase stimulates breast tumor growth in autocrine and paracrine actions.

1.2 TISSUE SPECIFIC PROMOTER FOR AROMATASE EXPRESSION

Aromatase is encoded by a single copy of *CYP19* gene, localized at chromosome 15q21.2 (Simpson *et al.*, 1994a; Chen, 1988). Transcriptional regulation of the CYP19 gene is complex and tissue specific. In human, the expression of CYP19 is regulated by alternative splicing (Harada *et al.*, 1993; Mahendroo *et al.*, 1993; Simpson *et al.*, 1994a). Each exon I is flanked by its own unique promoter region and is spliced onto a common splice junction immediately upstream of the start of translation. Hence, the opening reading frame of each transcript (exon II to X), as well as the CYP19 protein, is identical irrespective of the promoter used and site of expression (Figure 1.2 B). Up to this date, CYP19 is the only member of the cytochrome P450 gene superfamily. A schematic representation of the aromatase gene is shown in Figure 1.2. A.

In CYP19, there are ten distinct tissue-specific promoters: PI.1 (placenta, major), PI.2 (placenta, minor), PI.3 (adipose/ breast cancer), PI.4 (skin and adipose), PI.5 (fetal tissue), PI.6 (bone), PI.7 (endothelial), PI.f (brain), PII (ovary/breast cancer/endometriosis) and P2a (placenta, minor) (Shozu *et al.*, 1998; Sebastian *et al.*, 2001; Sebastian *et al.*, 2002) (Figure 1.2 A). In breast cancer, exons I.3 and II are the most frequently used, suggesting the promoter I.3 and II are the major promoters

directing aromatase expression in the malignant and surrounding tissue (Zhou C., *et al.*, 1996). This is different from exon I.4 used by the adipose stromal cells and fibroblasts in normal breast (Harada, 1993; Mahendroo *et al.*, 1993).

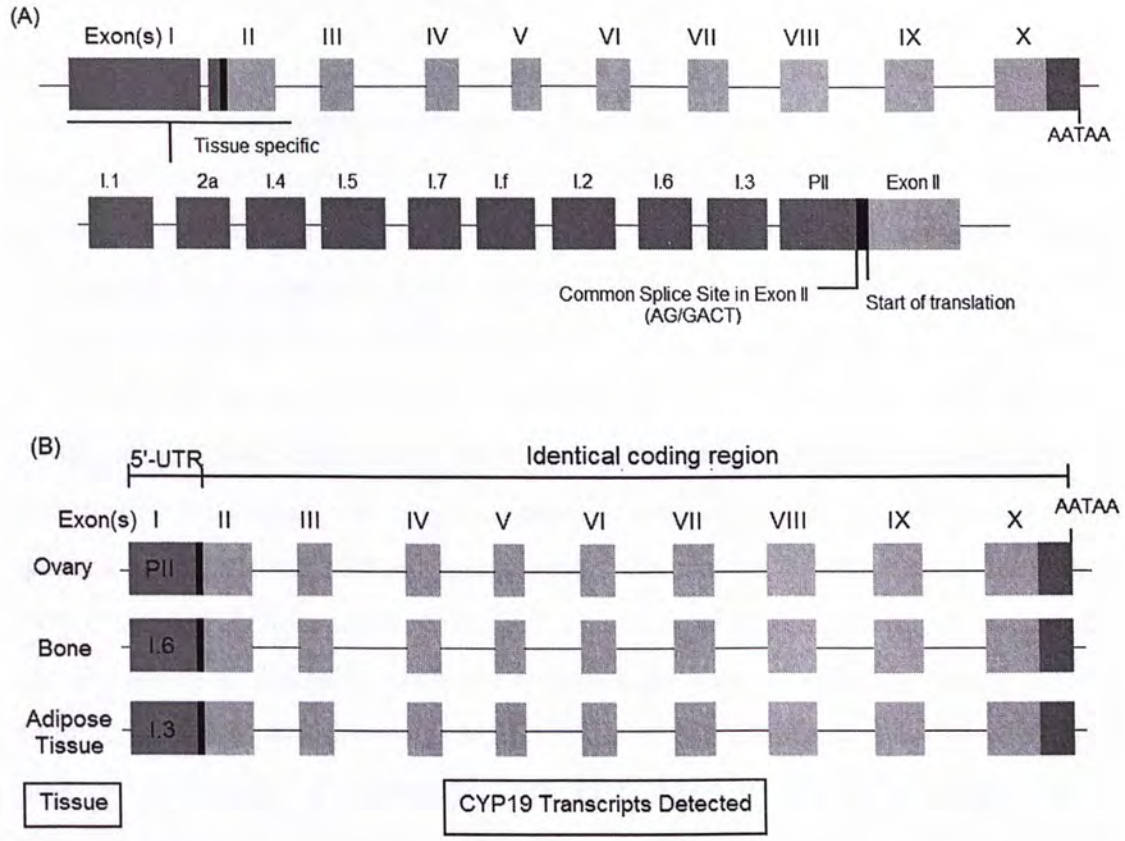

Figure 1.2. A. A schematic representation of the aromatase gene. A CYP19 overview shows the exons and multiple sub regions of exon I. Figure B illustrates that the coding region and the translated protein product are identical in all tissues, even though each tissue expresses a unique untranslated first exon 5'UTR. Dark gray shading, translated region; light gray shading, untranslated region; Black shading, common untranslated region. (Modified from Sebastian *et al.*, 2001 and Ellem *et al.*, 2004)

1.3 SIGNALING PATHWAY

MAP kinase is involved in the regulation of diverse processes ranging from transcription of protooncogenes to programmed cell death (Cobb, 1999). The MAPK cascade is generally sub-classified into three main branches: p38 kinases, c-Jun N-terminal kinases (JNKs), and extracellular signal-regulated kinases (ERKs) (Garrington and Johnson, 1999; Torres, 2003). Upon activation, a series of successively acting kinase would amplify signals that regulate diverse biologic functions, including cell growth, differentiation, proliferation and apoptosis (Molkentin, 2004). Generally, JNK and p38 kinase pathways may serve as transducers for injury or stress responses, whereas ERK pathways are more specialized for growth and mitogenic factor stimulation (Garrington and Johnson, 1999). Figure 1.3 illustrates a simplified scheme of MAPK signaling pathway. After signal coming at the cell membrane activates the Ras protein, Ras would directly couple to Raf and phosphorylates MEK1/2. These two proteins act as dual specificity kinases and directly phosphorylate the TEY motif in ERK1/2 kinases. Similar cascades have been observed in JNK and p38 kinase pathways. MKK4/7 can be activated by MEKK and then phosphorylate *JNK1/2/3*, while MKK 3/6 directly activate the genes, *p38α, p38β, p38δ, and p38γ* (Garrington and Johnson, 1999).

cAMP-dependent protein kinase, which is also known as protein kinase A (PKA), is allosterically activated by cAMP. The inactive form of PKA consists of two regulatory and two catalytic subunits. When cAMP binds to the regulatory subunit, the regulatory subunit would undergo conformational changes and yield two catalytic subunits. They in turn phosphorylate many enzymes at Ser or Thr sites, including CREB, glycogen synthase, phosphorylase b kinase and tyroine hydroxylase. Protein kinase C (PKC) is activated by elevated concentration of calcium. Phorbol esters, which are tumor promoters, are potent activators of PKC and affect the normal regulation of cell growth and division. Similar to PKA, PKC phosphorylates Ser or Thr residues of specific target protein and alters its catalytic activities (David, 2000).

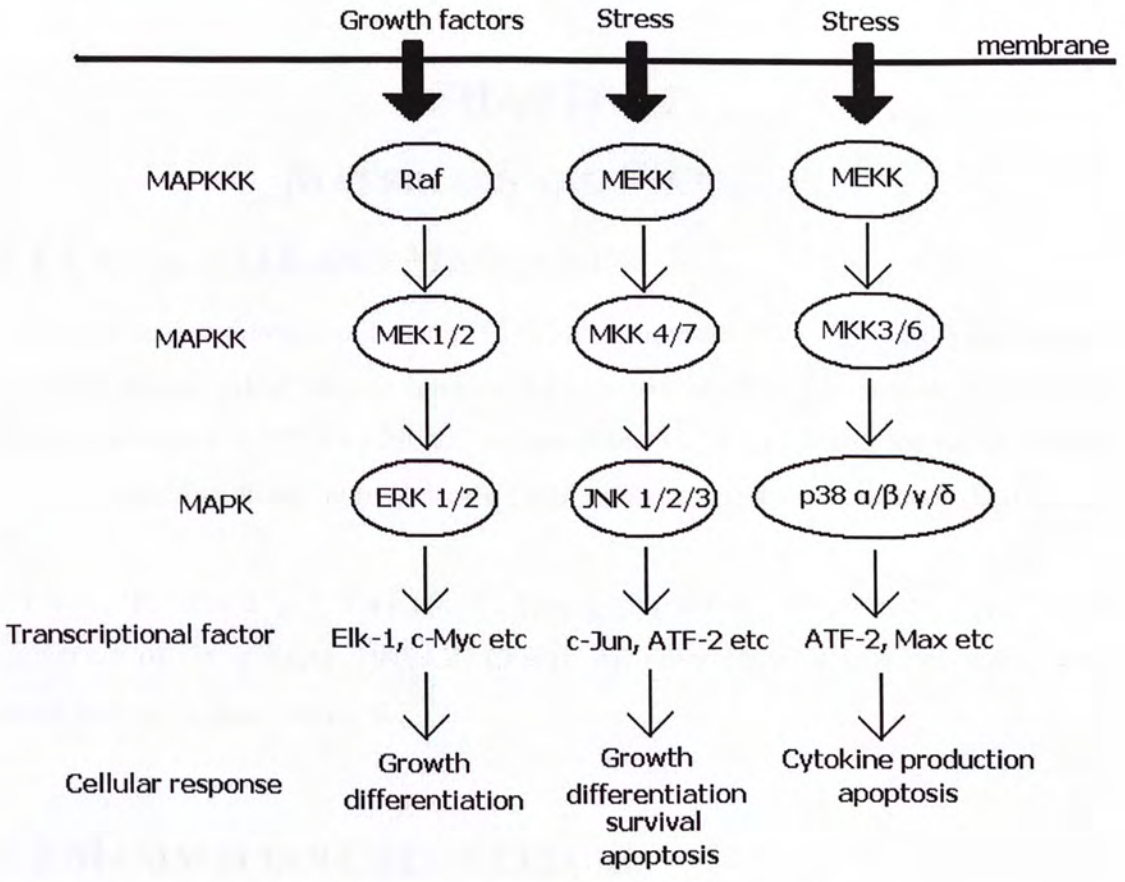

Figure 1.3 A simplified scheme illustrates MAPKs signaling pathway.
(Modified from Garrington and Johnson, 1999)

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS AND MATERIALS

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was purchased from Supelco. The mitogen-activated protein (MAP) kinase inhibitor U0126, p38 MAP kinase inhibitor SB203580, the protein kinase A (PKA) inhibitor myristoylated 14-22 amide and the protein kinase C (PKC) inhibitor bisindolylmaleimide I were obtained from Calbiochem (San Diego, CA).

ICI 182 780 was a gift from Dr. Y. Huang (Physiology Department, the Chinese University of Hong Kong, HKSAR, China). All other chemicals, if not stated, were acquired from Sigma Chemical.

2.2 MAMMALIAN CELL CULTURE

Two ER-positive human breast cancer cell lines MCF-7 and T47D, two ER-negative breast cancer cell lines SK-BR-3 and MDA-MB-231, a ER-negative hepatocellular carcinoma cell line HepG2 and a non-tumorigenic breast cell line MCF10A were used in the present study. SK-BR-3 cells were generously given by Dr. Richard K.W. Choy (Department of Obstetrics & Gynaecology, the Chinese University of Hong Kong, HKSAR, China). MCF-7 cells stably transfected with human CYP19 (MCF-7aro) or empty vector (MCF-7_{vec}) were kindly provided by Dr. S. Chen (Department of Surgical Research, Beckman Research Institute of the City of Hope, Duarte, CA91010, U.S.A.). MCF-7 cells stably transfected with ERK (MCF-7_{ERK}) were prepared in our lab by other postgraduate student. All other cell lines were purchased from American Type Cell Collection (Rockville, MD, USA)

2.2.1 Maintenance of cells

MCF-7, MDA-MB-231, T47D, MCF-7aro, MCF-7_{ERK} and MCF-7_{vec} were maintained in RPMI 1640 media, and the later two medium contained 500μg/ml selection antibiotics G418 (USB, Cleveland, OH, USA). HepG2 and MCF10A cells were maintained in phenol red free RPMI 1640 media and DMEM/F12 media (Gibco BRL, Rockville, MD, USA) respectively. All media were supplemented with 2 mol/L L-glutamine (Gibco BRL, Rockville, MD, USA), 1% Penicillin-Streptomycin (P/S) (Gibco BRL, Rockville, MD, U.S.A) and 10% fetal bovine serum (FBS) (Hyclone). SK-BR-3 cells were maintained in McCoy's 5A medium with 2 mol/L L-glutamine and 12.5% FBS. All the cell lines were incubated at 37°C, 5% carbon dioxide.

When cells reached about 80% confluence, the medium was discarded. The cell was washed with PBS and was trypsinized with trypsin-EDTA (Invitrogen) for 5 minutes. 10 minutes trypsinization was required in MCF10A cells. The cell suspension was transferred to a 15ml centrifuge tube containing 7ml PBS and centrifuged at 1,000 rpm at room temperature for 5 minutes. The cell pellet was resuspended with fresh growth medium, added in aliquot into new culture flasks and incubated as described above.

Phenol red is known to contain an estrogenic contaminant. Starting from the beginning of each assay, the cells were seeded with phenol-red free RPMI 1640 with 2 mol/L L-glutamine, 1% Penicillin-Streptomycin (P/S) and 5% charcoal/dextran treated fetal bovine serum (FBS) (Hyclone).

2.2.2 Preparation of cells stock

Semi-confluent cells in culture flask were rinsed with PBS and trypsinized as described above. After the centrifugation, the cell pellet was resuspended in 500μl of growth medium and transferred into a 2ml freezing tube containing 500μl of FBS and 200μl DMSO. The tubes were put inside an isopropanol filled freezing pot (Nalgene, USA). The pot was put into a -80 °C freezer so that the cell stock was frozen at a constant rate of nearly 1 °C / min. The frozen vials were stocked into liquid nitrogen for permanent storage.

2.2.3 Cell recovery from liquid nitrogen stock

The cell stock was taken out from the liquid nitrogen tank and thawed at once in a 37°C water bath. The cells were plated on a 60mm dish or a 25cm^2 culture flask with appropriate fresh growth medium. The cells were incubated at 37°C, 5% carbon dioxide. Due to the toxicity of DMSO, fresh medium was replaced after 6 hours incubation.

2.3 TRITIATED WATER RELEASE ASSAY

The aromatase enzyme activity was measured based on the tritiated water release assay, which measured the amount of tritiated water produced during the conversion of androstenedione to estrone by aromatase. As shown in the Figure 2.1, three molecules of NADPH and oxygen were required for each estrogen formed during the process. The final hydroxylation resulted in a loss of 1β-^{3}H of the androstenedione into the aqueous phase of the reaction. Then, it generated the tritiated water. The conversion rate determined by the isolation and the quantification of tritiated water represented the aromatase activity (Lephart & Simpson, 1991).

2.3.1 Aromatase Activity in Intact Cell

Cellular aromatase assays were performed as previously described (Ciolino et al., 2000). In short, cells were seeded in 6-well plates at a density of 5 x 10^5 per well for 24 hours. Tested compounds were administered with appropriate time points. The substrate [1β-^{3}H(N)]-androst-4-ene-3,17-dione (23.5 Ci/mmol, Perkin Elmer, Boston, MA, USA) in 1ml of serum-free medium after cells were rinsed with PBS. The final concentration of substrate was controlled at 25ηM. The reaction was incubated for 1 day at 37°C. Similar protocol was applied to assays performed on SK-BR-3, MCF-7aro and MCF-7$_{vec}$ cells, except that they were incubated with the substrate for 3 hours.

The medium was then removed and mixed with 1ml of chloroform. The mixture was then centrifuged at 10,000 x g at 4°C for 10 minutes to remove the unreacted substrate. 500μl of aqueous phase, which containing the tritiated water, was removed into a new eppendorf containing 500μl of 5% activated charcoal suspension. After 30 minutes incubation at room temperature, it was centrifuged at 15, 000 x g for 15 minutes. 625μl

of the supernatant fraction was transferred into vial with 2.5ml scnillation cocktail (Perkin Elmer) for the counting (Beckman, Fullerton, CA, USA).

On the other hand, the protein content of the cells was determined by using BCA kit after dissolving the cells in 300μl of 0.5mol/L NaOH. Results were expressed as radioactive counting recorded per hour per mg protein.

2.3.2 Aromatase assay on recombinant supersomes

In addition to the "in-cell" assay, aromatase assay was also performed on recombinant aromatase expressed in insect microsomes (human CYP19 Supersomes®, Gentest Corp, Woburn, MA, USA). Similar experimental procedures were applied except that the cells were replaced by the recombinant protein (Ciolino *et al.*, 2000). The assay performed in a total volume of 250μl with the following additions: 25ηM [1β-^{3}H(N)]-androst-4-ene-3,17-dione, 2ρmol Supersomes®, 3.3mM $MgCl_2$, 100mM KH_2PO_4 (pH 7.4), 2.5μl of testing compounds and 1.3mM NADPH. The reaction was initiated by adding NADPH and incubated at 37°C for 15 minutes. The reaction was stopped by adding 1.4ml of chloroform and 0.35ml 0.9% NaCl. After vortexing and centrifugation at 10,000 x g for 10 minutes, 400μl of the aqueous phase was removed and mixed with 400μl 5% activated charcoal in a new eppendorf tube. The charcoal-contacting aqueous extract was then vortex and incubated for 30 minutes at room temperature. The tubes were centrifuged at 15, 000 x g at at 4°C for 15 minutes. 625μl of the supernatant fraction was transferred into vial with 2.5ml scnillation cocktail for the counting.

2.4 RNA ISOLATION AND cDNA SYNTHESIS

Total RNA was isolated from cells grown in 12-well plates by TRIzol Reagent as directed by manufactures (Invitrogen Corp., California, USA). The concentration and purity of RNA were determined by spectrophotometry at 260/280nm. The integrity of RNA was assessed by agarose gel.

3μg of total RNA was denatured at 70°C for 5 minutes with 0.025μg/μl oligo-dT (Invitrogen). The samples were chilled on ice quickly. Complementary DNA (cDNA) was synthesized by using 100 units M-MLV Reverse Transcriptase (USB Corporation), 1X M-MLV reaction buffer and 0.5mM dNTP mixture in a total volume of 20ul. The contents was mixed and incubated at 37°C for 60 minutes and consequently inactivated at 70°C for 10 minutes. The cDNA generated was used as a template in PCR and real-time PCR.

2.5 SEMI-QUANTITATIVE PCR REACTION

The reaction mixture was set up as follows:

Sterile dH_2O	(16-x) μl
10X PCR reaction buffer with 50mM $MgCl_2$	2μl
10mM dNTP	0.4μl
10μM Forward primer	0.4μl
10μM Reverse primer	0.4μl
Template cDNA	x μl
Taq DNA polymerase (5U/μl)	0.2μl
Total	20μl

The primer sequences are listed in Table 2.1. 20 PCR cycles were performed for β actin and 40 cycles for aromatase exon I of amplification were performed with a denaturation temperature 94°C for 30 second. The annealing temperatures for β actin and aromatase exons were 60°C and 55°C respectively, followed by extension temperature of 72°C for 30 seconds. When the reaction was completed, the reaction mixture was analyzed by 1.5% agarose gel electrophoresis.

A

Gene and oligonucleotide	Sequence
β actin forward	TCA CCC ACA CTG TGC CCA TGT ACG A
β actin reverse	CAG CGG AAC CGC TCA TTG CCA ATG G
CYP 19 reverse (common antisense primer)	CTG ACA GAG CTT TCA TAA AGA AGG G

B

Gene and oligonucleotide	Sequence
I.1 forward	TGT GCT CGG GAT CTT CCA GAC
I.2 forward	TTC CAT TTC AGA TAT TCC CA
I.2 reverse	ATC CAT GGC TTG CTT GA
I.3 forward	GGG CTT CCT TGT TTT GAC TTG TAA
I.4 forward	AAC GTG ACC AAC TGG AGC CTG
I.5 forward	TTT GGA CAG TGG GCA CAG AG
I.6 forward	AGA CTA CCT ACC ATC CCT GAA A
I.7 forward	GGC TCC ATC TAC AAG GAT GA
I.f forward	TTA TAA AAG ATG GCA CAC GAA
2a forward	TGA AAT TCA GCC TGT GGA TT
II forward	CTC TGA AGC AAC AGG AGC TAT AGA T

Table 2.1 Primer sequences for (A) β actin and CYP19; (B) CYP19 multiple exon I

2.6 Quantitative Real Time PCR Using Taqman Probe

The target mRNA levels were determined by real-time PCR using the OpticonTM 2 System (MJ research, Waltham, MA, USA). The primers and FAM-labelled probes of CYP19, four CYP19 exons and internal control GADPH were all purchased from Applied Biosystems (Table 2.2).

The reaction mixture consisted of 10μl Taqman Universal PCR Master Mix (EastWin), 1ul FAM-labelled probe and 2μl cDNA in a final volume of 20μl. The thermocycling profile was 50°C for 2 minutes, 95°C for 10 minutes and 45 cycles of 95°C for 15 second and 60°C for 1 minute. The target and reference genes were amplified in separate wells. All samples were run in triplicate.

The gene expression of samples relative to control is determined by $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). The mean fold change in expression of target gene compared with control was calculated with the equation:

Relative target gene expression = $2^{-\Delta\Delta CT}$

where $\Delta\Delta CT = (CT_{target} - CT_{\text{house keeping}})_{sample} - (CT_{target} - CT_{\text{house keeping}})_{control}$

CYP19	Assay No	CYP19-F165
	Forward primer	GGAGAATTCATGCGAGTCTGGAT
	Reverse primer	GGAACATACTTGAGGACTTGCTGAT
	Reporter	TCTGGAGAGGAAACACTC
Exon I.1	Assay No	EXONIA.1-JUN
	Forward primer	CTGTGCTCGGGATCTTCCA
	Reverse primer	CATCTTGTGTTCCTTGACCTCAGA
	Reporter	ACGTCGCGACTCTAAAT
Exon I.3	Assay No	EXONI3-J68
	Forward primer	AAATTAGTCTTGCCTAAATGTCTGATCACA
	Reverse primer	CCAAAACCATCTTGTGTTCCTTGAC
	Reporter	TTATAAAACAGACTCTAAATTGCC
Exon I.4	Assay No	EXONI4-J60
	Forward primer	GTCCCTGGCACTGGTCAG
	Reverse primer	CATCTTGTGTTCCTTGACCTCAGA
	Reporter	CCCATCAAACCAGGACTC
Exon II	Assay No	EXONII-J79
	Forward primer	GCAACAGGAGCTATAGATGAACCTT
	Reverse primer	CATCTTGTGTTCCTTGACCTCAGA
	Reporter	CCACAGGACTCTAAATTG
Exon 2a	Assay No	EXONIA-J212
	Forward primer	CCGCACACACAAAGCAACATTT
	Reverse primer	CATCTTGTGTTCCTTGACCTCAGA
	Reporter	CCTGTGGACTCTAAATTG

Table 2.2 The forward primer, reverse primer and reporter sequences of CYP19 Taqman probes (Applied Biosystems).

