Reduction of Dioxin-Induced Expression of *cyp1a1* Gene through Repression of AhR/Arnt DNA Binding by Mek-1 inhibitor PD98059

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INTRODUCTION

Aryl hydrocarbons, environmental contaminants accumulate in tissue and pose potential risk in human health. 2,3,7,8-Tertachlorodibenzo-p-dioxin (TCDD) is known as a most potent toxicant among aryl hydrocarbons. TCDD elicits numerous toxic responses in experimental animals and human, including hepatic carcinoma, pulmonary and skin tumor in adult rodents, craniofacial abnormality during mouse embryogenesis, chloracne, reproductive abnormality, immunotoxicity, endocrine effects in exposed humans. One adaptive response is the transcriptional induction of the *cyp1a1* gene that encodes a microsomal enzyme, cytochrome P-4501A1, that oxygenates aryl hydrocarbon as part of detoxification process.

The aryl hydrocarbon receptor (AhR) is an intracellular, TCDD binding protein that functions as a transactivator for *cyp1a1* gene. Prior to binding its ligand such as TCDD, AhR is found in cytosol complexed with heat shock protein 90(hsp90). Liganded AhR dissociates from hsp90, translocates into nucleus and forms heterodimer with nuclear protein Arnt (AhR nuclear translocator). Liganed AhR/Arnt heterodimer specifically recognize dioxin-responsive elements in enhancer region of *cyp1a1*gene and increase transcription of *cyp1a1*gene. Both AhR and Arnt contain basic helix loop helix (bHLH) and PAS (Per, AhR, Sim) domain at their N-terminal half, which mediates dimerization and DRE binding.

Several studies have suggested that phosphorylational modulation is involved in activation process of AhR function; (i) unliganded AhR is a phosphoprotein; (ii) the DNA binding activity of the receptor is lost upon phosphatase treatment *in vitro*; (iii) Serum and mitogenic factors increased the expression of AhR in murine 3T3 Fibroblast, which is inhibited by treatment of tyrosine kinase inhibitor; (iv) in c-src deficient mice, in vivo toxicity of TCDD are not fully expressed and lethal dose of TCDD also increases; (v) tyrosine kinase inhibitor

reduced dioxin induced *cyp1a1* gene expression in human keratinocytes; (vi) protein kinase C is involved in the nuclear event that works in AhR binding to DRE site, thereby regulates *cyp1a1* gene expression. Here we investigated which protein kinase pathways are involved in activation process of AhR, and which steps of AhR activation is modulated by protein kinase by using specific protein kinase inhibitors.

RESULTS

1. Effects of kinase inhibitors on dioxin-induced expression of cyp1a1 gene

To investigate the possibility that specific kinases modulate dioxin-induced gene expression, we first measured the changes in mRNA level of *cyp1a1* gene in Hepa1c1c7 cells that were treated with several inhibitors prior to TCDD treatment. Pretreatments with Genistein, a tyrosine kinase inhibitor, Wortmannin and LY294002, PI3 kinase inhibitors, SB202190 a p38 kinase inhibitor do not significantly reduce the dioxin-induced expression of endogenous *cyp1a1* gene. In contrast, pretreatment with MEK-1 inhibitor, PD98059 decrease the level of dioxin-induced expression of cyp1a1 gene. In order to test whether treatment of PD98059 affects the enhancer activity of *cyp1a1* gene, we transfected Hepa1c1c7 cells with reporter plasmid,which encodes luciferase gene under control of dioxin-responsive elements, 484-bp fragment from the upstream region of the *cyp1a1* gene (containing four DREs) present directly upstream of the MMTV viral promoter. Treatment with PD98059 abolishes the dioxin-induced expression of reporter genes dose-dependently. Our results imply that Mek-1/MAP Kinase pathway is involved in dioxin-induced gene expression.

2. p42/p44 MAPK is necessary for DRE dependent gene expression

Since PD98059 is a specific inhibitor of MEK-1, we wanted to investigate if TCDD could ultimately activate p42/p44 MAPK, which are downstream protein kinases of MEK-1. Hepa1c1c7 cells were serum starved for 48 hours, and then exposed to TCDD for various times and doses. Whole cell lysates were immunoblotted with either an antibody specific for tyrosine-phosphorylated p42/p44 MAPK. Since phosphorylation of p42/p44 MAPK is a clear indication of activation, we used an anti-phospho-p42/p44 MAPK antibody to evaluate the activity of p42/p44 MAPK. Stimulation with 12-O-Tetradecanoylphorbol 13-acetate (TPA, 10ng/ml) plus

ionomycin (0.5μM) for 10 minutes induced an activation of p42/p44 MAPK. Two hour exposure to TCDD increased phosphorylation of p42/p44 MAPK maximum at 1nM. Phosphorylation of p42/p44 MAPK is increased for 30 minutes treatment of 1nM TCDD but is decreased after 6hours treatment of 1nM TCDD.

