[UCI]I804:24011-200000234537

Modulatory Effects of o,p'-dichlorodiphenyltrichloroethan on Genes Involved in Drug Metabolism and Inflammation

내분비계 장애물질인 유기염소계 살충제의 약물대사 및
염증 관련 유전자의 조절에 관한 연구

2005 年 8 月 日

朝 鮮 大 學 校 大 學 院

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Modulatory Effects of o,p'-dichlorodiphenyltrichloroethan on Genes Involved in Drug Metabolism and Inflammation

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이 論文을 藥學博士學位申請 論文으로 提出함.

2005年 4月 日

朝 鮮 大 學 校 大 學 院

藥 學 科

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DDT	dichlorodiphenyltrichloroethane
o,p'-DDT	o,p'-trichloro-2-(p-chlorophenyl)-2-(o-chlorophenyl) ethane
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
EROD	7-ethoxyresorufin *O*-deethylase
DRE	dioxin-response element
ERE	estrogen responsive element
AhR	aryl hydrocarbon receptor
CYP	cytochrome P450s
RT-PCR	reverse transcriptase-polymerase chain reaction
FBS	fetal bovin serum
PGE_2	prostaglandin E_2
PMA	phorbol 12-myristate 13-acetate
EP receptor	PGE_2 receptors
COX	cyclooxygenase
Aromatase	cytochrome P450 aromatase
EMSA	electrophoretic mobility shift assay
RIA	radioimmunoassay
P450scc	cytochrome P450 cholesterol side-chain cleavage enzyme
StAR	steroidogenic acute regulatory protein

3β-HSD	3β-hydroxysteroid dehydrogenase
StAR	steroidogenic acute regulatory
$P450_{17\alpha}$	P450 17α-hydroxylase
cAMP	cyclic AMP
CRE	cyclic AMP response elements
iNOS	inducible nitric oxide synthase
NO	nitric oxide
MAPKs	mitogen-activated protein kinases
ERK	extracellular signal regulated protein kinases
JNK	c-Jun N-terminal kinases
NF-κB	nuclear factor-κB
AP-1	activator protein 1
NF-IL6	nuclear factor-interleukin-6
PKC	protein kinase C

Modulatory effects of o,p’-dichlorodiphenyl -trichloroethan on genes involved in drug metabolism and inflammation

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Abstract

Down-regulation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced CYP1A1 gene expression by o,p’-DDT in murine heap-1c1c7 cells

Cultured mouse hepatoma Hepa-1c1c7 cells were treated with o,p'-DDT and/or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to assess the role of o,p'-DDT in CYP1A1 expression. o,p'-DDT alone did not affect CYP1A1-specific 7-ethoxyresorufin *O*-deethylase (EROD) activity. In contrast, TCDD-inducible EROD activities were markedly reduced upon concomitant treatment with TCDD and o,p’-DDT in a dose dependent manner. Treatment with ICI 182.780, an estrogen-receptor antagonist, did not affect the

suppressive effects of o,p'-DDT on TCDD-inducible EROD activity. TCDD-inducible CYP1A1 mRNA levels were markedly suppressed upon treatment with TCDD and o,p'-DDT, and this consistent with their effects on EROD activity. A transient transfection assay using dioxin-response element (DRE)-linked luciferase and an electrophoretic mobility shift assay revealed that o,p'-DDT reduced the transformation of the aryl hydrocarbons (Ah) receptor to a form capable of specifically binding to the DRE sequence in the promoter region of the CYP1A1 gene. These results suggest that the down regulation of TCDD-induced CYP1A1 gene expression by o,p'-DDT in Hepa-1c1c7 cells might be an antagonism of the DRE binding potential of the nuclear Ah receptor but is not mediated through the estradiol receptor.

o,p'-DDT reduced testosterone production via induction of aromatase (*CYP19*) gene expression in rat testicular Leydig cells

Various pesticides known or suspected to interfere with steroid hormone function were screened for determining effects on catalytic activity and mRNA expression of aromatase in leydig cells. Aromatase (*CYP19*) is the cytochrome P450 enzyme complex that converts C19 androgens to C18 estrogens. In this work, the effect of o,p'-DDT on steroid hormone through aromatase activity and its molecular mechanism were investigated in testicular leydig R2C cells by using radioimmunoassay (RIA). Treatment with o,p'-DDT caused a dose-dependent inhibition of testosterone (T) production in R2C cells. o,p'-DDT-induced inhibition of testosterone production is related to a decreased in the gene expression of cytochrome P450 17α-hydroxylase ($P450_{17\alpha}$) and 3β-hydroxysteroid dehydrogenase (3β-HSD). o,p'-DDT was found to increase aromatase activity and gene expression in R2C cell in a dose dependent manner. Furthermore, the inducible effects of o,p'-DDT on aromatase gene expression by the ERβ mediates the inducible effects of o,p'-DDT. Our hypothesis is that higher levels of COX-2 expression result in higher levels of prostaglandin E_2 (PGE_2), which in turn increases *CYP19* expression through increases in intracellular cyclic AMP levels. Therefore, whether o,p'-DDT in

Effect of o,p'-DDT on aromatase gene expression through cyclooxygenase-2 (COX-2) was investigated in R2C cells. T increased aromatase gene expression by o,p'-DDT is mediated through increased PGE_2 production by o,p'-DDT-induced COX-2 gene expression. Overall, by elevated levels of these factors in R2C cells o,p'-DDT could result in increased aromatase activity *via* autocrine mechanisms in R2C cells. From studies to determine whether effects of o,p'-DDT on aromatase gene expression might be influenced by EP, it was found that the EP_2 and EP_4 mediates the inducible effects of o,p'-DDT. In summary, these results demonstrated that o,p'-DDT-induced inhibition of T production in R2C cell might be mediated through aromatase gene expression *via* ERβ, EP_2, and EP_4 which might be influenced by COX-2 and PGE_2.

Up-regulation of cyclooxygenase-2 and iNOS gene expression in macrophages exposed to the o,p'-DDT

A number of reports have indicated that DDT may act as an endocrine disruptor and that DDT has possible carcinogenic effects. o,p'-DDT has been reported to possess immunomodulatory activity. However, its influence on cytokine production or the functions of the macrophages remains unclear. Macrophages are crucial for the inflammatory response because they can release a number of proinflammatory mediators. The purposes of this study were to test the hypothesis that o,p'-DDT induces COX-2 gene expression in macrophages and that this is regulated at the level of mitogen-activated protein kinases (MAPKs). In addition, effects of o,p'-DDT on the production of nitric oxide (NO) and proinflammatory cytokines (IL-1β, IL-6, TNF-α) were investigated to characterize the underlying molecular mechanism in mouse macrophages. Exposure of the murine macrophage cell line RAW 264.7 to o,p'-DDT for 24 h markedly enhanced the production of PGE_2, a major COX-2 metabolite. PGE_2 elevation was preceded by increases in the epression of COX-2 mRNA and COX-2 protein in o,p'-DDT-treated cells. o,p'-DDT induced rapid phosphorylation of ERK, p38 and JNK phosphorylation. To investigate the significant cis-acting regions in COX-2

promoter, transient transfection experiments were carried out using reporter vectors harboring deleted COX-2 promoters. The transcriptional factor binding sites for activator protein 1 (AP-1) appeared be important for the induction of COX-2 by o,p’-DDT. These results suggested that the induction of transcriptional activation of COX-2 by o,p’-DDT might be mediated through AP-1 activation.

The addition of o,p'-DDT to macrophages induced NO and proinflammatory cytokines production in a dose-dependent manner. o,p'-DDT also increased inducible nitric oxide synthase (iNOS) and proinflammatory cytokines expression levels in the cells. NF-κB sites were identified in the promoter of the iNOS and proinflammatory cytokine genes. Pretreating the cells with NF-κB pathway inhibitors suppressed the iNOS and proinflammatory cytokines expression induced by o,p'-DDT. The transient expression and electrophoretic mobility shift assays with the NF-κB binding sites revealed that o,p'-DDT-induced increase in the iNOS and proinflammatory cytokines expression level were mediated by the NF-κB transcription factor. However, pretreating the cells with o,p'-DDT and the NF-κB pathway inhibitors suppressed o,p’-DDT-induced NF-κB activation. These results demonstrated that o,p'-DDT stimulates the production of NO and proinflammatory cytokines and can up-regulate the gene expression

levels via NF-κB transactivation. Overall, the results of this study suggested for the first time that o,p'-DDT might possess an inflammatory potential that is previously unrecognized immunomodulating activity of o,p’-DDT.

Introduction

Down-regulation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced CYP1A1 gene expression by o,p'-DDT in murine heap-1c1c7 cells

DDT [1,1,1-trichloro-2,2-bis (*p*-chlorophenyl) ethane] was a commonly used pesticide in home gardens and in agriculture. Technical grade DDT contains 80% and 20% of the *p,p′*-DDT and *o,p′*-DDT isomers, respectively, and it has been used for almost 50 years, mainly for malaria control and other vector-ransmitted diseases. Although DDT has been banned in many countries (e.g. the USA in the 1970s) it is still prevalent in our ecosystem. The toxicological effects of DDT in animal models include neurotoxicity, hepatotoxicity, and reproductive and metabolic disruption. It also alters the activity of hepatic mixed function oxidase [Conney, 1967] and induces expression of cytochrome P450 (CYP) 2B and 3A subfamilies in rodents [Lewis and Lake; 1997]. Its current use is restricted in many countries; however, because of its high efficacy and low mammalian toxicity, it is still used for mosquito vector control for malaria prevention. Due to its lipophilic nature and its slow chemical and biological degradation, DDT tends to be taken up by biological membranes and tissues, is concentrated in organisms, and progresses up through the food chain; it is thus considered major environmental contaminant in addition to polyaromatic hydrocarbons such as dioxins and heavy metals. It

is assumed that these compounds, alone or in combination, may promote human diseases [Auger *et al*., 1995]. Although more evident toxic signs by an acute exposure to DDT occur in the central nervous system, a few studies have suggested that chronic exposure to DDT alters sexual steroidal hormone homeostasis in wildlife and experimental animals. For example, female rats given o,p'-DDT (1,1,1-trichloro-2-(p-chlorophenyl)-2-(o-chlorophenyl) ethane) as neonates exhibited advanced puberty and persistent vaginal estrus in later life [Soto *et al*., 1994]. Environmental estrogens are a class of natural and synthetic compounds, which can mimic the function or activity of the endogenous estrogen 17β-estradiol. DDT has been shown to mimic estrogen [Soto *et al*., 1994]. Therefore, DDT is an "endocrine disruptor" that has a significant influence on sexual and reproductive development [Sharpe *et al*., 1995]. Human exposure to environmental compounds with estrogenic activity and their potential effects on human health is the subject of an ongoing scientific debate. These environmental estrogens may function as endocrine disruptors both in wildlife and humans, and lead to developmental defects, disease and, potentially, cancer [Colburn *et al*., 1993]. However, the mechanism by which DDT causes these adverse effects is unclear.

Cytochrome P450s (CYP) are a superfamily of heme-containing monooxygenase enzymes that metabolize foreign chemicals, such as drugs

and environmental chemicals, and endogenous compounds, like steroids and fatty acids [Nelson *et al*., 1996]. CYP1A1 is one of the xenobiotic metabolizing enzymes, which is induced by polycyclic aromatic hydrocarbons (PAHs). The most potent inducer of CYP1A1 is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). In addition, TCDD induces a broad spectrum of biochemical and toxic effects, such as teratogenesis, immunosuppression and tumor promotion. Most, if not all, of the effects caused by TCDD and other PAHs are known to be mediated by AhR (aryl hydrocarbon receptor or dioxin receptor) which has a high binding affinity to TCDD. The liganded AhR translocates from cytoplasm to nuclei where it switches its partner molecule from Hsp90 to Arnt. Thus formed AhR/Arnt heterodimer binds a specific DNA sequence designated XRE in the promoter region of the target genes including CYP1A1, UDP-glucuronosyl transferase and others to enhance their expression (Fig. 1).

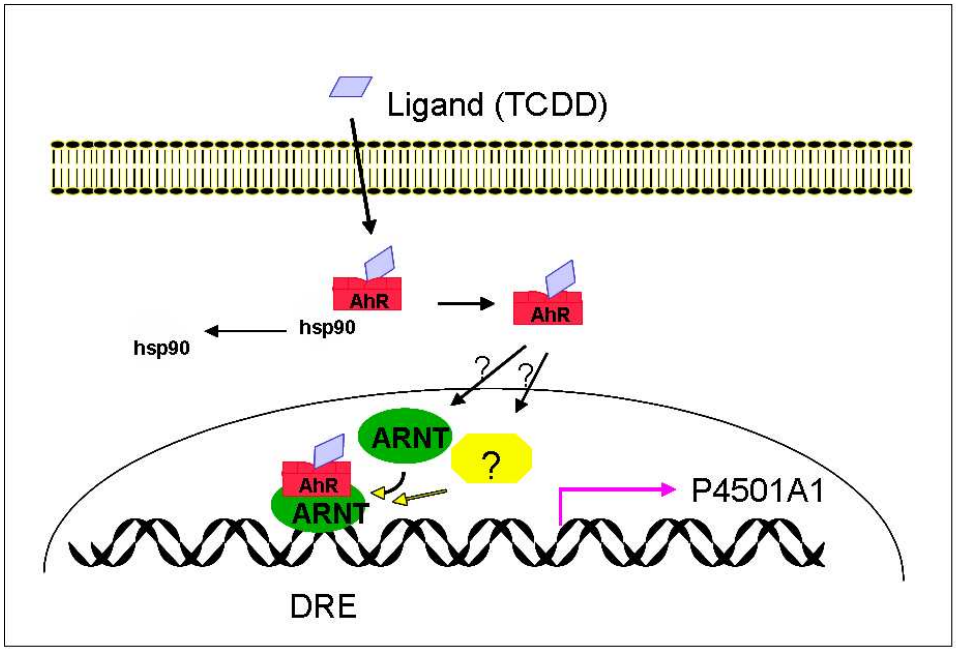


Fig. 1. Induction Pathway of Cytochrome P450 1A1

CYPs activate, in-activate, and facilitate the excretion of most xenobiotics, thus modulate both the durations and intensities of their respective toxicities. The levels of the gene expressions of these enzymes are influenced by a number of endogenous regulatory factors, such as hormones and xenobiotic substrates, including natural and synthetic chemicals [Nelson *et al*., 1996]. The effects of both natural and synthetic chemicals on the CYP enzymes are currently of considerable interests, as these effects may indicate a possible mechanism by which they affect the toxicity of environmental chemicals. It has been shown that CYP3A is normally responsible for steroid metabolism [Juchau, 1990], and there is also an indirect evidence which suggest that CYP1A is similarly involved [Aoyama *et al*., 1990]. Conversely, estradiol has been shown to inhibit CYP1A1 activity *in vitro* [Jeong and Lee, 1998]. CYP1A1 oxidatively biotransforms various polyaromatic hydrocarbons, like benzo(a)pyrene. Moreover, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a potent environmental contaminant that has been used as a model compound for investigating the mode of action of aryl hydrocarbons (Ah). The control and regulation of CYP1A1 gene expression have been extensively investigated, and it is currently believed that TCDD and its related Ah receptor agonists induce CYP1A1 gene expression through Ah receptor-mediated signal transduction. After binding with the ligand, the Ah receptor

forms a heterodimer with the Ah receptor nuclear translocator and binds to specific DNA recognition sequences known as dioxin-response elements (DREs), which are located upstream of the CYP1A1 transcription start site [Hankinson, 1995]. Binding to these enhancer sequences causes a change in the chromatin structure that facilitates the binding of the transcription factors to the CYP1A1 promoter [Ko *et al*., 1996].

DDT is a well-known inducer of microsomal monooxygenase systems in rodent liver. Based on the preferential induction of CYP2B and 3A subfamilies, DDT was classified as a Phenobarbital-type inducer [Nims *et al.*, 1998]. Furthermore, the preferential induction of CYP2B and 3A by DDT in a sex-related manner suggested that CYP regulation could play an important role in endocrine disruption [Sierra-Santoyo *et al*., 2000]. Studies on the expressions of CYPs are of considerable interests in understanding the toxicity of DDT on wildlife and human health. However, the molecular basis for the regulation of CYPs by DDT has not been well elucidated. o,p'-DDT is very similar in chemical structure to the synthetic estrogen, diethylstilbestrol. Moreover o,p'-DDT has been reported to have estrogenic activity [Soto *et al*., 1994]. In this regard reported that 17β-estradiol suppress CYP1A1 in Hepa-1c1c7 cells [Jeong and Lee, 1998]. However, the effects of o,p'-DDT on the regulation of CYP1A1 have not been investigated.

In the present study, the effect of o,p'-DDT on TCDD-induced CYP1A1 gene expression was investigated in mouse hepatoma Hepa-1c1c7 cells. The possible involvement of the estrogen receptor in this process was also investigated using the estradiol receptor antagonist, ICI 182,780. Our study suggested that o,p'-DDT down-regulates TCDD-induced CYP1A1 gene expression in Hepa-1c1c7 cells but do not act through the estrogen receptor in these cells.

o,p'-DDT reduced testosterone production via induction of aromatase (*CYP19*) gene expression in rat testicular Leydig cells

There are serious concerns that certain environmental contaminants and commercial products have the potential to disturb endocrine function in humans and wildlife, lead to impaired reproductive capacity, and have other toxic effects on sexual differentiation, growth, and development [Colborn *et al*., 1996]. Recently, Several studies have shown that exposure to DDT at early developmental stage results in altered sexual differentiation in male rats [Sanderson *et al*., 2002**]**. DDT, which has anti-androgenic properties [Kelce *et al*., 1997], has been reported to increase aromatase protein in rat [You *et al*., 2001].There is increasing evidence that certain environmental contaminants have the potential to disrupt endocrine processes, which may result in reproductive problems, certain cancers and other toxicities related to sexual differentiation, growth, and development. Testosterone biosynthesis requires five steroidogenic proteins: steroidogenic acute regulatory protein (StAR), cholesterol side chain cleavage enzyme (P450scc), CYP17, 17β-hydroxysteroid dehydrogenase (17β-HSD), and 3β-HSD. In the mitochondria, cholesterol is converted to pregnenolone by P450scc. Pregnenolone is then converted sequentially to progesterone by 3β-HSD, to 17α-hydroxyprogesteron and then to androstenedione by CYP17, and finally to testosterone by 17β-HSD (Fig. 2).

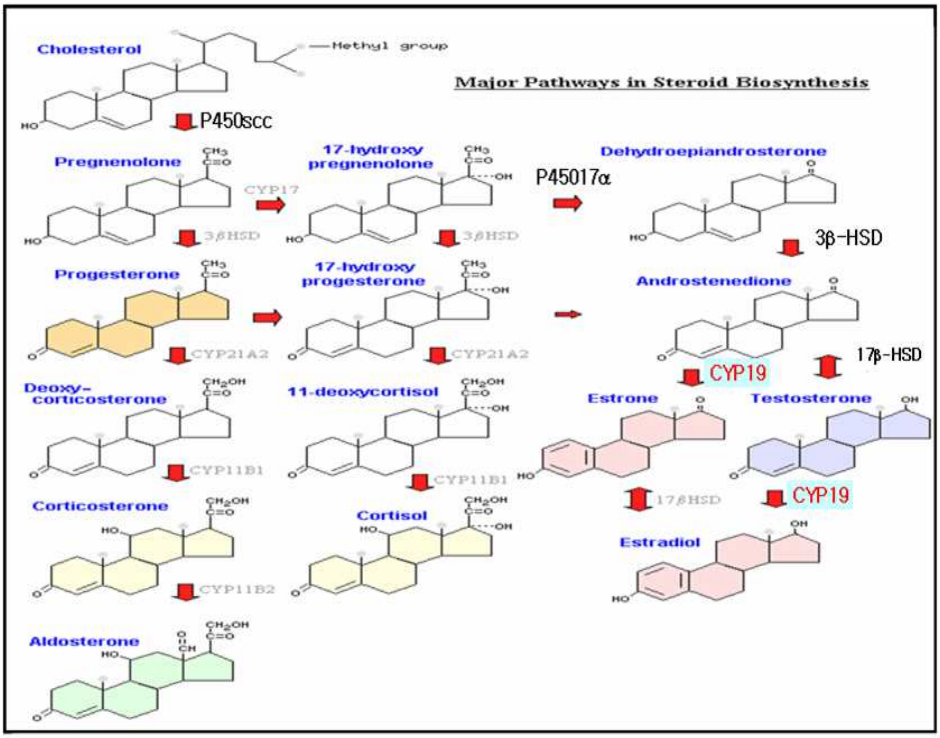


Fig. 2. Major pathways in steroid biosynthesis.

The biosynthesis of estrogens from testosteron is catalyzed by an enzyme complex which has been called aromatase (*CYP 19*, cytochrome P450arom) [Simpson *et al*., 1994]. P450arom is present in many tissues, including the gonads, brain, placenta, bone and adipose tissue [Simpson *et al*., 1994].