2.7 WESTERN BLOTTING

5 x 10^5 cells per well in a 6-well plate were seeded in RPMI 1640medium containing 5% charcoal/dextran treated FBS. After transfection of 0.7μg of expression vectors (pcDNA3.1 or pcDNA3.1- ERα) and/ or drug treatment for 2 days, the cells were harvested. Cells were rinsed with PBS and lysed by 50μl RIPA lysis buffer (25mM Tris-HCl pH 8.8, 50mM NaCl, 0.5% NP40, 0.5% Deoxycholate, 0.1% SDS). Then, the cells were scraped off with a cell disrupter. The lysates were then sonicated by a cell disruptor (Branson Ultrasonics Corp., Danbury, CT, USA) on ice for 10 seconds. It was then centrifuged at 4°C for 5 minutes. The supernatants were transferred into a new 1.5ml eppendorf for immediately use or storing at -80°C for long term storage.

Protein concentration of the supernatant was determined by Bicinochonic Acid Assay with bovine serum albumin (BSA) as standard. 50ug of sample protein was separated on 12.5% SDS-PAGE and transferred to an Immobilon PVDF membrane (Millipore, Bedford, MA, USA) for 90 minutes at 15V. The membrane was incubated in 5% non-fat milk powder in PBS for at least one hour at room temperature. Then, it was incubated in primary antibody overnight at 4°C. The membrane was washed three times with TBS/T. Then, the membrane was incubated with secondary antibodies conjugated with horseradish (Armoracia rusticana) peroxidase (Santa Cruz Biotechnology, Inc.) for at least one hour at room temperature. The Westsave Up™ (Labfrontier, Ewha University, Seoul, Korea) provided the chemi-luminence substrate for horseradish peroxidase. The targeted protein was visualized by exposure to X-ray film (Kodak, Rochester, NY, USA). β-actin was used for protein normalization.

Primary Antibody	Working dilution	Purchased by
β actin	1:5000	Sigma Chemical Co
Aromatase	1:400	Abcam, Cambridge, UK
pERK 1/2	1:250	Santa Cruz Biotechnology, Inc.
ERK 1/2	1:1000	Zymed Laboratories, South San Fransicso

2.8 MEASUREMENT OF PROMOTER ACTIVITY

2.8.1 Plasmid Preparation

The human ERα expression vector (pcDNA3.1-ERα), reporter plasmids for apolipoprotein A1 promoter, aromatase promoter I.1 and I.3/II were made in our lab by other postgraduate student. The pGL-3 basic reporter vector and AP-1 luciferase reporter plasmid were purchased from Promega and Clontech (Palo Alto, California, USA) respectively. ERE-luciferase reporter plasmid C_3-LUC was a gift from Dr. Donald McDonnell (Duke University, NC, USA).

2.8.2 Transient Transfection and Dual Luciferase Assay

1 x 10^5 cells were seeded in 24 well plate in RPMI 1640medium containing 5% charcoal/dextran treated FBS. In MCF-7, 0.2ug reporter plasmid (containing DNA fragments derived from aromatase promoter region) were pre-complexed with 2.5μl PLUS reagent and diluted into 25μl serum-free medium. The Renilla luciferase vector pRL-CMV (Promega) was cotransfected as an internal correction for transfection efficiency. Similar components are found in the transfection of HepG2 cells, except 0.2μg expression vectors (pcDNA3.1 or pcDNA3.1- ERα) and 1.5μl PLUS reagent were added in the reaction. The mixture was incubated at room temperature for 15 minutes. In a second tube, 1μl of LIPOFECTAMINE Reagent was diluted into 25μl medium without serum. It was mixed and incubated at room temperature for further 15 minutes. The cells were replaced with 200μl erum free RPMI 1640 medium. The DNAs-PLUS-LIOPFECTAMINE Reagent complexes were added into each well medium. After 4 hours incubation, cells were cultured in RPMI 1640medium containing 5% charcoal/dextran treated FBS. After one day, various concentration of testing compounds were added and incubated for 48 hours. The cells were lysed in 100μl lysis buffer (Promega). The cell lysates were stored at -80°C until assay. Dual Luciferase assays were performed according to manufacturer's instruction (Promega). By using a FLUOstar Galaxy plate reader (BMG Labtechnologies, Offenburg, Germany), the luciferase activity was read and expressed as relative light units of firefly/renilla.

2.9 STATISTICAL METHODS

A Prism® 3.0 (GraphPad Software, Inc., CA, USA) software package was utilized for statistical analysis. The results, whenever applicable, were analyzed by two-tailed Student's t-test to determine if significant ($P<0.05$) difference observed.

The results were expressed as means ±SEM.

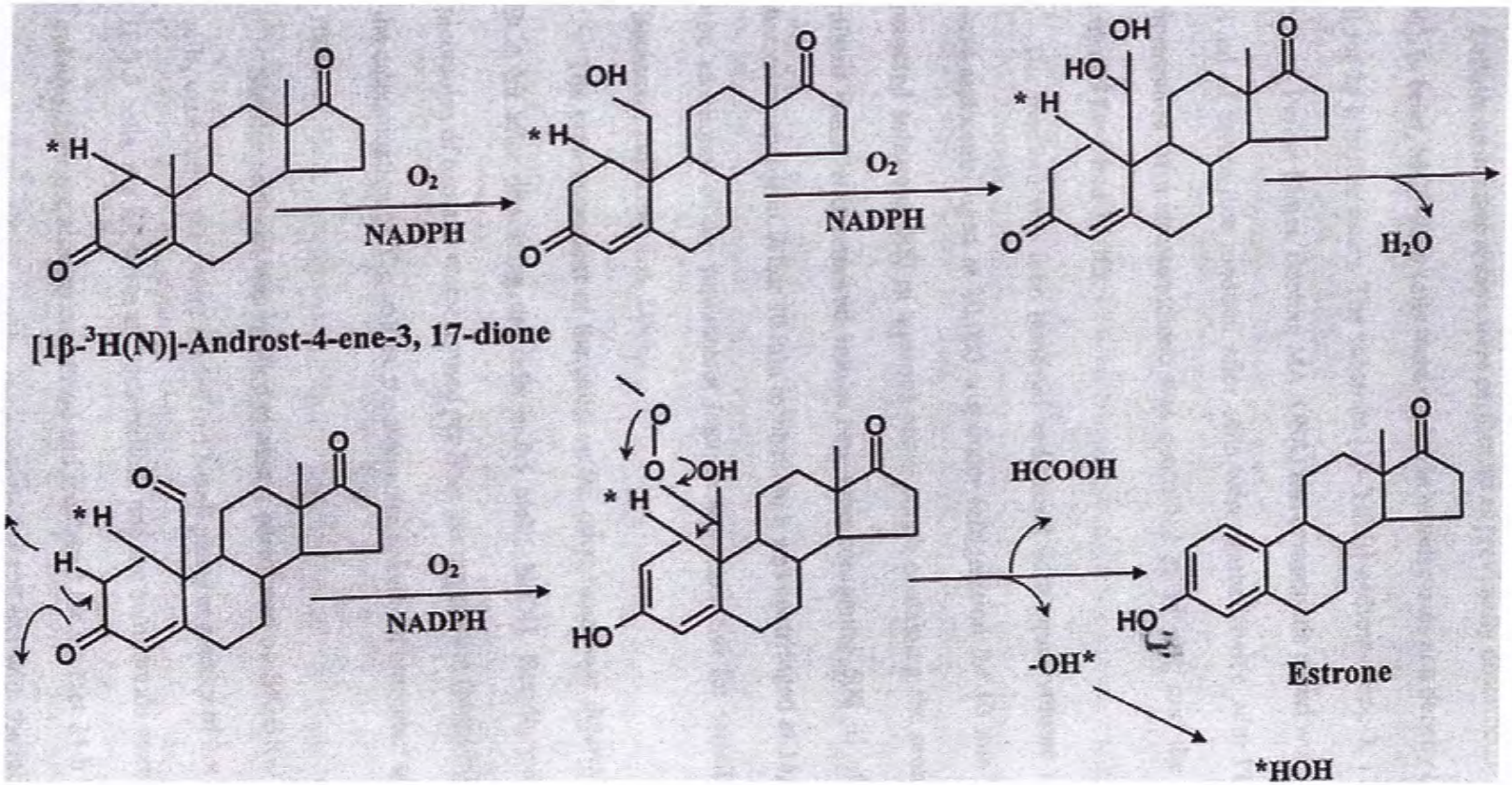

Figure 2.1 Aromatase reaction sequence shows the principle of tritated water release assay. During the reaction, the tritium atom in the 1β position is incorporated into water. Adopted from Lephart & Simpson (1991).

CHAPTER 3

GENISTEIN UP-REGULATES AROMATASE IN ESTROGEN RECEPTOR ALPHA-TRANSFECTED HEPG2 CELLS

3.1 INTRODUCTION

3.1.1 Cardiovascular diseases (CVD)

Diseases of the heart and blood vessel are collectively known as cardiovascular disease (CVD). CVD has been a leading cause of global morbidity and mortality and is responsible for one-in-three deaths. Recent projections suggest that CVD will be the leading cause of death in both developed and developing regions of the world by 2020 (Murray and Lopez, 1996). From 1981 to 2006, diseases of the heart were the second killer disease in Hong Kong (Department of Health, 2007). Epidemiologic studies have connected the consumption of an isoflavonoids-rich diet with a lower incidence of CVD (Adlercreutz, 2002). In normal postmenopausal women, dietary inclusion of whole soy foods containing 60 mg/d of isoflavones results in reductions in several key clinical risk factors for CVD (Scheiber *et al.,* 2001). Japanese men who consume large amounts of soy have nearly one-six the risk of CVD as their counterparts in America (Beaglehole, 1990). Other research group has also illustrated the inverse association between soy product intake and heart disease in Japan (Nagata, 2000).

3.1.2 Phytoestrogen

Phytoestrogens are widely studied in the mechanism in the prevention of cancers, heart disease, menopausal symptoms and osteoporosis (Setchell, 1998; Adlercreutz, 2002; Kronenberg and Fugh-Berman, 2002). High intake of dietary phytoestrogen may account for the lower rate of CVD in Asia than that in other Western country (Tikkanen and Adlercreutz, 2000; Tham *et al.,* 1998). Phytoestrogens are plant-derived chemicals, which have chemical structures similar to the mammalian estrogen 17β-estradiol (E_2) (Price and Fenwick, 1985; Knight and Eden, 1996; Mazur, 1998). They can be classified according to their differences in structure and functional group. One of the major classes of phytoestrogen is isoflavone, which has been the focus of many *in vitro*, *in vivo* and clinical research studies in relation to their health effects (Kingsbury, 1969).

The aromatic ring and the hydroxyl group of phytoestrogens are important for the binding to the estrogen receptors (ER) (Anstead *et al.,* 1997). The A and C rings of the isoflavones are similar to the A and H rings of estrogen (Figure 3.1.1). The phytoestrogens trigger both anti-estrogenic and estrogenic activities in the body. As estrogen antagonists, they may block or alter the ER binding and thus abolish estrogenic activity. On the other hand, they can mimic the effect of endogenous estrogen (Brzezinski and Debi, 1999). Based on their binding affinities to ER, the estrogenic activities of phytoestrogens are around 1/500 to 1/1000 of that of estrogen (Jefferson *et al.,* 2002; Joung *et al.,* 2003). Due to the slight structural differences among various phytoestrogens, the binding affinities towards isoforms of ER are also different. Among the soy isoflavones, genistein has the greatest binding affinitiy to ERα (Latonnelle *et al.,* 2002).

In this study, genistein, which is a major isoflavone isolated from soy bean, was investigated (Cheng E *et al.*, 1953). Soy beans have been reported in ancient Chinese herbals for the healthy functioning of the heart, kidneys, liver and stomach (Duke and Ayensu, 1985). Its structure is shown in Figure 3.1.1 C. Genistein is found as its glycosides in plants (Ibarreta *et al.,* 2001). They are readily degraded by gut enzymes to form aglycone and absorbed to the bloodstream. The plasma level of genistein in individuals consuming a high soy-containing diet was estimated as to be 1-4μmol/l (Adlercreutz *et al.*, 1995), or 0.1μmol/l in vegetarian women (Adlercreutz *et al.*, 1993). Though there has not been an extensive examination, the concentration of soy isoflavone in our body fluids is likely in the micromolar range.

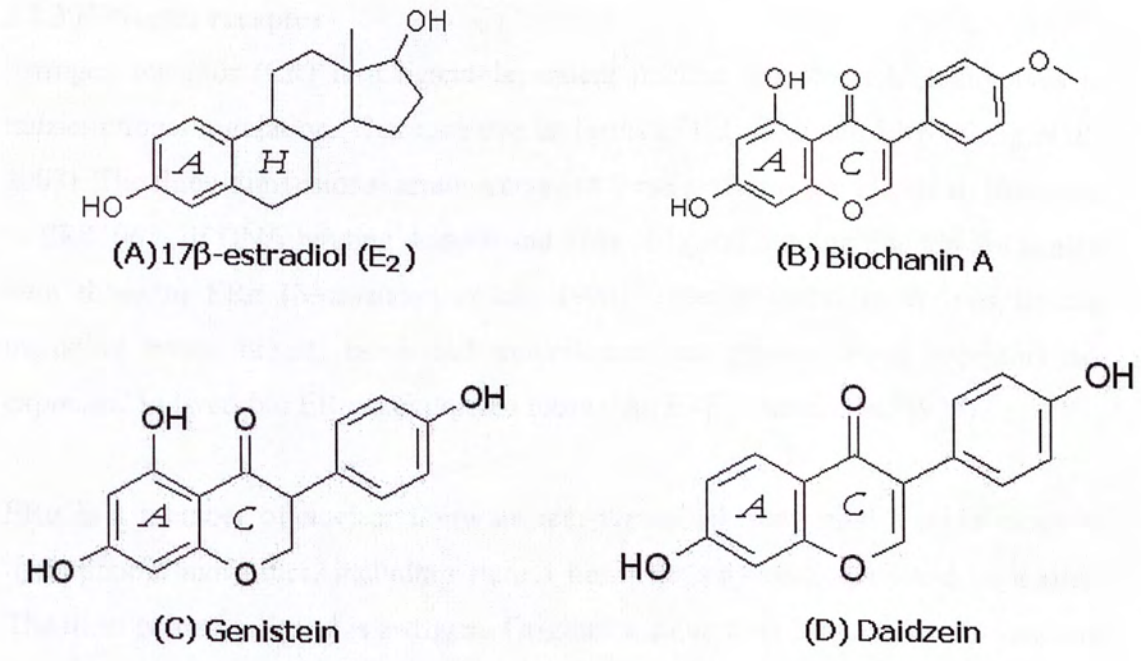

Figure 3.1.1 Structure of 17β-estradiol and isoflavones

3.1.3 Estrogen receptor

Estrogen receptor (ER) is a ligand-dependent nuclear receptor which involves in transcriptional regulation. There are two isoforms of ER: ERα and ERβ (Kong *et al.*, 2003). The three-dimensional arrangements of these isoforms are identical. However, in ERβ, 96% of DNA binding domain and 58% of ligand binding domain are similar with those in ERα (Mosselman *et al.*, 1996). ERα is found in various tissues, including bone, breast, heart and central nervous system. Both receptors are expressed in liver, but ERα is expressed more than ERβ (Gustafsson, 1999).

ERα is a member of nuclear hormone receptor which may bind a wide range of hydrophobic molecules, including steroid hormone, phytoestrogens and tamoxifen. The most common ligand is estrogen. Originally, more than 80% of ERα is localized in the nucleus. Upon estrogen stimulation, ERα is increasingly localized in the nucleus (Ylikomi *et al.*, 1992). The activation of ER depends on the ligand, dosage and tissue type. Raloxifene has shown an antiestrogenic effect in breast cancer tissue and the brain. On the other hand, it shows estrogen-like actions in bone and cardiovascular system.

Before being activated, heat shock proteins in dimeric forms block the DNA-binding domain of ERα so as to prevent the association of the receptor with DNA. Once the ligands bind to C-terminal domain of ERα, there is a conformational change of the receptor. The heat shock proteins would be released. The activated ERα, which is in its dimerized state, is translocated through nuclear pore into nucleus. After binding to estrogen response element (ERE), transcriptional coactivators (SRC-1) and cointegrators (p300/CBP) also interact with the complex. Transcription is activated and mRNA encoded by the gene is expressed (Parker *et al.*, 1993; Klinge, 2001).

ER can also activate gene transcription indirectly with promoter specific elements (Pettersson and Gustafsson, 2001). In some genes, estrogen has been shown to promote transcription through AP-1, a binding site for *Fos* and *Jun* (Webb *et al.*, 1995). In addition, ERα interacts with Sp1 and binds to Sp1 sites in LDLR promoter to enhance the transcription and translation of LDLR (Yien *et al.*, 1995). The interaction between ERα and Sp1 does not require the DNA-binding domain of ERα.

ER can also regulate gene expression in a ligand-independent manner by modulating several secondary signaling pathways. ICI 182 780, an estrogen antagonist, can abolish the 17β-estradiol-stimulated c-fos expression through the MAPK pathway (Hennessy *et al.,* 2005). Some non-genomic pathways can be activated by MAP kinase and PKA (Klinge *et al.,* 2005; Katzenellenbogen, 1996; Weigel and Zhang, 1997). Hence, the latter mechanisms of ER action enable a broader range of genes to be regulated other than the classical ER action.

3.1.4 Protective mechanism against CVD protective

Reducing platelet aggregation, inhibiting expression of tissue factor gene and lowering serum cholesterol would help to protect our bodies from getting CVD. High density lipoprotein (HDL) cholesterol lowers the serum cholesterol by reverse cholesterol transport (Mayne and Mayne, 1994; Fielding and Fielding, 1995; Breslow, 1995). Low density lipoprotein (LDL) cholesterol accounts for about 70% of the total cholesterol in plasma. It is regarded as bad cholesterol as it increases the serum cholesterol (Mayne and Mayne, 1994). Hence, increased HDL cholesterol and decreased LDL cholesterol are believed to lower the CVD risk. Serum LDL-cholesterol and HDL-cholesterol can be regulated by LDL receptor (LDLR) and apolipoprotein A-I (apoA1) respectively (Cortese *et al.,* 1983; Zabalawi *et al.*, 2003).

LDL is taken up by specific LDL receptors (LDLR), which are most abundant in the liver cell surface. LDLR recognizes apoB and apoE on LDL. The receptor-LDL complex would be internalized by endocytosis. The LDL particles would be broken down by lysosome after entering the cells. LDLR would return to plasma membrane and bind to another LDL. Hence, increasing the rate of LDLR synthesis would reduce the amount of intracellular cholesterol.

ApoA1 is the major protein of HDL. ApoA1 plays an important role in the reverse cholesterol transport pathway by activating LCAT for cholesterol esterification. It also interacts with ATP binding cassette transporter A1 cell membrane receptor and thus promotes cell cholesterol efflux. It carries the excess esterified cholesterol back to the liver for excretion (Rader, 2002).

3.1.5 Effects of genistein on LDL Receptor and Apolipoprotein A-I

Many research groups have studied the relationship between phytoestrogens and LDLR. Dietary isoflavones reduce plasma cholesterol and atherosclerosis in C57BL/6 mice but not LDL receptor-deficient mice (Kirk *et al.*, 1998). Soy protein with intact isoflavones has shown to increase serum HDL concentration (Sanders *et al.*, 2002).

Our group has investigated the effect of genistein on LDLR and apoA1. Similar to previous findings (Borradaile et al., 2002; Lamon-Fava *et al.*, 2004; Lamon-Fava, 2000), genistein was shown to up-regulate LDLR and ApoA1 expression through an ERα-dependent transcriptional control in HepG2 cells (Yuen, 2005). Lamon-Fava's group (2000, 2004) have suggested that the increase of apoA1 gene expression is probably not through the classical ER/ligand genomic activation. It may be due to the binding of transcriptional factors to -256 to -41 region of the apoA1 promoter. However, the underlying mechanism has not been fully elucidated.

3.1.6 Effects of estradiol on LDL Receptor and Apolipoprotein A-I

It is widely accepted that estrogen is critical in the prevention of CVD. According to a recent statistical figure from American Heart Association (2004), males have a higher rate of prevalence of CVD than females. Postmenopausal women have 2 times higher CVD incidence rate than premenopausal women at the same age in another study (Kannel *et al.*, 1976). The serum LDL-cholesterol levels decrease after treatment of aromatase inhibitor in men (Bagatell et al., 1994).

17β-estradiol (E_2) is the major form of estrogen, and has been shown to up-regulate apoA1 and LDLR in HepG2 cells. ApoA1 promoter transcription can be induced by 4-fold in the presence of 10μM E2 in HepG2 cells (Lamon-Fava *et al.*, 1999). Previously, our group has demonstrated that ERα is important in the gene regulation of LDLR and apoA1 under the physiological level of E2 in HepG2 cells (Yuen, 2005). Incubation of HepG2 with E_2 increases cell surface LDLR activity significantly *in vitro* (Semenkovich and Ostlund, 1987; Owen *et al.*, 2004). The mechanism could be upregulated through ERα (Bruning *et al.*, 2003) and tyrosine kinase (Distefano *et al.*, 2002) in these cells.

3.1.7 Aim of study and hypothesis

Though the interaction between genistein and LDLR or apoAI has been widely studied, the mechanism has not been fully elucidated. A schematic representation of the effect of genistein is shown in Figure 3.1.2. Genistein and estradiol were shown to increase LDLR and ApoA1 expression in HepG2 cells (Yuen, 2005; Lamon-Fava *et al.*, 1999). On the other hand, aromatase is a critical enzyme to convert androgen into estrogen in the body. Many researches have studied the effect of genistein on aromatase activity in other tissues, especially breast cancer tissue. 10μM genistein is found to inhibit aromatase in MCF-7 cells (Brooks and Thompson, 2005). Hence, this study investigated the effect of genistein on aromatase activity and its underlying mechanism in the hepatic cells HepG2. If genistein could regulate aromatase in HepG2 cells, it might alter the amount of E_2, and consequently affected LDLR and apoA1 expression. The current objective was concentrated on the effect of ER/genistein on the transcriptional control of aromatase.

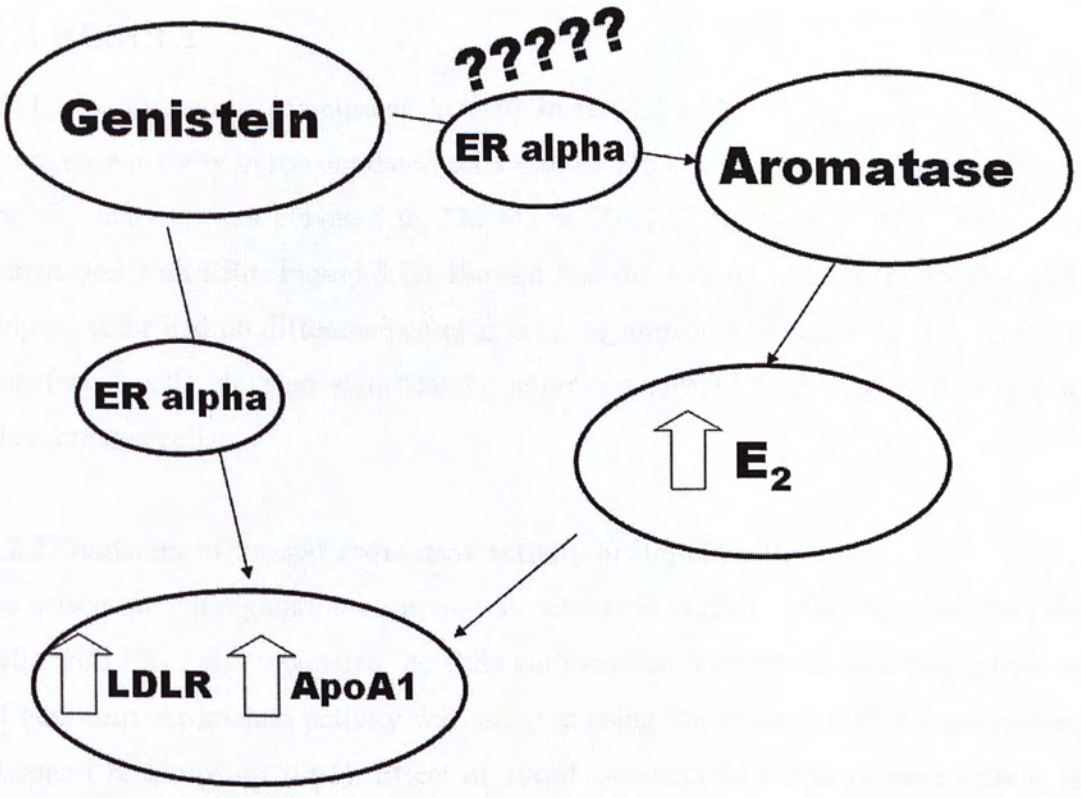

Figure 3.1.2 Schematic representation of the hypothesis on the estrogenic effect of genistein.

