LIVER SPECIFIC SUPPRESSION OF CARBONIC ANHYDRASE III BY A DIOXIN RELATED TOXIC COMPOUND, 3,3',4,4',5-PENTACHLOROBIPHENYL

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Introduction

A coplanar polychlorinated biphenyls (PCBs), 3,3',4,4',5-pentachlorobiphenyl (IUPAC PCB126) is the most toxic congener of all PCBs [1], well known to be one of the causal agents of Yusho [2] and is widespread environmental pollutants [3]. The mechanism of the toxicity induced by polychlorinated aromatic hydrocarbons, such as coplanar PCBs and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is assumed to be attributable to one of the PAS-family protein, Ah-receptor (AhR)-mediated reaction [4] but is not fully understood. Several enzymes, such as CYP1A1, are up-regulated *via* the xenobiotic responsive element (XRE) which AhR binds as the heterodimer complex with AhR nuclear translocator (Arnt) [5]. Unlike the activation of transcription, we know relatively little about the down-regulation of gene expression by TCDD or PCB126.

Oxidative stress is one of the toxicities caused by PCB126 [6]. Recently, CAIII was reported to play a role of an antioxidant that prevents from H_2O_2-inducible apoptosis [7]. In addition, CAIII was suggested as being one of the cytosolic protein tyrosine phosphatases (PTPs) involved in signal transduction [8]. Our current studies showed that PCB126 treatment on rat results in reduced glutathione peroxidase-redox system and catalase [6, 9]. We recently demonstrated that a coplanar PCB, PCB126, dramatically suppresses rat liver CAIII in protein and mRNA expression level [10]. CAIII is abundantly expressed in liver and muscle of rats [11]. The purification study suggested that there are some differences in CAIII protein sequence between liver and muscle [12]. CAIII cDNAs have been isolated from rat muscle [13] and Norway rat liver [14] and the deduced primary sequences showed 98% identity. We also cloned CAIII cDNA from Wistar rat liver and the deduced primary sequence was identical to that of Norway rat [10]. However, complete identity of rat CAIII between liver and muscle has not been confirmed using a single animal.

Thus, we compared CAIII cDNA sequence between liver and muscle of Wistar rat, suggest that the CAIII from both source is from the same gene. Then, the effects of coplanar PCB on CAIII expression in liver and muscle of rats were investigated. We demonstrate here that a coplanar PCB suppressed CAIIII in a manner with liver specific fashion.

Methods and Materials

Animals and treatment. Male Wistar rats (7 weeks) were given a single i.p. injection of PCB126 (corn oil), at a dose of 10 mg/kg. Free- and pair-fed controls were treated as described

previously [10]. The livers and soleus muscles were removed from the PCB126-treated and control animals 5 days after injection and none of the animals was fasted prior to sacrifice. Isolation of mRNA and preparation of cytosol were as described previously [10].

Comparison of CAIII cDNA between rat liver and soleus muscle by reverse transcription (RT)-PCR. mRNAs were isolated from Wistar rat liver and soleus muscle as described previously [10]. Amplification of liver and muscle CAIII cDNA by RT-PCR and following nested PCR were carried out according to Ikeda et al [10]. The 821 bp products covering the full length of the open reading frame (ORF) of rat liver and muscle CAIII cDNA were subcloned and sequenced as described previously [10].

Immunoblotting. Cytos .ic proteins were immunoblotted using the isoform-selective antibody toward CAIII [15].

Northern blotting. Northern blotting of liver and muscle CAIII was performed as described previously [10]. Normalization was performed using rat ß-actin cDNA as a probe.