The expression of aromatase is controlled by regulatory pathways involving gonadotropins, steroid hormones, and growth factors [Roselli and Resko, 1997]. In addition to steroidogenic tissues, this reaction occurs at a number of nonsteroidogenic sites and serves various physiological roles through the production of estrogens. Similar to other CYP enzymes, aromatase expression is also responsive to environmental factors, including nutritional elements and chemical exposures [Roselli and Resko, 1997]. A number of synthetic compounds have been shown to affect aromatase, both *in vivo* and *in vitro* [Smith., 1996]. Aromatase was expressed in a variety of cell types, including tumor, stromal, adipose, and endothelial cells. Prostaglandin G/H endoperoxide synthase, also known as Cyclooxygenase (COX), is a key enzyme which catalyzes the conversion of arachidonic acid to prostaglandins. Two isoforms have been identified, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) [Smith., 1996]. Even though COX-1 is present at a constant level in most cells and tissues and is believed to play a housekeeping role, some studies have shown that COX-1 activity and

expression is elevated in human breast cancer tumors [Hwang *et al*., 1998]. Most studies have shown that COX-2 is present in breast cancer tissue samples but not in the normal breast tissue [Hwang *et al*., 1998]. Prostaglandins produced by COX-2, predominantly prostaglandin E_2 (PGE_2), induce inflammation and are potent mediators of a number of signal transduction pathways that are implicated in cancer development.

The biological activities of PG are mediated by the activation of specific cell surface receptors. PGE_2 receptors (EP receptors) are pharmacologically distinguished into four different subtypes (EP_1-EP_4), each receptor encoded by a distinct gene and differently expressed in diverse target cells [Fedyk *et al*., 1996]. The PGE_2 receptor family, a member of the G protein-coupled receptor family, has been characterized as having four subtypes, EP_1-EP_4 [Funk *et al*., 1993]. The EP_2 and EP_4 receptors have been shown to couple with the G protein and to stimulate increases in cAMP [An *et al*, 1993; Bastien *et al*., 1994]. EP_3 receptors have been primarily shown to either couple to Ga;, thus inhibiting cAMP accumulation, or to modulate intracellular Ca changes, depending on the alternatively spliced form studied [Kunapuli *et al*., 1994]. EP_1 induces changes in intracellular calcium levels that are believed to be mediated either by the PLC signaling pathway, most likely via Ga_n, or via direct activation of a calcium channel

independent of PLC activation [Breyer *et al*., 2001]. Prostaglandins increases intracellular cAMP levels and stimulates estrogen biosynthesis [Zhao *et al*., 1996]. Local production of PGE_2 via the COX isozymes may influence estrogen biosynthesis. o,p'-DDT could result in increased aromatase activity via autocrine mechanisms in R2C cells (Fig. 3).

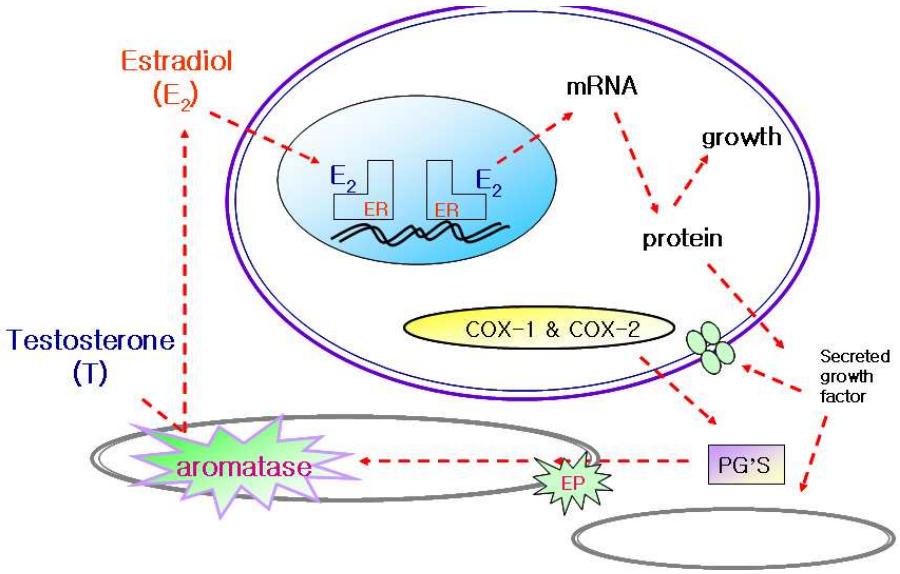


Fig. 3. Model of autocrine pathways in the regulation of expression of aromatase, COX-1, and COX-2.

Aromatase was strongly correlated with COX-2 expression. This finding suggests a possibility that the feminization seen in o,p'-DDT-exposed R2C cells may also be involved in decreased testosterone production. The current study suggested the possibility that o,p'-DDT inhibition of T production and in R2C cell is mediated through aromatase gene expression via ER and EP influenced by COX-2 and PGE_2 mediates.

Therefore, the objectives of the present study was to determine whether o,p'-DDT can affect the inhibition of T production through aromatase. Moreover, the inducible effects of o,p'-DDT on aromatase gene expression might be influenced by medicated COX-2 and PGE_2 in testicular leydig cell, R2C.

Up-regulatory effects of o,p'-DDT on cyclooxygenase-2 and iNOS gene expression in RAW 264.7 cells

Cyclooxygenase (COX) catalyzes the synthesis of prostaglandins (PGs) from arachidonic acid. Two isozymes, COX-1 and COX-2, have been identified which are encoded by separate genes. The COX-1 isozyme is believed to be a housekeeping protein in most tissues and appears to catalyze the synthesis of prostaglandins for normal physiological functions. In contrast, COX-2 is not present under normal physiological conditions but is rapidly induced in various cell types by tumor promoters, growth factors, cytokines and mitogens [Simon, 1999; Prescott and Fitzpatrick, 2000]. Increased expression of COX-2 in malignancy is likely to occur via multiple routes. COX-2 induction by lipopolysaccharide (LPS) has been shown to occur through both the MAPK and protein kinase C (PKC) pathways [Ridley *et al*., 1998]. It has also been shown that ceramide-stimulated activation of MAPK can activate c-Jun N-terminal kinase (JNK), which in turn can lead to increased COX-2 gene expression. This occurs via a cAMP response element (CRE) in the COX-2 promoter. Transient transfection experiments have demonstrated that nuclear factor-κB (NF-κB), nuclear factor IL-6 (NF-IL6) and CRE promoter sites mediate gene transcription independently in response to LPS treatment [Inoue *et al*, 1994; Inoue *et al*, 1995]. LPS can activate different pathways to induce COX-2 gene transcription: through NF-κB via

extracellular signal related kinase (ERK-2), p38 and JNK pathways, through NF-IL6 via a p38 pathway, and through CRE via ERK-2 and JNK pathways. Moreover, PKC signalling seems to mediate transcription after LPS treatment through all three promoter sites. Therefore, individual signalling pathways, such as ERK, p38, JNK or PKC, appear to be sufficient to mediate COX-2 gene transcription by virtue of their ability to recruit transcription factors to at least two promoter sites. This may indicate redundancy in the signalling pathways and promoter elements regulating COX-2 transcription, at least in endotoxin-treated cells of macrophage/monocyte lineage. Several inflammatory stimuli that induce COX-2 gene expression also activate the MAPKs [Guan *et al*., 1998; Scherle *et al*., 1998; Ridley *et al*., 1998; Xie *et al*., 1994]. MAPKs are a group of serine/threonine protein kinases comprising three well-characterized subfamilies: the p42/p44 extracellular regulated kinases ERKs, which also known as p42/p44 MAPKs; the c-Jun N-terminal kinases JNKs, which are also known as stress-activated protein kinases, and the p38 MAPKs [Mestre *et al*., 2001]. The MAPKs are important intermediates in various signaling pathways in many physiological processes, including cell growth, differentiation, and apoptosis [Wadleigh *et al*., 2000]. A major consequence of MAPK phosphorylation is the activation of these transcription factors [Wadleigh *et al*., 2000], which serve as immediate or downstream substrates of these kinases. ERK1/2 MAPK stimulates AP-1 activity by inducing c-Fos, which heterodimerizes with c-Jun

(Karin, 1995). p38 MAPK induces AP-1 activity by phosphorylating ATF-2. A heterodimer comprised of phospho-ATF-2 and c-Jun can induce c-Jun expression [whitmarsh and Davis, 1996]. JNK induces the expression and phosphorylation of c-Jun [Karin, 1995]. A recent report suggested that a tumor promotor, phorbol esters up-regulates PKC, stimulating MAPKs [Inoue *et al*., 1995]. Tumor promoting phorbol esters induce COX-2 gene expression by activating the PKC signal transduction pathway [Zhang *et al*., 1998]. More specifically, the AP-1 transcription factor is a downstream target of activated PKC that is implicated in inducing COX-2 and promoting carcinogenesis [Xie and Herschman, 1995].

It is reasonable to suggest that the capacity of o,p'-DDT to activate MAPKs may contribute to transcriptional activation of COX-2 genes. Here, we hypothesized that MAPKs and transcription factors are activated prior to or concurrently with COX-2 gene up-regulation. To test this hypothesis, RAW 264.7 cells were treated with o,p'-DDT for MAPK phosphorylation as well as for activation of nuclear proteins binding to four different consensus transcriptional control motifs associated with COX-2 promoters. Response elements associated with NF-κB, C/EBP, CREB and AP-1 were selected based on the presence of these DNA sequences in the COX-2 promoters as well as on the recognized capacities of these transcription factors to mediate proinflammatory cytokine gene transactivation *in vitro*. In the COX-2 gene,

promoter elements for NF-κB (-223/-214) and nuclear factor NF-IL6 (-132/-124) and CRE (-59/-53) have been found to be important in regulating transcription [Inoue *et al.*, 1994; Inoue *et al.*, 1995; Kosaka *et al*., 1994; Mestre *et al*., 2001; Subbaramaiah *et al*., 2002]. The CRE appears to be the crucial site in epithelial cells [Subbaramaiah *et al*., 1998; Xie and Herschman, 1995] whereas other promoter elements, such as those for NF-κB and NF-IL6, seem to have a role in regulating COX-2 gene transcription in macrophage-like cells [Hwang *et al.*, 1997; Inoue and Tanabe, 1998; Wadleigh *et al*., 2000]. Therefore, the purpose of this study was to investigate that o,p'-DDT may induce COX-2 gene expression in macrophages, and that this was regulated at the level of MAPKs and transcriptional factors.

Nitric oxide (NO) mediates a diverse range of functions, including vasodilatation, neurotransmission, the inhibition of platelet aggregation and other homeostatic mechanisms [MacMicking *et al*., 1997]. Following the exposure to interferon (IFN)-γ, lipopolysaccharide (LPS) as well as a variety of proinflammatory cytokines, inducible nitric oxide synthase (iNOS) can be induced in various cells such as macrophages, Kupffer cells, smooth muscle cells, and hepatocytes [MacMicking *et al*., 1997]. iNOS catalyzes the formation of a large amount of NO, which plays a key role in a variety of pathophysiological processes including various forms of circulatory shock, inflammation and carcinogenesis [MacMicking *et al*., 1997; Maeda and

Akaike, 1998]. Therefore, NO production by iNOS may reflect the degree of inflammation and provide a measure for assessing the effect of chemicals on the inflammatory process. In addition, proinflammatory cytokines such as IL-1β, IL-6 and TNF-α appear to play an active role in the pathogenesis of many diseases with autoimmune or inflammatory components, including rheumatoid and osteoarthritis, atherosclerosis, and osteoporosis [Elenkov and Chrousos, 2002]. Macrophages are involved in inflammatory reactions by synthesizing and releasing a variety of mediator molecules, including NO, arachidonic acid metabolites and cytokines [Coleman *et al*., 2001]. The nuclear transcription factor, NF-κB, is the central regulator for the expression of various genes involved in inflammation, infections and immune response including the genes encoding iNOS and IL-1β, IL-6, TNF-α and the leukocyte adhesion molecules [Baeuerle and Baichwal, 1997; Ghosh *et al*., 1998]. Because these proinflammatory molecules are regulated at the transcription level, NF-κB is a critical intracellular mediator of the inflammatory cascade [Baeuerle and Baichwal, 1997].

Several studies investigate the effects of DDT on the immune system. DDT decreases the anti-ovalbumin serum antibody titers and the number of mast cells in animals [Banerjee, *et a1*., 1986]. Futhermore, it was reported that mice exposed to DDT exhibited suppressed primary and secondary humoral immune responses to thymus-dependent and -independent antigens

in addition to decreased cell mediated responses [Banerjee, *et a1*., 1986, 1997]. The mechanism of how DDT results in the apparent selective reduction of a particular cell population and humoral and cellular immune responses has not been explained. Although DDT is widely used as a pesticide and despite evidence that DDT can modulate the immune functions, little is known regarding its effects on inflammation. Studies on the production of NO and inflammatory cytokines are of considerable interest toward understanding the toxicities of DDT on wildlife as well as human health. o,p'-DDT has a similar chemical structure to the synthetic estrogen, diethylstilbestrol. Moreover, o,p'-DDT has been reported to exhibit estrogenic activity [Banerjee, *et a1*., 1986]. Recently, it was reported that 4-nonylphenol and bisphenol A, which like o,p'-DDT are well known as xeno-estrogens, suppress the LPS-induced iNOS and TNF-α expression level in LPS-activated macrophages [You *et al*., 2002; Kim and Jeong, 2003]. However, few studies have examined the effects of o,p'-DDT on the regulation of iNOS and proinflammatory cytokines. Therefore, this study investigated the effect of o,p'-DDT on NO and proinflammatory cytokines production in mouse macrophages. In addition, the roles of NF-κB in o,p'-DDT-mediated iNOS and proinflammatory cytokine expression were examined. It was observed that o,p'-DDT up-regulates iNOS and

proinflammatory cytokines expression in macrophages, and this induction was mediated in part via NF-κB sites of these genes

II. Materials and Methods

1. Materials

Chemicals and cell culture materials were obtained from the following sources: o,p'-DDT (AccuStandard); ICI 182,780 (Tocris); 7-ethoxyresorufin and resorufin (Pierce Chemical Co.); TCDD (Chemsyn Science Lab.); LipofectAMINE Plus, αMEM and fetal bovien serum (FBS) (Gibco BRL); Penicillin-streptomycin solution, and trypsin (Life Technologies, Inc); pCMV-β-gal, and the luciferase assay system (Promega); PGE_2 Enzyme Immunoassay kit (cayman Co); RIA kits (Diagnostic Systems Laboratories); Antibodies to COX-2, Aromatase, and β-actin (Santa Cruz Biotechnology, Inc.); Western blotting detection reagents (ECL) (Amersham Phamacia Biotech.); *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) (Sigma Co.); pGL3-4κB-Luc, pGL3-3AP-1-Luc and the luciferase assay (Promega); pCMV-β-gal (Clonetech); MTT-based colorimetric assay kit (Roche Co.); Enzyme-linked immunosorbent assay (ELISA) kit for IL-1β, IL-6, and TNF-α (R&D Systems); MAPKs Primary antibodies (anti-phospho-MAPK (Erkl/2) (Thr202/ Tyr204), anti-phospho-p38 MAPK (Thr180/ Tyr182), and anti-phospho-SAPK/JNK (Thr183/ Tyr185) and Secondary antibody (HRP-linked anti-rabbit IgG) (Cell Signaling Technology); All other chemicals were of the highest commercial grade available.

2. Cell culture and treatment

Mouse hepatoma Hepa-1c1c7 cells, human breast MCF-7 cells and rat testicular leydig R2C cells were obtained from the American Type Culture Collection (Rockville, MD). Hepa-1c1c7 cells and R2C cells were cultured in an α-MEM and RPMI 1640 supplemented with 10% fetal bovine serum at 37^{o}C in a humidified 5% CO_2 incubator. MCF-7 cells were cultured in a MDEM. o,p'-DDT, TCDD, and ICI 182,780 were dissolved in dimethylsulfoxide, and stock solutions of these chemicals were added directly to culture media and incubated with o,p'-DDT and/or TCDD. The cell were incubated with different concentration of DDT dissolved in ethanol absolute (EtOH) or with EtOH as vehicle control (the final EtOH concentration was 0.1%, which is ineffective by itself).

3. 7-Ethoxyresorufin O-deethylase assay

Hepa-1c1c7 cells were incubated with 0.5 nM TCDD in the presence of dimethylsulfoxide (the vehicle control), or o,p'-DDT for 18 h. Ethoxyresorufin-*O*-deethylase (EROD) activity was determined in intact cells grown in 24-well plates, as described previously [Ciolino *et al*., 1998].

4. RNA preparation and CYP1A1 mRNA analysis by RT-PCR

Hepa-1c1c7 cells were incubated with 0.5 nM TCDD and/or o,p'-DDT for 6 h. Total cellular RNA was isolated by the acidic phenol extraction procedure as described previously [Kim *et al.*, 2004]. cDNA synthesis, semiquantitative RT-PCR for CYP1A1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs, and the results were analyzed as described previously [Oinonen et al., 1994]. cDNA was synthesized from 2 μg of total RNA using an Omniscript RT-PCR kit as instructed. Cycle numbers that fell within the exponential range of both the CYP1A1 (302 bp, 26 cycles) and GAPDH (983 bp, 17 cycles) responses were used.

5. Transfection and luciferase and β-galactosidase assays of DRE

Hepa-1c1c7 cells (2.5×10^5) were plated in each well of a 12-well plate in α-MEM supplemented with 10% FBS. After 12 h, the cells were transfected using LipofectAMINE Plus. Subsequently, the cells were co-transfected with 0.2 μg of pCMV-β-gal and 1 μg of DRE-regulated luciferase reporter gene, pCYP1A1-Luc, or estrogen responsive element (ERE)-regulated luciferase reporter gene pGE3-ERE3-Luc per well. The reporter gene pCYP1A1-Luc contains a fragment of the murine CYP1A1 gene upstream region (482bp: -1306~-824), which contains four DREs (TTGCGTGAGA) located upstream

of the mouse mammary tumor virus promoter that is linked to the firefly luciferase gene. pGE3-ERE3-Luc reporter gene was constructed by conjugation of the three consensus ERE-binding sites (AGCTTGTCAGGTCAGCGTGACCTCCAA). Four hours after transfection, a fresh medium containing 10% FBS was added to the cells, which were treated with TCDD or o,p'-DDT. After 18 h exposure, the cells were washed with 2 ml of PBS and lysed. The lysed cell preparations were then centrifuged, and the supernatants were assayed for luciferase and β-galactosidase activity. Luciferase activity was determined using the luciferase assay system (Promega) in accord with the manufacturer's instructions using a luminometer. The β-galactosidase assay was carried out in 250 μl of assay buffer containing 0.12 M Na_2HPO_4, 0.08 M NaH_2PO_4, 0.02 M KCl, 0.002 M $MgCl_2$, 0.1 M β-mercaptoethanol, 50 μg of *o*-nitrophenyl-β-galactoside, and 100 μg of the cell extract. Luciferase activity was normalized using β-galactosidase activity and is expressed as a proportion of the activity detected in the vehicle controls.

6. Electrophoretic mobility shift analysis of DRE

Nuclear extracts were prepared and electrophoretic mobility shift analysis performed according to the procedure previously described [Jeong *et al*., 1997; Jeong and Lee, 1998]. Briefly, the nuclear extract (5 μl/10 μg protein)

was mixed with 15 μl HEDG (HEPES-EDTA-DTT-Glycerol) containing 1 mM dithiothreitol, 0.1 mM PMSF and 1.0 μg of poly(dI·dC), and incubated for 20 min at 20°C before the addition of 1.0 μl of ^{32}P-labeled synthetic oligonucleotide (100,000 dpm). After incubating for an additional 20 min, samples were run on a 4% polyacrylamide gel with recirculating 1X TAE buffer (6.7 mM Tris-HCl, pH 8, containing 3.3 mM sodium acetate and 1.0 mM EDTA). The gel was then vacuum dried and exposed at -80°C to X-film.

7. Testosterone determination

Testosterone was assayed in duplicate by using a ^{125}I (Coat-A-Count)-radioimmunoassay (RIA) kit. Details of the hormonal assays have been published previously (Harman *et al*., 2001**).** T levels were determined in duplicate using ^{125}I double antibody RIA kits obtained from Diagnostic Systems Laboratories (Webster, TX). The radioactivity of ^{125}I was quantified by a gamma-counter. All experiments were repeated at least three times.

8. RT-PCR assay of stoidogenic genes

Steady-state mRNA levels for P450scc were measured after immature Leydig cells were incubated for 12 h. Total RNA was isolated by a single-step method after cells were lysed in culture plates without detachment using phenol and

guanidinium thiocyanate in accordance with the manufacturer's instructions. The sense/antisense primers used were: aromatase,5'-GGTCACAGTCTGTGCTGAATCC-3'/5'-CTCGAGTCTGTGCAT-CCTTA3'; cytochrome P450 side-chain cleavage (P450scc). Amplification products were electrophoresed in 2% agarose gel and stained with ethidium bromide. The gel images were captured on a gel doc image analysis system (Kodak) and the yield of PCR products was normalized to GAPDH after quantitative estimation using the NIH Image software.