In order to test whether increased p42/p44 MAPK activity is involved in the TCDD-induced transcription of genes that are under the control of DREs, we cotransfected Hepa1c1c7 cells with the DRE-driven reporter plasmid together with increasing amounts of plasmids that encode dominant-negative p44 and p42 MAPK mutants, pERK1-KR and pERK2-KR, respectively. Cotransfection of 100ng of pERK2-KR, dominant negative mutant of p42 MAPK, dramatically reduced TCDD dependent transcription of the DRE-reporter gene, whereas cotransfection of pERK1-KR, dominant negative mutant of p44 MAPK, does not significantly influences the dioxin-induced gene expression of DRE-reporter plasmid. The inhibition of p42 MAPK more dramatically reduces the dioxin-induced gene activation. Thus, the activity of p42 MAPK is required for dioxin-dependent transcription.

3. Effects of PD98059 on dioxin-induced stability and nuclear localization of AhR

It was proposed that AhR protein undergoes the ligand-induced degradation in cytoplasm following nuclear export suggesting that novel mechanism regulating AhR function. In order to investigate whether PD98059 regulate the stability and nuclear concentration of AhR, we measured the protein level of AhR in nuclear fraction. The results indicated that treatment of PD98059 did not change either nuclear localization or the concentration of both AhR and Arnt. Treatment of PD98059 without TCDD did not cause changes in localization and concentration of AhR protein.

4. Effects of PD98059 on dioxin-induced DNA binding ability of AhR/Arnt complex

To investigate the inhibitory effect of PD98059 on the ability of dioxin-induced protein binding on DRE in response to dioxin, we prepared nuclear extracts from Hepa1c1c7 cells that were pretreated with inhibitors. The nuclear extracts were mixed with a radiolabeled oligonucleotide, DRE, which contains the DRE sequence from the 5' enhancer region of the cyp1a1gene (-1026 to –999 of the promoter element of the rat cytochrome p-4501A1gene), and then were subjected to electrophoretic mobility shift assay (EMSA). Our results revealed dioxin-induced, constitutive

and nonspecific complexes. Dioxin-induced complex was detected specifically when nuclear extracts from dioxin-treated Hepa1c1c7 cells were assayed. Constitutive complexes were present when either induced or uninduced extracts were assayed and therefore are due to the constitutively expressed factors. To examine the composition of the hypoxia-induced complexes, nuclear extracts were mixed with anti-Arnt antibody and then subjected to EMSA. Supershifts confirm the presence of Arnt in the complex. In accord with the results, PD98059 decreases the dioxin-induced DNA binding of the AhR/Arnt complex. Reiners *et al.* demonstrated that PD98059 acts as a partial agonist for AhR and it induced DRE binding of AhR/Arnt in absence of dioxin. In contrast, our results showed that PD98059 itself does not induce AhR/Arnt binding to DRE in response to dioxin. Also we examined whether PD98059 induced *cyp1a1* gene expression in absence of dioxin by using DRE-driven reporter plasmid. Our results showed that PD98059 itself fails to induce induction of DRE-driven reporter. The results showing that the expression of dominant negative p42 MAPK dramatically repressed TCDD-induced expression of DRE driven reporter gene implied that PD98059 reduced TCDD-induced gene expression by inhibiting MEK and its subsequent substrate MAPK. Mechanistically, the results suggest that PD98059 specifically repressed the TCDD-induced interaction between DRE elements and AhR/Arnt. In order to test whether PD98059 directly blocks the interaction between DRE and AhR/Arnt, we measured the effect of PD98059 on interaction between DRE and *in vitro* translated AhR and Arnt in vitro. We mixed in vitro translated AhR and Arnt protein with labeled DRE oligo in presence of TCDD (20nM) and PD98059, then analyzed protein/DNA interaction in EMSA. In vitro translated AhR and Arnt make a specific complex with DRE in presence of TCDD and this protein/DNA complex is vanished or supershifted by pretreatment with AhR and Arnt antibodies, respectively. Interestingly, the presence of PD98059 (1 to 100μM) does not affect the *in vitro* interaction between DRE and AhR/Arnt, although pretreatement of PD98059 on the hepa1c1c7 cells does affect the DRE binding function of the endogenous AhR and Arnt. These results indicated that PD98059 has a negative indirect effect on interaction between DRE and AhR/Arnt in an intact cell-context. In contrast, it has less direct effect on in vitro interaction. Therefore, our results suggest that target of PD98059, MEK/MAPK pathway but not PD98059 itself repressed TCDD-induced interaction between DRE and AhR/Arnt, leading repression of TCDD-induced gene expression.