3.2 RESULT

3.2.1 ERα increased Aromatase Activity in HepG2 cells

Aromatase activity in the untreated cells was 10.315 ± 2.9628 cpm/ μg protein/ hour, and the activity was elevated to 386.445 ± 10.3733 cpm/ μg protein/ hour after transfected with ERα. Figure 3.2.1 showed that the activity in cells transfected with empty vector had no difference compared to the untreated one. The activity in ERα-transfected cells elevated significantly, approximately 37 times higher than that in the untreated cells.

3.2.2 Genistein increased aromatase activity in HepG2 cells

To determine the regulation of aromatase activity in HepG2 cells, we transfected the cells with ERα, and incubated the cells with various time points and concentrations of genistein. Aromatase activity was assessed using the tritiated H_2O release method (Lephart & Simpson, 1991). Effect of 10μM genistein on the aromatase activity in HepG2 cells was investigated over a 48 hour period (Figure 3.2.2). 10μM genistein would increase the aromatase activity after more than 24 hours. In the presence of ERα, the aromatase activity was induced significantly, nearly 10 fold higher than that in the absence of ERα. Aromatase activity was significantly induced as early as 6 hours and reached a maximum at 48 hours after the addition of genistein. Hence, in the following experimental design, the cells would be treated with genistein for 48 hours.

Genistein could induce the aromatase activity in a dose-dependent manner in HepG2 cells (Figure 3.2.3). It induced the activity by 50% at 10μM and such induction could be abolished by estrogen antagonists ICI 182 780. In the presence of ERα, genistein could increase aromatase activity in a dose-dependent manner. Genistein induced the activity by 200% at 0.1μM while over 3-fold increase was observed at 10μM (Figure 3.2.4). Such induction could be suppressed by ICI 182 780 (Figure 3.2.5).

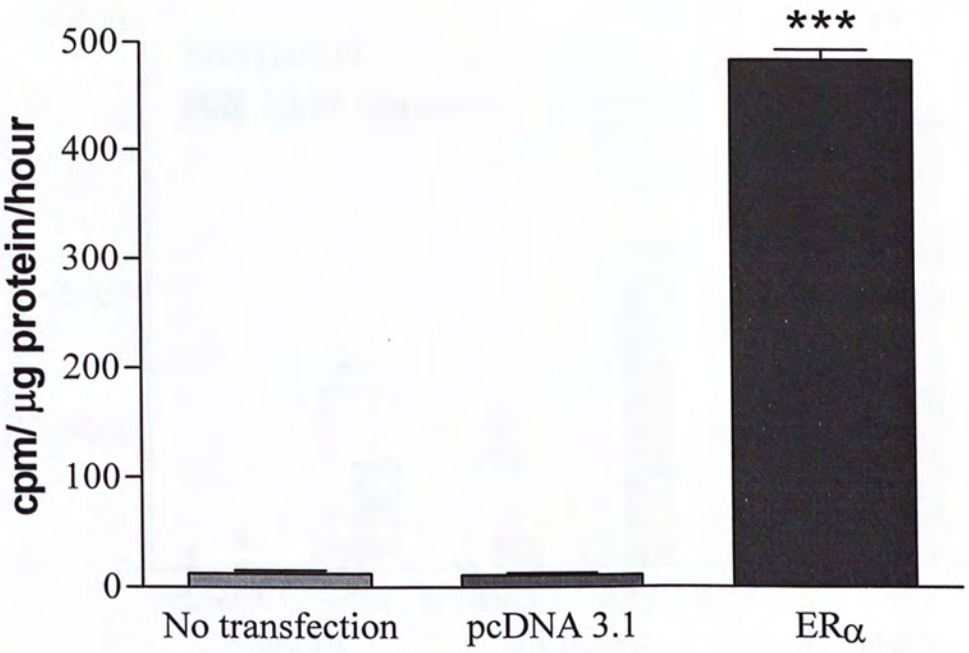

Figure 3.2.1 Aromatase activity in HepG2 cells. HepG2 cells were transfected with ERα expression vector and control plasmid pcDNA3.1. The cells were further incubated with 25ηM [1β-^{3}H(N)]-androst-4-ene-3,17-dione for 24 hours. Aromatase activity was determined by using tritiated water release assay. Values are means ±SEM, n=3. (***P<0.0005)

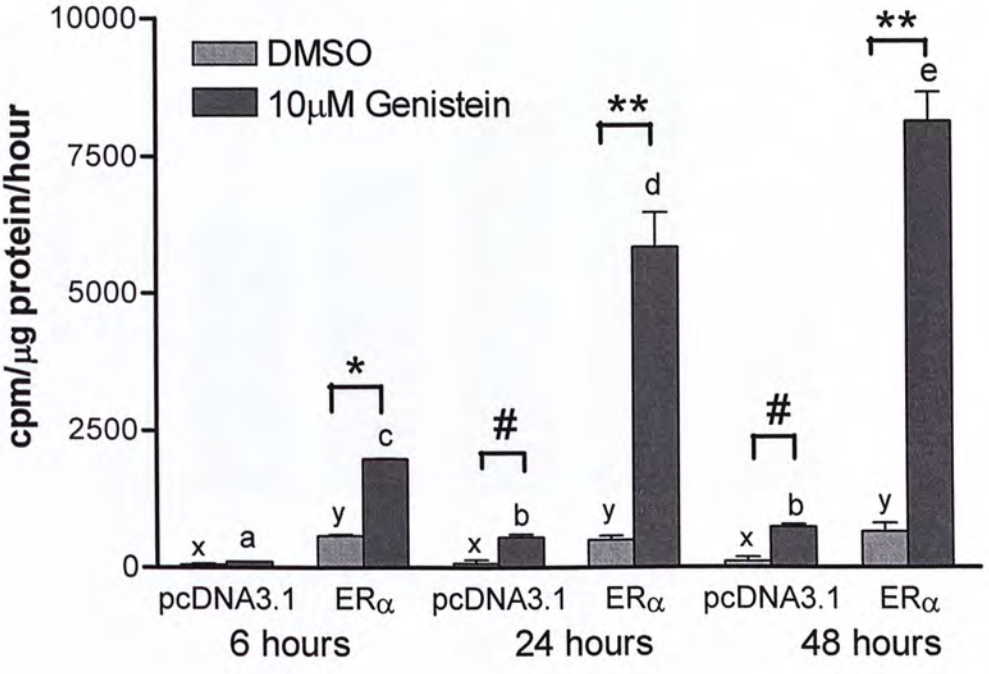

Figure 3.2.2 Time course of genistein stimulation of the aromatase activity in cultured HepG2 cells. Cells were transfected with ERα expression vector and control plasmid pcDNA3.1, and treated with 10μM genistein for 6, 24 and 48 hours. The cells were further incubated with 25ηM [1β-^{3}H(N)]-androst-4-ene-3,17-dione for 24 hours. Aromatase activity was determined by using tritiated water release assay. Values are means ±SEM, n=3. Symbols a to e are used for the 10μM genistein group and the order is e > d >c >b > a. Symbols x and y are used for the DMSO group and the order is y > x. (*/ # $P<0.05$; **$P<0.005$)

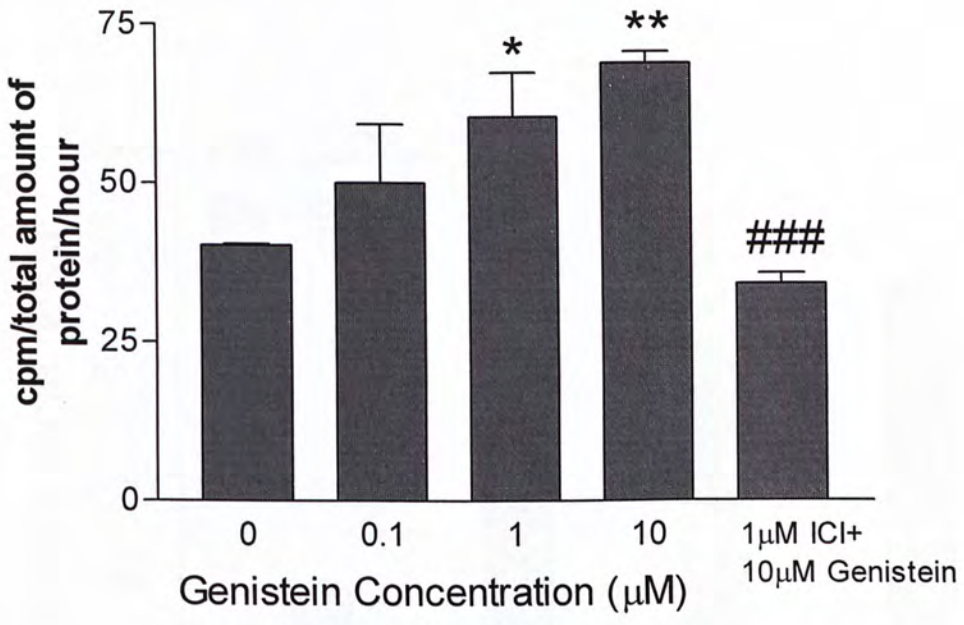

Figure 3.2.3 Dose-dependent elevation of aromatase activity induced by genistein in HepG2 cells. The cells were pretreated with 1μM ICI 182 780 for 3 hours before adding genistein for further 48 hours. The cells were further incubated with 25ηM [1β-^{3}H(N)]-androst-4-ene-3,17-dione for 24 hours. Aromatase activity was determined by using tritiated water release assay. Values are means ±SEM, n=3. * represents values different from control; # represents values different from 10μM genistein. (*P<0.05; **P<0.005; ###P<0.0005)

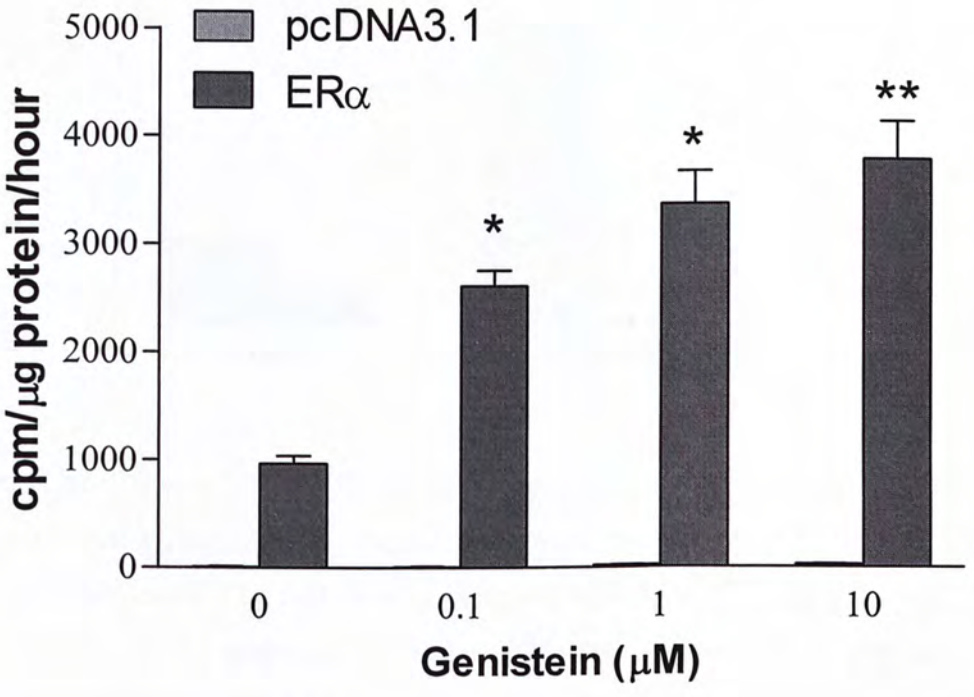

Figure 3.2.4 Dose-dependent elevation of aromatase activity induced by genistein in ERα-transfected HepG2 cells. Cells were transfected with ERα expression vector and control plasmid pcDNA3.1, and treated with 10μM genistein for 48 hour. The cells were further incubated with 25ηM [1β-^{3}H(N)]-androst-4-ene-3,17-dione for 24 hours. Aromatase activity was determined by using tritiated water release assay. Values are means ±SEM, n=3. (*P<0.05; **P<0.005)

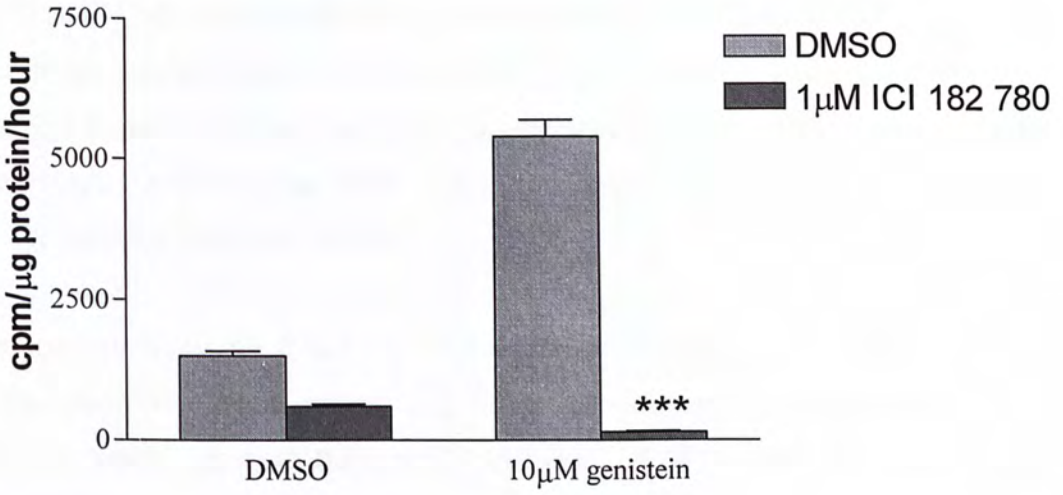

Figure 3.2.5 Effect of 1μM ICI 182 780 on genistein-induced aromatase activity in ERα transfected HepG2 cells. HepG2 cells were transfected with ERα expression vector for 24 hours. The cells were pretreated with 1μM ICI 182 780 for 3 hours before adding 10μM genistein for further 48 hours. The cells were incubated with [1β-^{3}H(N)]-androst-4-ene-3,17-dione for 1 day. Aromatase activity was determined by using tritiated water release assay. Values are means ±SEM, n=3. *** $P<0.0005$ vs. cells treated with 10μM genistein only.

3.2.3 Differential effect of MAP kinase inhibitors

U0126 and PD98059 are specific inhibitors of MAP kinase kinase (MEK). They both block phosphorylation and activation of ERK 1/2. A research group has shown that PD98059 would stimulate the ERE transcription, displaced radiolabeled estradiol from receptor and stimulate ERα- coactivator interactions (Long X. *et al.*, 2001). PD98059 exhibit estrogenic activity.

In the present study, we found out that U0126 and PD98059 exert differential effect on apolipoprotein A1 promoter activity in ERα-transfected HepG2 cells. 1μM PD98059 would increase the apoA1 promoter activity. 10μM PD98059 would double the promoter activity, nearly to the same extent as 1ηM estradiol (Figure 3.2.6 A). However, U0126 would not show any inductive in apoA1 promoter activity (Figure 3.2.6 B). At high concentration (10μM), the promoter activity was even suppressed. As PD98059 consists of a flavonoid backbone, it might interact with ER to increase the apoA1 promoter activity. To prevent confounding interpretation by the estrogenicity of PD98059, U0126 was chosen as the inhibitor of MAP kinase in this project.

3.2.4 Role of MAP Kinase, PKA and PKC in genistein induced aromatase activity in ERα-transfected HepG2 cells

To investigate the possible involvement of signal transduction pathway on aromatase activity, ERK (U0126), JNK (JNK inhibitor), p38 (SB203580), PKA (14,22-amide) and PKC (BI) inhibitors were used. None of the inhibitors tested, except SB203580, showed reduced aromatase activity in the presence of ERα (Figure 3.2.7 A). The genistein-induced aromatase activity could be suppressed by 1μg/μl SB203580 (Figure 3.2.7 B). This result suggested that genistein could up-regulate aromatase activity through p38 pathway.

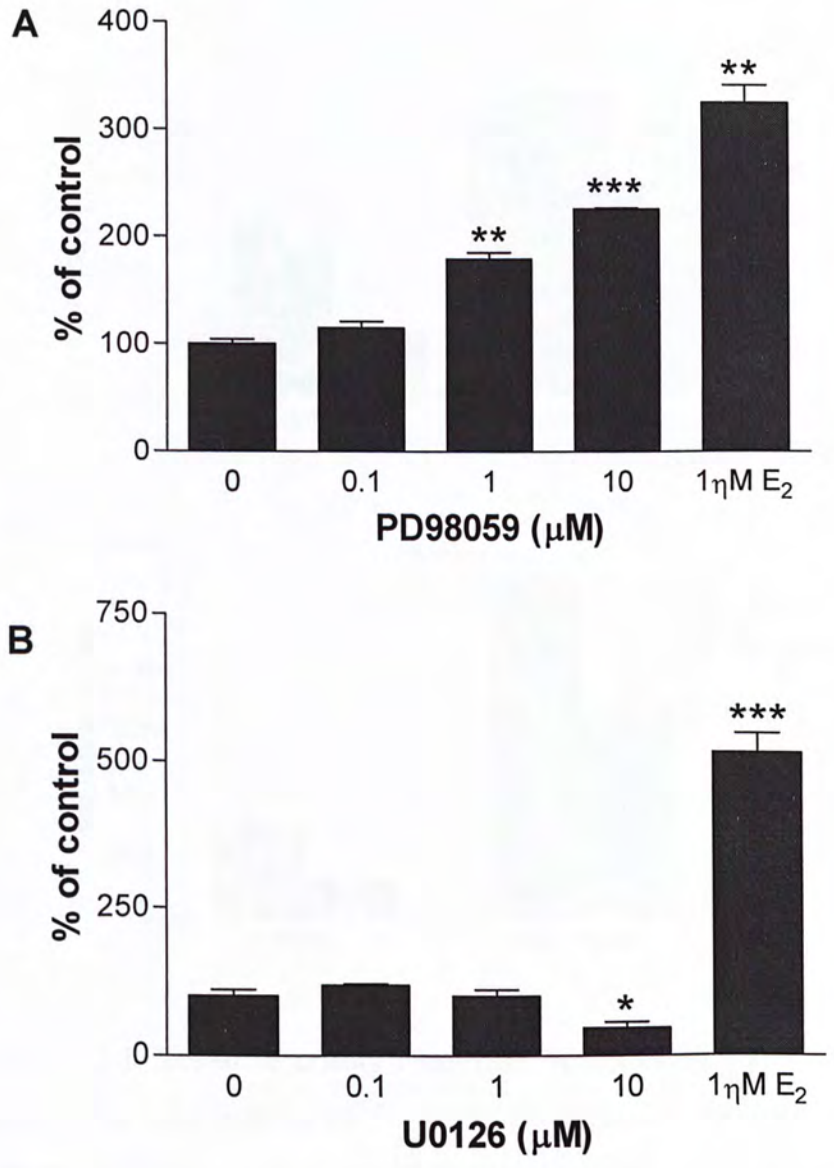

Figure 3.2.6 apoA1 promoter activity in ERα-transfected HepG2 cells, treated with (A) PD98059; (B) U0126. Cells were transfected with apoA1 reporter plasmid, PRL-CMV control vector and ERα expression plasmid. Then the cells were incubated with different concentration of (A) PD98059 and (B) U0126 for 1 day and dual-luciferase activity was measured. The relative luciferase activity was calculated by normalizing the light unit of firefly by that of renilla. Values are means ±SEM, n=3. (*P<0.05; **P<0.005; ***P<0.0005)

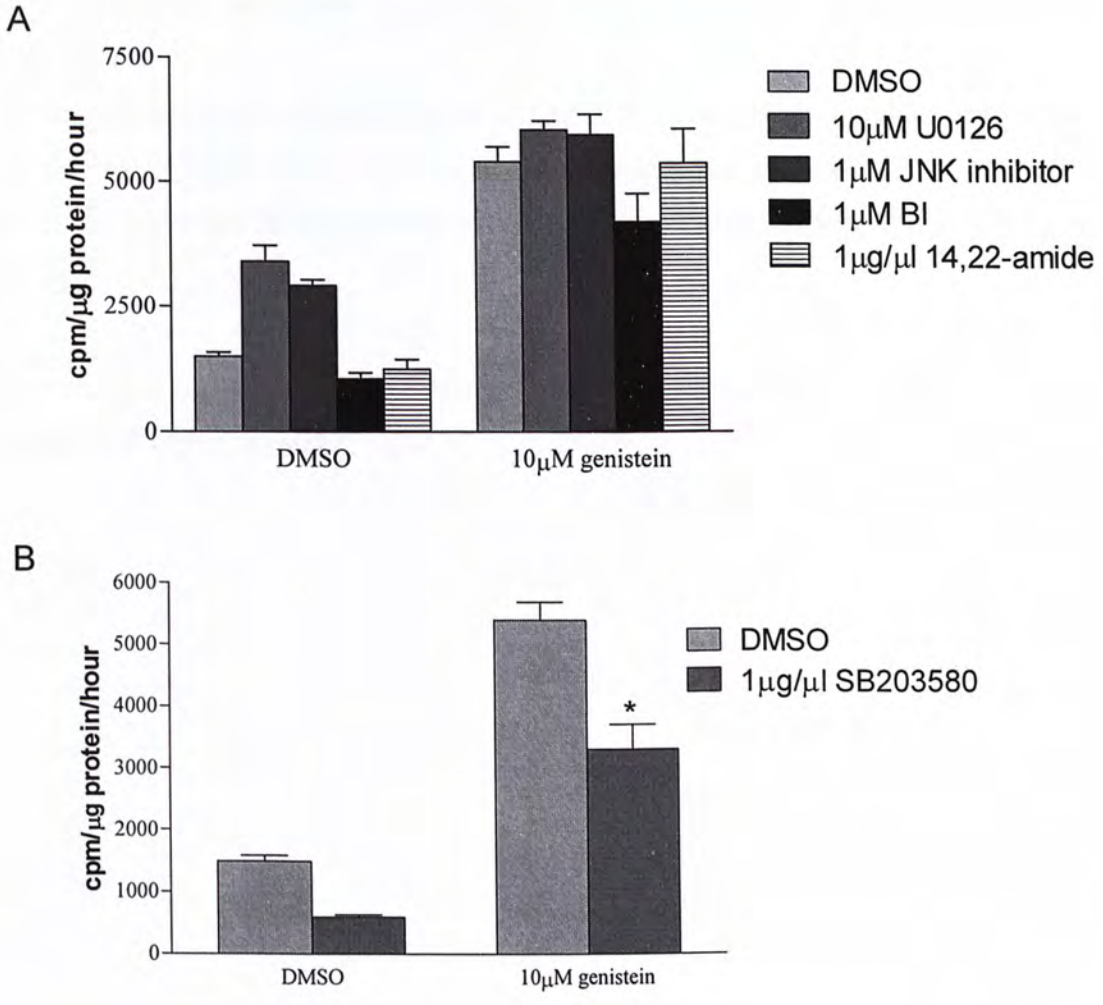

Fig 3.2.7 Effect of secondary messenger inhibitors on genistein-induced aromatase activity in ERα-transfected HepG2 cells. HepG2 cells were transfected with ER alpha expression vector for 24 hours. The cells were pretreated with different inhibitors for 3 hours before adding 10μM genistein for further 48 hours. The cells were further incubated with [1β-^{3}H(N)]-androst-4-ene-3,17-dione for 1 day. Aromatase activity was determined by using tritiated water release assay. Values are means ±SEM, n=3. (* $P < 0.05$)

3.2.5 Genistein increased aromatase protein expression in ERα-transfected HepG2 cells

To investigate the role of genistein on aromatase expression, western blot was carried out. It was found that ERα could induce aromatase expression in HepG2 cells. With genistein treatment, the aromatase protein expression was further elevated (Figure 3.2.8).