9. Aromatase activity assay

Aromatase activity was determined using the [^{3}H] H_2O release method that was reported previously [Zhou *et al*., 1990]. In the "In-cell" aromatase assay, the cells grown in 6-well culture plates were washed twice with PBS. After a 3-h incubation at 37°C, the reaction mixture was removed and extracted with an equal volume of chloroform to extract unused substrate and further treated with dextran-treated charcoal. After centrifugation, supernatant containing the product, [^{3}H] H_2O, counted in a liquid scintillation counter. The protein concentration was determined after dissolving cells in 0.5 N NaOH [Bradford, 1976]. Aromatase activity was calculated as pmol/μg protein/h.

10. PGE_2 immunoassay using ELISA

PGE_2 in the culture medium was measured with an enzyme immunoassay kit (Cayman Chemical Co., Inc.). The assay was performed according to the manufacturer's instructions. Briefly, 25 or 50 µl of the medium, along with a serial dilution of PGE_2 standard samples, were mixed with appropriate amounts of acetylcholinesterase-labeled tracer and PGE_2 antiserum and incubated at room temperature for 18 h. After the wells were emptied and rinsed with wash buffer, 200 µl of Ellman's reagent containing substrate for acetylcholinesterase were added. The enzyme reaction was carried out on a slow shaker at room temperature for 1 h. The plates were read at 415 nm, and the results were analyzed.

11. Aromatase, COX-2, EP, and ER gene expression by RT-PCR assay

R2C cells were incubated with o,p'-DDT for COX-2 (2 h), estrogen receptor and aromatase (6 h). Total cellular RNA was isolated by the acidic phenol extraction procedure. For COX-2 and aromatase, its cDNA, which was reverse transcribed from total RNA, was amplified by PCR. The sense/antisense primers used were: aromatase, 5'-GGT/CAC/AGT/CTG/TGC/TGA/ATC/C-3'/5'-CTC/GAG/TCT/GTG/CAT/CC

T/TA-3'; COX-2 5'-ACT/CAC/TCA/GTT/TGT/TGA/GTC/ATT/C-3'/5'-TTT/GAT/TAG/TAC/TGT/AGG/GTA/AAT/G-3'ERα- 5'TAT/GGG/GTC/TGG/TCC/TGT/GA-3'/5'-GGG/CGG/GGC/TAT/TCT/TCT/TA-3', ERβ 5'-TCC/CTC/TTT/GCG/TTT/GGA/CT-3'/5'-TTC/CCG/GCA/GCA/CCA/GTA/AC-3', $EP_1$5'-CGC/AGG/GTT/CAC/GCA/CAC/GA-3'/5'-CAC/TGT/GCC/GGG/AGC/TAC/GC-3' [Fedyk *et al*., 1996; Okuda-Ashitaka *et al*., 1996]; EP_2, 5'-TTC/AAT/GAC/TCC/AGG/CGA/GT-3'/5'-AGG/ACA/GTA/GGG/GAC/GTA/CT-3 [Nemoto *et al*., 1997]; EP_3, 5'-GCC/CGG/CAC/GTG/GTG/CTT/CAT-3'/5'-TAG/CAG/CAG/ATA/AAC/CCA/GG-3' [Kasugai *et al*., 1995]; EP_4,5'-TTC/CGC/TCG/TGG/TGC/GAG/TGT/TC-3'/5'-GAG/GTG/GTG/TCT/GCT/TGG/GTC/AG-3' [Fedyk *et al*., 1996]. glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sense: 5'-TGA/AGG/TCG/GAG/TCA/ACG/GAT/TTG-3'/5'-CAT/GTG/GGC/CAT/GAG/GTC/CAC/CAC-3'). Amplification products were electrophoresed in 2% agarose gel and stained with ethidium bromide. The gel images were captured on a gel doc image analysis system (Kodak) and the yield of PCR products was normalized to GAPDH after quantitative estimation using the NIH Image software.

12. Transient transfection and reporter gene assays of aromatase

R2C cells, rat testicular leydig cells ere transfected for 22 h with 1.5 μg of a chimeric DNA reporter gene construct containing 774 bp of the 5'-regulatory region upstream of promoter I.4 of the human *CYP19* gene fused to a Luc reporter gene in the pGL3 vector (a generous gift from Dr. Makio Shozu, Kanazawa University, Kanazawa, Japan), CRE gene fused to a Luc reporter gene in the pGL3 vector (a generous gift from Dr. Kumiko Saeki, Research Institute, International Medical Center, Japan), and pCMV-β-galactosidase control vector (Promega, Sydney, New South Wales, Australia) using LipofectAMINE tansfection reagent according to manufacturer's instructions. After serum starvation, cells were stimulated with o,p'-DDT for 18 h. Cells were washed with 2 ml of PBS and lysed. The lysed cell preparations were then centrifuged, and the supernatants were assayed for luciferase and β-galactosidase activity. Luciferase activity was determined using the luciferase assay system (Promega) in accord with the manufacturer's instructions using a luminometer. The β-galactosidase assay was carried out in 250 μl of assay buffer containing 0.12 M Na_2HPO_4, 0.08 M NaH_2PO_4, 0.02 M KCl, 0.002 M $MgCl_2$, 0.1 M β-mercaptoethanol, 50 μg of *o*-nitrophenyl-β-galactoside, and 100 μg of the cell extract. Luciferase activity was normalized using β-galactosidase activity and is expressed as a proportion of the activity detected in the vehicle controls.

13. Preparation of peritoneal macrophages and cell cultures

Peritoneal macrophages were isolated from specific pathogen free-BALB/c mice (females, 5-7 weeks old), which were obtained from KRIBB (South Korea), and cultured as described previously [Verdot *et al*., 1999]. The RAW 264.7 cells, a mouse macrophage cell line, were obtained from the American Type Culture Collection (Bethesda, MD), and grown in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37^{o}C in a 5% CO_2 humidified incubator. The o,p'-DDT was dissolved in DMSO, and stock solutions of these chemicals were added directly to the culture media. The control cells were treated only with the solvent, with a final concentration that never exceeded 0.1%, which is a concentration that did not have any effect on the assay systems. The cell viability was assessed by a MTT assay according to the manufacturer's instructions.

14. Plasminds and COX-2 promoter constructs

The COX-2 promoter constructs (-1432/+59, -327/+59, -220/+59, -124/+59, -52/-59, KBM, ILM, CRM, and CRM-ILM) were a gift from Dr. Chieko Yokoyama (National Cardiovascular Center Research Institute, Osaka, Japan).

15. PGE_2 assay in RAW 264.7 cells

The cells were incubated with the o,p'-DDT and/or LPS (0.5 μg/ml). After incubating the cells for 24 h, the culture medium was collected and the level of PGE_2 released into culture media was measured using a specific enzyme immunoassay according to the manufacturer's instructions.

16. Nitrite assay

The peritoneal macrophages (2 X 10^5 cells/ml) or RAW 264.7 cells (5 X 10^5 cells/ml) were cultured in 48-well plates. After incubating for the cells for 24 h, the level of NO production was determined by assaying the culture supernatants for nitrite, which is the stable reaction product of a reaction of NO with molecular oxygen, using a Griess reagent as described previously [Choi *et al*., 2001].

17. RNA preparation and COX-2 and iNOS mRNA analysis by reverse RT-PCR in RAW 264.7 cells

RAW 264.7 cells were cultured with either o,p'-DDT or LPS for either 2 or 6 h. The total cellular RNA was isolated using the acidic phenol extraction

procedure described previously [Chomczynski and Sacchi, 1987]. cDNA synthesis, semiquantitative RT-PCR for COX-2, IL-lβ, IL-6, TNFα, GAPDH mRNA, and the analysis of the results were performed as described previously [Choi *et al*., 2001]. The PCR products were electrophoresed through a 2% agarose gel and visualized with ethidium bromide staining and UV irradiation.

18. Western blotting of COX-2

RAW 264.7 cells were cultured with the chemicals and/or LPS (0.5 μg/ml) for 24 h and equal amounts of the total cellular proteins (50 μg) were resolved by 10% SDS–PAGE and transferred onto polyvinylidene difluoride membranes. After blocking, the membranes were incubated with COX-2 polyclonal antiserum or monoclonal anti-β-actin antibody. The secondary antibody to IgG conjugated to horseradish peroxidase was used. The blots were probed with the ECL Western blot detection system according to the manufacturer's instructions.

19. Detection of MAPK phosphorylation

Phosphorylation of MAPKs was assayed by Western blot, using rabbit

polyclonal antibodies specific for phospho-JNK 1/2, phospho-ERK 1/2, and phospho-p38 MAPK (Cell Signaling, Beverly, MA) as described in manufacturer's instruction. RAW 264.7 cells were dispersed in SDS lysis buffer (1% SDS, 1 mM sodium orthovanadate, 10 mM Tris, pH 7.4), transferred to a microcentrifuge tube, boiled for 5 min, and then sonicated briefly. The lysates was centrifuged at 15,000 g for 15 min at 4°C to pellet insoluble material. Protein concentration of the resultant supernatant was determined using a Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Inc., Melville, NY). Total cellular proteins were resolved by 10% (w/v) SDS PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham, Arlington Heights, IL). After blocking with 5% (w/v) nonfat dry milk, the immobilized proteins were incubated with phospho-specific antibodies followed by horseradish peroxidase-conjugated anti-rabbit IgG antibodies (Amersham). Bound peroxidase was determined using an ECL chemiluminescence detection kit (Amersham). To assess loading, membranes were stripped and reprobed with specific antibodies that recognize both phosphorylated and unphosphorylated forms (Cell Signaling) of each MAPK.

20. COX-2 promoter constructs transfection and luciferase assays

RAW 264.7 cells (5 X 10^5 cells/ml) were plated in each well of a 12-well

plate, and 12 h later transiently co-transfected with the plasmids using LipofectAMINE Plus according to the manufacturer's protocol. The constructs used were as follows : the COX-2 promoter construct (−327/+59), its NF-κB mutant constructs (designated as KBM, −223/−214), its CEBP mutant constructs (designated as ILM, −132/−124) and its CRE mutant constructs (designated as CRM, −59/−53), have been described previously [Inoue *et al*., 1995], and were generous gifts from Dr. Tadashi Tanabe and Chieko Yokoyama (National Cardiovascular Center Research Institute, Osaka, Japan), pGL3-4κB-Luc, and pCMV-β-gal using LipofectAMINE Plus according to the manufacturer's protocol. After 18 h, the cells were treated with the chemicals and/or LPS (0.5 μg/ml) for 12 h, which were then lysed. The luciferase and β-galactosidase activity were determined as described previously [Jeong and Kim, 2002] The luciferase activity was normalized with respect to the β-galactosidase activity and was expressed relative ratio to the activity of the control.

21. Enzyme-linked immunosorbent assay (ELISA) of proinflammatory cytokines

For the cytokine immunoassay, the RAW 264.7 cells (2 X 10^5 cells/ml) were cultured in 24-well plates. The supernatants were removed at the designated

times and the level of TNFα, IL-lβ, and IL-6 production was measured using a sandwich ELISA according to the protocol supplied by R&D Systems.

22. Electrophoretic mobility shift assay of NF-κB

The nuclear extracts were prepared as previously described [Jeon *et al*., 1999]. Two double-stranded deoxyoligonucleotides containing the NF-κB binding site (5'-GGGGACTTTCC-3') [Ghosh *et al*., 1998] were end-labeled with [γ-^{32}P]dATP. The nuclear extracts (5 μg) were incubated with 2 μg of poly (dI-dC) and the ^{32}P-labeled DNA probe in a binding buffer (100 mM NaCl, 30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 1mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of aprotinin and leupeptin) for 10 min on ice. The DNA was separated from the free probe using a 4.8% polyacrylamide gel in a 0.5X TBE buffer (44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA). Following electrophoresis, the gel was dried and autoradiographed.

23. Statistical analysis

All experiments were repeated at least three times to ensure reproducibility. Results are reported as means ± S.D. ANOVA was used to evaluate the difference between multiple groups. If a significant difference was observed

between groups, Dunnet's 't' test was used to compare the means of two specific groups; $P < 0.05$ was considered significant.

III. Results

Down-regulation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced CYP1A1 gene expression by o,p'-DDT in murine heap-1c1c7 cells

1. Suppressive effects of o,p'-DDT on TCDD-inducible EROD activity

EROD activities were measured in Hepa-1c1c7 cells that were treated with vehicle (VH), TCDD (0.5 nM) or/and various concentrations of o,p'-DDT (0.1 μM ~ 20 μM) for 18 h. Subsequent to treating cells with 0.5 nM TCDD, there was a marked increase in EROD activity compared to the control (Fig. 4). Maximum activities were detected 18 h after the addition of the inducer (data not shown). However, TCDD-inducible EROD activities were significantly reduced in cultures co-treated with both o,p'-DDT and TCDD versus induced cultures treated with TCDD alone (Fig. 4).

2. Estrogenic activity of 17β-estradiol and o,p'-DDT was examined in MCF-7 cells

Estrogenic activity of 17β-estradiol and o,p'-DDT was examined in MCF-7 cells. Using an estrogen-responsive reporter gene assay, 17β-estradiol or o,p'-DDT treatment was shown to result in a increase in luciferase activity (Fig. 5). Addition of the anti-estrogen ICI 182,780 with either 17β-estradiol or o,p'-

DDT reduced luciferase activity, indicating that the effect of o,p'-DDT on ERE-mediated transcriptional activity was dependent on the estrogen receptor in MCF-7 cells (Fig. 5).

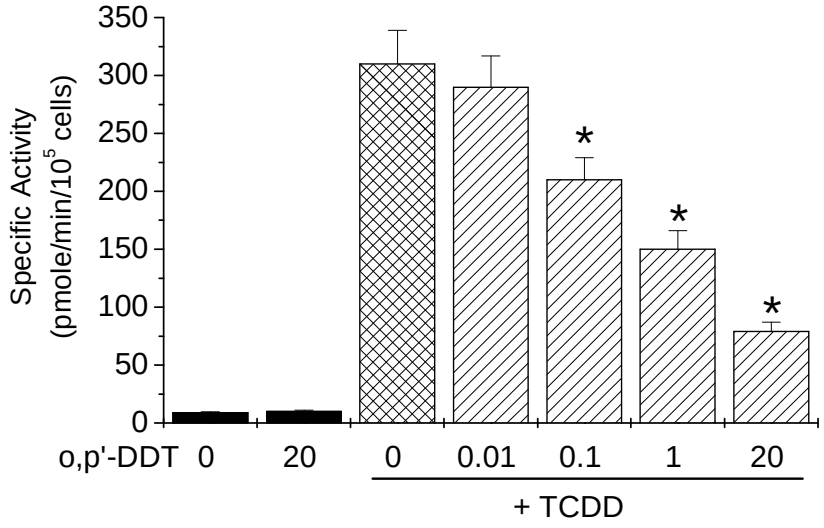


Fig. 4. Effects of o,p'-DDT on TCDD-induced EROD activity in Hepa-1c1c7 cells. EROD activities were measured in Hepa-1c1c7 cells that were treated with vehicle (VH), TCDD (0.5 nM) or/and various concentrations of o,p'-DDT (0.1 μM ~ 20 μM) for 18 h, as described in Materials and Methods. The values are presented as the means ± S.D. of triplicate cultures. * $P < 0.05$, significantly different from TCDD.

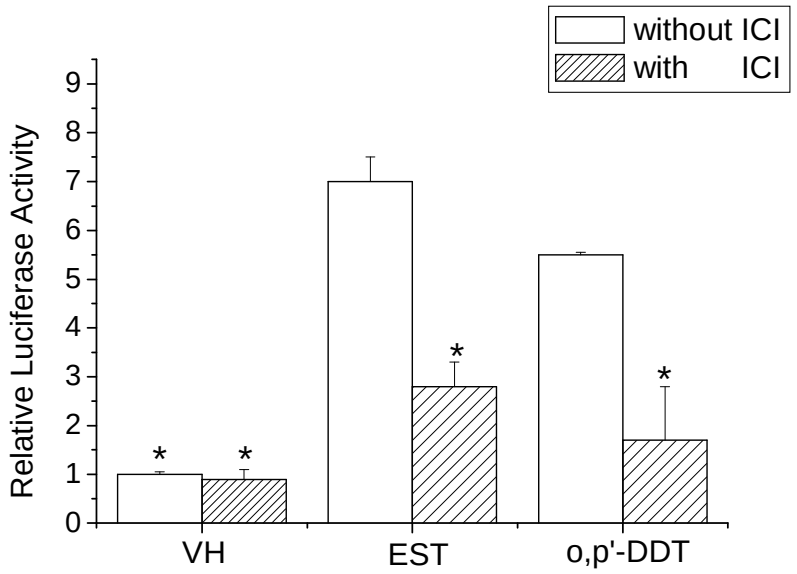


Fig. 5. Effects of o,p'-DDT on luciferase activity in MCF-7 cells transiently transfected with pGL3-ERE3-Luc. MCF-7 cells were transiently transfected with pGL3-ERE3-Luc, and then treated with 17β-estradiol (EST; 1 nM), o,p'-DDT (DDT; 100 nM), ICI 182,780 (ICI; 100 nM), or in combination for 18 h. The cells were harvested, and their luciferase activities determined, as described in Materials and Methods. Values shown are means ± S.D., each performed in triplicate. Enzyme activities are expressed relative to those of the vehicle (VH) alone. * $P < 0.05$, significantly different 17β-estradiol.

3. Effects of 17β-estradiol or o,p'-DDT on TCDD-inducible EROD activity

In order to assess whether the suppressive effects of o,p'-DDT on TCDD-inducible EROD activity might be influenced by the estrogen receptor, ICI 182,780, was used. However, ICI 182,780 was found to be no effect on suppressing TCDD-induced EROD activity by either 17β-estradiol or o,p'-DDT (Fig. 6), which suggests that the suppressive effect of o,p'-DDT and 17β-estradiol on TCDD-induced EROD activity might not be mediated by the estrogen receptor in Hepa-1c1c-7 cells. The action of the o,p'-DDT appears to be cell specific.

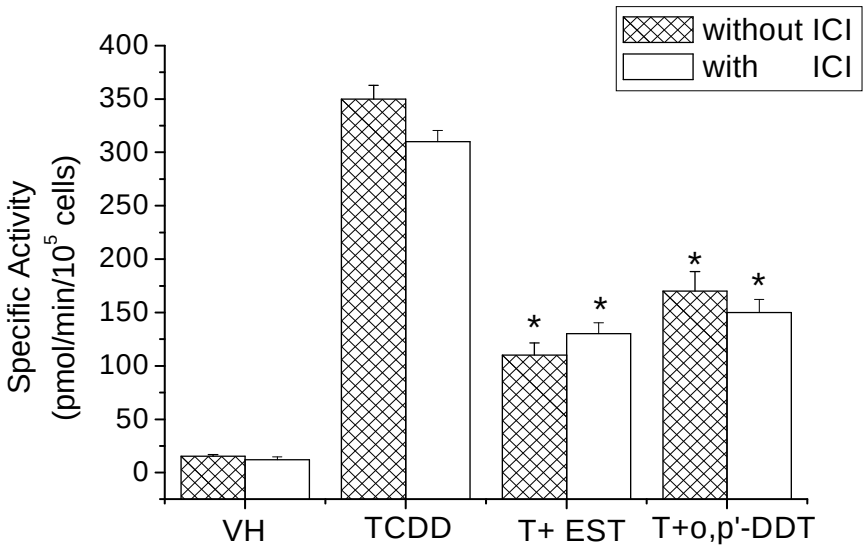


Fig. 6. Effects of ICI 182,780 on EROD activity in Hepa-1c1c7 cells. EROD activities were measured in Hepa-1c1c7 cells that were treated with vehicle (VH), TCDD (0.5 nM), 17β-estradiol (EST; 10 μM), o,p'-DDT (10 μM), ICI 182,780 (ICI; 100 nM), or in combination for 18 h. The values are presented as the means ± S.D. of triplicate cultures. * $P < 0.05$, significantly different from the TCDD.

4. Down regulation of CYP1A1 gene expression by o,p'-DDT

Hepa-1c1c7 cells were treated with TCDD (0.5 nM) and/or o,p'-DDT (1 ~ 20 μM) for 6 h. Total cellular RNA was isolated from the cells. The effects of o,p'-DDT on the TCDD-induced induction of CYP1A1 mRNA levels were determined by RT-PCR. Consistent with the results obtained from the EROD activity assay, CYP1A1 mRNA levels were markedly suppressed by co-treatment with o,p'-DDT and TCDD (Fig. 7).