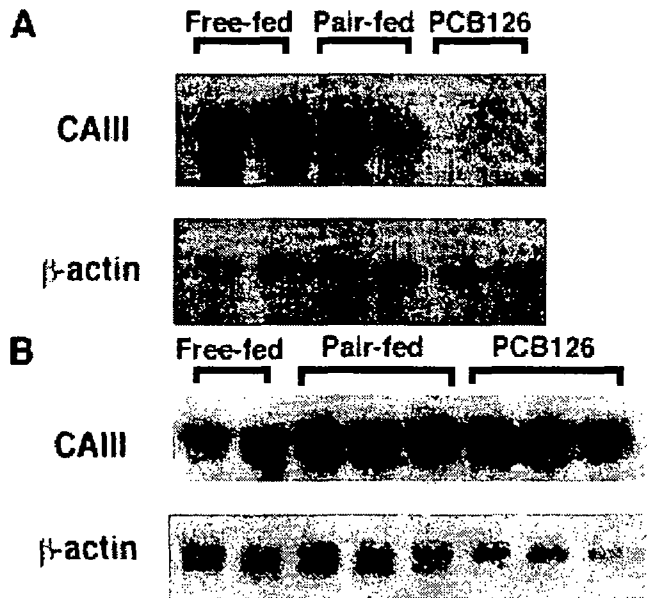

Fig. 1 Effect of PCB126 on the level of CAIII mRNA in rat liver and soleus muscle. mRNA was isolated from rat liver (A) and muscle (B) of free- and pair-fed control, and PCB126-treated groups and subjected to northern blot analysis. Each lane contains 1.0 μg (A) and 0.5 μg (B) of mRNA. A β -actin probe was used for normalization of mRNA amount and transfer efficiency.

Results and Discussion

Results from this study, comparison of CAIII cDNAs in male Wistar rat liver and muscle using RT-PCR, show that an ORF of muscle CAIII cDNA is identical to that of liver. Some differences observed in CAIII protein sequence between liver and muscle [11] may be due to strain difference.

CAIII in liver cytosol of Wistar rats was analyzed by immunoblotting using isoform-selective anti-CAIII antibody. The immunoreactive band with anti-CAIII antibody was markedly reduced by PCB126. The CAIII levels were comparable within free- and pair-fed animals. The mRNA level of CAIII in rat liver was dramatically suppressed by PCB126 (Fig. 1A). Thus, the reduced protein level of CAIII in rat liver by PCB126 is due to the decreased mRNA level. On the contrary, Fig. 2 shows that the CAIII mRNA in rat muscle was not decreased by PCB126-treatment. Although CAIII was abundantly expressed in liver and muscle of rat, the effect of PCB126 on the expression was different. The suppression of CAIII by PCB126 was only observed in the liver but not in the muscle.

Recently, involvement of AhR and Arnt in the down-regulation of major histcompatibility $Q1^b$ (MHC $Q1^b$) gene by TCDD has been suggested [16]. AhR may be involved in the suppression of CAIII in the rat liver because that was observed to take place in a manner which was PCB126-dose responsive [10]. The C/EBP binding motif and IL-6 responsive elements are conserved in the proximal promoter of CAIII gene between mouse and human [17], although the involvement of C/EBPα and C/EBPβ in the expression of CAIII have not yet been demonstrated. C/EBPα was originally shown to be involved in the expression of liver-specific gene [18]. Although CAIII is abundantly expressed in mouse [19] and rat [20] liver, the expression level in human liver is quite low [21]. Although the regulatory region of rat CAIII gene remains to be characterized, further investigations are needed to clarify the involvement of the changes in the levels of these transcription factors or cytokines as well as the Ah-receptor as determinants of the suppression of rat liver CAIII.

We know relatively little about the mechanism of muscle toxicity by dioxins. Wasting syndrome, one of toxic manifestation caused by TCDD, is considered to be due to decreased food consumption. Specific biochemical alterations such as an elevation of glutamine synthetase activity, occur in skeletal muscle in the wasting syndrome [22]. However, the mechanism remains to be elucidated. It is interesting that the suppression of CAIII by PCB126 was only observed in the liver but not in the muscle. This may be due to the differential constitutive regulation of CAIII gene between liver and muscle. Possibly, CAIII mRNA is regulated through the AU-rich element (AURE) in the 3'-untranslated region [10] which are reported to function as mRNA-destabilizing signals where Tristetraprolin, a CCCH zinc finger protein, interacts with [23]. However the effect of PCB126 on the turn over of CAIII mRNA or the level of destabilizing signal related factors in liver and muscle when rats received PCB126 remain to be investigated.

Thus, coplanar PCB suppressed CAIII in a manner with liver specific fashion. Further study is needed to clarify the tissue specific suppression of CAIII.