These results suggested that genistein could up-regulate aromatase protein expression through ER alpha in HepG2 cells.

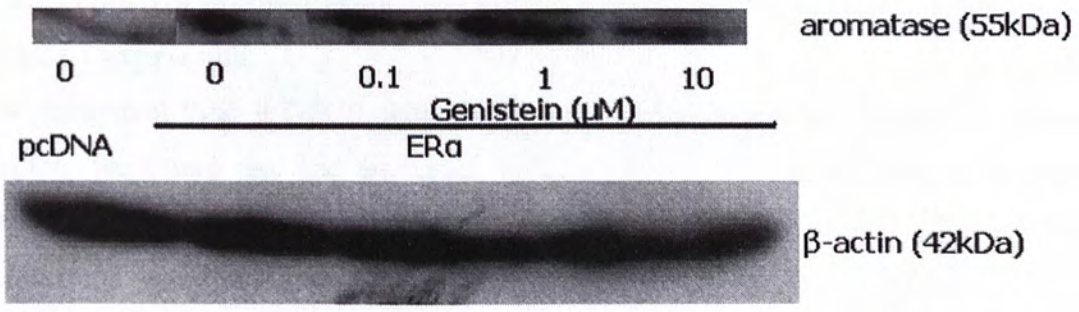

Figure 3.2.8 Detection of aromatase protein In HepG2 cells. HepG2 cells were transfected with ERα expression vector and incubated with genistein for 2 days. Total protein was collected and the amount of aromatase was detected using Western Blot. The molecular weight of aromatase and β-actin was 55kDa and 42kDa respectively.

3.2.6 Genistein induced aromatase mRNA expression attributed to induction of exon I.1 expression

By using real time RT-PCR with probes constructed within the aromatase coding region, we found that the aromatase mRNA expression was elevated in a dose-dependent manner in the presence of ERα (Figure 3.2.9). The aromatase mRNA expression was increased by about 3 times and 16 times with 0.1μM and 10μM genistein treatment, respectively. Slight inductions were also observed in high dosages of genistein in the absence of ERα.

The expression of aromatase is regulated by alternate splicing. To characterize the regulation of gene expression in HepG2, we first determined its promoter specificity by real time PCR using Taqman probing. In the absence and presence of ERα, the dominant usage was exon II. Only in the presence of ERα and 10μM genistein, the mRNA expression of exon I.1 was increased, while that of exon II decreased significantly (Figure 3.2.10). As mention in the general introduction, there are ten distinct tissue-specific promoters in the exons I of aromatase gene. To further confirm that the other promoters would not participate strongly in the regulation of gene expression in HepG2, we further determined its promoter specificity by RT-PCR using various sense primers located in exon I and a common antisense primer in coding region of aromatase gene. From figure 3.2.11, the PCR products corresponding to exon I.1 and II were relatively abundant in HepG2 cells, while there was no band detected in the other eight exons. The real time RT-PCR suggested that exons I.3 and PII were the two major mRNA species present in ERα positive or negative HepG2 cells. After the addition of genistein in ERα-transfected HepG2 cells, there was a switch of the regulatory mechanism of aromatase expression from exons I.3 and PII to exon Ia.

The aromatase activity and protein expression, induced by genistein in the presence of ERα was consistent with the abundance of mRNA, which was mainly driven by promoter I.1.

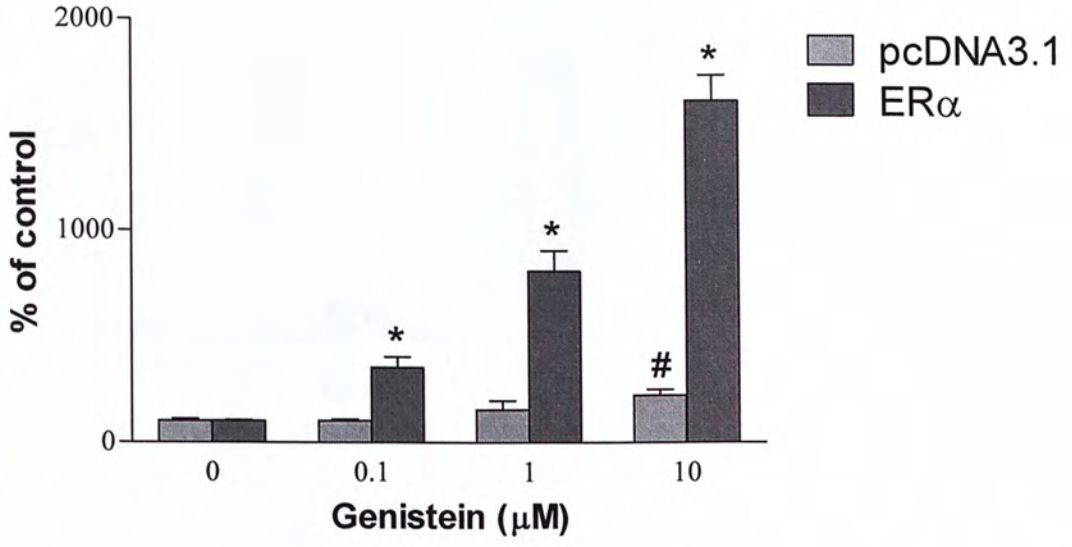

Figure 3.2.9 Effect of genistein on CYP19 mRNA expression in HepG2 cells. HepG2 cells were transfected with ERα expression vector and control plasmid pcDNA3.1, and treated with 10μM genistein for 48 hour. The amount of CYP19 mRNA was determined by relative quantitative real time PCR. The expression of CYP19 mRNA was normalized by GAPDH. Values are means ±SEM, n=3. * or # represents values different from control. (*/# $P < 0.05$)

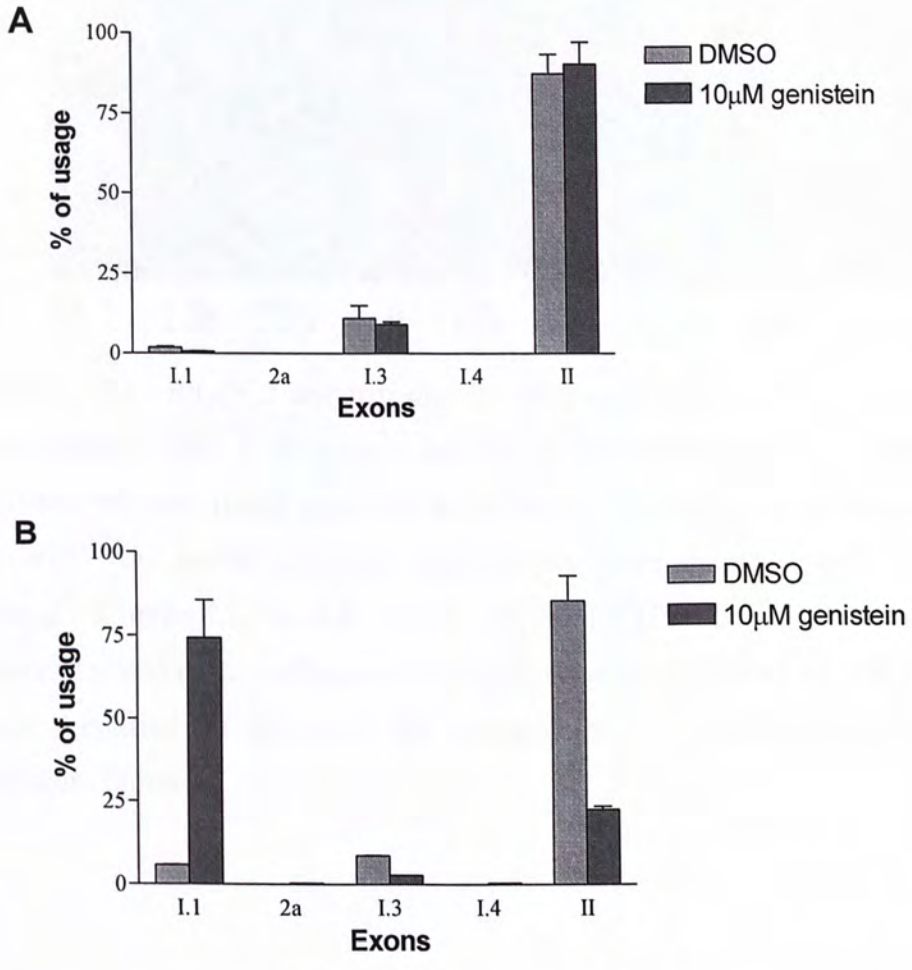

Figure 3.2.10 Exon I-specific real time PCR in HepG2 cells transfected with control plasmid (A) and ERα-transfected HepG2 cells (B). Cells were treated with 10μM genistein for 48 hours. Then total RNA was extracted and reverse transcripted. Real time PCR was performed using Taqman probe (Table 2.2). GAPDH was amplified as a house keeping gene. Promoter usage levels were calculated by using $2^{-\Delta\Delta CT}$ method and expressed as percentage of total CYP19 expression. Values are means ±SEM, n=3.

Figure 3.2.11 RT-PCR amplification of alternate sites in exon I of aromatase gene from HepG2 cells. Cells were transiently transfected with ERα expression plasmid and treated with 10μM genistein for 48 hours. Then total RNA was extracted and RT-PCR was carried out. The spliced exons were amplified using sense primers specific to exon I.1, I.2, I.3, I.4, I.5, I.6, I.7 I.f, II or 2a and a common antisense primer located in the coding region of aromatase gene (Table 2.1). The PCR products were amplified for 40 cycles and separated on 1.5% agarose gel and stained with ethidium bromide.

3.2.7 Genistein induced promoter I.1 transcriptional activity in ERα-transfected HepG2 cells

We determined that exon I.1 mRNA was elevated by genistein in ERα-transfected HepG2 cells. Next, we investigated whether genistein could up-regulate the transcriptional activity of promoter I.1. As shown in Figure 3.2.12, the result paralleled the mRNA data. However, genistein could not change the promoter activity in the absence of ERα. Such induction could be suppressed by estrogen antagonists ICI 182 780 (Figure 3.2.13). These results were consistent with the results of aromatase activity.

To locate the sequence responsible for the induction, a series of truncate promoters were generated from the -700bp of promoter I.1, and inserted into the pGL3-basic reporter plasmid (Figure 3.2.14 A). These constructs were co-transfected into HepG2 cells with ERα and control plasmid. Transfected cells were treated with or without 10μM genistein for two days and cell lysates were prepared for assay of luciferase activity assay. As shown in Figure 3.2.14 B, significant decreases in promoter activity in the -190 to -212, -260 to -280 and -280 to -300 deletions were observed. Hence, the response regions of promoter I.1 induced by genistein could be located in these three sequences.

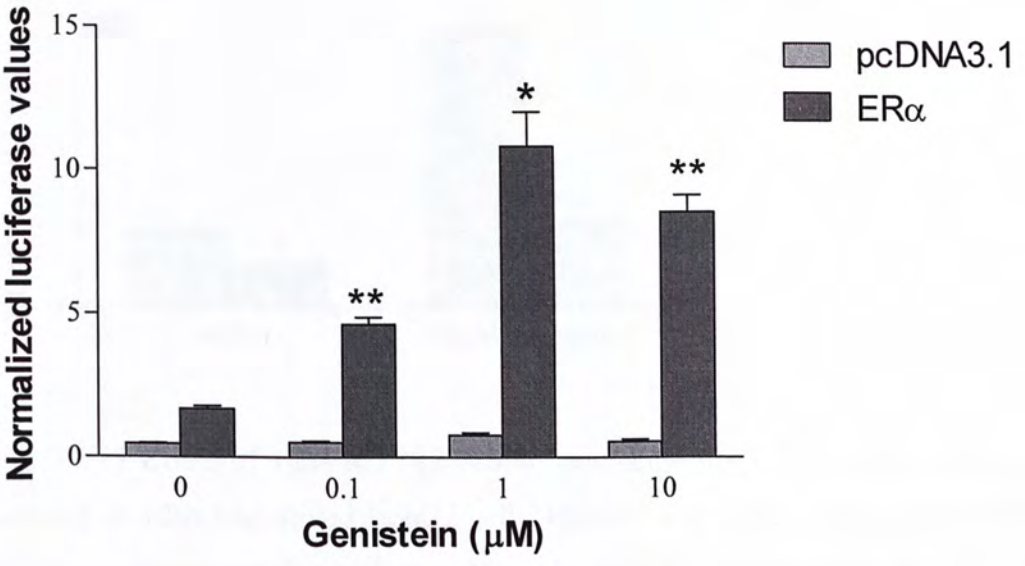

Figure 3.2.12 Transcriptional activity of aromatase promoter I.1 in HepG2 cells. Cells were transfected with promoter I.1 reporter plasmid, PRL-CMV control vector and ERα or pcDNA3.1 expression plasmid. Then the cells were incubated with 10μM genistein for 48 hours and dual-luciferase activity was measured. The relative luciferase activity was calculated by normalizing the light unit of firefly by that of renilla. Values are means ±SEM, n=3. (*P<0.05; **P<0.005)

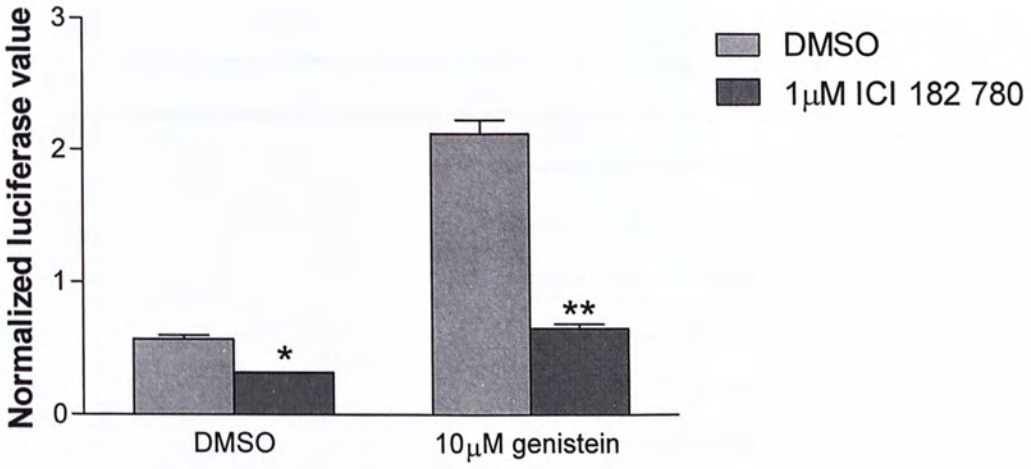

Figure 3.2.13 Effect of 1μM ICI 182 780 on genistein-induced aromatase promoter I.1 activity in ERα-transfected HepG2 cells. HepG2 cells were transfected with ER alpha expression vector for 24 hours. The cells were pretreated with 1μM ICI 182 780 for 3 hours before adding 10μM genistein for further 48 hours and dual-luciferase activity was measured. The relative luciferase activity was calculated by normalizing the light unit of firefly by that of renilla. Values are means ±SEM, n=3. (*P<0.05; **P<0.005)

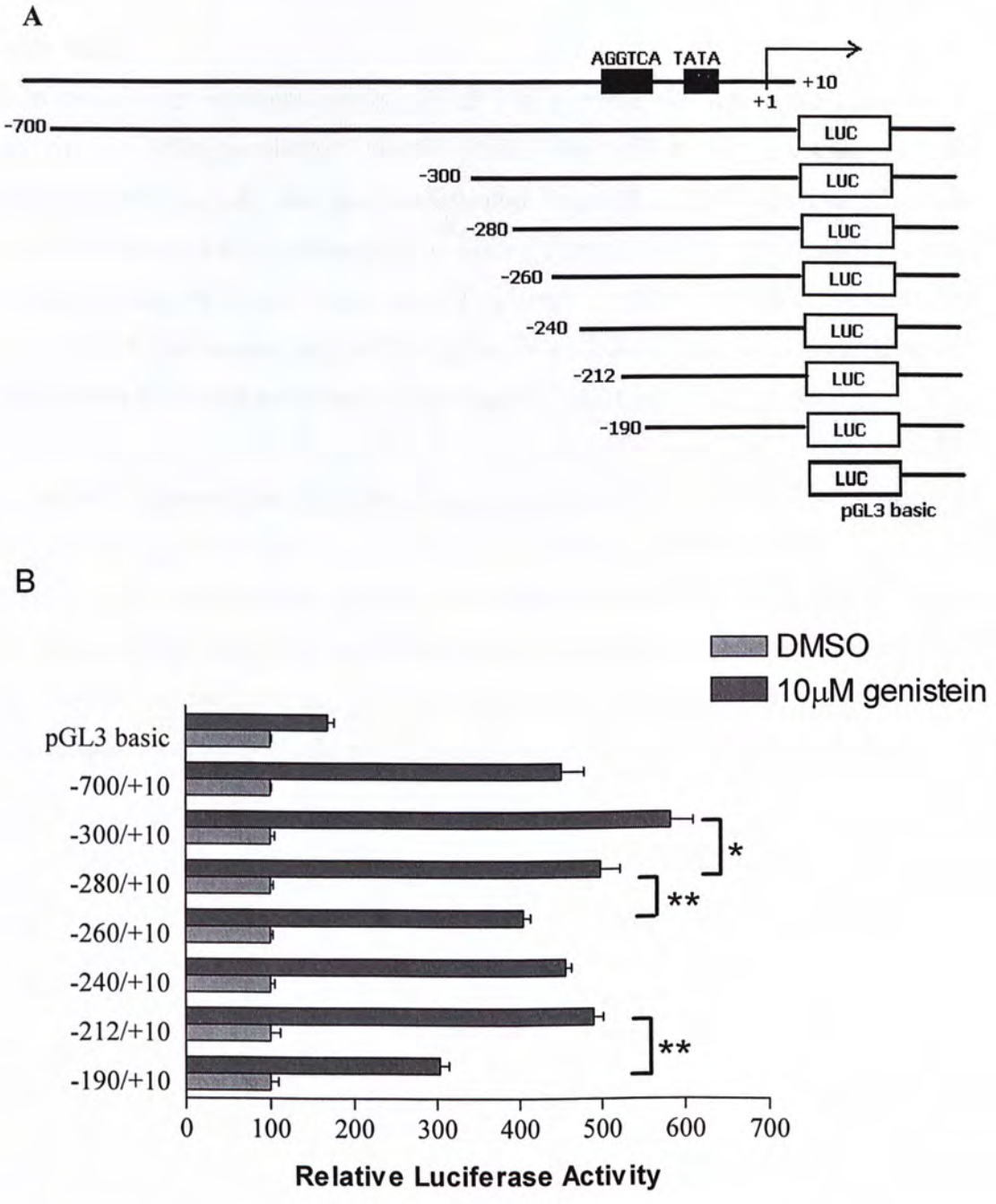

Figure 3.2.14 (A) A schematic diagram of the 5'-deleted mutants used in this experiment. (B) Transcriptional activity of a series of 5'-deleted promoter fragments in ERα-transfected HepG2 cells. Cells were transfected with pGL3-luciferase reporter constructs of promoter I.1, ERα expression vector and PRL-CMV control vector for 24 hours. Then the cells were incubated with 10μM genistein for further 48 hours and dual-luciferase activity was measured. The results of genistein group were normalized by the corresponding results of DMSO group. Values are means ±SEM, n=3. (*P<0.05; **P<0.005)

3.2.8 Genistein increased ERE and AP-1 reporter activity through interaction with ERα

Genistein is an estrogen agonist, which can increase the aromatase promoter I.1 activity in ERα-transfected HepG2 cells. The effect of genistein on ERE transactivation activity was also investigated. Figure 3.2.15 showed that ERα alone could not increase ERE transactivation activity. However, the activity would increase in the presence of ligand. Even 0.1μM genistein could induce ERE transactivation activity in ERα-transfected HepG2 cells by about 2 fold. The transactivation activity was further increased when the cells treated with 1μM and 10μM genistein.

It has been reported that ERα can interact with some transcription factors, including AP-1 (Cheung *et al.*, 2005). We also investigated the effect of genistein on AP-1 in HepG2 cells. Even without genistein, ERα alone could induce AP-1 binding (Figure 3.2.16). After treating with 1μM and 10μM genistein, AP-1 binding transactivation activity had doubled. Hence, the result suggested that genistein induced promoter I.1 transcriptional activity might be associated with interaction of ERα and AP-1.