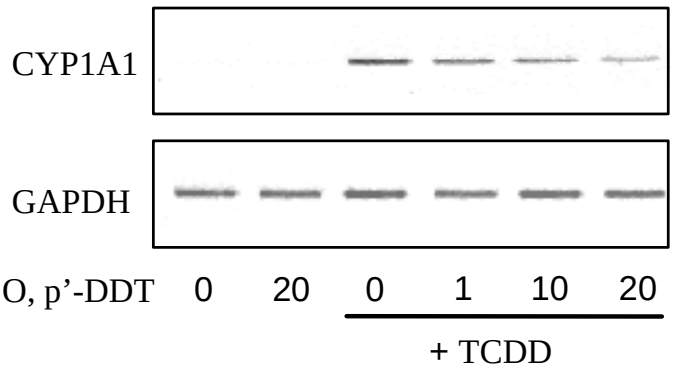


Fig. 7. RT-PCR analysis of CYP1A1 mRNA in Hepa-1c1c7 cells. Cells were treated with TCDD (0.5 nM) and/or o,p'-DDT (1 ~ 20 μM) for 6 h. Total cellular RNA was isolated from the cells. For CYP1A1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression, their cDNAs, which were reverse transcribed from the total RNA, were amplified by PCR. Amplification products were electrophoresed in 3% agarose gel and stained with ethidium bromide, as described in Materials and Methods.

5. Effects of o,p'-DDT on luciferase activity in Hepa-1c1c7 cells.

Hepa-1c1c7 cells were transiently transfected with pCYP1A1-Luctreated with TCDD and/or o,p'-DDT and luciferase activities determined. TCDD treatment resulted in an increase in luciferase activity compared with control. However, when the cells were treated simultaneously with TCDD and o,p'-DDT, the luciferase activity was significantly lower than in the cells treated with TCDD alone (Fig. 8).

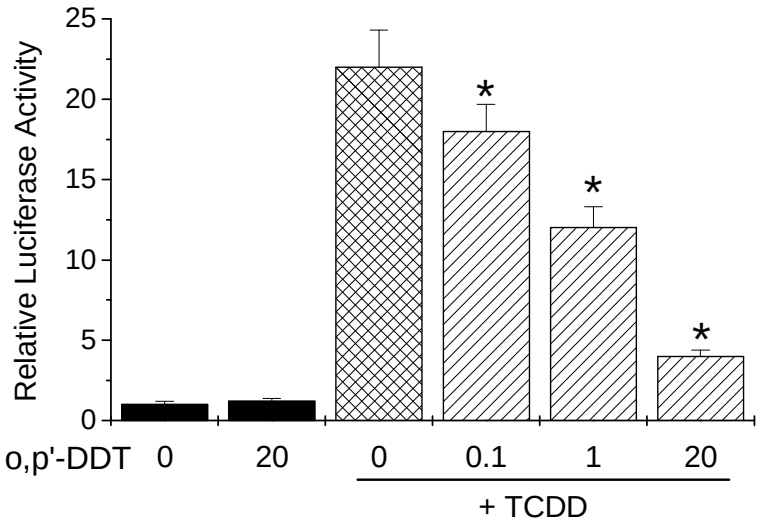


Fig. 8. Effects of o,p'-DDT on luciferase activity in Hepa-1c1c7 cells transiently transfected with pCYP1A1-Luc. Hepa-1c1c7 cells were transiently transfected with pCYP1A1-Luc, which contains the 5'-regulating region of the CYP1A1 gene, and then treated with TCDD (0.5 nM) and/or o,p'-DDT (1 ~ 20 μM) for 18 h. The cells were harvested, and their luciferase activities determined, as described in Materials and Methods. Values shown are means ± S.D., each performed in triplicate. Enzyme activities are expressed relative to those of vehicle alone. * $P < 0.05$, significantly different from the TCDD.

6. Effects of o,p'-DDT on TCDD-induced transformation of a Ah receptor /^{32}P-DRE complex

An electrophoretic mobility shift assay was performed to determine whether o,p'-DDT is capable of reducing the transformation of the Ah receptor, which are capable of specific binding to ^{32}P-labeled double-stranded oligonucleotides containing DRE sequence. After treatment of Hepa1c1c7 cells with TCDD and/or o,p'-DDT to Hepa-1c1c7 cells, nuclear extracts were isolated and the electrophoretic mobility shift assay was performed. o,p'-DDT reduced the TCDD-induced transformation of a Ah receptor/^{32}P-DRE complex (Fig. 9).

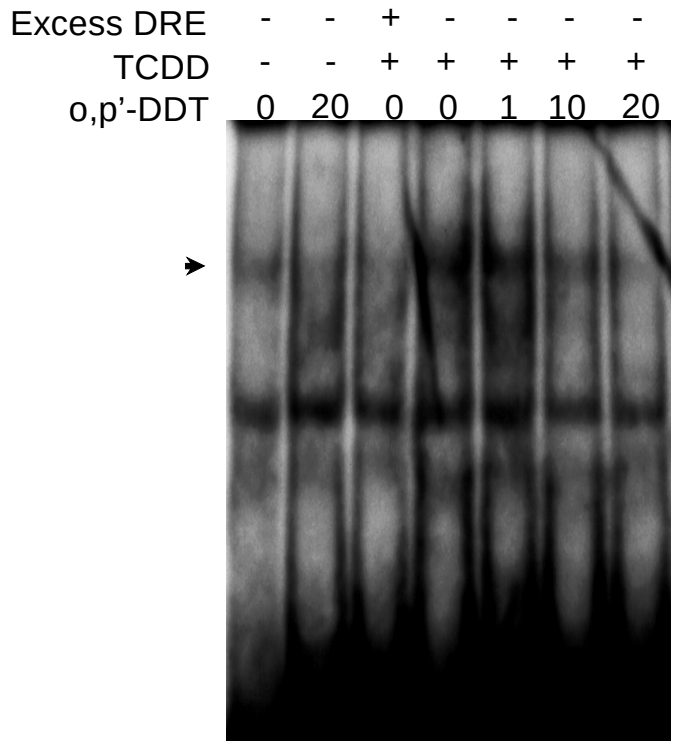


Fig. 9. Electrophoretic mobility shift assay of DRE-binding proteins in Hepa-1c1c7 cells. Cells were treated with TCDD (0.5 nM) and/or o,p'-DDT (1 ~ 20 μM) for 3 h. Nuclear extracts were isolated and used in a electrophoretic mobility shift assay with ^{32}P-labeled DRE oligonucleotide as probe, as described in Materials and Methods. The arrow indicates the Ah receptor-DRE complex. Excess DRE; 200-fold excess of nonlabeled DRE.

o,p'-DDT reduced testosterone production via induction of aromatase (CYP19) gene expression in rat testicular Leydig cells

7. Suppressive effects of o,p'-DDT on testosterone production in R2C cells

Testosterone (T) production by immature Leydig cells, incubated in fresh media for 3 h after treatment with 1-10 μM of o,p'-DDT for 18 h, is shown in Figure 8. o,p'-DDT caused a dose-dependent inhibition of T production in R2C cells. Inhibition of T production commenced at 1 μM, while the highest concentration of o,p'-DDT (10 μM) reduced T production to as low as 68% of control (Fig. 10). Assessment of cell viability by the trypan blue exclusion test showed that the fraction of vehicle control and o,p'-DDT-treated Leydig cells taking up the blue stain was less than 10% in all cases, indicating that the concentrations of o,p'-DDT used were not overtly cytotoxic.

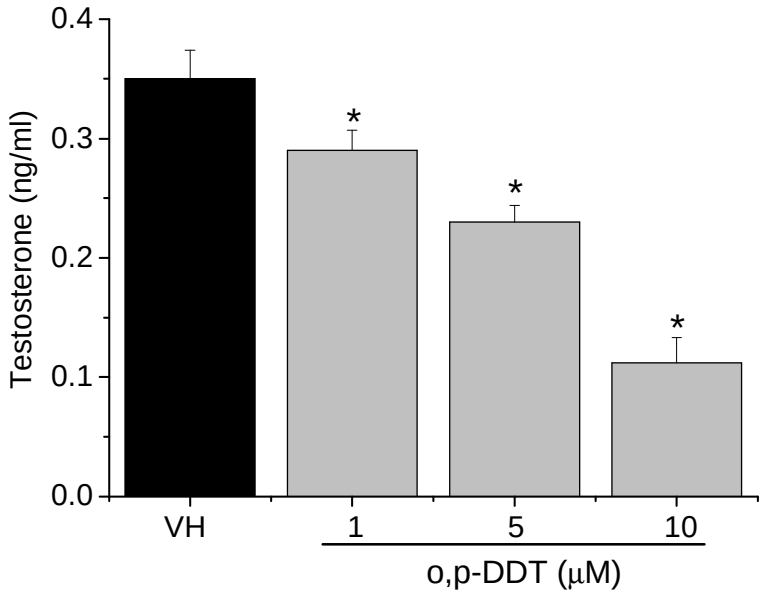


Fig. 10. Effects of o,p'-DDT on testosterone production in rat testicular Leydig R2C cells. After cells were cultured in the presence of o,p'-DDT (1-10 μM) for 18 h, testosterone concentrations were measured by RIA. Three experiments were conducted for this determination. * $P < 0.05$, significantly different from the VH.

8. Steady-state mRNA levels of P450scc, 3β-HSD, CYP17α and 17β-HSD in o,p'-DDT-treated R2C cells

R2C cells were treated with o,p'-DDT (0.1 ~ 10 μM) for 6 h. Treatment of the R2C cells with o,p'-DDT significantly decreased the $P450_{17\alpha}$ (17α-hydroxylase) and 3β-HSD (3β-hydroxysteroid dehydrogenase), which one related with T biosynthesis. Meanwhile, 17β-HSD and P450scc were no affected by o,p'-DDT (Fig. 11). o,p'-DDT causes a direct inhibition of T biosynthesis by leydig cells and this inhibition seems to be derived from a decrease in the activity of $P450_{17\alpha}$ and 3β-HSD.

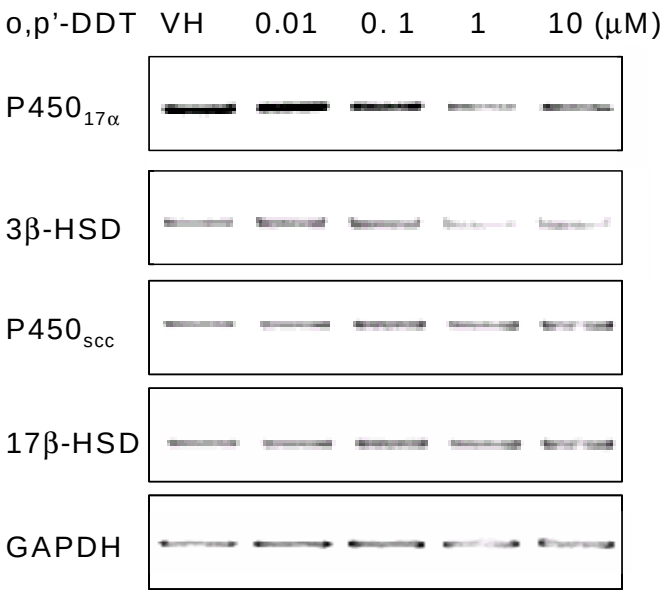


Fig. 11. Effects of o,p'-DDT on steady-state mRNA levels of $P450_{17\alpha}$, 3β-HSD, $P450_{SCC}$, and 17β-HSD in o,p'-DDT-treated R2C cells. R2C cells were lysed and total RNA was prepared for RT-PCR analysis of gene expression. The ratio of RT-PCR product of $P450_{17\alpha}$, 3β-HSD, $P450_{SCC}$, and 17β-HSD to GAPDH was determined. Amplification products were electrophoresed in 3% agarose gel and stained with ethidium bromide, as described in Materials and Methods.

9. Effects of o,p'-DDT on aromatase activity, mRNA and protein expression in R2C cells

Various pesticides known or suspected to interfere with steroid hormone function were screened for determining effects on catalytic activity and mRNA expression of aromatase in R2C cells. o,p'-DDT induced aromatase activity in R2C cells in a concentration-dependent manner. The effects PGE_2 and phorbol ester TPA, known regulators of aromatase activity, of o,p'-DDT on aromatase activity were determined (Fig. 12). o,p'-DDT significantly stimulated aromatase activity in R2C cells in a dose dependent manner. TPA significantly stimulated aromatase activity over control levels ($p < 0.05$). TPA was identified to be the most potent inducer of activity seen (mean activity, 0.095 ± 0.098 pmol/h per 10^6 cells).

The increase in T biosynthesis was associated with induction of aromatase gene expression, because steady state aromatase mRNA level were induced in o,p'-DDT-treated leydig cells compared with control (Fig. 13). The patterns of aromatase mRNA expression are generally consistent with those of aromatase activity. Western blotting analysis was performed to determine aromatase protein expression (Fig. 14). Using an equine aromatase polyclonal antibody, aromatase protein was detected primary as a doublet of 55,000 molecular mass and quantified using β–actin protein as a control. These results indicated

that o,p'-DDT-mediated inhibition of T production in R2C cell might be through the increase in aromatase enzymes.

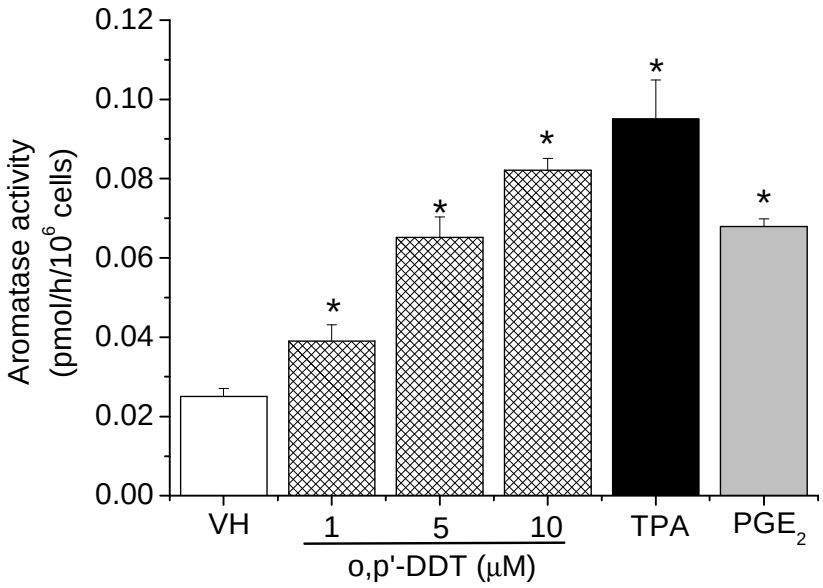


Fig. 12. Effects of o,p'-DDT on aromatase activity in R2C cells. Aromatase activity was determined in cells treated with o,p'-DDT (1-10 μM), TPA (10 nM), PGE_2 (1 μM), or vehicle (control) for 18 h. The amount of inhibition was determined by measuring the tritium released as ^3H_2O from [1β-^{3}H] androst-4-ene-3,17-dione, as described in Materials and Methods. * $P < 0.05$, significantly different from the VH.

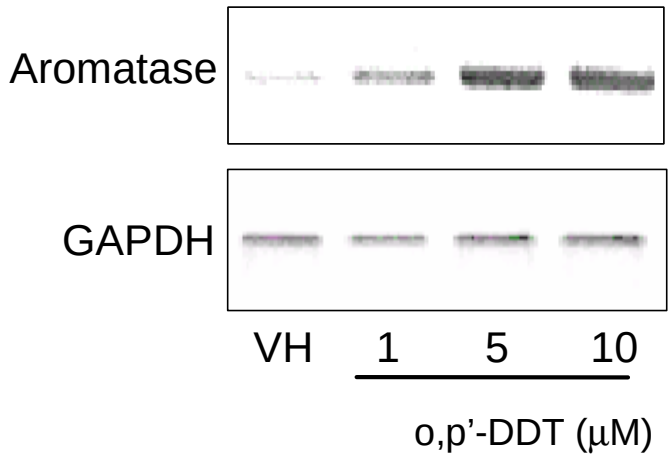


Fig. 13. Effects of o,p'-DDT on aromatase mRNA expression in R2C cells. R2C cells ($1x10^6$ cells/ml) were cultured for 6 h in the presence of media alone, with the indicated concentrations of o,p'-DDT. R2C cells were lysed and total RNA was prepared for RT-PCR analysis of gene expression.

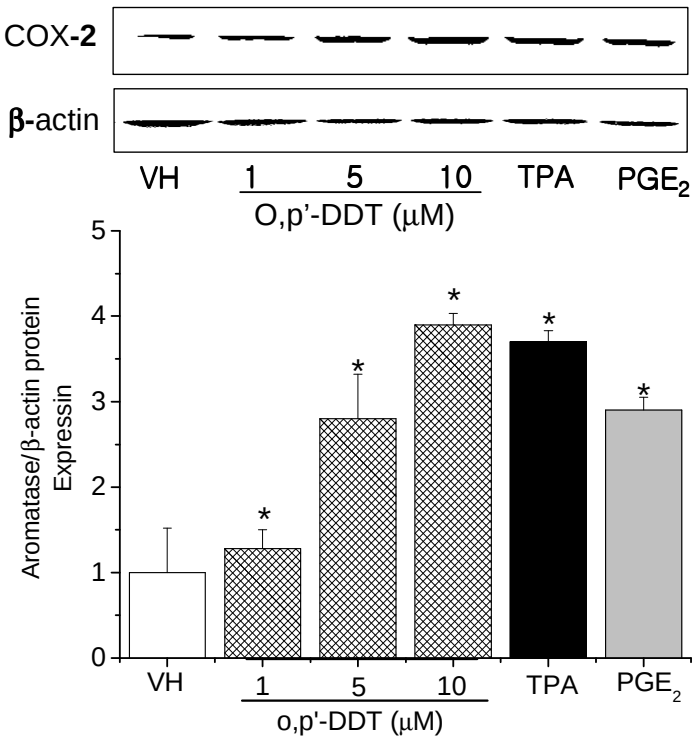


Fig. 14. Effects of o,p'-DDT on aromatase protein expression in R2C cells. Cells (1 X 10^6 cells/ml) were cultured for 24h in the presence of media alone, with the indicated concentrations of o,p'-DDT. Thirty microgram whole cell lysates were analyzed by western blotting with an equine aromatase polychlonal antibody and a mouse β-actin monoclonal antibody. (A) Quantified aromatase levels are shown as aromatase/ β-actin protein expression (B) and reported as mean ± SD. * $P < 0.05$, significantly different from the VH.

10. Effects of o,p'-DDT on transcriptional activation of aromatase expression in R2C cells

To gain further insight into how o,p'-DDT interfere with the transcription of the aromatase gene, Cells were transfected with pGL3 vector containing 5'-regulatory region between -774 and +14 or with pGL3-basic vector. Fig. 15A demonstrated that in the absence of treatment, basal levels of *Luc* gene expression were observed in cells transfected with both pGL3-basic vector and -774/+14 P450-I.4/Luc. Treatment with 10 μM of o,p'-DDT (which up-regulate aromatase expression through promoter 1.4) caused a 4.5-fold induction in aromatase-*Luc* gene expression (Fig. 15B). This result was consistent with the effect of o,p'-DDT on aromatase activity (Fig. 12) and expression (Fig. 13). Treatment with o,p'-DDT had no effect on basal *Luc* or β-galactosidase expression, and treatment with 0.1% DMSO vehicle did not affect Luc activity (data not shown).

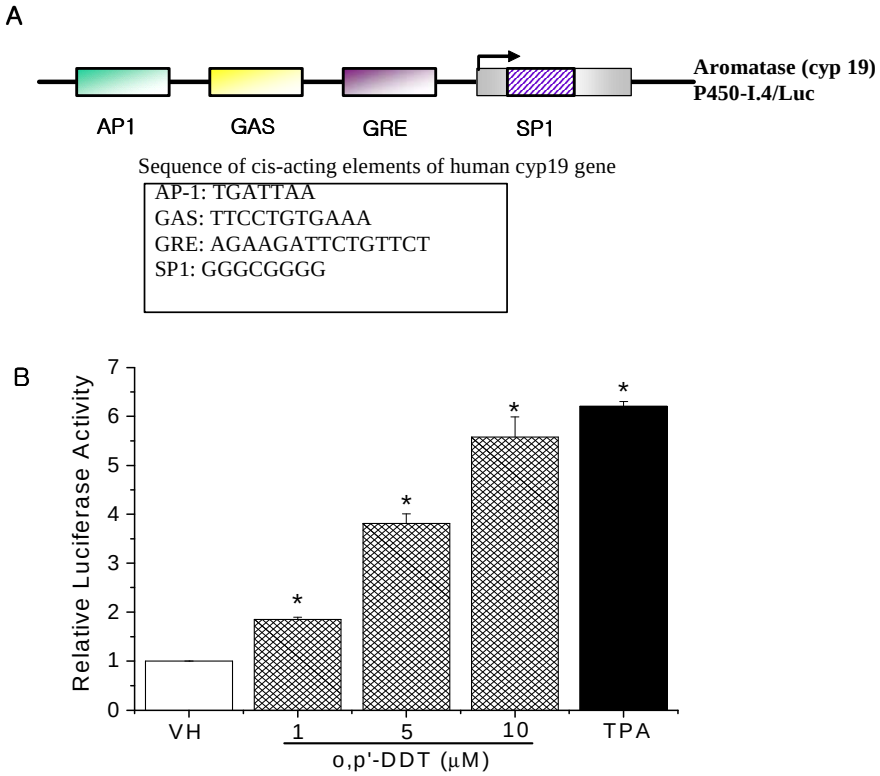


Fig. 15. Effects of o,p'-DDT on aromatase gene transcription via promoter I.4. Cells (1 X 10^6 cells/ml) were transfected for 22 h with 1 μg of the *Luc* reporter gene construct containing 774 bp of the 5'-regulatory gene upstream of the aromatase gene promoter I.4 (-774/+14 P450-I.4/*Luc*) or pGL3-basic vector and cotransfected with 0.5 μg of pCMV-β-galactosidase control vector. Cell were stimulated for 12 h with o,p'-DDT (1-10 μM) or TPA. Cells were lysed, and *Luc* activity was determined and normalized to β–galactosidase activity. * $P < 0.05$, significantly different from the VH.