DISCUSSION

Our results demonstrated that Mek-1 specific inhibitor PD98059 reduced TCDD-induced CYP1A1 expression in mouse hepa1c1c7 cells and it specifically reduced interaction between AhR/Arnt heterodimer with DRE. The findings that TCDD treatment increases phosphorylation of p42/p44 MAPK and that dominant negative mutant of p42 (K52R) MAP Kinase specifically inhibited TCDD-induced expression of DRE-driven reporter plasmid indicated that PD98059 modulated function of AhR/Arnt by blocking Mek-1/p42/p44 MAPK pathway. Consistently, PD98059 inhibits DRE binding ability of the endogenous AhR and Arnt more intensively than *in vitro* translated AhR and Arnt, indicating that PD98059 repressed TCDD function by inhibiting other cellular targets other than AhR or Arnt. Instead of using intact cells, Reiners *et al.* transformed rat liver extract by treating with TCDD and measured the gain of DRE binding ability of cytosolic AhR by EMSA. This *in vitro* transformation assay showed that the addition of PD98059 to rat liver cytosol prior to exposure of TCDD, suppressed TCDD-induced AhR/DRE complex formation (IC_{50} = 1 μM). By using sucrose gradient analyses of AhR/[H^3]TCDD, they also proved the possibility that PD98059 competed with TCDD for binding to AhR (IC_{50} = 4 μM) as a partial agonist. Indeed, AhR antagonists or partial agonists such as as flavone, flavonone and 7,8-benzoflavone-α-naphthoflavone have similar chemical structure with PD98059. Their results suggested that PD98059 functions as an AhR antagonist leading to reduction of TCDD-induced DRE binding and target gene expression by transformed AhR. In addition to antagonistic effect of PD98059, We proposed that PD98059 suppressed TCDD-induced activation of AhR by inhibiting Mek-1/MAPK pathway based on the following findings. First, the expression of dominant-negative mutant of p42 (K52R) MAPK repressed TCDD-induced expression of DRE-driven reporter gene. Second, inhibitory effects of PD98059 on DRE/AhR/Arnt interaction is more dramatic when it is added in medium on the intact hepa1c1c7 cells than when it is added in the mixture of *in vitro* translated AhR, Arnt and labeled DRE Third, TCDD also activates p42/p44 MAPK

Recently Tan *et al.* also demonstrated that TCDD activated p42/p44 MAPKs (extracellular signal-regulated kinases) and the Jun N-terminal kinases, but not the p38 MAPKs in mouse Hepa-1 hepatoma cells, and that TCDD-induced gene expression was repressed by either treatment of U0126, MEK-specific inhibitor or expression of dominant negative mutant of

MEK-1. MAPK is a serine/threonine kinase. The treatment of inhibitors of serine/threonine-specific protein phosphatase such as calyculin A, cyclosporin A, okadaic acid increase TCDD-induced expression of DRE driven reporter gene without affecting TCDD independent basal level expression. This finding emphasized that TCDD induced phosphorylation of serine/threonine residue is important for TCDD-induced gene expression. In mouse lung tumors caused by N-nitrosodimethylamine (NDMA), tumor suppressive membrane-associated K-ras p21 was decreased but total raf-1 and phosphoform of ERK-1 and 2 (p42/p44 MAPK) were increased compared to normal control lung. TCDD treatment (5nmol/kg) of NDMA tumor resulted in 2.4-fold increase in tumor multiplicity, greater decrease of membrane associated K-ras p21, and increase in total and membrane associated raf-1, indicating that TCDD may promote tumors by contributing to down-regulation of membrane-associated K-ras p21 and stimulation of raf-1.

TCDD was found to inhibit apoptosis stimulated by withdrawal of growth factor in human mammary epithelial cell line MCF-10A. TCDD increase PI3K activity leading phosphorylation of it substrate Akt, a serine/threonine kinase that has antiapoptotic activity. Protein Kinase C (PKC) activator, phorbol-12 myristate 13 acetate (PMA) enhanced TCDD induced expression of DRE driven reporter gene and inhibitors of PKC blocks PMA effect. PMA treatment did not change either protein level of AhR and Arnt or DRE binding ability of AhR/Arnt heterodimer. PKC could modulate the transactivation capability following DRE binding process. Several studies have suggested that protein kinase pathways could modulate TCDD-induced gene expression and also TCDD could modulate some protein kinase pathways that are involved in proliferation or apoptosis. However it remains to investigate how the cross-talk between TCDD signaling and protein kinase signaling affects the physiological and toxic effects of TCDD such as tumor promotion, teratogenesis and infertility.

Backlund *et al.* showed that pretreatment rat hepatoma H4IIE cells with Genistein reduced OME (omeprazole)-mediated induction of CYP1A1 but failed to reduce TCDD-mediated induction of CYP1A1, rather it potentiated the formation of the TCDD-induced DRE binding of AhR complex. In contrast, in human keratinocyte, Genistein inhibited TCDD-induced expression of *cyp1a1* gene. Our results showed that pretreatment of Genistein failed to reduce TCDD-induced expression of *cyp1a1* gene in mouse hepa1c1c7 cells suggesting that effect of Genistein could be different depending on the type of treated cell lines.

AhR activation process has previously been shown to depend on phosphorylation

processes. Our results indicated that MEK-1/p24/p44 MAP kinase pathway but not either PI3 kinase or p38 kinase, protein tyrosine kinase is involved on TCDD-induced gene expression pathways. We speculate that MEK-1/p42/p44 MAP kinase pathway is specifically involved in a process of DRE-AhR/Arnt binding. It is further investigated whether p42/p44 MAP kinase phosphorylates AhR or Arnt directly or whether it phosphorylated the other regulatory proteins for activation of AhR/Arnt heterodimer.