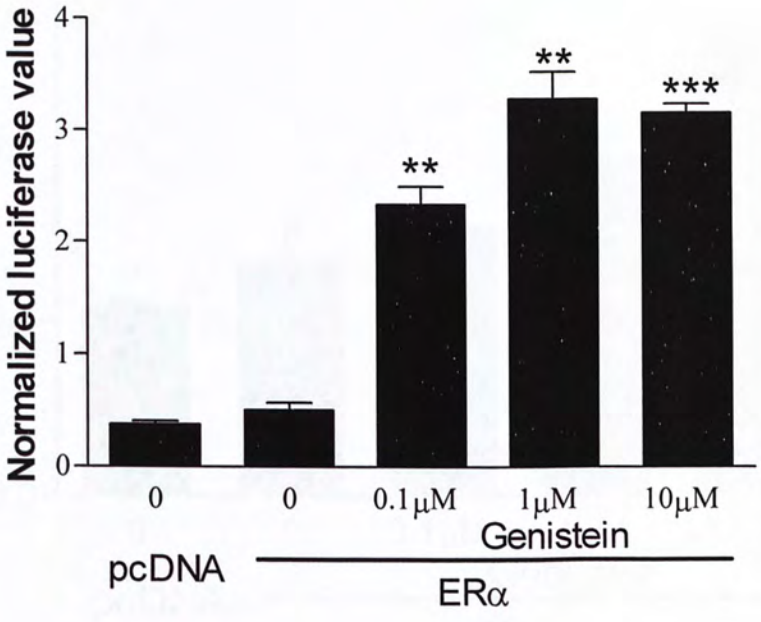

Figure 3.2.15 Effect of genistein on ERE promoter transcription activity in HepG2 cells. Cells were transfected with ERE reporter plasmid, PRL-CMV control vector and ERα or pcDNA3.1 expression plasmid. Then the cells were incubated with 10μM genistein for 48 hours and dual-luciferase activity was measured. The relative luciferase activity was calculated by normalizing the light unit of firefly by that of renilla. Values are means ±SEM, n=3. (** $P< 0.005$; *** $P<0.0005$)

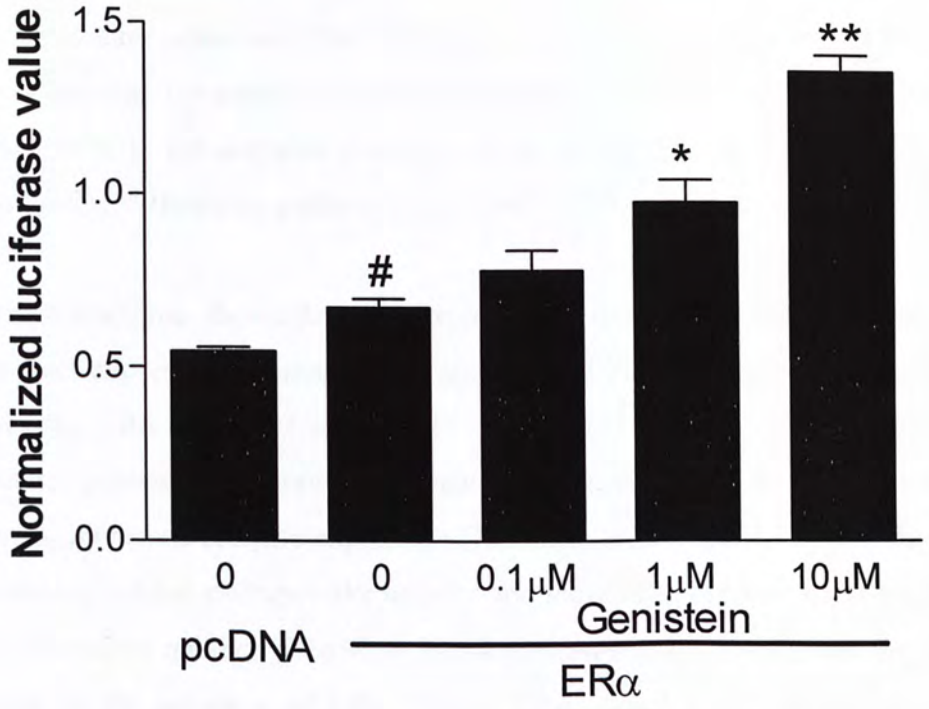

Figure 3.2.16 Effect of genistein on AP-1 promoter transcription activity in HepG2 cells. Cells were transfected with AP-1 reporter plasmid, PRL-CMV control vector and ERα or pcDNA3.1 expression plasmid. Then the cells were incubated with 10μM genistein for 48 hours and dual-luciferase activity was measured. The relative luciferase activity was calculated by normalizing the light unit of firefly by that of renilla. Values are means ±SEM, n=3. (#) represents value different from cells with control vector; (*) represents value different from cells with ERα and without genistein treatment. (*/# $P< 0.05$; ** $P<0.005$)

3.3 DISCUSSION

The hepatocarcinoma cell line, HepG2, is a widely accepted model for studying human hepatic lipoprotein metabolism (Javitt, 1990). It has been reported that HepG2 cells is ER-negative (Harnish *et al.*, 1998). Hence, they can be used for investigating differential pathways controlled by ER.

Previous study has shown that ERα expression is increased in the mammary tissue of mice over-expressing aromatase (Kirma *et al.*, 2001). Our data indicated that over-expressing ERα in HepG2 cells would significantly increase the aromatase activity. Recently, genistein was found to increase aromatase activity in endometrial stromal cells via increased enzyme expression (Edmunds *et al.*, 2005). In the present study, genistein exhibited estrogen-like activity and induced aromatase activity in dose and time-dependent manner. 0.1μM to 10μM genistein could up-regulate the aromatase activity in the presence of ERα. Hence, ERα played a critical role in genistein-mediated aromatase activity. The induction of aromatase activity was consistent with the up-regulations in aromatase protein expression, mRNA expression and promoter activity.

Previously, Chen and Kinoshita (2003) have found that E_2 induces aromatase expression in ER-positive SK-BR-3 cells through binding to the region of -300 and -280 bp. Though promoter I.1 was regarded as placenta-specific promoter, we identified this promoter usage was dominant in genistein-treated ERα positive HepG2 cells. From the promoter deletion analysis, we found that the sequences between -212 to -190 and -300 to -260 bp upstream of exon I.1 might be responsible for the activity induced by genistein. These two regions might account for the increase of promoter I.1 activity in ERα-transfected HepG2 cells.

The classical mechanism of ER involves ligand binding to receptors in the nucleus, after which the receptors dimerize and bind to specific response elements (ERE) located in the promoter of target gene. The estrogen antagonist ICI 182 780 blocks the activation of AF-1 and AF-2 domain in ERα (Lu *et al.*, 2002). ICI 182 780 completely abolished the genistein-induced aromatase activity and promoter activity, indicating that ERα activation regulated the gene transcription. Though the addition

of genistein could increase the ERE transactivation activity, there was no ERE consensus sequence found within promoter I.1. Previous findings suggested that ERα can regulate the gene expression without binding to the DNA directly. Instead, it can interact with other transcription factors, including AP-1, Sp1, NF-κB. The transcription factors would bind to their corresponding response element to trigger the gene transcription, instead of binding to ERE (Paech *et al.,* 1997; Porter *et al.*, 1997; Ray *et al.,* 1997; Webb *et al.,* 1999; Bjornstrom & Sjoberg, 2005). Our lab previously has demonstrated that genistein would not affect Sp1 mRNA expression in ERα-transfected HepG2 cells (Yuen, 2005). In the present study, the results suggested that genistein would up-regulate the AP-1 site in a dose dependent manner. Within the regions of promoter I.1, several AP-1 sites are detected, including -498 and -935 in promoter II and I.3 (Zhou *et al.,* 1996). Two putative AP-1 sites have been found between -70 bp and the start site of exon I.1 (Chen and Kinoshita, 2003). -212 to -190 region of exon I.1 was important in the genistein-mediated aromatase activity. It may due to an AP-1 binding site (TGTGGGTCATA) located in -211 to -201 of this region.

Emerging evidence indicated the importance of signaling pathway in the actions of ER. The extracellular-regulated kinase (ERK) and p38 MAPK pathways are shown to initiate ER phosphorylation at key positions (McClelland *et al.,* 2001; Knowlden *et al.,* 2003). Other research groups have shown that MAPK mediated cross talk occurs between growth factor and estrogen receptor (Bunone *et al.,* 1996; Font de Mora and Brown, 2000). Estrogen can activate the MAPK pathway in mammalian cells by interacting with membrane or cytosol ER (Migliaccio *et al.,* 1996; Watters *et al.,* 1997; Manolagas & Kousteni, 2001; Lu *et al.,* 2004). On the other hand, genistein can inhibit protein tyrosine kinase (Akiyama *et al.*, 1987 ; Barnes *et al.*, 2000). It inhibits the nongenomic effect of estradiol on protein kinase C (PKC) activity in chondrocytes isolated from female rats (Sylvia *et al.,* 2000). However, ERK, JNK, PKA and PKC pathways were not involved in the genistein-induced aromatase activity in the present study. Only inhibition of p38 MAPK pathway could partly abolish the induction. Lee and Bai (2002) have suggested that the kinase responsible for phosphorylating ER at Thr^{311} may be a member of the p38 family. Genistein rapidly and significantly activates p38 in immortalized human mammary epithelial cells (Frey and Singletary, 2003). The possibility that genistein

phosphorylates ER through p38 and promotes aromatase activity needs further investigation.

In the present study, we demonstrated that genistein could significantly increase the aromatase activity in the ERα-transfected HepG2 cells. Genistein could increase the promoter activity, mRNA and protein expression of aromatase in ERα-transfected HepG2 cells. -300 to -260 and -212 to -190 regions in promoter I.1 were critical in the up-regulation of aromatase activity. Such induction was possibly mediated through AP-1 activation pathway. As estrogen can trigger a wide-range of gene transcriptions in liver, this study provided a new insight for the gene-regulatory mechanism of genistein.

CHAPTER 4

EFFECT OF 2,3,7,8-TETRACHLORODIBENZO-*PARA*-TCDD (TCDD) ON AROMATASE IN MCF-7 CELLS

4.1 INTRODUCTION

4.1.1 Breast Cancer

In Hong Kong, breast cancer is the most common cancer affecting female. It is ranked the third major cause of cancer death (Hong Kong Cancer Registry, 2004). There are several risk factors which contribute to the etiology of breast cancer, including age, race, genetic, physical activity and diet (American Cancer Society).

Nearly 60% of pre-menopausal and 75% of postmenopausal breast cancer patients have hormone-dependent tumors. Lifetime exposure to estrogens is a major risk factor for both breast and endometrial cancer in women (Hulka, 1997; Hulka *et al.*, 1994). The positive correlation between estrogen exposure and breast cancer has been demonstrated in cell and animal models (Colditz, 1999; Yoshidome *et al.*, 2000).

4.1.2 TCDD

Environmental pollution is getting increasingly serious nowadays. Polycyclic aromatic hydrocarbons (PAHs) belong to a class of environmental contaminants that could be found in diesel exhaust, tobacco smoke and over-heated cooking oil (IARC, 1983; EPC, 1990). PAHs have been widely studied on the effect of DNA adduct formation and carcinogensis. The compound 2,3,7,8-tetrachlorodibenzo-*para*-TCDD (TCDD) is considered to be the most toxic member of a class of planar, halogenated aromatic hydrocarbons (Birnbaum, 1994; Birnbaum, 1995). Its chemical structure is contains two aromatic rings connected through a pair of oxygen atoms. Four chlorine atoms, two on each aromatic ring, are attached at positions 2, 3, 7 and 8.

TCDD is a widespread environmental contaminant produced by different chemical reactions, waste incineration and incidental formation in pesticides producing plants (Zook *et al,* 2003; Tuppurainen *et al.*, 2003). As TCDD is extremely stable and highly lipophilic, it can accumulate in the food chain (Birnbaum, 1994). The half life

of TCDD in human is about 7-9 years (Pirkle *et al.*, 1989). Animal products in our diet account for 90% of our TCDD exposure (Baars *et al.*, 2004). The persistence and ubiquity in the environment make it difficult to determine the pollutant's impact on human health. In 1997, the International Agency for Research on Cancer (IARC) classified TCDD as a Group I carcinogen, which can increase the mortality for all cancers. It is shown that TCDD is a multi-site carcinogen in both animal and human studies (IARC, 1997). Moreover, TCDD affects the male and female reproductive system and on sexual and learning behavior in rats and monkeys (Peterson *et al.*, 1993; Kociba *et al.*, 1976; Mably *et al.*, 1992a,b).

4.1.3 CYP enzymes

There are at least 25 distinct classes of CYP enzymes identified (Guengerich, 1994). Based on the similarities in the amino acid sequences, CYP enzymes are divided into families and subfamilies. Generally, CYP 1 to 4 families are responsible for the metabolism of xenobiotics. CYP 5 to 27 families are involved in endogenous substrates synthesis and metabolism (Nelson *et al*, 1993). Most researches of TCDD are concentrated on the effect on certain aryl hydrocarbon receptor (AhR) activated CYP enzyme, including CYP1A1, CYP1A2 and CYP1B1, which catalyze the activation of a wide range of chemical carcinogens (Chen *et al*, 2004; Bofinger *et al*, 2001; Safe, 1994). Upon TCDD binding, HSP90 will be released from AhR. The activated AhR will then translocate to the nucleus. AhR forms heterodimer complex with the structurally related AhR nuclear translocator protein (ARNT). The activated complex binds to specific gene regulatory sequences, known as xenobiotic response element (XRE). This binding activates transcription of several genes, especially CYPs which are involved in xenobiotic compound metabolism (Schrenk, 1998; Whitlock, 1990; Swanson and Bradfield, 1993; Whitlock, 1993; Hankinson, 1995; Whitlock *et al.*, 1997). However, the effect of TCDD on aromatase activity is seldomly investigated in human breast cancer cells.

4.1.4 TCDD and breast cancer

TCDD is involved in the modulation of estrogen in many paradigms. In rats, 17 β-estradiol-induced uterine responses, uterine weight, peroxidase activity, estrogen receptor, and progesterone receptor were decreased after the treatment of TCDD (Astroff and Safe, 1990; Romkes and Safe, 1988). Several studies have shown that TCDD inhibits diverse E2-induced responses (Safe, 1995). TCDD acts as an antiestrogen by suppressing estrogen-stimulated growth of human MCF-7 breast cancer xenografts (Gierthy *et al.*, 1993). It also reduces the level of estrogen receptor in MCF-7 (Harris *et al*, 1990). However, other group has indicated that TCDD does not affect the total estrogen receptor levels (Gierthy *et al.*, 1987).

Environmental toxicants are thought to play a role in several estrogen-dependent diseases including breast cancer. In 1976, an industrial explosion in Seveso, Italy, resulted in high TCDD exposure to the residential populations (Mocarelli and Pocchiari, 1988). In the Seveso Women's Health study, individual's serum TCDD level was associated with breast cancer risk, contrasting to its antiestrogenic effect in breast cancer cells (Warner *et al.*, 2002). The anti-estrogenic activity of TCDD could not account for the increased breast cancer risk. The possibility that TCDD possesses estrogenic effect should be considered. Due to the functional role of aromatase to convert androgen into estrogen, it may play a role in the TCDD-induced breast cancer.

4.1.5 Aim of study

The aim of the present study was to examine whether TCDD could increase aromatase activity in human breast cells and might therefore increase the risk of breast cancer. In our experiment design, we employed different human breast cancer cell models for this purpose.

4.2 RESULT

4.2.1 Effect of TCDD on aromatase activity in different cell lines

In the present study, six breast cancer cell lines (MCF-7, T47D, MCF-10A, MDA-MB-231and SK-BR-3) and one liver cancer cell line (HepG2) were screened for the aromatase induction by 10ηM TCDD. As shown in figure 4.2.1, 10ηM TCDD could not induce aromatase activity in T47D, MCF-10A and MDA-MB-231cell lines.

Aromatase activity of untreated SK-BR-3 cells was 10.450 ± 0.2828 cpm/ μg protein/ hour, and the activity was elevated to 2204.603 ± 527.3202 cpm/ μg protein/ hour after transfected with ERα (Figure 4.2.2). The activity of ERα-transfected cells elevated significantly, nearly 220 times higher than that in the untreated cells. Similar to HepG2 cells, ERα transfection increased aromatase activity in SK-BR-3 cells. However, 10ηM TCDD could not further induce the aromatase activity in the presence or absence of ERα (Figure 4.2.3).

In the absence of ERα, 10ηM TCDD would not increase the aromatase activity in HepG2 cells. In the presence of ERα , 10ηM TCDD elevated the activity by 2-fold and such induction could be suppressed by ICI 182 780 (Figure 4.2.4). Hence, TCDD could induce aromatase activity via ERα in HepG2 cells.

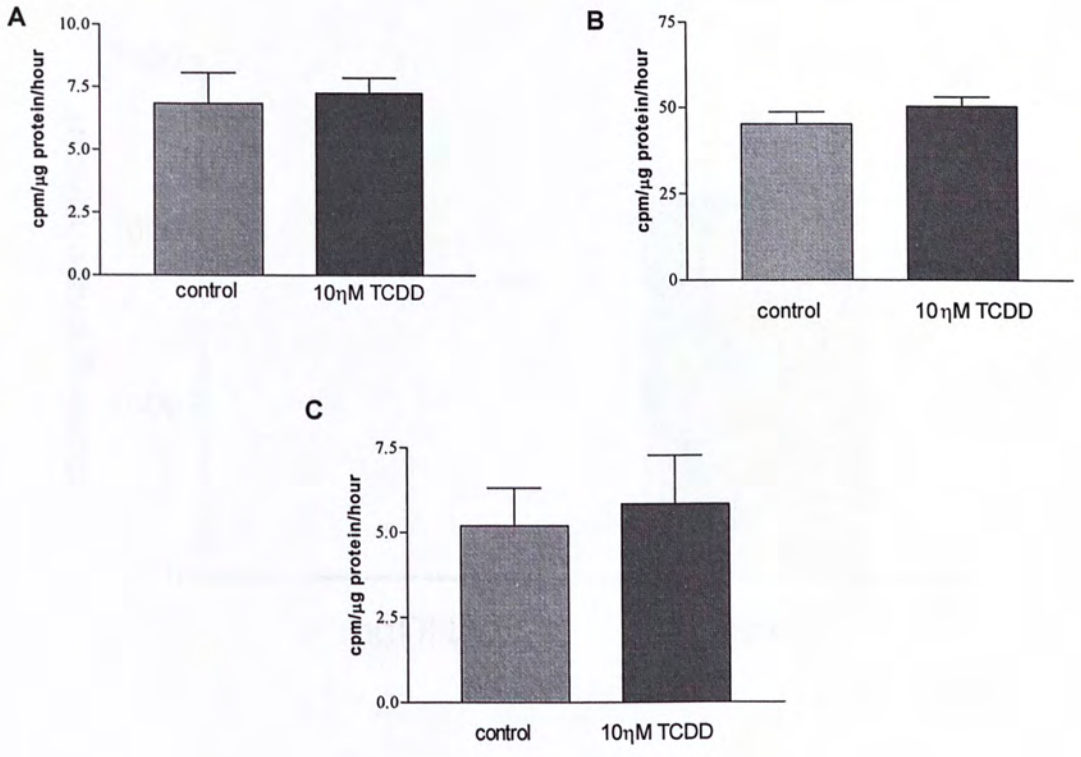

Figure 4.2.1 Aromatase activity in (A) T47D, (B)MCF10A and (C) MDA-MB-231. Cells were treated for 48 hour 10ηM TCDD and were then further incubated with [1β-^{3}H(N)]-androst-4-ene-3,17-dione for 1 day. Aromatase activity was determined by using tritiated water release assay. Values are means ±SEM, n=3.

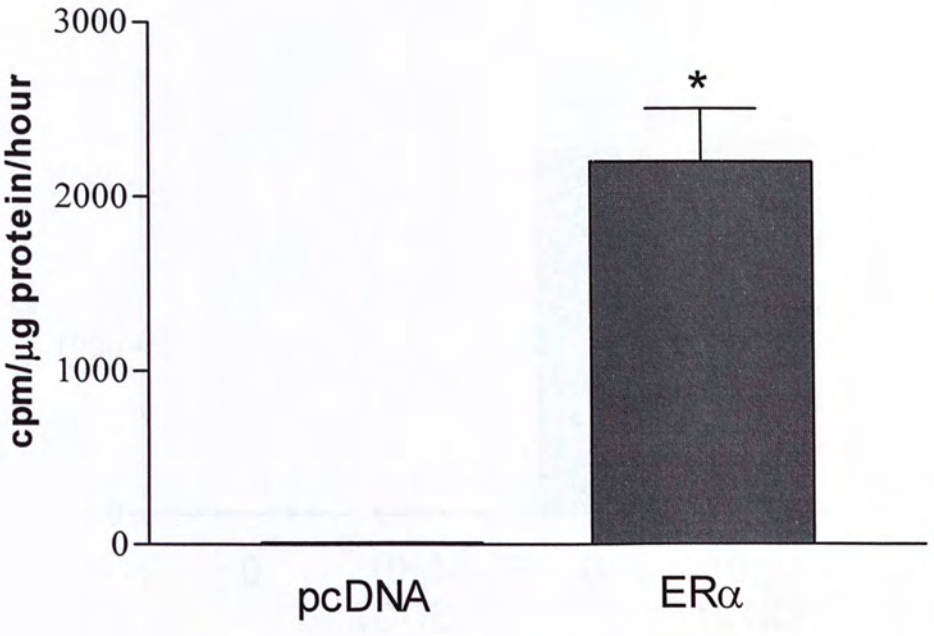

Figure 4.2.2 Aromatase activity in SK-BR-3 cells. SK-BR-3 cells were transfected with ER alpha expression vector and control plasmid pcDNA3.1. The cells were further incubated with 25ηM [1β-^{3}H(N)]-androst-4-ene-3,17-dione for 3 hours. Aromatase activity was determined by using tritiated water release assay. Values are means ±SEM, n=3. *P<0.05 vs. control plasmid cells.

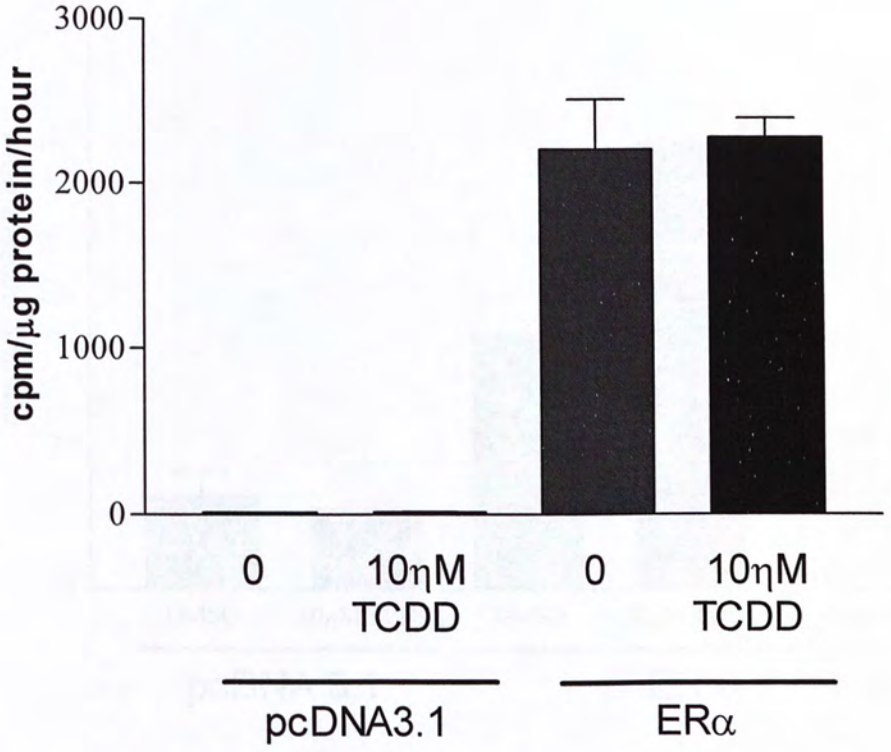

Figure 4.2.3 Aromatase activity in SK-BR-3 cells. SK-BR-3 cells were transfected with ER alpha expression vector and control plasmid pcDNA3.1, and treated with 10ηM TCDD for 48 hour. The cells were further incubated with 25ηM [1β-^{3}H(N)]-androst-4-ene-3,17-dione for 3 hours. Aromatase activity was determined by using tritiated water release assay. Values are means ±SEM, n=3.