11. Effects of o,p'-DDT on functional ERα and ERβ in R2C cells

o,p'-DDT was found to increase aromatase activity and gene expression in R2C cells in a dose dependent manner. In order to assess whether the increased effects of o,p'-DDT on aromatase gene expression might be influenced by the ER (estrogen receptor). Stimulation of R2C cells with o,p'-DDT for 6 h induced a marked increase in ERβ mRNA (Fig. 16). In particular, the increase of ERβ in R2C cells after stimulation with 1-10 μM of o,p'-DDT was 4-fold higher compared to the increase in ERβ but not ERα. Therefore, inducible effects of o,p'-DDT on aromatase gene expression might be influenced by the ERβ.

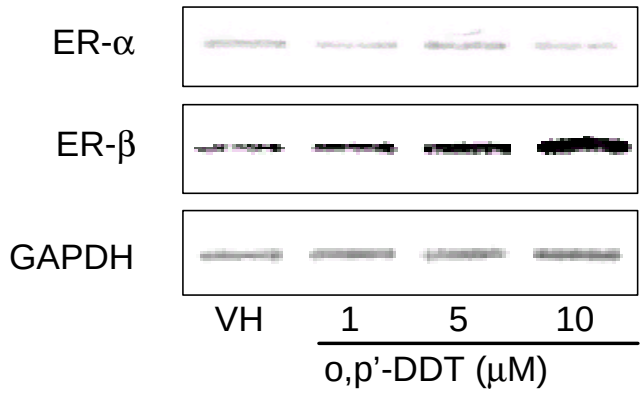


Fig. 16. Effects of o,p'-DDT on ER-α and ER-β mRNA expression in R2C cells. Cells (1 X 10^6 cells/ml) were cultured for 6 h with o,p'-DDT (1-10 μM). Total cellular RNA was isolated from the cells. For ER-α, ER-β and GAPDH expression, their cDNAs, which were reverse transcribed from the total RNA, were amplified by PCR. Amplification products were electrophoresed in 2.5% agarose gel and stained with ethidium bromide, as described in Materials and Methods.

12. Effects of o,p'-DDT on COX-2 activity and it's gene expression in R2C cells

Effects of o,p'-DDT on COX-2 activity and expression were therefore assessed in R2C cells. Exposure of the cells to 1 μM ~ 10 μM o,p'-DDT for 24 h markedly enhanced the production of PGE_2, one of the major COX-2 metabolites (Fig. 17). The veracity of the assay was confirmed using LPS, which is well known to induce COX-2 in R2C cells including RAW 264.7 cells. To explain PGE_2 elevation by o,p'-DDT, protein and mRNA levels of COX-2 were determined following treatment with o,p'-DDT using, Western-blot analysis and RT-competitive PCR, respectively. o,p'-DDT caused a dose dependent increase in levels of COX-2 mRNA. o,p'-DDT at a dose dependent increase in amounts of COX-2 mRNA in R2C cells (Fig. 18A). Treatment with 1~10 μM o,p'-DDT for 18 h caused dose-dependent increases in COX-2 protein (Fig. 18B).Thus, o,p'-DDT markedly up-regulated COX-2 gene expression and activity in R2C cells. Since PGE_2 is known to regulate aromatase gene expression and is the product of COX-2, an enzyme frequently over-expressed in R2C cells, aromatase was believed to be strongly correlated with COX-2 expression. This finding suggested a possibility that the feminization seen in o,p'-DDT-exposed R2C cells may also be involved in decreased T production.

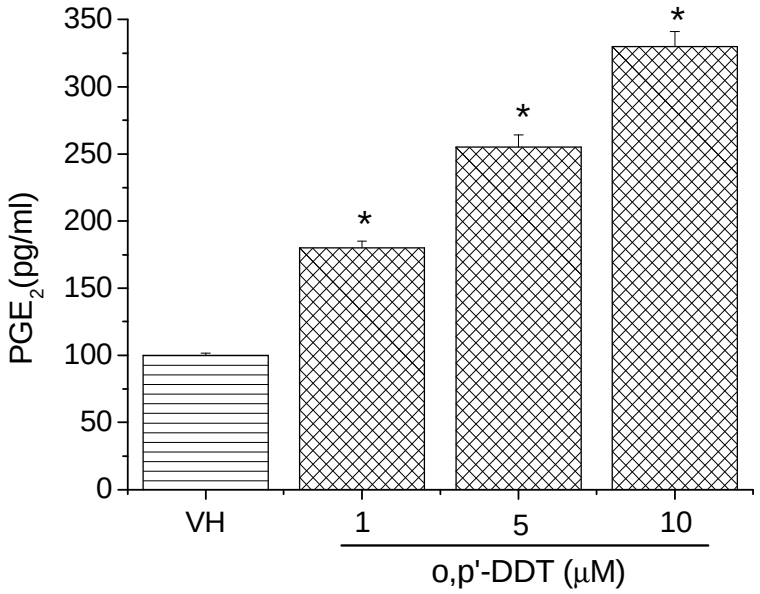


Fig. 17. Effects of o,p'-DDT on PGE_2 production in R2C cells. Cells (1 X 10^6 cells/ml) were cultured for 24 h with o,p'-DDT. The medium was removed and the production of PGE_2 was measured by PGE_2 enzyme immunoassay. * $P < 0.05$, significantly different from the VH.

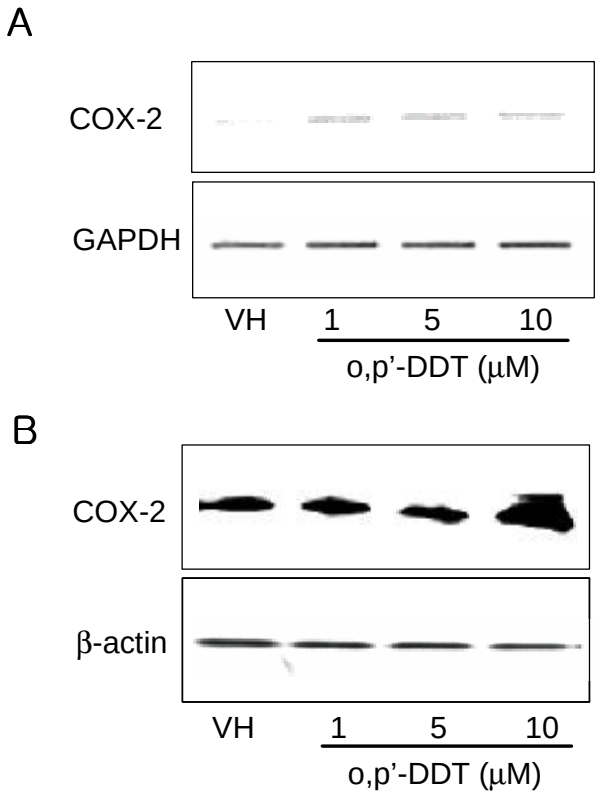


Fig. 18. Effects of o,p'-DDT on COX-2 mRNA and protein expression in R2C cells. (A) Cells (1 X 10^6 cells/ml) were cultured for 2 h with o,p'-DDT. Cells were lysed and total RNA was prepared for RT-PCR analysis of gene expression. Total cellular RNA was isolated from the cells. For COX-2 and GAPDH expression, their cDNAs, which were reverse transcribed from the total RNA, were amplified by PCR. Amplification products were electrophoresed in 2.5 % agarose gel and stained with ethidium bromide, as described in Materials and Methods. (B) Cells (1 X 10^6 cells/ml) were cultured for 18 h with o,p'-DDT. Cells were lysed and total protein was prepared for Western blotting.

13. Expression of EP receptors in R2C cells and effects of o,p'-DDT on EP receptor expression in R2C cells

Effects of o,p'-DDT on the expression of EP_1, EP_2, EP_3, and EP_4 receptor mRNAs were assessed in R2C cells using RT-PCR analysis. The mRNAs of EP_{1-4} subtype receptors were detected in R2C cells. Upon o,p'-DDT treatment for 6 h, the mRNA level of EP_2 and EP_4 subtype receptor was evidently increased (Fig. 19), Whereas other subtypes of EP receptor mRNAs remained unchanged (data not shown).

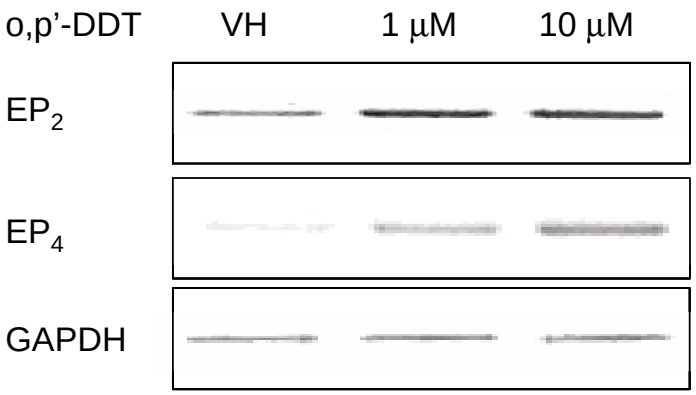


Fig. 19. Effects of o,p'-DDT on EP receptors mRNA expression in R2C cells. Cells ($1x10^6$ cells/ml) were cultured for 6 h in the presence of media alone or with the indicated concentrations of o,p'-DDT. Total cellular RNA was isolated from the cells. For EP_{1-4} receptor and GAPDH expression, their cDNAs, which were reverse transcribed from the total RNA, were amplified by PCR. Amplification products were electrophoresed in 2.5% agarose gel and stained with ethidium bromide, as described in Materials and Methods.

14. Effect of o,p'-DDT on the intracellular cAMP production

In this wok, treatment with o,p'-DDT for 1 h resulted in dose dependent increase of cAMP levels in the R2C cells (Fig. 20). PGE_2, a potent activator of intracellular cAMP production activator, was used as the positive control. Subsequently, examine the role of putative *cis*-element in cAMP-induced transcriptional activity, the site were mutated CRE (cAMP responsible element) of *CYP19* promoter I.4 were constructed with luciferase gene. Mutation of CRE decreased o,p'-DDT-induced basal promoter by approximately 70% (Fig. 21). Therefore, it was concluded that cAMP-induced transcription from promoter I.4 in R2C cells might require CRE.

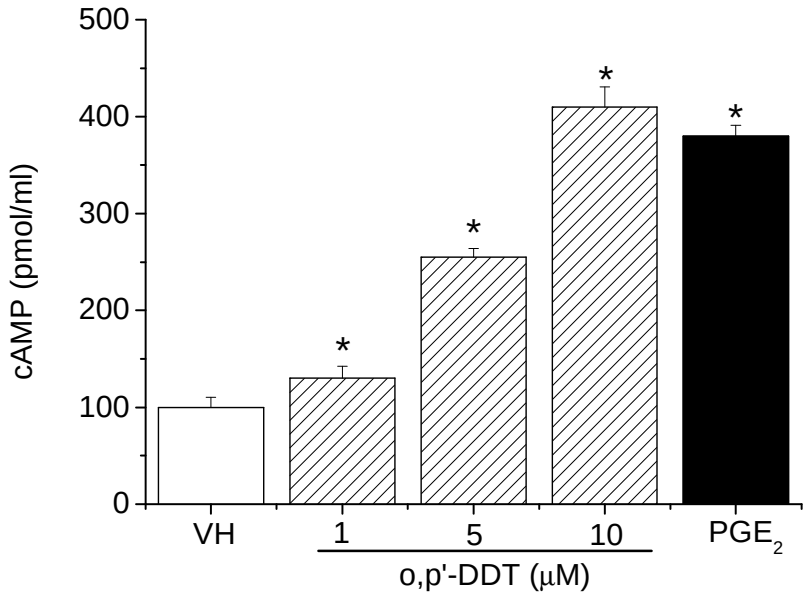


Fig. 20. Effects of o,p'-DDT on intracellular cAMP production in R2C cells. Cells (5 X 10^5 cells/ml) were cultured for 1 h in the presence of media alone or with the indicated concentrations of o,p'-DDT (1-10 μM) or PGE_2 (1 μM). Intracellular accumulation of cAMP was determined by ELISA. o,p'-DDT dose dependently increased intracellular cAMP concentration in R2C cells. Data points represent means ± S.D. of duplicate determinations from three independent experiments. Error bars standard deviations. * $P < 0.05$, Significantly different from the VH.

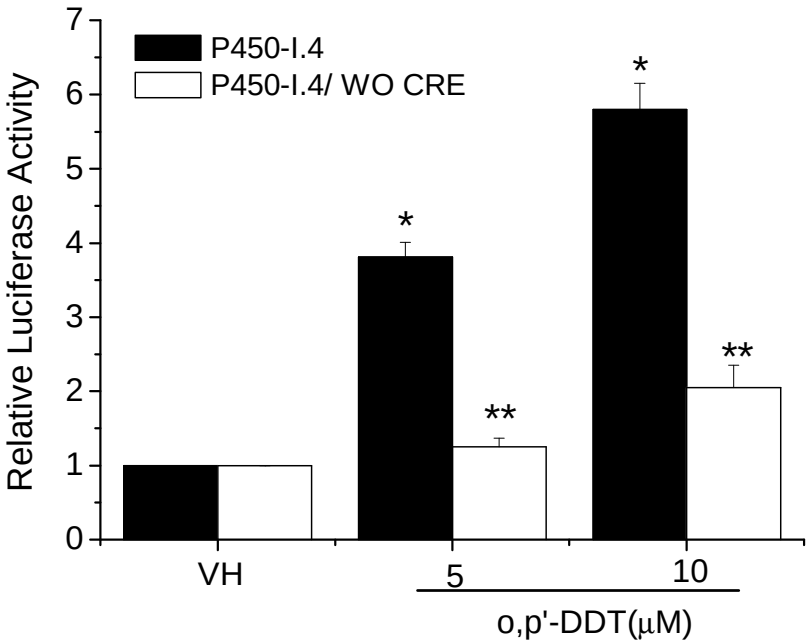


Fig. 21. Effects of mutation in CRE-like sequence within *CYP19* promoter I.4. on o,p'-DDT-induced luciferase activity in R2C cells. R2C cells were transfected with the *CYP19* promoter I.4. Reporter construct depicted above, harboring mutation in CRE site, and incubated in the presence or absence of o,p'-DDT (5, 10 μM) for 12 h. Cells were lysed, and *Luc* activity was determined and normalized to β-galactosidase activity. * $P < 0.05$, Significantly different from the VH.

Up-regulatory effects of o,p'-DDT on cyclooxygenase-2 and iNOS gene expression in RAW 264.7 cells

15. Effects of o,p'-DDT on COX-2 activity in RAW 264.7 cells

The effects of o,p'-DDT on COX-2 activity and expression were therefore assessed in RAW 264.7 cells. Exposure to 0.01 μM ~ 1 μM o,p'-DDT for 24 h markedly enhanced the production of PGE_2, one of the major COX-2 metabolites (Fig. 22). The veracity of the assay was confirmed using LPS, which is well known to induce COX-2 in macrophage cells including RAW 264.7 cells.

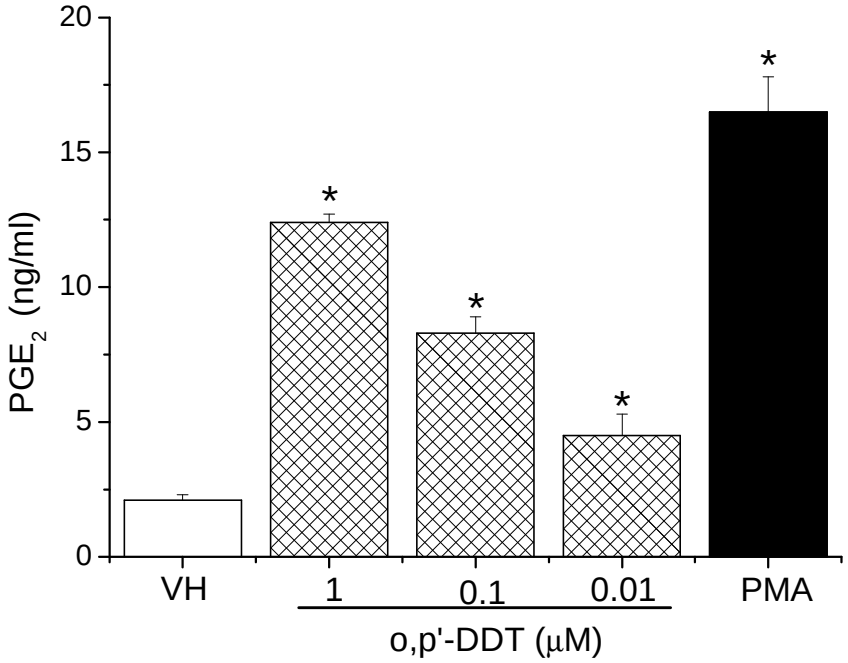


Fig. 22. Effects of o,p'-DDT on PGE_2 production in RAW 264.7 cells. RAW 264.7 cells were treated with 0.01 μM ~ 1 μM o,p'-DDT for 24 h. The medium was then replaced with fresh medium containing 5 μM sodium arachidonate. 30 min later, the medium was collected to determine the synthesis of PGE_2. Production of PGE_2 was determined by enzyme immunoassay. One of three representative experiments is shown. Each bar shows the mean ± S.D. of three independent experiments, performed in triplicate. * $P < 0.05$, significantly different from the VH.

16. Effects of o,p'-DDT on COX-2 gene expression in RAW 264.7 cells

To explain PGE_2 elevation by o,p'-DDT, protein and mRNA levels of COX-2 were measured using, Western blot analysis and RT-competitive PCR, respectively. Treatment with 0.01 μM ~ 1 μM o,p'-DDT for 18 h caused dose-dependent increases of COX-2 protein (Fig. 23A). For mRNA expression study, cells were treated with o,p'-DDT for 2h. o,p'-DDT caused a dose dependent increase in levels of COX-2 mRNA. o,p'-DDT at a dose of 1 μM lead to about 3-fold increase in amounts of COX-2 mRNA in RAW 264.7 cells (Fig. 23B). Thus, o,p'-DDT markedly up-regulated *COX-2* gene expression and activity in RAW 264.7 cells.

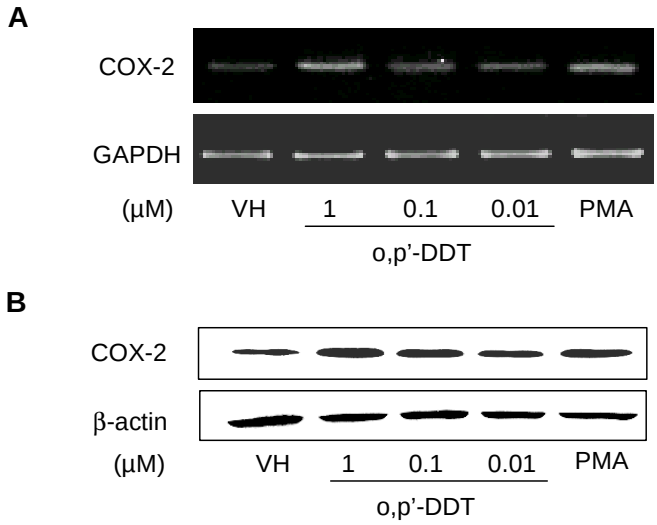


Fig. 23. Effects of o,p'-DDT on COX-2 mRNA and protein expression in RAW 264.7 cells. (A) Cells (5 X 10^5 cells/ml) were treated with o,p'-DDT for 2 h. Total cellular RNA was then extracted for RT-PCR with specific mouse COX-2 primers. Quantitation of bands normalized to GAPDH showed o,p'-DDT (1 μM) increased COX-2 mRNA 3.5-fold compared with control. The results in each panel represent one of three separate experiments with similar findings. (B) Cells (5 X 10^5 cells/ml) were treated with o,p'-DDT for 24 h. Western blot analysis was performed as described in "Materials and Methods." The membrane was probed with a COX-2-specific antibody, and bands were visualized with ECL reagents. o,p'-DDT induced COX-2 in a dose-dependent manner with a maximal effect at 1 μM. Each blot in this figure is representative of three separate experiments independently performed, with similar results.