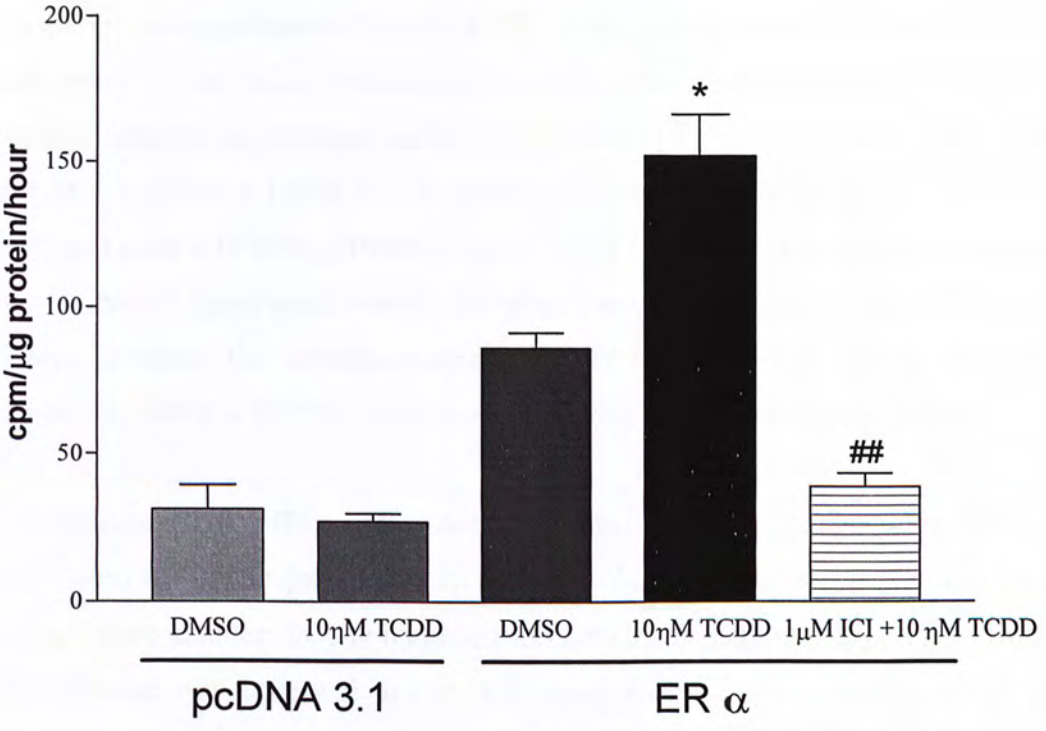

Figure 4.2.4 Effect of 10ηM TCDD on aromatase activity in HepG2 cells. HepG2 cells were transfected with ERα expression vector for 24 hours. The cells were pretreated with 1μM ICI 182 780 for 3 hours before adding 10ηM TCDD for further 48 hours. The cells were further incubated with [1β-^{3}H(N)]-androst-4-ene-3,17-dione for 1 day. Aromatase activity was determined by using tritiated water release assay. Values are means ±SEM, n=3. * $P<0.05$ vs. cells transfected with ERα only; ## $P<0.005$ vs. cells treated with TCDD in the presence of ERα.

4.2.2 TCDD increased aromatase activity in MCF-7cells

The regulation of aromatase activity in MCF-7 cells was determined by titrated water release assay. The cells were incubated with various concentration of TCDD. Aromatase activity was assessed using the release of $[^3H]H_2O$ into media. The time course of the effect of 10ηM TCDD on the aromatase activity in MCF-7 cells was investigated over a 48 hour period (Figure 4.2.5). TCDD could not induce aromatase activity in both 6 hours and 24 hours. However, incubating the cells with TCDD for 48 hours increased the aromatase activity nearly by two times. Hence, the cells would be incubated in TCDD for 48 hours in the following experimental design.

The dose-dependent effect was studied by culturing the cells with various concentration of TCDD for 48 hours. TCDD could induce aromatase activity in a dose-dependent manner. TCDD increased the activity slightly at 0.1ηM while about 2-fold increase was observed at 10ηM (Figure 4.2.6). Such induction could be suppressed by estrogen antagonists ICI 182 780 (Figure 4.2.7).

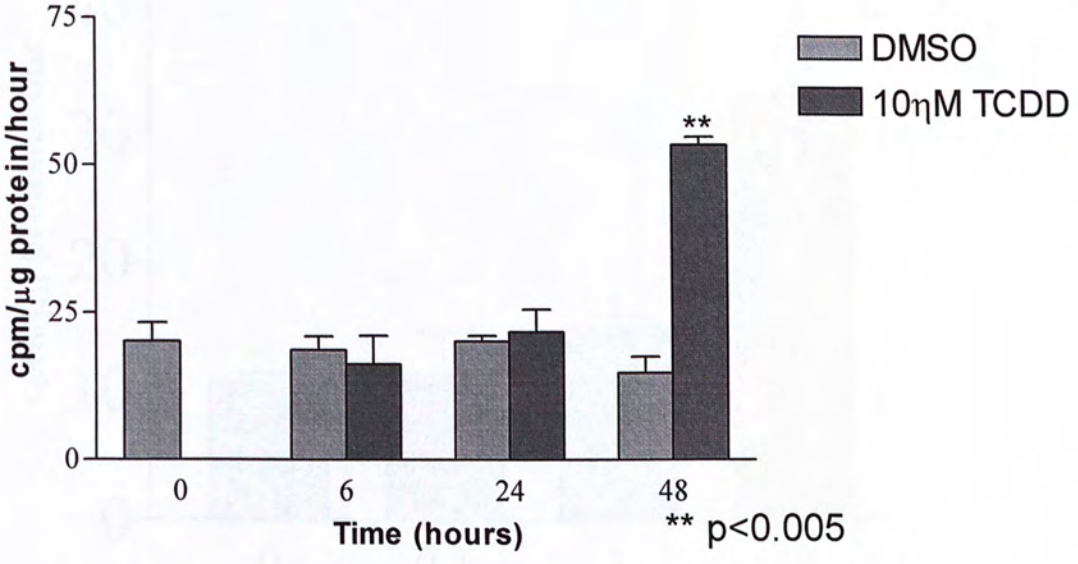

Figure 4.2.5 Time course of TCDD stimulation of the aromatase activity in cultured MCF-7 cells. MCF-7 cells were treated for various time periods with TCDD at 10ηM. Values are means ±SEM, n=3. **P<0.005 vs. control cells treated with DMSO.

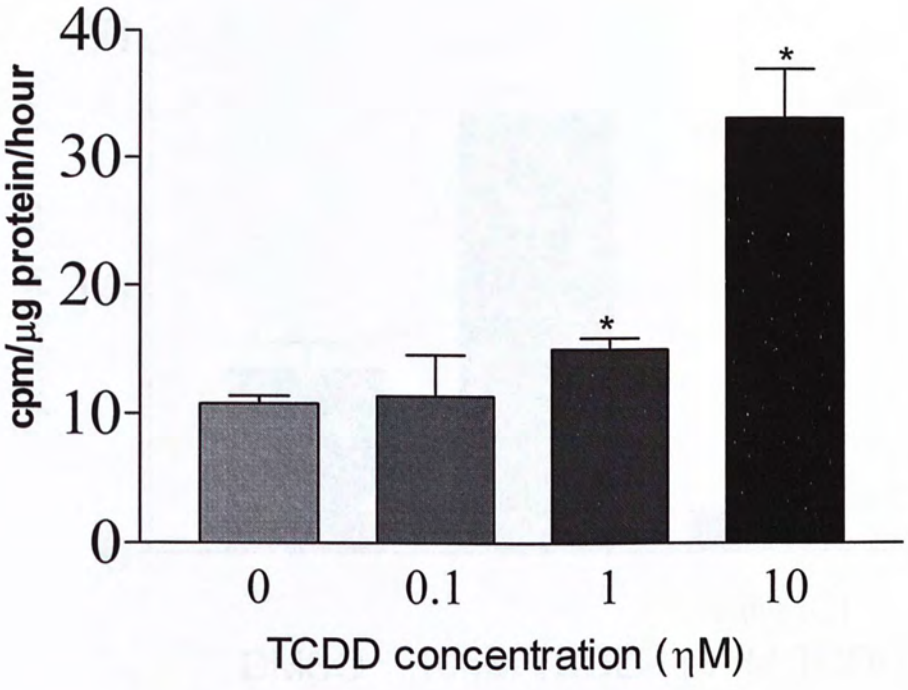

Figure 4.2.6 Dose-response of TCDD stimulation of the aromatase activity in cultured MCF-7. MCF-7 cells were treated for 48 hour with different concentration of TCDD and were then further incubated with [1β-^{3}H(N)]-androst-4-ene-3,17-dione for 1 day. Aromatase activity was determined by using tritiated water release assay. Values the means ±SEM, n=3. * $P<0.05$ vs. control cells treated with DMSO.

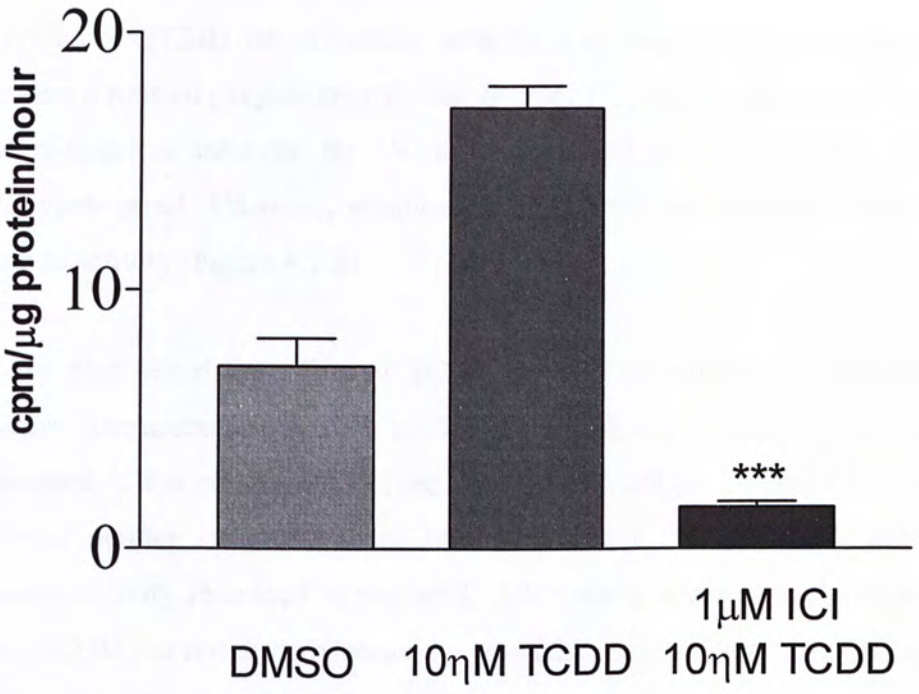

Figure 4.2.7 Effect of 1μM ICI 182 780 on TCDD-induced aromatase activity in MCF-7 cells. The cells were pretreated with 1μM ICI 182 780 for 3 hours before adding 10ηM TCDD for further 48 hours. The cells were further incubated with [1β-^{3}H(N)]-androst-4-ene-3,17-dione for 1 day. Aromatase activity was determined by using tritiated water release assay. Values are means ±SEM, n=3. *** $P<0.005$ vs. cells treated with 10ηM TCDD.

4.2.3 Effect of TCDD on human CYP19 recombinant Supersomes® and MCF-7 aro cells

The effect of TCDD on aromatase activity was also determined using CYP19 recombinant protein (Supersomes®). Incubation of 2 pmol Supersomes® with 25nM of the radioactive substrate for 15 minutes resulted in an activity of 14607.51 ± 613.65 cpm/ pmol. However, addition of TCDD did not show any effect on the aromatase activity (Figure 4.2.8).

We have also tested the effect of TCDD on MCF-7 cells stably transfected with aromatase. The aromatase activity in MCF-7aro cells was about 40 times higher than that assayed in the control MCF-7vec cells (Wang, 2005). Upon TCDD treatment, there was neither stimulatory nor inhibitory effect on aromatase activity. The aromatase activity remained at around 3, 300 cpm/μg protein/ hour (Figure 4.2.9). Hence, TCDD did not directly stimulate aromatase enzyme complex. Other possible factors and mechanisms should be considered.

4.2.4 TCDD increase aromatase protein expression in MCF-7 cells

To investigate the role of TCDD on aromatase expression, western blot was carried out. It was found that TCDD could dose-dependently induce aromatase expression in MCF-7 cells (Figure 4.2.10). These results suggested that TCDD could up-regulate aromatase protein expression, so as to increase the aromatase activity in MCF-7 cells.

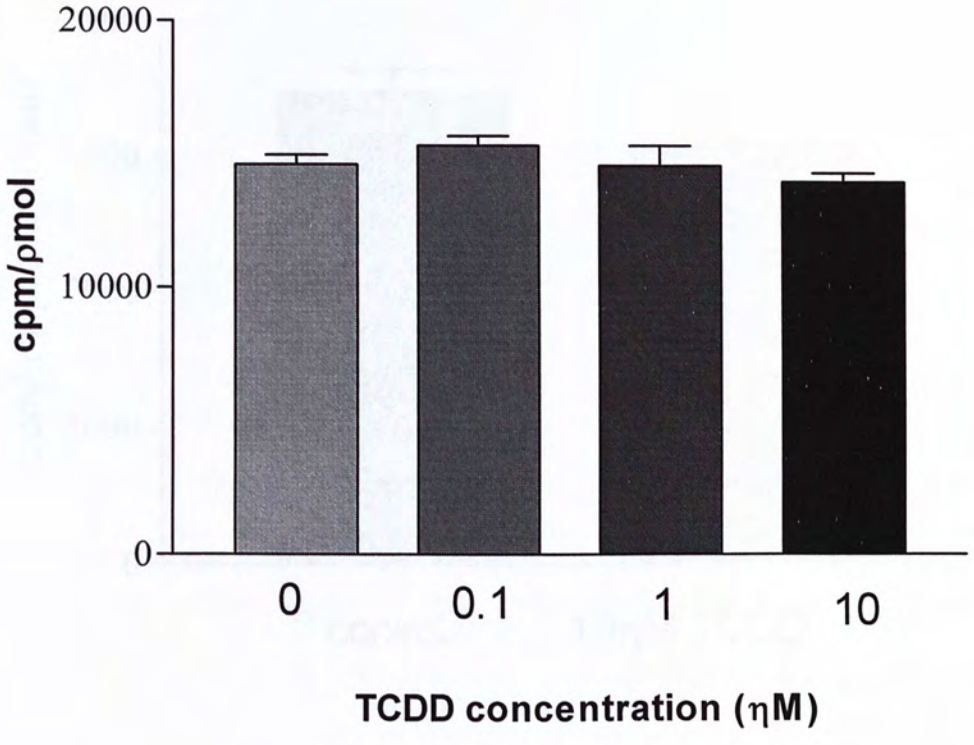

Figure 4.2.8 aromatase enzyme assay performed on hCYP19 Supersomes®. Incubation of 2ρmol hCYP19 Supersomes® with 25 ηM of the substrate [1β-^{3}H(N)]-androst-4-ene-3,17-dione and different concentration of TCDD for 15mins. Aromatase activity was determined by using tritiated water release assay. Values are means ±SEM, n=3.

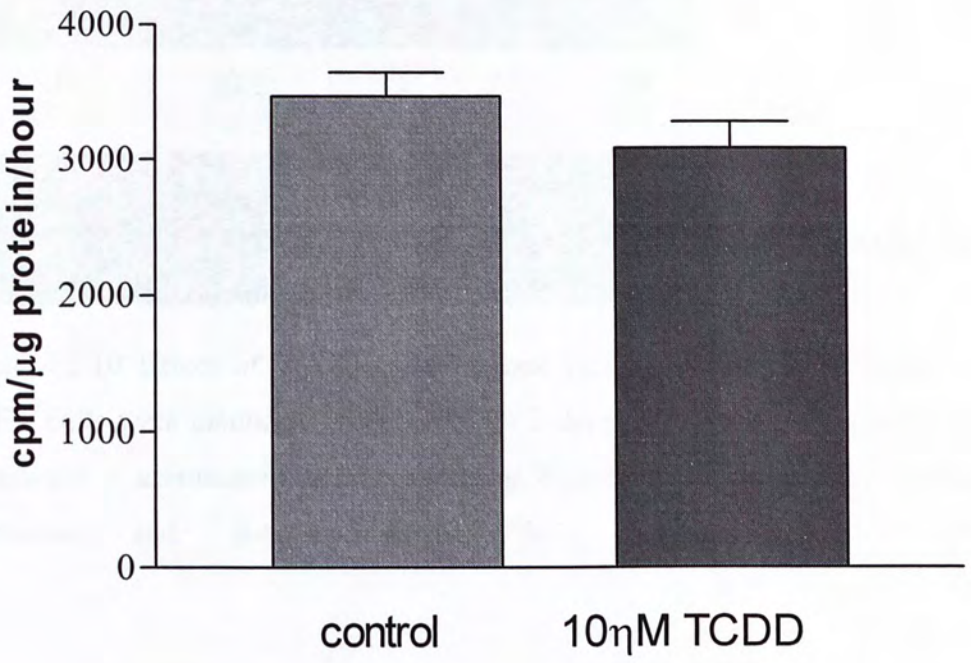

Figure 4.2.9 Aromatase activity in MCF-7 aro cells. Cells were treated for 48 hour 10ηM TCDD and were then further incubated with [1β-^{3}H(N)]-androst-4-ene-3,17-dione for 1 day. Aromatase activity was determined by using tritiated water release assay. Values are means ±SEM, n=3.

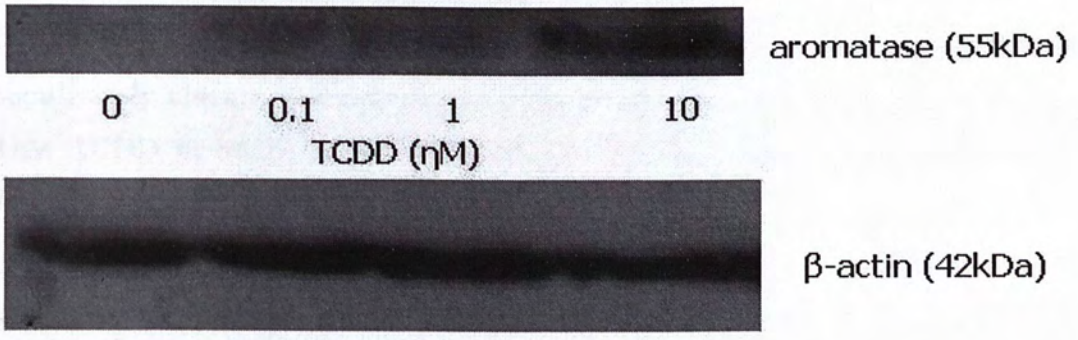

Figure 4.2.10 Effect of TCDD on aromatase protein expression in MCF-7 cells. MCF-7 cells were incubated with TCDD for 2 days. Total protein was collected and the amount of aromatase was detected using Western Blot. The molecular weight of aromatase and β-actin were 55kDa and 42kDa respectively.

4.2.5 Effect of TCDD in aromatase mRNA expression in MCF-7 cells

By using Taqman probing within the aromatase coding region, there was no significantly change in the aromatase mRNA expression after treatment of 0.1 and 1ηM TCDD in MCF-7 cells. However, the mRNA expression was increased by roughly 300% at 10ηM TCDD (Figure 4.2.11).

By performing real time PCR, we noticed that the dominant promoter usage in MCF-7 were exons II and I.3, contributing for 90% and 6% of total transcriptional activity respectively. Exons I.1 contributed for only 4%. After the addition of TCDD, the promoter usage did not show any significant changes (Figure 4.2.12).

4.2.6 Effect of TCDD in CYP19 promoter and AP-1 promoter activity in MCF-7 cells

The proximal promoter II located in the immediate upstream region of the translation start site, while promoter I.3 is about 200bp proximal to promoter II (Sebastian and Bulun, 2001). They share common regulatory elements (Mahendroo *et al.,* 1993). As the dominant promoters in MCF-7 are I.1, I.3 and II, we further investigated the effect of TCDD on promoters I.1 and I.3/II. As shown in figure 4.2.13, treatment with 10ηM TCDD for 2 days would not change the promoter activities.

AP-1 is another response element that ER could interact with (Cheung *et. al.,* 2005). Previous finding has shown that promoter I.3/II bears the consensus sequences of AP-1 at -498 and -935 (Zhou D., *et al.*, 1996). Effect of TCDD on AP-1 interaction was investigated in the present study. Figure 4.2.14 showed that various concentrations of TCDD could not change the reporter activity of AP-1 in MCF-7 cells. This result suggested that TCDD-induced aromatase activity was not associated with interaction of AP-1. Hence, the induction of aromatase activity and protein expression by TCDD might not be mediated through transcriptional control.

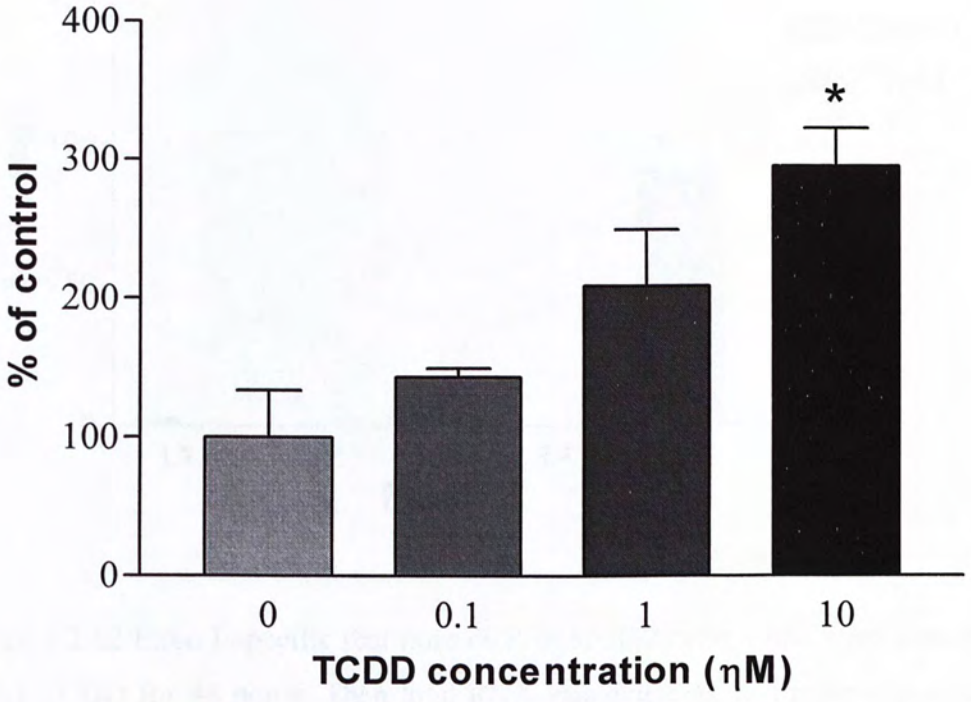

Figure 4.2.11 Effect of TCDD on CYP19 mRNA expression in MCF-7 cells. Cells were treated with TCDD for 48 hours. The amount of CYP19 mRNA was determined by relative quantitative real time PCR. The expression of CYP19 mRNA was normalized by GAPDH. Values are means ±SEM, n=3. *P<0.05

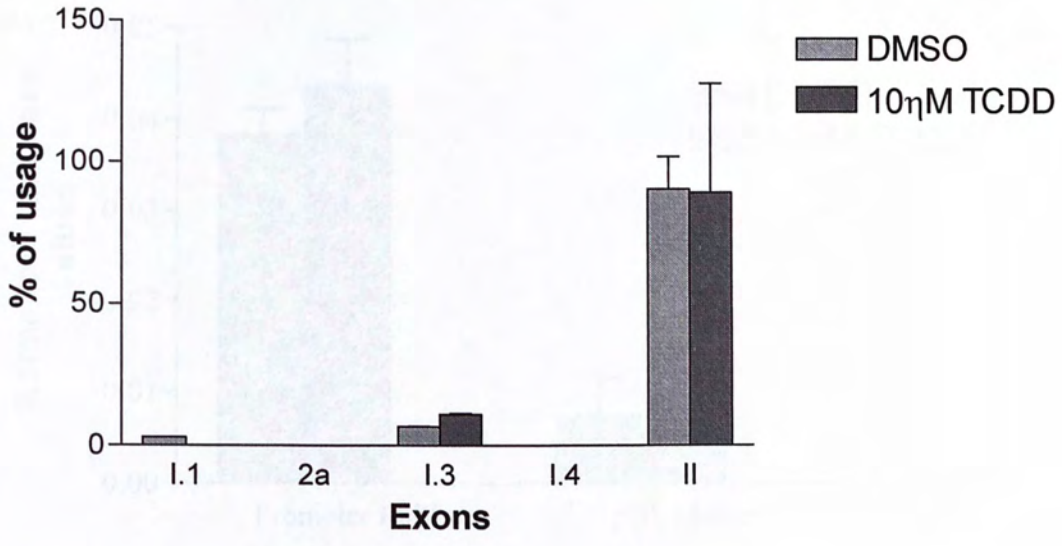

Figure 4.2.12 Exon I-specific real time PCR in MCF-7 cells. Cells were treated with 10ηM TCDD for 48 hours. Then total RNA was extracted and reverse transcribed. Real time PCR was performed using Taqman probe (Table 2.2). GAPDH was amplified as a house keeping gene. Promoter usage levels were calculated by using $2^{-\Delta\Delta CT}$ method and expressed as percentage of total CYP19 expression. Values are means ±SEM, n=3.