17. Effects of o,p'-DDT on MAPK activation

MAPKs are known to be important signaling modulators in COX-2 expression and these had been recently shown to be activated by DDT [Shinomiya and Shinomiya, 2003; Robinson and Dickenson, 2001; Tessier and Matsumura, 2001]. Therefore, the involvement of MAPK-signaling pathways in o,p'-DDT-induced COX-2 expression was investigated. When the effects of o,p'-DDT on phosphorylation of ERK, p38, and JNK were analyzed by Western blot analysis, the o,p'-DDT was found to enhance phosphorylation of all 3 MAPKs, with maximal effects being observed 30 min after 0.01 μM ~1 μM o,p'-DDT exposure (Fig. 24). The levels of non-phosphorylated MAPKs were unaffected by o,p'-DDT treatment.

Treatment with MAPK-signaling inhibitors PD98059 (an MEK1/2 inhibitor that inhibits ERK activation) or SB203580 (a p38 MAPK inhibitor) or SP600125 (a SAPK/JNK MAPK inhibitor) significantly reduced o,p'-DDT-induced phosphorylation of all 3 MAPKs (Fig. 25). At the concentrations employed, PD98059, SB203580 and SP600125 inhibited ERK, p38 and JNK activities, respectively, without having cytotoxic effects (data not shown).

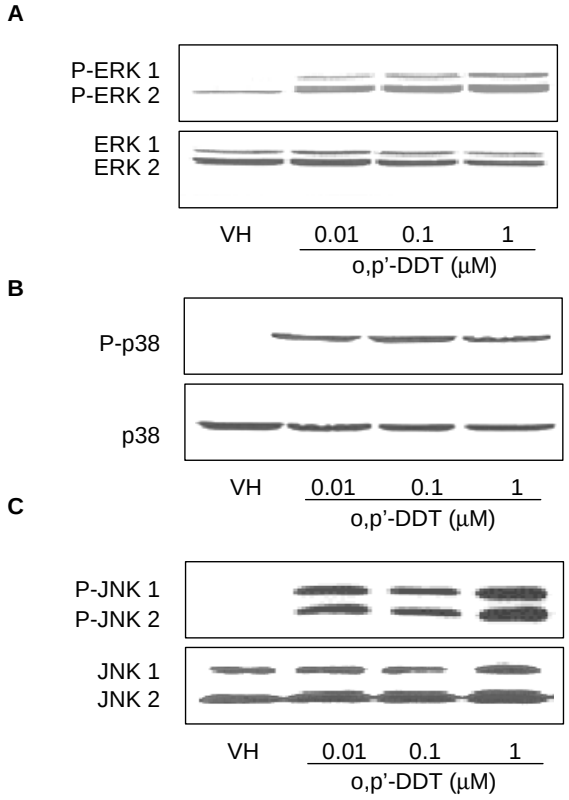


Fig. 24. Effects of o,p'-DDT on MAPK phosphorylation in RAW 264.7 cells. Cells (5 X 10^5 cells/ml) were treated with vehicle, o,p'-DDT or LPS. 15 min After, stimulation with various concentrations (0.01 μM ~ 1 μM) of o,p'-DDT, cells were harvested and suspended in the lysis buffer. In each lane, cell lysates from 1 X 10^5 cells were separated by 10% SDS-PAGE and examined by Western blot analysis. Extracts were analyzed for MAPK activation by Western analysis using antibodies to phosphorylated JNK, ERK, and p38.

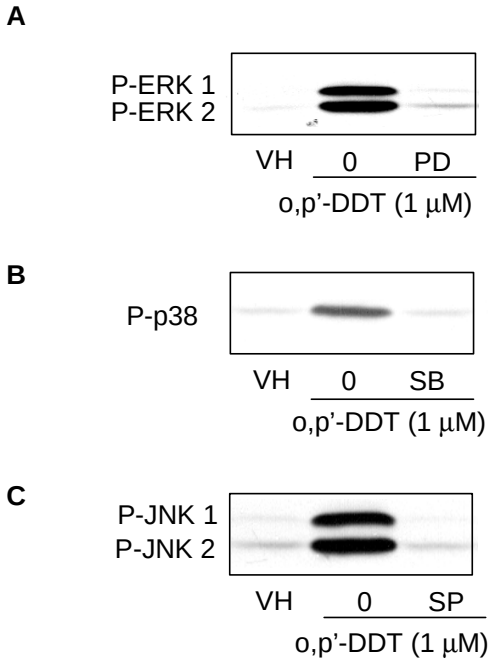


Fig. 25. Effects of inhibitors on o,p'-DDT-induced MAPK phosphorylation in RAW 264.7 cells. Cells (5 X 10^5 cells/ml) were treated with vehicle, o,p'-DDT (1 μM), PD98059, SB203580 or SP600125. After 15 min, cells were harvested and suspended in the lysis buffer. In each lane, cell lysate from 1 X 10^5 cells were separated by 10% SDS-PAGE and examined by Western blot analysis. Extracts were analyzed for MAPK activation by Western analysis using antibodies to phosphorylated JNK, ERK, and p38.

18. Effects of o,p'-DDT on AP-1 activation

Since o,p'-DDT has previously been shown to induce NF-κB activation in the RAW 264.7 macrophage cell line [Kim *et al*., 2004]. This suggests that the o,p'-DDT-induced stimulation of COX-2 expression in RAW 264.7 cells may require NF-κB. To obtain further insights into the molecular mechanism of o,p'-DDT action during COX-2 induction, I was determined the effects on transcription factors such as AP-1, since they are known to affect COX-2 promoter activity. AP-1 activation was analyzed by transactivational activity following transient transfection. RAW 264.7 cells were also stimulated with 100 nM PMA as positive control for AP-1 activity. Cells were transfected with an AP-1 luciferase reporter construct and the luciferase activity was determined following a 24 h incubation in the absence or presence of o,p'-DDT. To ascertain whether AP-1 activation is required in o,p'-DDT-mediated COX-2 expression, we treated RAW 264.7 cells with curcumin, which is an inhibitor of AP-1 binding [Guo *et al*., 2001; Mohan *et al*., 2000]. RAW 264.7 cells were transiently transfected with a pGL3-AP-luc plasmid containing COX-2 promoter coupled with reporter gene and pretreated with or without curcumin for 0.5 h and then stimulated with 0.01 - 1 μM of o,p'-DDT for 24 h. Curcumin (40 μM) completely abolished AP-1 induction induced by o,p'-DDT (Fig. 26). Thus, these findings demonstrated that the AP-1 is an important intermediate in the o,p'-DDT-mediated COX-2 gene expression in

RAW 264.7 cells. These results suggested that NF-κB and AP-1 is required for the transcriptional induction of COX-2 in o,p'-DDT-stimulated RAW 264.7 cells.

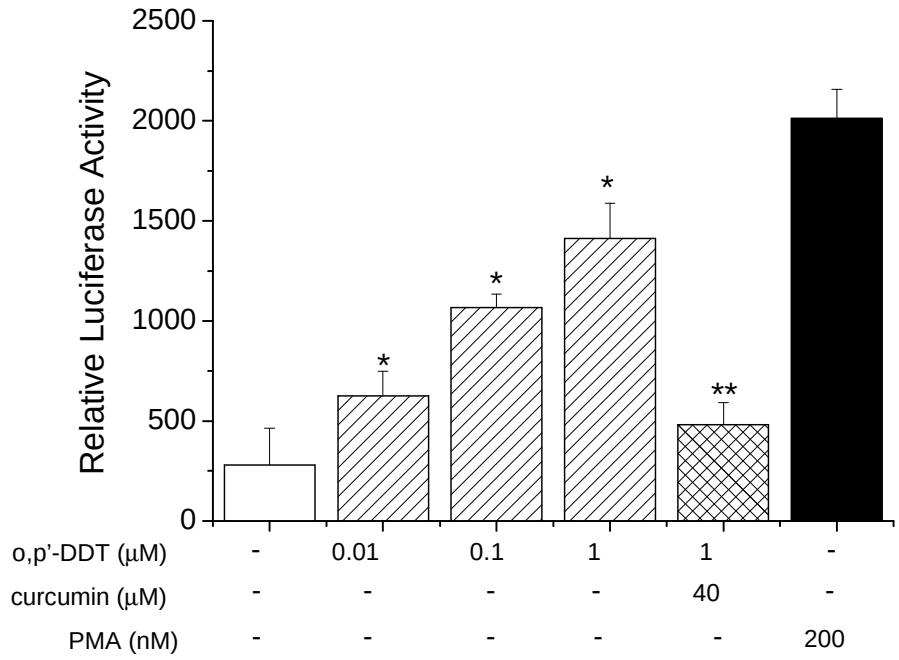


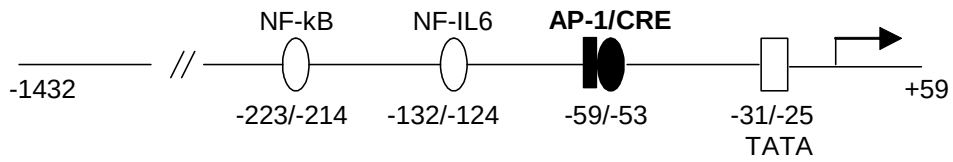
Fig. 26. Effects of o,p'-DDT on AP-1-dependent luciferase gene expression in RAW 264.7 cells. The RAW 264.7 cells (5 X 10^5 cells/ml) were transiently co-transfected with pGL3-3AP1-Luc and pCMV-β-gal. After 18 h, the cells were pretreated with curcumin (40 μM) for 15 min. Then was added o,p'-DDT or PMA (200 nM) for 18 h. The cells were then harvested, and their luciferase and β-galactosidase activities were determined. The luciferase activity was normalized with respect to the β-galactosidase activity. Each bar shows the mean ± S.D. of three independent experiments, performed in triplicate. *P < 0.05; significantly different from the control. **P < 0.05; significantly different from the o,p'-DDT.

19. Effects of o,p'-DDT on transcriptional activation of COX-2 expression.

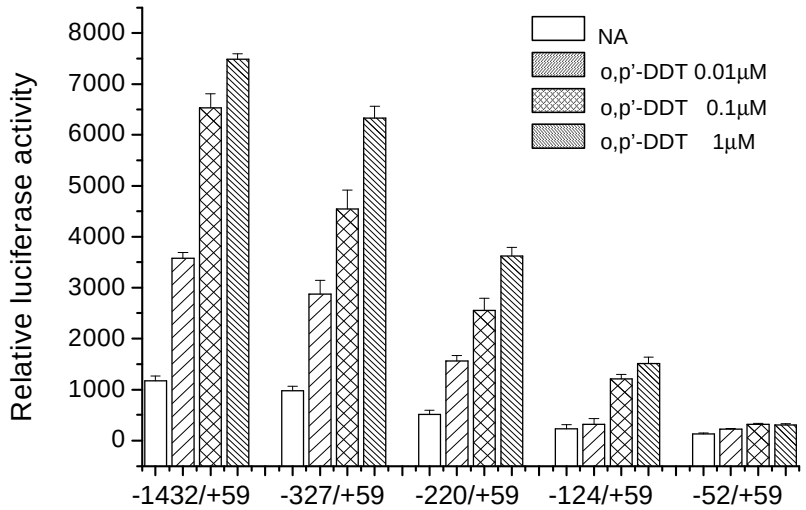
Subsequently the regions of the COX-2 promoter that was important for mediating the inductive effects of o,p'-DDT were identified. Transient transfections were performed with a series of human COX-2 5'-promoter-deletion constructs (Fig. 27A). In agreement with the results shown in Fig 21A, treatment of cells with o,p'-DDT led to a doubling o*f* COX-2 promoter activity when a –1432/+59 COX-2 promoter construct was utilized (Fig. 27B). A stepwise decrease in basal COX-2 promoter activity was observed when shorter constructs were used. We carried out transient transfections to further elucidate the effects of o,p'-DDT on COX-2 transcription. PMA at 100 nM was used as the positive control, because it has also been shown to activate COX-2 promoter activity in RAW 264.7 cells [Paul *et al*., 1999]. o,p'-DDT, at concentrations of 0.01 ~ 1 μM, significantly increased luciferase expression dose-dependently in the transfected cells at 18 h. However, the magnitude of induction by o,p'-DDT remained essentially constant with all promoter deletion constructs except the –52/+59 construct (Fig. 27C). The –52/+59 COX-2 promoter construct was not stimulated by o,p'-DDT. This result implied that one or more promoter elements lying between –53 and –123 are necessary for o,p'-DDT-mediated induction of COX-2. The fact that a CRE is present between nucleotides –59 and –53 raising the possibility that this element could be involved in mediating the inductive effects of o,p'-DDT. To

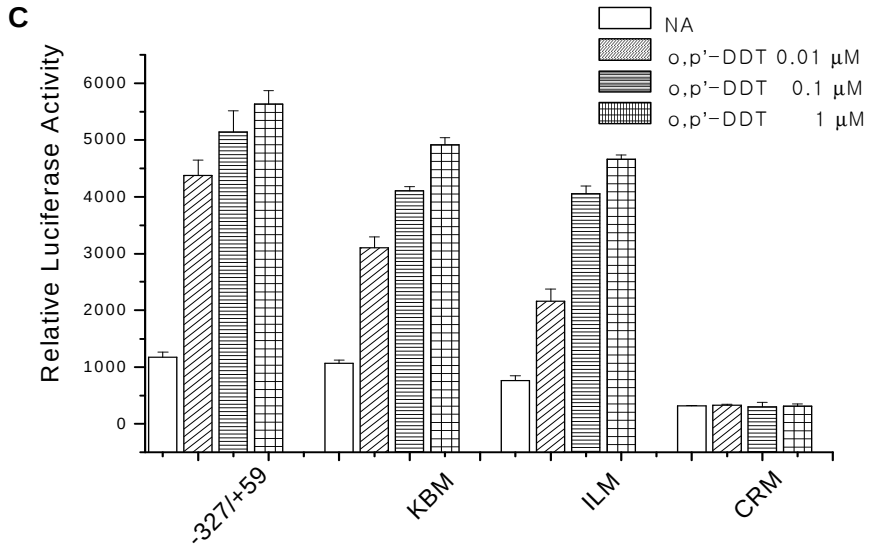
test this hypothesis, transient transfections were performed utilizing *COX-2* promoter constructs in which specific known enhancer elements including the CRE were mutated. As shown in Fig. 25B, mutation in the CRE site abrogated o,p'-DDT-mediated stimulation of COX-2 promoter activity. By contrast, mutation in the NF-IL6 sites had no effect on *COX-2* promoter function. In addition, the involvement of MAPKs in o,p'-DDT-induced transcriptional activation was investigated. The MAPKs inhibitors (PD98059, SB203580 or SP600125) significantly suppressed luciferase reporter induction in o,p'-DDT-treated transfected cells (Fig. 27D). Taken together, the results indicate that p38<ERK<JNK MAPKs activation and by o,p'-DDT contributed to transcriptional activation of the COX-2 gene.

A


NF-kB
NF-IL6
AP-1/CRE
-1432
-223/-214
-132/-124
-59/-53
-31/-25
TATA
+59

B


Relative luciferase activity
8000
7000
6000
5000
4000
3000
2000
1000
0
NA
o,p'-DDT 0.01μM
o,p'-DDT 0.1μM
o,p'-DDT 1μM
-1432/+59
-327/+59
-220/+59
-124/+59
-52/+59


C
NA
o,p'-DDT 0.01 μM
o,p'-DDT 0.1 μM
o,p'-DDT 1 μM
Relative Luciferase Activity
6000
5000
4000
3000
2000
1000
0
-327/+59
KBM
ILM
CRM

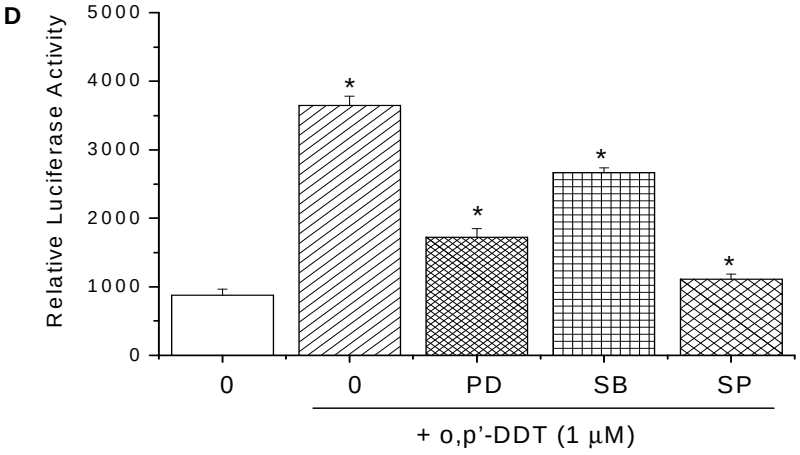

D
Relative Luciferase Activity
5000
4000
3000
2000
1000
0
*
0
0
PD
SB
SP
+ o,p'-DDT (1 μM)

Fig. 27. Localization of the *COX-2* promoter region that mediates the effects of o,p'-DDT. **(A**) A scheme of the human *COX-2* promoter. (**B**) RAW 264.7 cells were transfected with 1 μg of a series of human *COX-2* promoter deletion constructs ligated to luciferase (-1432/+59, -327/+59, -220/+59, -124/+59, or -52/+59) and 0.5 μg of pCM-ß-gal. (**C)** RAW 264.7 cells were transfected with 1 μg of a series of human *COX-2* promoter-luciferase constructs (-327/+59, KBM, ILM, CRM, and CRM-ILM) and 0.5 μg of pCMV-ß-gal. KBM represents the -327/+59 *COX-2* promoter construct in which the NF-κB site was mutated; ILM represents the -327/+59 *COX-2* promoter construct in which the NF-IL6 site was mutated; CRM refers to the -327/+59 COX-2 promoter construct in which the CRE was mutated; CRM-ILM represents the -327/+59 COX-2 promoter construct in which both the NF-IL6 and CRE site were mutagenized. After transfection, cells were treated with vehicle, PMA (200 nM), or various concentrations (0.01 μM ~ 1 μM) of o,p'-DDT. Reporter activities were measured in cellular extract 6 h later. (**D)** RAW 264.7 cells were transfected with 1 μg of -327/+59 and 0.5 μg of pCMV-ß-gal. After transfection, cells were pretreated with 10 μM PD98059, 10 μM SB203580 or 10 μM SP600125 for 30 min. Then, 1 μM of o,p'-DDT was added. Reporter activities were measured in cellular extract 12 h later. Luciferase activity represents data that have been normalized with ß-galactosidase activity. The values are presented as the mean ± S.D., each performed in triplicate. **P* < 0.05; significantly different from the control.

20. Effects of o,p'-DDT on NO production

Peritoneal macrophages and RAW 264.7 cells, a mouse macrophage cell line, were incubated with o,p'-DDT for 24 h, and the NO concentrations in the culture supernatants were assessed using the Griess reaction. o,p'-DDT had a significant effect on NO production from a dose of 0.2 μM. Upon o,p'-DDT stimulation, NO production by the macrophages increased in a dose-dependent manner (Fig. 28). The cytotoxicity of the o,p'-DDT in macrophages was assessed by a MTT assay, which indicated that the concentration of o,p'-DDT used in these experiments did not decrease the cell viability in the cells (> 95% cell viability, Fig. 26).