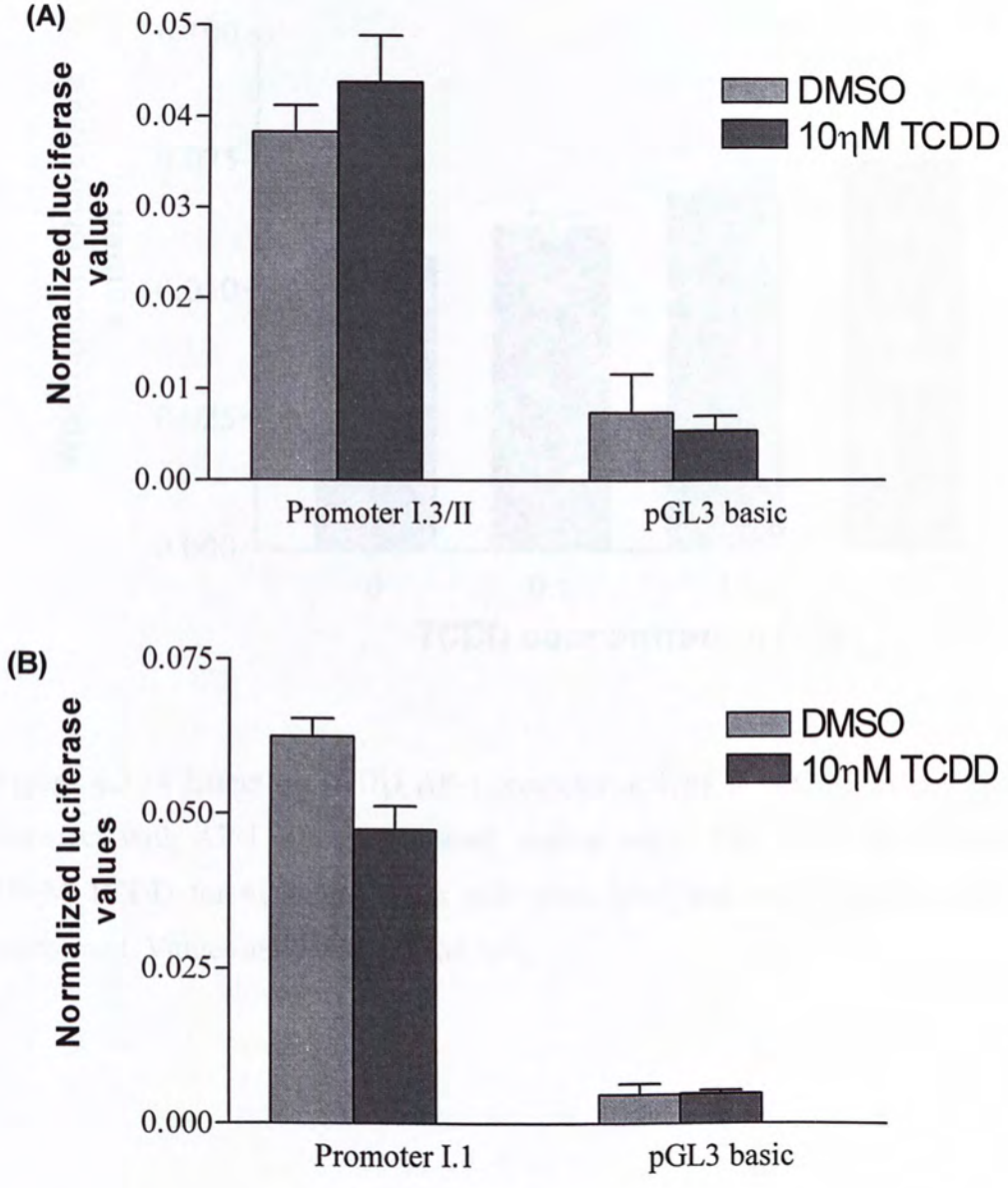

Figure 4.2.13 Effect of TCDD on (A) CYP19 promoter I.3/II and (B) CYP19 promoter I.1 activity in MCF-7 cells. Cells were transfect with (A) promoter I.3/II and (B) promoter I.1 reporter plasmid, control vector PRL-CMV and treated with 10ηM TCDD for 48 hours. Then cells were lysed and dual luciferase assay was performed. Values are means ±SEM, n=3.

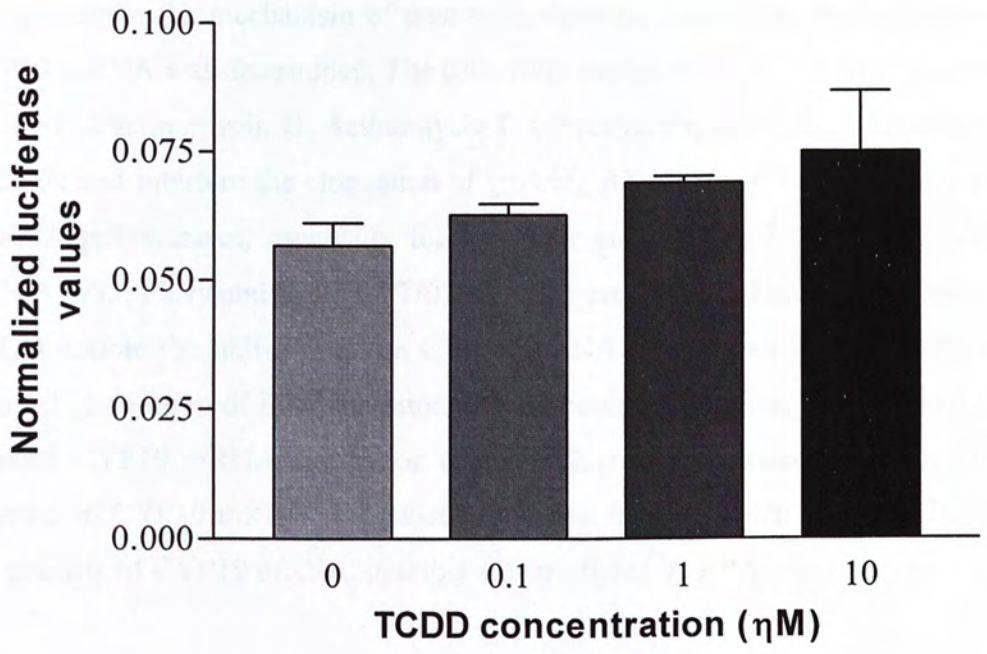

Figure 4.2.14 Effect of TCDD AP-1 promoter activity in MCF-7 cells. Cells were transfect with AP-1 reporter plasmid, control vector PRL-CMV and treated with 10ηM TCDD for 48 hours. Then cells were lysed and dual luciferase assay was performed. Values are means ±SEM, n=3.

4.2.7 Effect of TCDD in CYP19 mRNA Half-life

To investigate the mechanism of post-transcriptional regulation, the half-life of the CYP19 mRNA was determined. The cells were treated with TCDD for 2 days before addition of actinomycin D. Actinomycin D intercalates into DNA to immobilize the complex and interfere the elongation of growing RNA chains. Thus, the progression of RNA polymerases, especially for the RNA polymerases I, would be inhibited (Sobell 1985; Perry and Kelley, 1970). The data presented in Figure 4.2.15 show that TCDD double the half-life of the CYP19 mRNA by approximately 5 hours to 10 hours. The addition of ERK inhibitor, U0126, could successfully abolish the TCDD-induced CYP19 mRNA expression (Figure 4.2.16). These data suggested that the increase in CYP19 mRNA was caused by a post-transcriptional effect of TCDD on the stability of CYP19 mRNA, possibly was mediated by ERK.

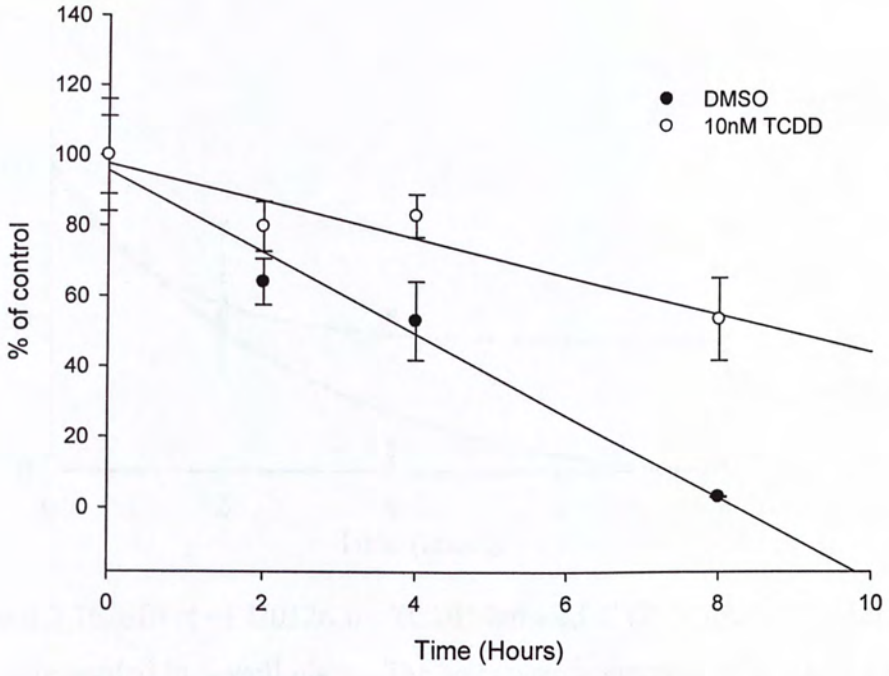

Figure 4.2.15 Effect of TCDD on CYP19 mRNA half-life. MCF-7 cells were seeded in 6-well plates. After 48-hour treatment with 10ηM TCDD, transcription was stopped by 4μM actinomycin D. Total RNA was isolated and analyzed by real time PCR as described in Figure 4.2.11. Values are presented as percentage of RNA at 0 hour. Values are means ±SEM, n=3.

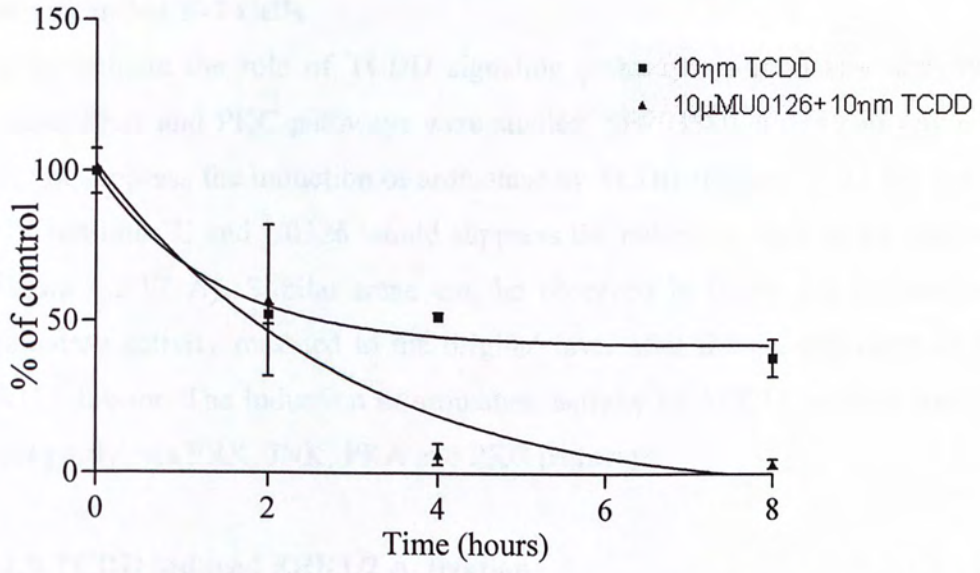

Figure 4.2.16 Effect of U0126 on TCDD-induced CYP19 mRNA half-life. MCF-7 cells were seeded in 6-well plates. The cells were pretreated with 10μM U0126 for 3 hours before adding 10ηM TCDD for further 48 hours. The transcription was stopped by 4μM actinomycin D. Total RNA was isolated and analyzed by real time PCR as described in Figure 4.2.11. Values are presented as percentage of RNA at 0 hour. Values are means ±SEM, n=3.

4.2.8 Role of MAP Kinase, PKA and PKC in genistein induced aromatase activity in MCF-7 Cells

To investigate the role of TCDD signaling pathway on aromatase activity, MAP kinase, PKA and PKC pathways were studied. SB203580, a p38 pathway inhibitor, did not suppress the induction of aromatase by TCDD (Figure 4.2.17 B). In contrast, JNK inhibitor II and U0126 would suppress the induction back to the original level (Figure 4.2.17 A). Similar trend can be observed in figure 4.2.18, the increased aromatase activity returned to the original level after the pre-treatment of PKA or PKC inhibitor. The induction of aromatase activity by TCDD could be mediated, at least partly, via ERK, JNK, PKA and PKC pathways.

4.2.9 TCDD induced ERK1/2 Activation

To further study the role of TCDD in ERK signaling pathway, ERK 1/2 phosphorylation was studied. It was found that TCDD could not induce the inactive form of ERK, but increase the phosphorylation of ERK 1/2 in a dose dependent manner in MCF-7 cells (Figure 4.2.19). This suggested that TCDD might up-regulate aromatase expression through of ERK 1/2 pathway.

4.2.10 Induction of aromatase activity in MCF-7_{ERK} cells

To investigate the relationship between ERK and aromatase, ERK over-expressing MCF-7 cells (MCF-7_{ERK}) and the vector control cells (MCF-7_{vec}) had been studied. When MCF-7_{ERK} cells were incubated with [1β-^{3}H(N)]-androst-4-ene-3,17-dione for 1 day, the aromatase activity was around 1.5 times higher than the control vector cells (Figure 4.2.20). The aromatase protein expression (Figure 4.2.21) and mRNA expression (Figure 4.2.22) in MCF-7_{ERK} cells were higher than those in the MCF-7_{vec} cells. The mRNA expression in MCF-7_{ERK} cells was nearly 5 times higher than the control. However, the aromatase promoter activities in MCF-7_{ERK} and MCF-7_{VEC} cells are nearly the same (Figure 4.2.23). The half life of CYP19 mRNA in MCF-7_{ERK} cells was slightly higher than the control cells (Figure 4.2.24). The induction mechanism would be through post-transcriptional control. Hence, over-expressing ERK in MCF-7 cells would induce the expression of aromatase. This implicated that ERK played an important role in the regulation of aromatase in MCF-7 cells.

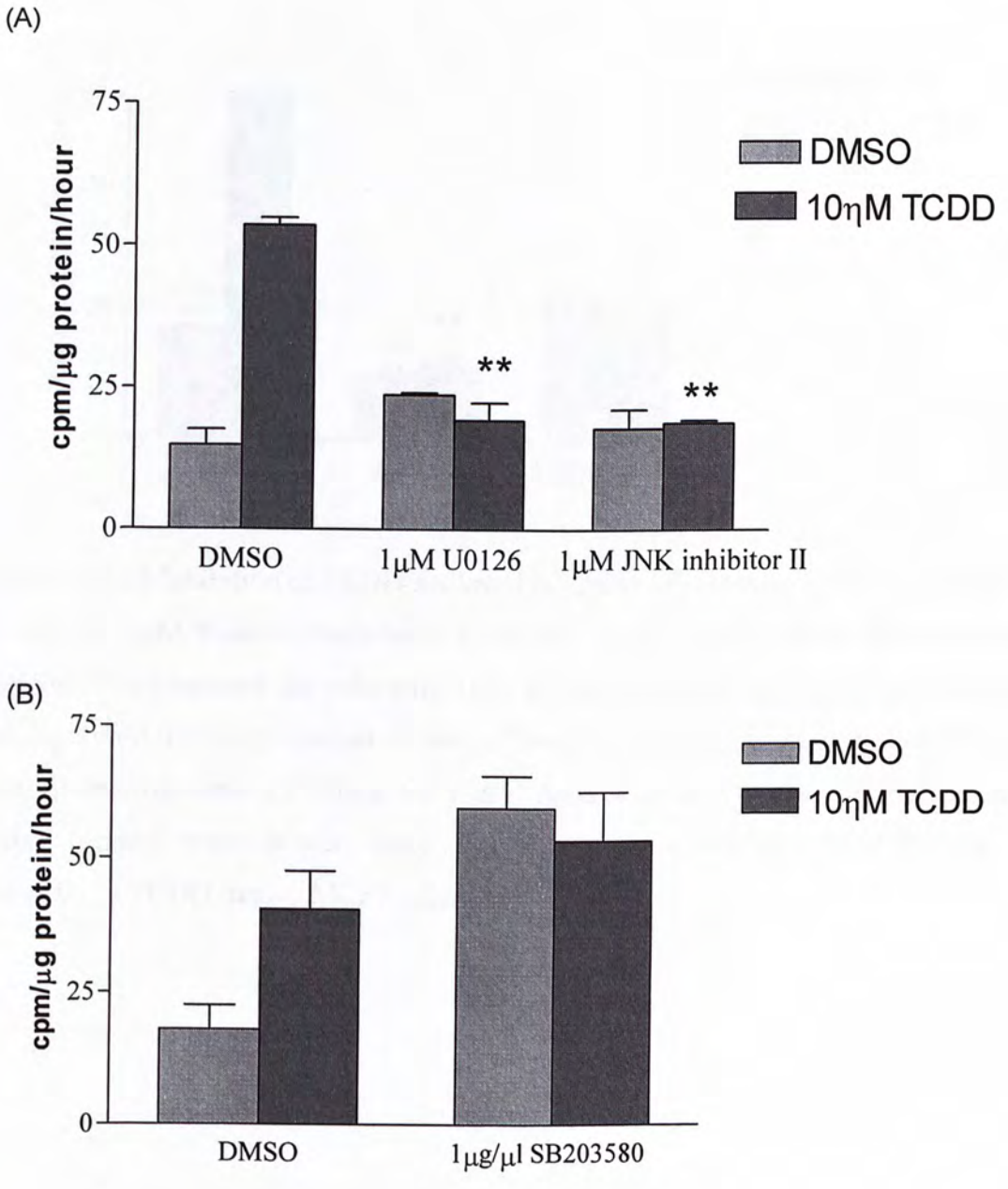

Figure 4.2.17 Inhibition of TCDD-mediated induction of aromatase activity in MCF-7 cells by (A) U0126 and JNK inhibitor II; (B) 1μg/μl SB203580. Pre-treatment the cells with (A) 1μM U0126 and 1μM JNK inhibitor II; (B) 1μg/μl SB203580 for 3 hours before adding 10ηM TCDD for further 48 hours. The cells were further incubated with [1β-^{3}H(N)]-androst-4-ene-3,17-dione for 1 day. Aromatase activity was determined by using tritiated water release assay. Values are means ±SEM, n=3. (**P<0.005)

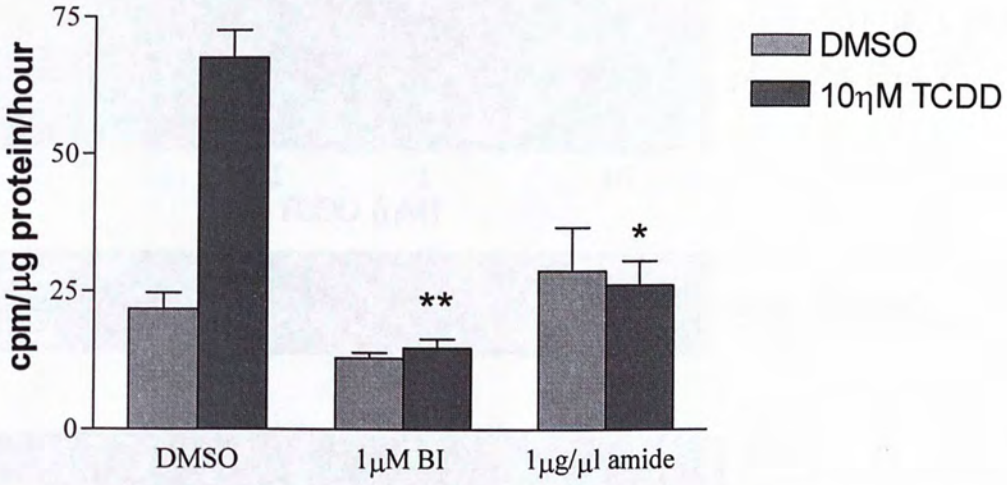

Figure 4.2.18 Inhibition of TCDD-mediated induction of aromatase activity in MCF-7 cells by 1μM bisindolylmaleimide I (BI) and 1μg/μl myristoylated 14-22 amide (amide). Pre-treatment the cells with 1μM BI and 1μg/μl amide for 3 hours before adding 10ηM TCDD for further 48 hours. The cells were further incubated with [1β-^{3}H(N)]-androst-4-ene-3,17-dione for 1 day. Aromatase activity was determined by using tritiated water release assay. Values are means ±SEM, n=3. **P<0.005; *P<0.05 vs TCDD treated MCF7 cells.

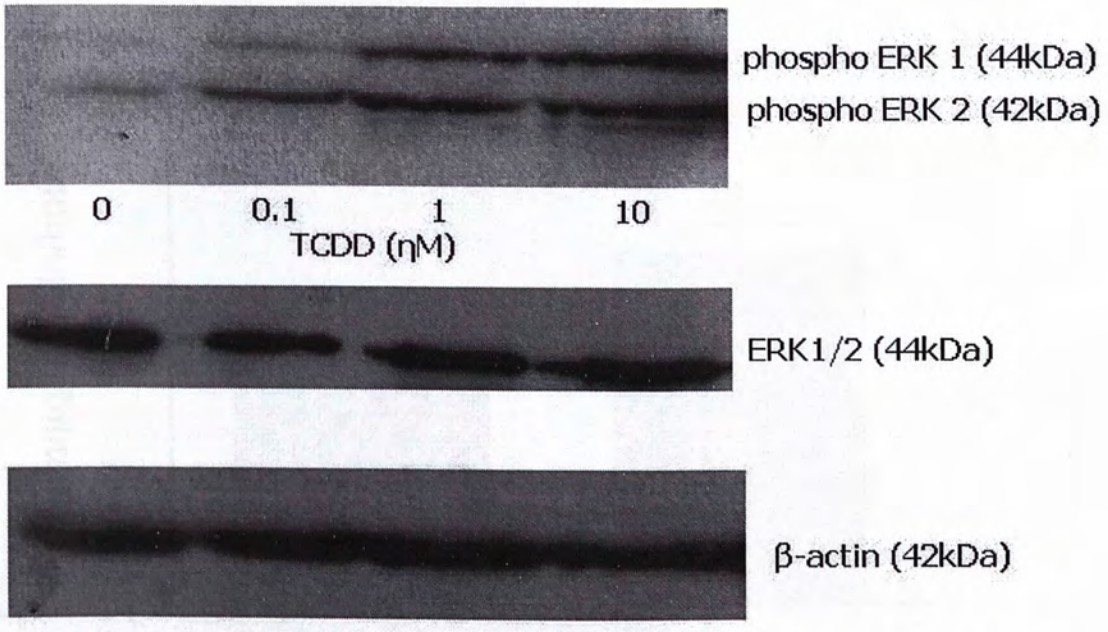

Figure 4.2.19 Effect of TCDD on phospho-ERK1/2 expression. MCF-7 cells were treated with various concentration of TCDD for 48 hours. Total protein was collected and the amount of phospho-ERK1/2 was detected using Western Blot. The molecular weight of phospho-ERK1/2, ERK1/2 and β-actin were 44/42kDa, 44kDa and 42kDa respectively.