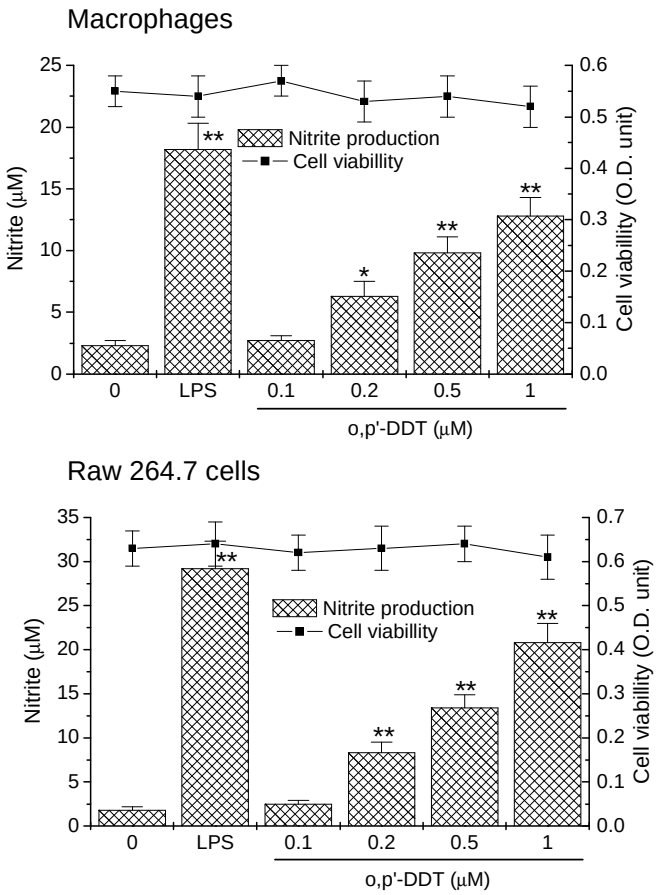


Fig. 28. Effects of o,p'-DDT on NO production in macrophages. Murine peritoneal macrophages (2 X 10^5 cells/ml) or RAW 264.7 cells (5 X 10^5 cells/ml) were cultured for 24 h in the presence of the media alone, with the indicated concentrations of o,p'-DDT, or with LPS (0.1 μg/ml). The level of NO production was determined by measuring the accumulation of nitrite in the culture medium. The cell viability was assessed by a MTT assay. Each bar shows the mean ± S.D. of three independent experiments, performed in triplicate. *$P < 0.05$; **$P < 0.005$, significantly different from the control.

21. Effects of o,p'-DDT on macrophage-related proinflammatory cytokines production

In order to assess the effects of o,p'-DDT on TNF-α, IL-1β and IL-6 production, RAW 264.7 cells were incubated in the presence of o,p'-DDT and the quantities of the these cytokines secreted into the culture supernatants were then monitored by ELISA. o,p'-DDT increased in the levels of TNF-α and IL-6 secretion in the supernatant at 6 h in a dose-dependent manner. However, the level of IL-lβ secretion in the supernatant 6 h after o,p'-DDT treatment was slightly increased without showing a significant difference in IL-lβ secretion between o,p'-DDT and control. Thereafter, the cells were cultured for either 6 h (TNF-α and IL-6) or 12 h (IL-1β) in the presence of media with o,p'-DDT and the amount of TNF-α, IL-1β, and IL-6 released to the culture medium were measured by an immunoassay. In this study, o,p'-DDT increased the levels of TNF-α, IL-1β, and IL-6 secretion in the supernatant in a dose-dependent manner and in a similar manner to that observed for NO production (Table 1). LPS, a known potent macrophage activator, was used as a positive control. The secretion of these cytokines might be due to the activation of the macrophages via the autocrine or paracrine action of the secreted TNF-α, IL-lβ or IL-6 by o,p'-DDT.

Table 1. Effects of o,p'-DDT on the production of cytokines in RAW 264.7 cells

Treatment[a]	TNF-α (ng/ml)	IL-1β (ng/ml)	IL-6 (ng/ml)
Control	0.62 ± 0.09[b]	0.31 ± 0.05[b]	0.28 ± 0.05[b]
o,p'-DDT (0.2 μM)	2.13 ± 0.29*	1.33 ± 0.17*	0.83 ± 0.11*
o,p'-DDT (0.5 μM)	6.72 ± 0.87**	2.24 ± 0.31**	1.67 ± 0.19**
o,p'-DDT (1 μM)	12.73 ± 1.41**	3.72 ± 0.42**	2.85 ± 0.34**
LPS (0.1 μg/ml)	18.22 ± 2.12**	5.58 ± 0.73**	3.98 ± 0.54**

[a]The RAW 264.7 cells (5 X 10^5 cells/ml) were cultured for either 6 h (TNF-α and IL-6) or 12 h (IL-1β) in the presence of media alone, with op'-DDT, or LPS. The amount of TNF-α, IL-1β, and IL-6 released to the culture medium were measured using an immunoassay. [b]The Results are expressed as a mean ± S.D. of four independent experiments, performed in triplicate. *$P < 0.05$; **$P < 0.005$, significantly different from control.

22. Effects of o,p'-DDT on the gene expression of iNOS and proinflammatory cytokines

As described above, o,p'-DDT induced the macrophage secretion of NO, TNF-α, IL-lβ, and IL-6. A RT-PCR assay was conducted in order to determine whether or not o,p'-DDT regulates NO as well as the secretion of these cytokines at the mRNA level. LPS, a known potent macrophage activator, was used as the positive control. Consistent with the results obtained from the NO and cytokines secretion immunoassays, the iNOS, TNF-α, IL-lβ, and IL-6 mRNA levels were markedly increased by the o,p'-DDT treatment (Fig. 29). Control β-actin was constitutively expressed and was unaffected by o,p'-DDT treatment. Therefore, the increase in iNOS, TNF-α, IL-lβ, and IL-6 by o,p'-DDT is believed to be regulated by the transcriptional activation.

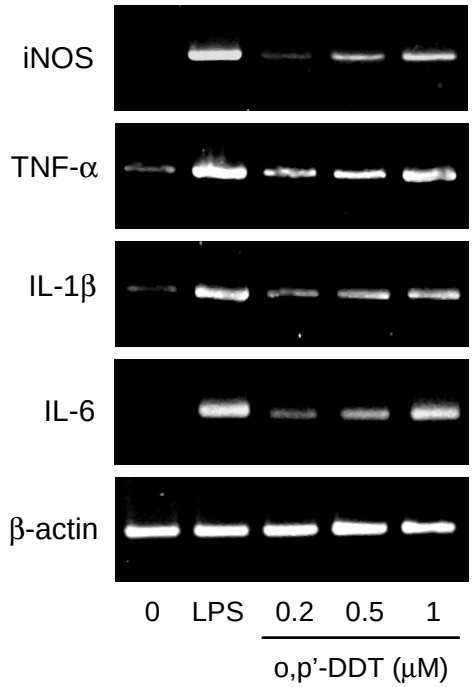


Fig. 29. Effects of o,p'-DDT on iNOS, TNF-α, IL-1β, and IL-6 mRNA expression in RAW 264.7 cells. RAW 264.7 cells (1 X 10^6 cells/ml) were cultured for 6 h in the presence of media alone, with the indicated concentrations of o,p'-DDT, or with LPS (0.1 μg/ml). The cells were lysed and the total RNA was prepared for analysis of gene expression by RT-PCR. PCR amplification of the housekeeping gene, β-actin, was performed for each sample. The PCR amplification products were electrophoresed in 2.5% agarose gel and stained with ethidium bromide. One of three representative experiments is shown.

23. Effects of the o,p'-DDT on the activation of NF-kB

The transcription factor, NF-κB, is the central regulator for the expression of the genes encoding iNOS and the proinflammatory cytokines, and NF-κB was identified as a critical intracellular mediator of the inflammatory cascade [Baeuerle and Baichwal, 1997; Ghosh *et al*., 1998]. In order to further investigate the role of o,p'-DDT on iNOS gene expression, the effect of o,p'-DDT on the NF-κB-dependent gene expression level was assessed by using the luciferase reporter gene assay. The macrophages were transiently transfected with a plasmid containing 4 copies of the NF-κB binding sites, and the luciferase activities were measured. LPS was then used as the positive control. Consistent with NO and proinflammatory cytokine production and these mRNA expression, o,p'-DDT also significantly increased the NF-κB-dependent luciferase activities in a dose dependent manner (Fig. 30). In order to further investigate the putative mechanism, the effects of o,p'-DDT on the activation of a family of transcription factors was monitored by an electrophoretic mobility gel shift assay (EMSA). As shown in Fig. 31, incubating the cells with o,p'-DDT for 1 h markedly increased the level of NF-κB binding at its conserved site, which was visualized as a distinct band (indicated by an arrow). The addition of an excess of an unlabeled wild type probe completely prevented the NF-κB binding, indicating the

binding specificity of the NF-κB complex. In order to further confirm the role of NF-κB in iNOS expression caused by o,p'-DDT, we used BAY 11-7082, an IκBα kinase inhibitor, which specifically inhibits NF-κB activation by inhibiting the phosphorylation and the subsequent degradation of IκBα, an endogenous NF-κB inhibitor [Pierce *et al*., 1997]. As shown in Fig. 32, pretreating the macrophages with BAY 11-7082 effectively inhibited the NO production and iNOS expression induced by o,p'-DDT. This suggests that the induction of iNOS, TNF-α, IL-lβ, and IL-6 expression by o,p'-DDT occurred via NF-κB activation.

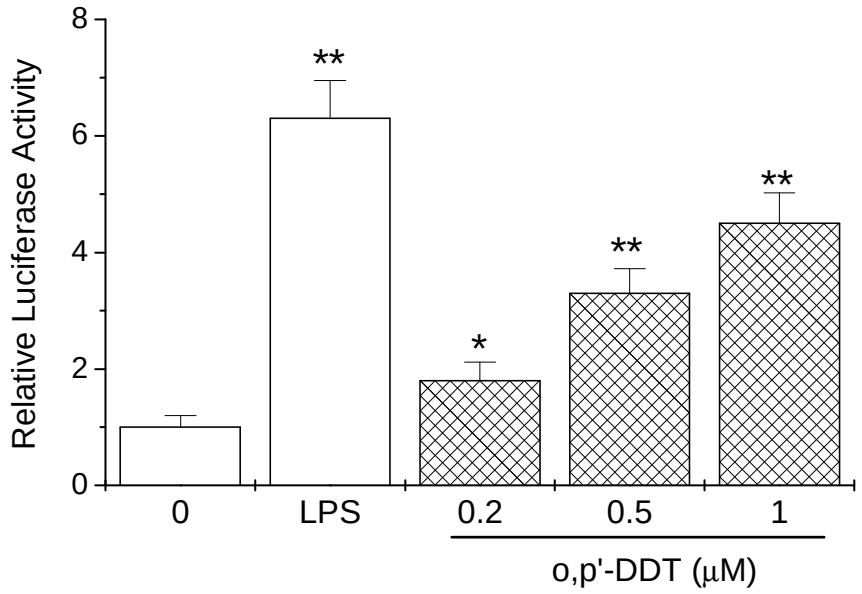


Fig. 30. Effects of o,p'-DDT on NF-κB-dependent luciferase gene expression in RAW 264.7 cells. RAW 264.7 cells (5 X 10^5 cells/ml) were transiently co-transfected with pGL3-4κB-Luc and pCMV-β-gal. After 18 h, the cells were treated with the indicated concentrations of o,p'-DDT or LPS (0.1 μg/ml) for 12 h. The cells were then harvested, and their luciferase and β-galactosidase activities were determined. The luciferase activity was normalized with respect to the β-galactosidase activity and expressed relative ratio to the activity of the control. Each bar shows the mean ± S.D. of three independent experiments, performed in triplicate. *P < 0.05; **P < 0.005, significantly different from the control.

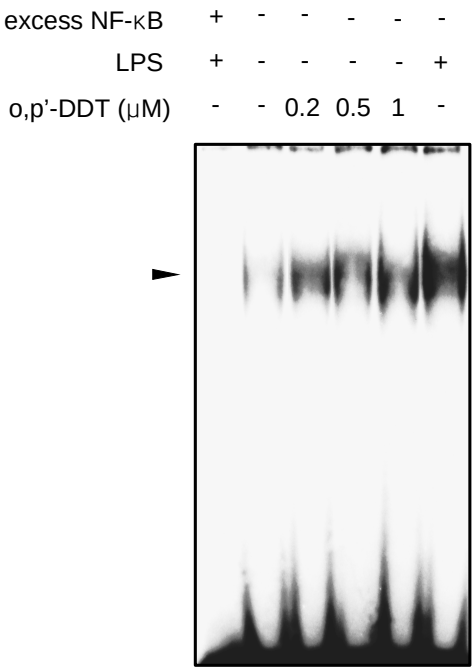


Fig. 31. Effects of o,p'-DDT on NF-κB-binding in RAW 264.7 cells. RAW 264.7 cells were treated with o,p'-DDT or LPS (1 μg/ml) for 1 h. The nuclear extracts were isolated and used in an electrophoretic mobility shift assay with ^{32}P-labeled NF-κB oligonucleotide as a probe, as described in Materials and Methods. The arrow indicates the NF-κB binding complex. Excess NF-κB; 200-fold molar excess of non-labeled NF-κB probe. One of three representative experiments is shown.

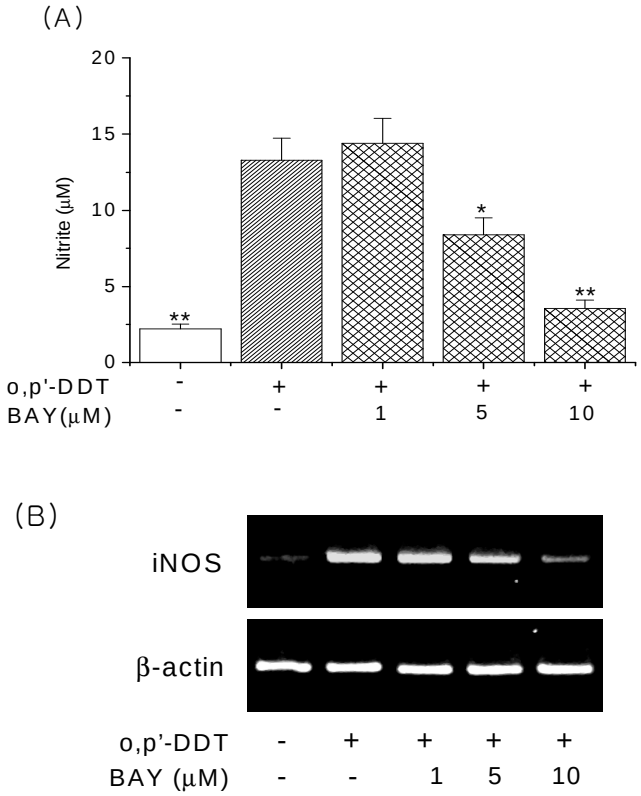


Fig. 32. Effects of NF-κB inhibition on NO production and iNOS expression by o,p'-DDT in RAW 264.7 cells. RAW 264.7 cells (5 X 10^5 cells/ml) were pretreated with BAY 11-7082 for 1 h and then cultured for 24 h in the presence of the media alone or with o,p'-DDT (1 μM). The level of NO production was determined by measuring the accumulation of nitrite in the incubation medium. Each bar shows the mean ± S.D. of three independent experiments, performed in triplicate. *P < 0.05; **P < 0.005, significantly different from the op'-DDT. (B) The RAW 264.7 cells (1 X 10^6 cells/ml) were cultured for 6 h in the presence of media alone, with o,p'-DDT (1 μM). The cells were lysed and the total RNA was prepared for the RT-PCR analysis of gene expression. PCR amplification of β-actin was performed for each sample.

IV. Discussion

Down-regulation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced CYP1A1 gene expression by o,p'-DDT in murine heap-1c1c7 cells

The present results indicated that the inhibitory action of o,p'-DDT on TCDD-induced CYP1A1 gene expression might be a result from decreased Ah receptor-dependent transcriptional activation, caused by an alteration in the DRE binding potential of either the nuclear Ah receptor or a blockage of the Ah receptor in transport to the nucleus. However, other possibilities such as the possibility that o,p'-DDT exert an indirect transcriptional interference between other transcription factors binding to the negative regulatory element region in the CYP1A1 promoter region, or changes in the phosphorylation state of the cytosolic receptor complex could not be excluded [Reiners *et al*., 1993]. It is also possible that the inhibitory effect of o,p'-DDT on TCDD-inducible CYP1A1 gene expression may be the result of one or more of its metabolites irreversibly inactivating an essential component of the CYP1A1 gene expression system. Additional studies are needed to answer these questions, and further studies to elucidate the mechanism are in progress. To date, a number of chemicals have been reported to antagonize some of the biological and toxicological effects of TCDD. These include 2,2',4,4',5,5'-hexachlorobiphenyl [Biegel *et al*., 1989], 6-methyl-1,3,8-trichlorodiben-

zofuran [Piskoska-Pliszczynzka, 1991], and thyroid hormone [Hong *et al*., 1987]. Previous studies have shown that in Ah-responsive mammalian cells in culture α-naphthoflavone antagonizes TCDD-induced CYP1A1 gene expression [Gasiewicz and Rucci, 1991].

The estrogen receptor and the Ah receptor are co-expressed in several Ah and estrogen-responsive cell lines. Some studies have shown that the estrogen receptor is important for Ah receptor-mediated transactivation in breast cancer cells, such as in MCF-7 cells [Thomsen *et al*., 1994]. Another study reported that estradiol inhibited Ah responsiveness in mouse Hepa-1c1c7 cells, and therefore, demonstrated the presence of two-way cross-talk between the intracellular signaling pathways involving the estrogen and Ah receptors [Kharat and Saatcioglu, 1996]. To assess the possibility that the suppressive effects of o,p'-DDT on TCDD-inducible EROD activities might be related with the estrogen receptor, ICI 182,780, an antiestrogen that acts through the estrogen receptor was used. ICI 182,780 treatment had no effect on the influence of o,p'-DDT on TCDD-induced EROD activity. Thus, the antagonism of TCDD-induced CYP1A1 gene expression in Hepa-1c1c7 cells by o,p'-DDT is not mediated through the estrogen receptor in this cell line. This suggests that o,p'-DDT action might be independent of the classical estradiol receptor pathway. One possible receptor that could mediate the effect

of endosulfan is the pregnane X receptor or a related receptor. These receptors can bind several steroids and xenobiotics. Recent studies have shown that organochlorine pesticides can bind and activate this receptor [Schuetz *et al*., 1998]. Furthermore, the pregnane X receptor is expressed in liver cells. Treatment of the Hepa-1c1c7 cells with rifampicin, a typical activator of pregnane X receptor, did not affect the basal EROD activity but suppressed TCDD-inducible EROD activity like as o,p'-DDT (data not shown). Thus, this result suggests that the pregnane X receptor rather than the estrogen receptor could be implicated in the o,p'-DDT effect in Hepa-1c1c7 cells. Additional studies are required to firmly establish this mechanism.

This work showed that o,p'-DDT inhibits the TCDD-inducible expression of CYP1A1. Thus, o,p'-DDT may serve to modulate response to xenobiotics. Several studies have shown that o,p'-DDT induces the CYP3A gene, which codes for an enzyme that is capable of metabolizing steroids including 17β-estradiol [Juchau, 1990]. There is also indirect evidence which suggest that CYP1A is similarly involved in steroid metabolism [Aoyama *et al*., 1990]. Thus, in liver derived cells, addition of o,p'-DDT to TCDD leads to a shift in the ratio of steroid-metabolizing enzymes by increasing CYP3A and decreasing CYP1A1. The physiological consequences of this shift remain unclear. It is well known that humans usually are contaminated by a com-

bination of several xenobiotics. It is likely that the interaction between these contaminants, synergy or antagonism, will be relevant for the final toxic effect [Auger *et al*., 1995]. Our data suggest that the regulation of the CYP1A1 gene may constitute an important aspect of the toxicity of a combination of dioxins and o,p'-DDT. Such a combination of contaminants may not be uncommon because dioxins are produced during the synthesis of numerous pesticides and because both compounds contaminate food.

o,p'-DDT reduced testosterone production via induction of aromatase (*CYP19*) gene expression in rat testicular Leydig cells

In this study, we have found that o,p'-DDT-caused inhibition of T production in R2C cell is mediated through aromatase gene expression via ERβ and $EP_{2,4}$ and might be influenced by COX-2 and PGE_2. There is increasing evidence that diverse environmental contaminants have the potential to disrupt endocrine processes, which may result in reproductive problems, certain cancers and other toxicities related to sexual differentiation, growth, and development [McLachlan, 2001]. Leydig cells are specialized interstitial cells in the testis that produce the testosterone required for spermatogenesis [Zhao *et al*., 1996]. The present study demonstrated that exposure to environmentally relevant level of o,p'-DDT has adverse effects on testicular function and reducing Leydig cell steroidogenesis.

In the present study, we tested the effects of o,p'-DDT on T synthesis in R2C cells. Steroidogenesis in leydig cells is initiated with cholesterol transfer into the mitochondria, which is mediated by the steroidogenic acute regulatory (StAR) protein. Steroidogenesis in leydig cells primarily regulated by LH through the production of the intracellular second messenger cAMP [Akingbemi *et al*., 2003]. cAMP stimulates steroidogenesis by increasing the expression of steroidogenic-enzyme genes [Dufau, 1988]. Studies on

steroidogenic enzyme gene expression by RT-PCR indicated that o,p'-DDT caused specific inhibition of the $P450_{17\alpha}$ enzyme, which is known to be inhibited by environmental estrogens [Akingbemi *et al*., 2003]. In the present study, treatment of the R2C cells with o,p'-DDT significantly decreased the $P450_{17\alpha}$, 3β-HSD, and relative genes of T synthesis. These results suggested that o,p'-DDT causes a direct inhibition of T biosynthesis by leydig cells and that o,p'-DDT-induced inhibition of T production is related to a decrease in the activity of $P450_{17\alpha}$ and 3β-HSD.Various pesticides known or suspected to interfere with steroid hormone function were screened for effects on catalytic activity, mRNA and protein expression of aromatase in leydig cells [Sanderson *et al*., 2002]. o,p'-DDT increased aromatase activity and gene expression in rat testicular leydig R2C cells in a concentration-dependent manner. These results indicated that o,p'-DDT-induced inhibition of T production in R2C cell is mediated through aromatase. In addition, it was observed that, o,p'-DDT increased the aromatase transcriptional activation in R2C cells by using aromatase gene promoter I.4 transient transfection. The change in T biosynthesis and related aromatase gene expression by o,p'-DDT were associated with changing in T hormones in rat leydig cell line, R2C cells. These results suggested that the inductive effects of o,p'-DDT on the T biosynthesis and related aromatase gene expression may, therefore,

partly explain the induction of imposex.

ERβ expression which is thought to be significantly lower than ERα has been known to up-regulate both ER subtypes in the pitutary and cause transcriptional activation through both forms of the ER [Pennie *et al*., 1998]. In the present study o,p'-DDT treatment increased steady state level of pituitary ERβ mRNA, but not ERα (Fig. 16). This observation supported the hypothesis that o,p'-DDT has a greater potency for ER-mediated activity than was previously thought [Kuiper *et al*., 1997]. Also, activation of the ER by endocrine disruptors is known to be moderated by estrogen-responsive elements, transcription factors, and the transcription-regulating domains of the target gene [Kuiper *et al*., 1997].

Therefore, o,p'-DDT-induced inhibition of T production was ERβ - mediated, and o,p'-DDT-induced suppression of the CYP17 gene, which encodes the $P450_{17\alpha}$ enzyme, was presumably mediated by ERβ as previously suggested [Akingbemi *et al*., 2003]. As T is required for male reproductive tract development and function, suppression of steroid synthesis may be responsible for testicular abnomalies associated with endocrine disruptor, such as pesticides, in consumer products. The fact that humans are chronically exposed to endocrine disruptors warrants the continued investigation of this compound at low dose exposure levels for the purpose of

risk assessment. PGE_2 is known to regulate aromatase gene expression and is the product of COX-2, an enzyme frequently over-expressed in R2C cells. The expression of aromatase was strongly correlated with COX-2 expression. This finding suggested a possibility that the feminization seen in o,p'-DDT-exposed R2C cells may also be related with the decrease in testosterone production. Treatment with o,p'-DDT (1~10 μM) caused dose-dependent increases of COX-2 mRNA and protein expression. As PGE_2 exerts its biological activity through the different G protein-coupled receptors (EP_1, EP_2, EP_3, and EP_4), it was important to eveluate their expression in R2C cells [Zhao *et al*., 1996]. PGs increases intracellular cAMP levels and stimulates estrogen biosynthesis [Zhao *et al*., 1996]. Local production of PGE_2 via the COX isozymes may influence estrogen biosynthesis. When R2C cells were treated with o,p'-DDT for 6 h, the mRNA level of EP_2 and EP_4 subtype receptor was evidently increased, whereas other subtypes EP receptor mRNA remained unchanged (data not shown). In addition, o,p'-DDT increased cAMP levels by CRE transcriptional activation in the R2C cells. These results suggested that the o,p'-DDT-induced induction of aromatase expression level in R2C cells is mediated partly through the transactivation.

In conclusion, this study demonstrated that o,p'-DDT-induced inhibition of T production in R2C cell is mediated through the induction of aromatase

activity and gene expression through at least three receptor subtypes, EP_2, EP_4, and ER by COX-2 and PGE_2 mediates. This present study indicated that the organochlorlines, such as DDT and methoxychlor, which have been studied during recent years as a major issue of concern for the toxicity may result from T production through the modulation of T biosynthsis-related genes.

Up-regulatory effects of o,p'-DDT on cyclooxygenase-2 and iNOS gene expression in RAW 264.7 cells

Critical finding in this study was the establishment of a definitive link between MAPKs phosphorylation and transcription factor activation in macrophages exposed to o,p'-DDT. This study clearly showed that o,p'-DDT induces COX-2 gene expression in macrophages and that this is regulated at the level of MAPKs. Overall, these data were consistent with a model whereby o,p'-DDT induces MAPK phosphorylation, which, in turn, activate AP-1 and CRE transcription factors. MAPKs are known to be important signaling modulators in COX-2 expression and these have been recently shown to be activated by DDT [Shinomiya and Shinomiya, 2003; Robinson and Dickenson, 2001; Tessier and Matsumura, 2001]. Our data clearly showed that o,p'-DDT induces COX-2 mRNA, protein, and prostaglandin synthesis in macrophages. Transient transfections demonstrated that o,p'-DDT treatment of macrophages increased in the rate of COX-2 transcription. o,p'-DDT-mediated activation of NF-κB has recently been demonstrated [Kim *et al*., 2004]. This becomes important, especially for up-regulation of COX-2, since activation of both NF-κB and AP-1 is necessary for o,p'-DDT-induced COX-2 expression.

MAPKs, including three structurally related subfamilies of the ERKs, the

JNKs, and the p38 MAPKs (p38), are known to be key regulators of NF-κB and AP-1 proteins. MAPKs, including ERK, p38, and JNK, are the most important protein kinases that mediate the post-translational modification of AP-1 proteins [Niederberger *et al.*, 2003; Glinghammar *et al.*, 2002; Allport *et al.*, 2000]. These data suggested that activation of ERK, p38, and JNK were critical in o,p'-DDT-induced up-regulation of *COX-2* gene expression. Human *COX-2* gene promoter contains several sequences that have been shown to act as positive regulatory elements for the *COX-2* gene transcription in different cell types [Smith *et al*., 2000]. p38 is also known to mediate xenobiotic- or endogenous factor-induced COX-2 expression by transcriptional and post-transcriptional mechanisms [Vogel, 2000]. As shown here, p38 contributed to o,p'-DDT-induced COX-2 expression by both mechanisms. o,p'-DDT-induced transactivation of the COX-2 gene may result from JNK-mediated activation of AP-1. The COX-2 promoter contains multiple potential cis-activating elements. To date, AP-1, CRE, E-box, NF-IL6 (C/EBPβ), and NF-κB transcriptional elements have been reported to be involved in COX-2 expression [Guo *et al*., 2001; Xie and Herschman, 1995; Yamamoto *et al*., 1995; Zhou *et al*., 2003]. As shown by our results, o,p'-DDT plays a role in up-regulating the expression of COX-2 in macrophages. Investigations are currently under way to determine the identity of the

transcription factors involved in o,p'-DDT-regulated transcription activity of the COX-2 promoter in macrophages.

The regulation of cytokine production is a potential target for environmental toxicants that are capable of modulating the immune function. In vitro studies using established cell lines have been useful for characterizing the effects of chemicals on the immune function alteration. o,p'-DDT is still a widely distributed pesticide and has been linked to adversely affects on the humoral and cellular immune responses [Banerjee, *et a1*., 1997]. Therefore, it was necessary to determine whether or not o,p'-DDT affects the production of NO and proinflammatory cytokines as well as their expression in macrophages. Murine macrophages can be stimulated *in vitro* with LPS, a bacterial product, to secrete high levels of various cytokines, which may influence the outcome of the immune response during an infection [MacMicking *et al*., 1997]. LPS was used in this study as non-specific inducer of macrophage activation and cytokine gene expression. This study clearly showed that o,p'-DDT increases in NO and proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 production and expression of these genes in macrophages. The macrophage-derived excess NO levels may play a key role in the inflammatory response [Coleman *et al*., 2001]. Proinflammatory cytokines also play an important role in the regulation of the inflammatory

responses: IL-1β and/or TNF-α have been shown to correlate with the induction of inflammatory mediators in various cells [Elenkov and Chrousos, 2002]. Furthermore, it has been demonstrated that there is a correlation between the proinflammatory cytokines, such as IL-6 and TNF-α, and several chronic inflammatory diseases, including asthma, rheumatoid arthritis and inflammatory bowel and allergic diseases [Eigler *et al*., 1997; Ohkubo *et al*., 1998]. o.p'-DDT increased in the production of NO and the proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, in macrophages. However, it has been reported that the DDTs inhibit NO, IL-1β, TNF-α production and decreases in the IFN-γ-induced iNOS, IL-1β, TNF-α expression in the IFN-γ-activated macrophages [Nunez *et al*., 2002]. In the previous study, they used more than 2.5 μg/ml (about 7 μM) DDT and they did not report the effects of DDTs alone on iNOS, IL-1β, TNF-α expression in the resting or non-stimulated macrophages. This study also observed that op'-DDT at high concentrations (more than 10 μM) decreased LPS-induced NO production and the iNOS expression level in macrophages (data not shown). However, op'-DDT at low concentrations (less than 1 μM), unlike in a previous study [Nunez *et al*., 2002], significantly elicited a dose-dependent increase in NO, TNF-α, IL-1β, IL-6 production in the absence of any stimulator. These suggests that op'-DDT can induce an increase in the level of NO TNF-α, IL-

1β, and IL-6 production and expression even though op'-DDT causes a reduction in the LPS-inducible NO, TNF-α, IL-1β, IL-6 production and expression level. The biological significance of these different effects of o,p'-DDT between the resting and LPS- or IFN-γ-stimulated state need to be further examined. Previous studies revealed that 4-nonylphenol and bisphenol A, which are also known as xeno-estrogens, suppress LPS-induced iNOS and TNF-α expression in LPS-activated macrophages [You *et al*., 2002; Kim and Jeong, 2003]. However, either 4-nonylphenol or bisphenol A alone did not affect the iNOS and TNF-α expression level in the absence of LPS. The rational for the different effects of o,p'-DDT, 4-nonylphenol and bisphenol A on the between resting and stimulated macrophages are unknown. Moreover, this o,p'-DDT-induced NO production was reversed when the cells were treated with both o,p'-DDT and the N-nitro-L-arginine methyl ester, which is a competitive NOS enzyme inhibitor (data not shown). Therefore, o,p'-DDT unlike 4-nonylphenol and bisphenol A, has the ability to increase the NO production level alone in the resting macrophages. NF-κB appears to play a primary role in the transcriptional regulation of various genes such as iNOS and the proinflammatory cytokines [Baeuerle and Baichwal, 1997; Ghosh *et al*., 1998]. In the resting cells, the NF-κB dimer is held in the cytosol via an interaction with the inhibitor κB (IκB) inhibitory proteins [Baeuerle and

Baichwal, 1997]. After exposure to proinflammatory stimuli, IκB becomes phosphorylated by IκB kinase α and β, ubiquitinated, and then degraded. Therefore, the liberated NF-κB dimers are translocated to the nucleus, where the transcription of the target gene is induced [MacMicking *et al*., 1997]. This study found that o,p'-DDT increases the NF-κB activation in macrophages using transient transfection (Fig. 30) and EMSA (Fig. 31). These effects of o,p'-DDT confirm with the BAY 11-7082, an IκBα kinase inhibitor, which specifically inhibits NF-κB activation by preventing the phosphorylation and subsequent degradation of IκBα, an endogenous inhibitor of NF-κB [Pierce *et al*., 1997]. As shown in Fig. 5, NO production and iNOS expression were reduced by BAY 11-7082, indicating that o,p'-DDT may induce the gene transcription mediated by NF-κB. Furthermore, when the macrophages were treated with o,p'-DDT for 20 min, the IκB rapidly degraded (data not shown). Therefore, the o,p'-DDT-induced NF-κB activation occurs by modulating IκB degradation.

In this study, it was revealed that o,p'-DDT increases the NF-κB binding activity. Although the precise molecular mechanism of the inducible effects of iNOS and the proinflammatory cytokines by o,p'-DDT are unclear, these results suggest that the o,p'-DDT induction of iNOS and the proinflammatory cytokines expression level in macrophages is mediated

partly through the NF-κB sites of these genes. However, it cannot be completely ruled out that o,p'-DDT, in addition to the induction of the NF-κB-dependent activity, modulates iNOS and the proinflammatory cytokines mRNA levels by influencing the activity of other the transcription factors that are important for cytokine induction, such as AP-1 and C/EBP [Giri *et al*., 2002], or at the posttranscriptional level. Additional studies will be needed to answer these questions and further clarify the mechanisms involved. The current study suggests the possibility that o,p'-DDT might act as an inflammatory immunomodulator, which is a new insight and a previously unrecognized phenomenon. Although information concerning the level of exposure to o,p'-DDT in humans is limited, further in vivo studies are necessary to understand whether or not o,p'-DDT affects the production of NO and proinflammatory cytokines and ultimately alters the immune function. In conclusion, these results demonstrated that o,p'-DDT stimulates the production of PGE_2 and can up-regulate the *COX-2* gene expression levels via AP-1 and CRE transactivation by activation of all 3 MAPKs. In addition, o,p'-DDT induces the iNOS and proinflammatory cytokines in the macrophages. In addition, these results indicate that this induction is mediated through NF-κB transactivation in the macrophages, which possibly provides o,p'-DDT with the means to alter the immune response.

V. References

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[국문초록]

내분비계 장애물질인 유기염소계살충제의 약물대사 및 염증 관련 유전자의 조절에 관한 연구

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본 연구에서는 내분비계 장애물질로 분류되어 있는 유기 염소계 살충제 (Dichlorodiphenyltrichloroethan; o,p'- DDT)의 약물대사 및 염증 관련 유전자의 발현 조절 및 작용기전을 조사하였다. 약물대사 효소 cytochrome P450 1A1(CYP1A1) 유전자 발현 및 작용기전에 대한 o,p'-DDT 의 영향을 조사하기 위하여 마우스 간암 세포주 Hepa-1c1c7 세포에서 o,p'-DDT 를 처리하였을 때 o,p'-DDT 그 자체는 CYP1A1 에 특이적인 효소반응인 Ethoxyresorufin-*O*-deethylase (EROD) 활성도에 영향을 주지 않았으나 CYP1A1 유전자 발현의 강력한 유도제로 잘 알려진 2,3,7,8-Tetrachloro-dibenzo-*p*-dioxin (TCDD)와 o,p'-DDT 를 동시 처리하였을 때 TCDD 에 의해 증가된 EROD 활성도가 o,p'-DDT 처리 농도에 의존적으로 감소하였다. EROD 활성도의 감소가 estrogen 수용체를 경유하는지를 알아보기 위하여 estrogen 수용체를 봉쇄시키는

효과를 가진 ICI 182.780 를 처리한 결과, 본 연구에 이용된 Hepa 1c1c7 세포에서는 o,p'-DDT 에 의한 TCDD 유도성 P4501A1 억제는 estrogen 수용체는 경유하지 않는다는 것을 알 수 있었다. TCDD 에 의해 유도되어 증가된 CYP1A1 의 mRNA 발현이 o,p'-DDT 의 농도 의존적으로 감소하였고, pCYP1A1-*Luc* 을 이용한 luciferase 활성도 역시 o,p'-DDT 의 농도 의존적으로 감소하였다. 또한, o,p'-DDT 는 TCDD 에 의해 증가된 Ah 수용체와 ^{32}P-DRE (Dioxin Responsive Element) 복합체를 감소시켰으며 이는 DRE 전사인자의 활성을 저하시킴을 알 수 있었다. 이와 같은 결과를 종합해 볼때 o,p'-DDT 에 의한 CYP1A1 의 발현 억제는 CYP1A1 의 발현과 관련된 전사 조절인자인 DRE 활성과 Ah 수용체와의 결합능 감소에 의한 것으로 사료된다.

o,p'-DDT 의 testosterone 생성 및 관련효소의 발현에 대한 영향을 웅성 생식 leydig 세포주 (R2C)에서 조사하였다. 또한, 성호르몬 전환에 관련된 효소인 aromatase (*CYP19*)의 발현 및 활성을 측정하고 aromatase 의 조절과 관련된 COX-2 와 PGE_2 의 효소의 발현 및 조절 기전에 대한 영향을 조사하였다. 미성숙 흰쥐 정소세포에 o,p'-DDT 를 처리하였을 때 o,p'-DDT 의 농도 의존적으로 testosterone 의 양이 감소하였다. 이러한 testosterone 호르몬 조절 관련 기전을 규명하기 위해 남성호르몬의 생성에 관련된 효소 $P450_{scc}$ (cholesterol side chain cleavage), 3β-HSD (3β-hydroxysteroid dehydrogenase), $P450_{17\alpha}$ (17α-hydroxylase), 17β-HSD (17β-hydroxysteroid dehydrogenase)의 유전자 발현에 대한 o,p'-DDT 의 영향을 조사한 결과, o,p'-DDT 의 농도 의존적으로 3β-HSD, $P450_{17a}$ 유전자 발현을 감소시켰으나 P450scc, 17β-HSD 유전자 발현에는 영향이 없었다. Testosterone 으로부터 estradiol 의 전환에 관여하는 aromatase 효소 활성 및 aromatase 발현에 대한 영향을 조사한 결과 o,p'-DDT 의 농도 의존적으로 aromatase 의 효소활성 및 발현이 증가함을 확인하였다. Aromatase 의 mRNA 발현이 o,p'-DDT 의 농도

의존적으로 증가하였고, P450-I.4-*Luc* 를 이용한 luciferase 활성도 역시 o,p'-DDT 의 농도 의존적으로 증가하였다. 이러한 aromatase 의 유전자 발현이 estradiol receptor (ER)인 ERα, ERβ를 경유하여 발현되는지를 확인한 결과 o,p'-DDT 는 ERβ를 경유하여 aromatase 의 유전자 발현을 증가시킴을 확인할 수 있었다. Aromatase 에 영향을 미칠 수 있는 cyclooxygenase-2 (COX-2)의 유전자 및 단백질 발현에 대한 영향을 조사한 결과, o,p'-DDT 는 COX-2 의 발현 및 prostaglandin E_2 (PGE_2)의 양을 농도 의존적으로 증가시켰다. 또한, R2C Leydig 세포에서 prostaglandin E (EP) receptor 2 및 4 가 존재하며 o,p'-DDT 에 의해 생성된 PGE_2 가 EP receptor 를 경유한 cAMP 양의 증가를 확인하였다. Aromatase 의 promoter 에 존재하는 CRE (cAMP Responsive Element)의 결합부위를 중심으로 luciferase reporter gene 을 포함한 luciferase 활성을 측정한 결과 o,p'-DDT 가 CRE-luciferase 활성도를 증가시킴을 확인하였다. 이러한 결과를 종합해 볼 때 R2C Leydig 세포에서 성 호르몬 생성조절에 관련된 aromatase 유전자 발현은 EP receptor 와 결합하여 PGE_2 생성량이 증가하며 이러한 PGE_2 의 증가로 인한 cAMP 양을 증가시킴으로써 aromatase 의 promoter 상에 CRE 의 촉진에 의한 유전자 발현이 증가됨을 확인 하였다. 또한, aromatase 발현의 증가로 testosterone 생성량 및 testosterone 생성 관련 효소 (3β-HSD, $P450_{17\alpha}$)의 발현을 감소시킨 것으로 사료된다.

R2C Leydig 세포에서 성 호르몬 생성조절에 관련된 aromatase 유전자 발현에 의해 PGE_2 생성량 및 COX-2 의 발현이 증가함을 확인하고 이에 o,p'-DDT 에 의한 염증관련 COX-2, iNOS 및 각종 염증성 cytokines 의 발현 및 조절에 대한 영향을 조사하였다. 마우스 대식세포주인 RAW 264.7 세포에서 PGE_2 및 NO 의 생성량이 o,p'-DDT 의 농도 의존적으로 증가하였으며 COX-2, iNOS 및 염증성 cytokines 의 mRNA 발현이 o,p'-DDT 의 농도 의존적으로 증가함을 확인하였다. 또한, iNOS 의 mRNA 발현이 o,p'-DDT 의 농도 의존적으로 증가하였고, NF-κB-*Luc* 를 이용한 luciferase

활성도 역시 o,p'-DDT 의 농도 의존적으로 증가하였다. COX-2 의 발현 증가에 관여하는 COX-2 promoter 에 존재하는 전사조절 인자를 조사한 결과 o,p'-DDT 에 의해 AP-1 결합부위의 활성화로 인한 COX-2 발현이 증가하는 것을 확인할 수 있었다. COX-2 의 발현과 관련된 mitogen-activated protein kinase (MAPK)과 같은 세포 내 상위 신호전달계 효소의 활성화에 대한 영향을 RAW 264.7 세포를 이용하여 조사한 결과 o,p'-DDT 는 MAPK 의 ERK, P38, JNK 를 모두 활성화 시킴을 확인할 수 있었다. 이러한 결과를 종합해 볼 때 o,p'-DDT 에 의한 COX-2 및 iNOS 의 발현 증가는 상위 신호전달체계인 ERK, P38, JNK MAPK 의 활성화를 통하여 전사인자 AP-1 및 NF-κB 를 활성화 시킴으로써 염증관련 COX-2, iNOS 및 각종 염증성 cytokines 의 발현을 증가시킴을 확인할 수 있었다.