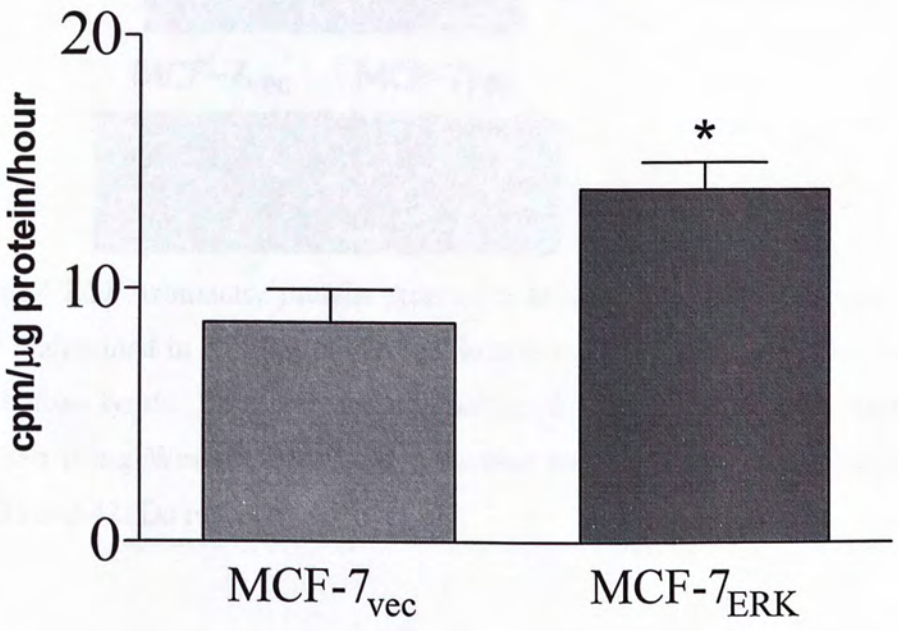

Figure 4.2.20 Aromatase activity in MCF-7_{vec} and MCF-7_{ERK} cells. Cells were maintained in RPMI medium and switch to charcoal treated RPMI medium one day before doing the assay. In the next day, the cells would incubate in serum-free medium with the addition of [1β-^{3}H(N)]-androst-4-ene-3,17-dione for 1 day. The activity was determined by tritiated water release assay. Values are the means ±SEM, n=3. (* $P<0.05$)

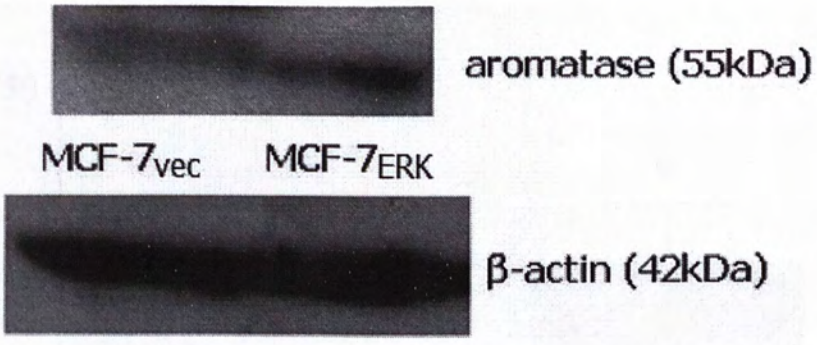

Figure 4.2.21 Aromatase protein expression in MCF-7_{vec} and MCF-7_{ERK} cells. Cells were maintained in RPMI medium and switch to charcoal treated RPMI medium one day before lysate. Total protein was collected and the amount of aromatase was detected using Western Blot. The molecular weights of aromatase and β-actin are 55kDa and 42kDa respectively.

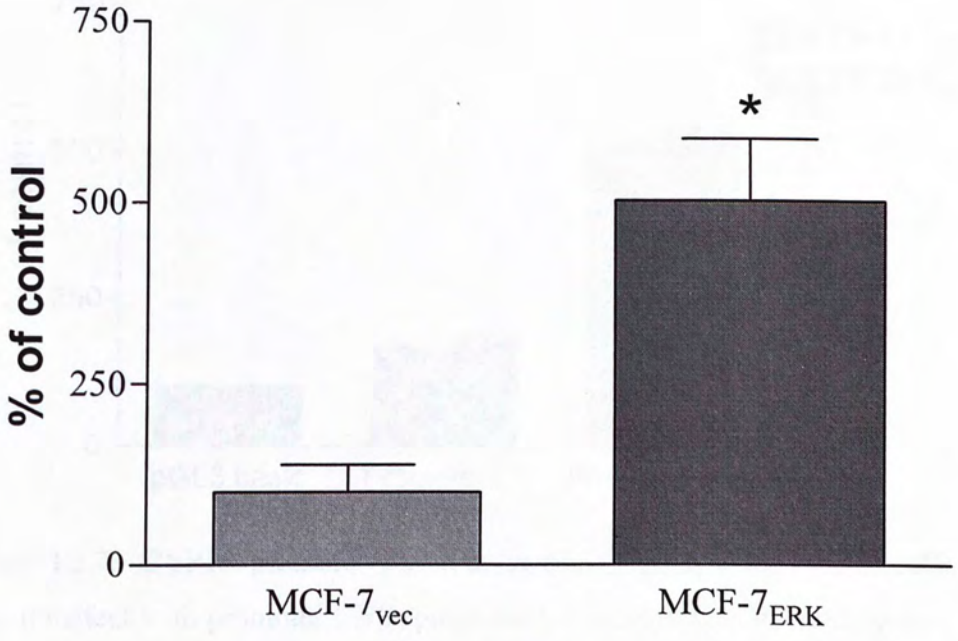

Figure 4.2.22 CYP19 mRNA expression in MCF-7_{ERK} cells. mRNA were extracted in MCF-7_{vec} and MCF-7_{ERK} cells. The amount of CYP19 mRNA was determined by relative quantitative real time PCR. The expression of CYP19 mRNA was normalized by GAPDH. Values are means ±SEM, n=3.

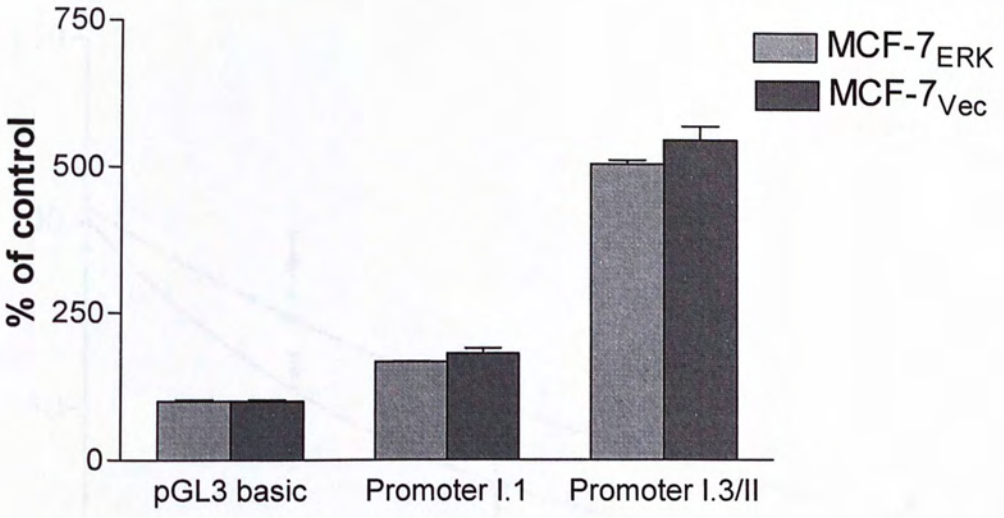

Figure 4.2.23 CYP19 promoter activities in MCF-7_{vec} and MCF-7_{ERK} cells. Cells were transfect with promoter I.3/II, promoter I.1 reporter plasmid and control vector PRL-CMV. After 24 hours, the cells were lysed and dual luciferase assay was performed. Values are means ±SEM, n=3.

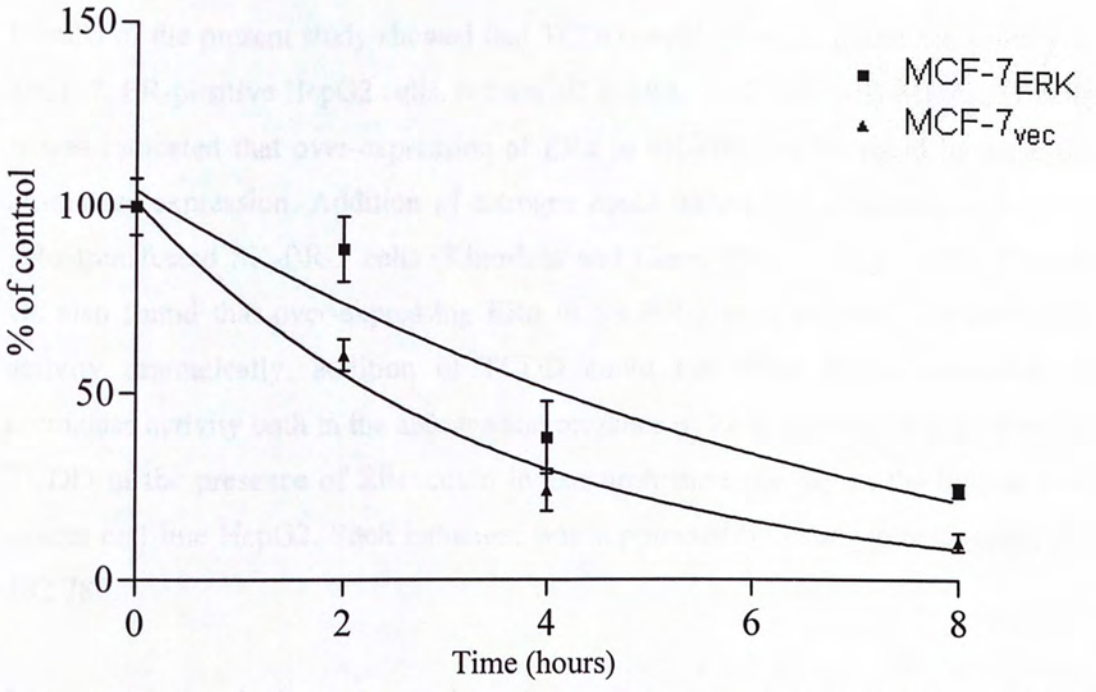

Figure 4.2.24 The CYP19 mRNA half-life in MCF-7_{ERK} cells. MCF-7_{vec} and MCF-7_{ERK} cells were seeded in 6-well plates for 24 hours. The transcription was stopped by 4μM actinomycin D. Total RNA was isolated and analyzed by real time PCR as described in Figure 4.2.11. Values are presented as percentage of RNA at 0 hour. Values are means ±SEM, n=3.

4.3 DISCUSSION

Results of the present study showed that TCDD could increase aromatase activity in MCF-7, ER-positive HepG2 cells, but not MCF-10A, T47D and MB-MDA-231 cells. It was indicated that over-expression of ERα in SK-BR-3 cells would increase the aromatase expression. Addition of estrogen could induce the aromatase activity in ERα-transfected SK-BR-3 cells (Kinoshita and Chen, 2003; Wang, 2005). Though we also found that over-expressing ERα in SK-BR-3 cells elevated the aromatase activity dramatically, addition of TCDD could not show further induction of aromatase activity both in the absence and presence of ERα. Surprisingly, addition of TCDD in the presence of ERα could induce aromatase activity in the human liver cancer cell line HepG2. Such induction was suppressed by estrogen antagonist, ICI 182 780.

Most researches, both *in vitro* and *in vivo* studies, showed the anti-estrogenicity of TCDD (Safe *et al.,* 1991; Gallo *et al.,* 1986; Gierthy *et al.,* 1987). It has been documented that TCDD decreases aromatase activity in human choriocarcinoman cells (Drenth *et al.,* 1998). However, the estrogenic effect of TCDD has seldomly been reported. Recently, an environmental toxicants 2,2-bis(p-chlorophenyl)ethylene (p,p'-DDE) and TCDD have been shown to increase aromatase activity in cultures of endometrial stromal cells (Holloway *et al.*, 2005; Stys *et al.,* 2005). Results in this study showed that TCDD could also induce aromatase activity in MCF-7 cells. The cells treated with 10ηM TCDD for two days had double or triple aromatase activity than the control. Induction of aromatase expression by TCDD occurred within the same concentration range. Additional experiments performed with human CYP19 Supersomes® indicated that this induction was unlikely a direct stimulatory effect of TCDD on the aromatase enzyme complex. This result is similar to the one performed in microsomal fractions of human placental tissue (Drenth *et al.,* 1998).

TCDD has previously been found to increase aromatase mRNA expression in endometrial stromal cells (Stys *et al.,* 2005). In this study, we observed comparable results in MCF-7 cells. It is demonstrated that promoters II and I.3 are the major promoters driving aromatase expression in MCF-7 cells (Mu *et al.,* 2000), bearing consensus sequences of AP-1 at -498 and -935 (Zhou D *et al.*, 1996). In our study,

MCF-7 was shown to use promoter II, I.3 and I.1. However, the exon I.1, II and I.3 expressions surprisingly were not up-regulated. There was no induction observed in promoters I.1; I.3/II activities nor AP-1 transactivation activity after the treatment of TCDD. Hence, it appeared that TCDD mediated aromatase activity through an atypical transcriptional control in MCF-7 cells.

Post transcriptional regulations, which encompass nuclear RNA processing, exporting RNA from the nucleus and mRNA degradation, have become increasingly important in the regulation of cytoplasmic mRNA levels. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) decreases ER levels through a post-transcriptional destabilization of the ER mRNA (Saceda *et al.,* 1991). Estradiol decreases ER expression in MCF-7 cells predominantly by a post-transcriptional mechanism (Saceda *et al.,* 1998). The data in present study suggested that TCDD could increase the half-life of CYP19 mRNA. The mechanism of CYP19 mRNA decay needs further investigation.

MCF-7 is a ER-positive cell line derived from a metastatic adenocarcinoma of human breast, which is widely used for studying estrogen-dependent mechanism. Addition of estrogen antagonist ICI 182 780 could abolish the induction of aromatase by TCDD. This suggested ERα might play an important role in the induction mechanism. However, TCDD could not show induce the activity in T47D, which is also a ERα-positive breast cancer cell line. Hence, ERα alone could not give a whole picture of the story. It is widely accepted that the major effect of TCDD is mediated through the activation of aryl hydrocarbon receptor (AhR) (Nebert *et al.,* 1993; Okey *et al.,* 1994; Chen *et al.,* 2004). Wild-type MCF-7 and Hepa/1c1c7 cells are Ah-responsive. TCDD decreases ER levels in these cells, but not in two mutant Ah non-responsive Hepa 1c1c7 cell lines (Harris *et al.,* 1990; Zacharewski *et al.,* 1991). Baba *et al.* (2005) has demonstrated that AhR is crucial in female reproduction by regulating the expression of ovarian aromatase. AhR cooperates with an orphan nuclear receptor, Ad4BP/SF-1, to activate aromatase gene transcription in ovarian granulosa cells. Functional cross talk between AhR and ER has been reported (Wormke *et al.,* 2000; Ohtake *et al.,* 2003). The ligand-bound AhR activates the ligand-less ER to interact with ERE in target gene promoter (Ohtake *et al.,* 2003). By

studying the role of AhR in TCDD-induced aromatase might give us some clues in the complex mechanism in the future.

ERα is phosphorylated and activated by several signaling kinases, including ERK 1/2, p38 and PKA (Kato *et al.,* 1995; Lee and Bai, 2002; Cho and Katzenellenbogen, 1993; Chen D. *et al.,* 1999; Loven *et al.,* 2004). Estrogen can cause rapid activation of MAPK pathway in mammalian cells through ligand-binding with ER (Lu *et al.,* 2004). Our lab also reported that estrogen could induce the phosphorylation of ERK 1/2 in ERα-transfected SK-BR 3 cells (Wang, 2005). Hence, the importance of signaling kinase should not be ignored. Previous studies on interaction of TCDD with aromatase have not investigated the involvement of signaling pathways (Drenth *et al.,* 1998; Stys *et al.,* 2005). In this project, we investigated the MAP kinase and protein kinase pathways by adding the corresponding inhibitors. The results showed that ERK, JNK, PKA and PKC could take part in the regulation process.

Brodie *et al.* (2005) have implanted aromatase over-expressing MCF-7 cells into ovariectomized and nude mice. They found that tumor cells adapt to estrogen deprivation during letrozole treatment by activation of p-Raf, p-MEK1/2 and p-MAPK. Previously, our lab successfully established an ERK over-expressing MCF-7 cell line. The aromatase activity, protein, mRNA expression and mRNA stability in MCF-7_{ERK} cells were higher than that of the control cells. Hence, ERK is a regulatory factor of aromatase in MCF-7 cells. TCDD could increase the ERK protein expression in MCF-7 in a dose-dependent manner. Addition of ERK inhibitors would abolish the TCDD-induced aromatase activity in MCF-7 cells. Hence, the observed modulation of the aromatase activity could be ERK-mediated.

To summarize, TCDD could increase the aromatase activity in MCF-7 cells. Though TCDD could not up-regulate CYP19 transcriptional activity, it increased the mRNA stability by way of ERK activation. Hence, the aromatase mRNA, protein expression and enzyme activities were increased. Further investigation should be carried out in order to elucidate the mechanism.

CHAPTER 5 SUMMARY

Aromatase catalyzes the hydroxylation reactions converting androstenedione and testosterone into estrone and estradiol respectively (Simpson *et al.*, 1994). It is generally accepted that estrogen is important in the prevention of the cardiovascular disease. 17β-estradiol (E_2) has been shown to up-regulate apoA1 and LDLR in HepG2 cells (Yuen, 2005; Lamon-Fava *et al.*, 1999). In the present study, we demonstrated that genistein could significantly increase the aromatase activity in the ERα-transfected HepG2 cells. AP-1 and p38 pathway might be involved in the induced aromatase activity in ERα-transfected HepG2 cells (Figure 5.1.A). As estrogen can trigger a wide-range of gene transcriptions in liver, including apoA1 and LDLR, this study provided a new insight for the gene-regulatory mechanism of genistein.

On the other hand, estrogen exposure has been proven to be a negative component in breast cancers. In the present study, effect of TCDD on aromatase was evaluated. Among all the breast cancer cells tested, TCDD could only increase the aromatase activity in MCF-7 cells possibly through a post-transcriptional regulation (Figure 5.1.B). The mRNA degradation results showed that TCDD could increase the aromatase mRNA stability. In studying the ERK over-expressing MCF-7 cells, there was a close relationship between ERK and aromatase activity. The observed modulation of the aromatase activity by TCDD could be ERK-mediated.

To conclude, genistein and TCDD could mediate the aromatase activities in HepG2 and MCF-7 cells respectively. The results obtained provide a better insight of the genistein and TCDD induced aromatase mechanisms. The findings could better our understanding of the estrogenic effect of genistein and the pathway involved in TCDD-induced breast cancer. These may rationalize the use of genistein for post-menopausal application.

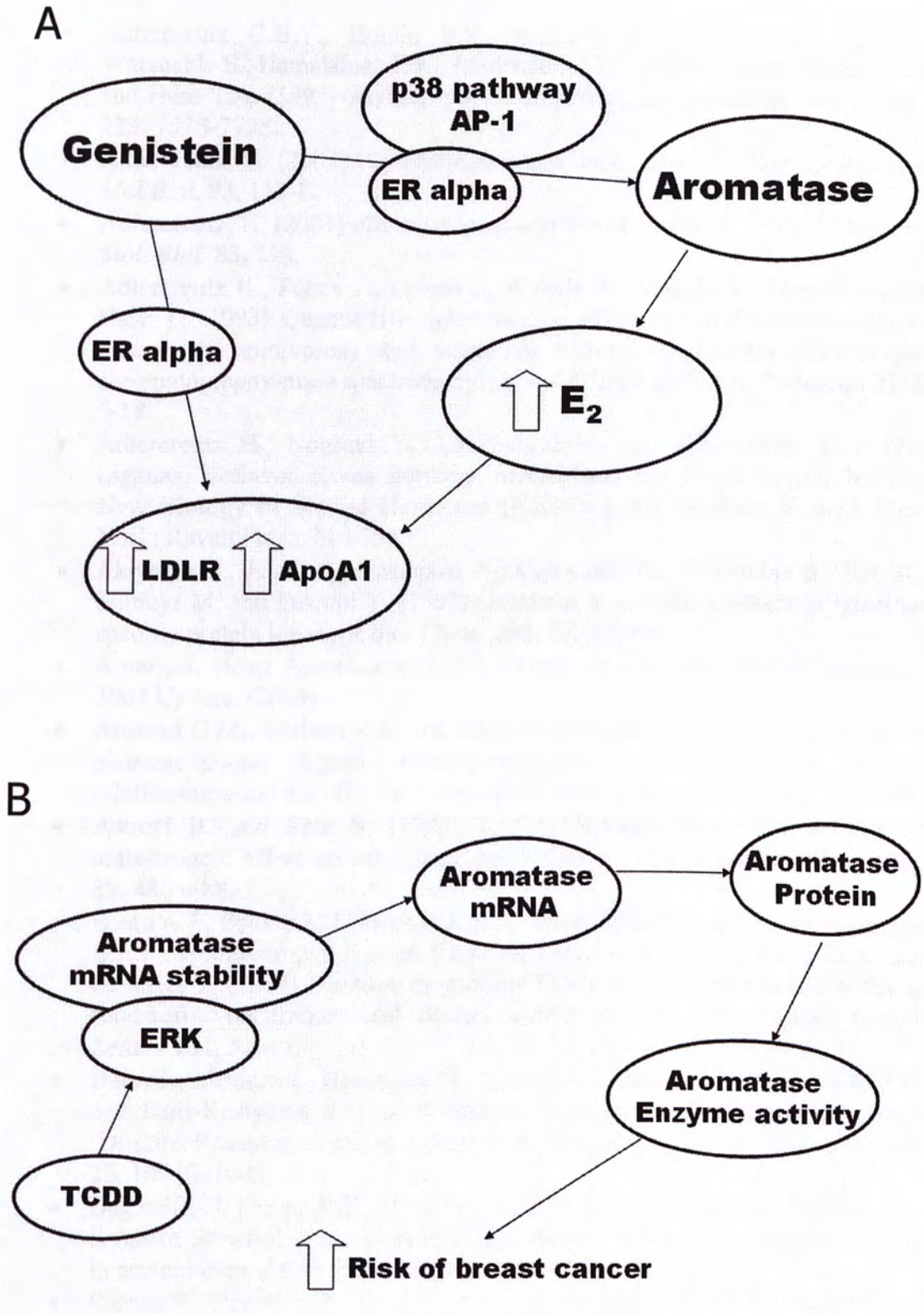

Figure 5.1 Schematic representation of the effect of (A) genistein and (B) TCDD on aromatase.

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