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HYPOXIC AGENTS INHIBIT TCDD STIMULATED *Cyp1a1* PROMOTER ACTIVITY

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Introduction

The cytochrome P450-dependent monooxygenase system catalyzes oxidative metabolism of a wide variety of substrates including endogenous as well as exogenous compounds. As a preliminary detoxification step, many compounds are first converted to polar metabolites by cytochrome P450, which facilitates their elimination. However, some compounds may also be inadvertently bioactivated by cytochrome P450 to reactive intermediates that produce adverse biological effects[1,2]. For example, carcinogenic polycyclic aromatic hydrocarbons (PAH) such as benzo(a)pyrene (B[a]P) undergo metabolic activation by cytochrome P450 and epoxide hydrolase to chemically reactive ultimate carcinogen diol epoxides [3]. The *CYP1* family, which consists of at least three enzymes, *CYP1A1, CYP1A2* and *CYP1B1* has been shown to be important in the metabolism of several xenobiotics such as PAH and heterocyclic amines, and the expression of these enzymes is inducible by PAHs such as TCDD. TCDD induction of *CYP1* transcription is mediated by the cytosolic AhR, which is known as a ligand-activated transcription factor. The activation of AhR involves ligand binding, dissociation of heat-shock protein-90, nuclear translocation, and dimerization with the Arnt followed by binding to dioxin responsive element (DRE, or XRE) enhancer elements in the 5'-noncoding region of the responsive gene [4~6]. The mechanism of action of this compound is to activate the AhR to a form that binds to specific gene regulatory sequence elements, called XREs, through heterodimerization with Arnt [7~9]. AhR and Arnt have a similar overall structure and belong to the basic helix-loop-helix class of transcription factors [10,11]. Members in this class of factors are characterized by a bHLH motif contiguous with a region, PAS (Per-Arnt-Sim), which is conserved between the *Drosophila* neural cell developmental regulator, Sim, the *Drosophila* circadian rhythm regulatory protein, Per, and Arnt [12]. Upon binding XREs, the AhR-Arnt complex activates transcription of adjacent structural genes which encode enzymes that are involved in the oxidative metabolism of these compounds [13,14]. HIF-1α) has recently been reported to mediate transcriptional responses to hypoxia by binding to hypoxia-inducible enhancer motif (hypoxia responsive elements; HREs) of target genes [15]. HRE core sequences are asymmetric E-box motifs that have been characterized in erythropoietin (EPO) gene[16], genes encoding vascular endothelial growth factor (VEGF) [17,18] and a number of glycolytic enzymes [19]. HIF-1α binds to HRE motif as a heterodimeric complex, termed HIF-1, with the transcription factor, Arnt [20]. Thus, Arnt seems to be critical for both dioxin receptor mediated and HIF-1α mediated signaling pathway. In our effort to understand the mechanism of the regulation of *Cyp1a1* gene expression, we demonstrate here that hypoxic agents such as cobalt chloride, desferrioxamine, and picolinic acid inhibit the TCDD induced *Cyp1a1* promoter activity based on the determination of luciferase activity in Hepa I cells transfected with pm*Cyp1a1*-Luc.

Methods and Materials

Cell culture and transfection

Hepa I (Hepa 1c1c7) mouse liver cell lines were transfected with pm*Cyp1a1*-Luc and LipofecTAMINE according to supplier's manual.

Chemical treatment

Hepa I cells were rinsed with serum-free medium twice before the administration of various chemicals in serum free medium. Stock solutions of chemicals were made in DMSO as a vehicle and control cells were treated with 0.1% DMSO

Luciferase reporter assay

The luminescents were measured using luciferin.

Results and Discussion

TCDD concentration dependent induction of pm*Cyp1a1*-Luc expression

Mouse Hepa I cells were transfected with pm*Cyp1a1*-Luc construct containing 1.6Kb DNA of mouse *Cyp1a1* 5' flanking region. Transfected Hepa I cells were treated with various concentration of TCDD (1 pM~1 nM) for 24 hours and lysed for luciferase activity measurement. 1 pM TCDD increased luciferase activity 850-fold over that of control and as the TCDD concentration increased, luciferase activity was also increased with dose dependent manner. The maximal stimulation of luciferase activity with 1 nM TCDD treatment was 6810 fold over that of control. This result is in agreement with the previous data that 1 nM TCDD brought about maximal responses in stimulation of ethoxyresorufin deethylase activity, and *Cyp1a1* mRNA increases [21]. Thus, this reporter gene system can be very useful tool to study the mechanism of the regulation of *Cyp1a1* gene expression.

Time dependent induction of pm*Cyp1a1*-Luc expression by TCDD.

1 nM TCDD which results in maximal biological response was treated into Hepa I cells that were transfected with pm*Cyp1a1*-Luc for various periods of time. From the 6 hour treatment with TCDD, luciferase activity began to increase and reached the maximal level at 24 hour. 48 Hour treatment with TCDD brought the luciferase activity back to near the untreated level and 72 hour and 96 hour treatments show little luciferase activities possibly due to the cell death with TCDD treatment. This result shows the time dependent induction of *Cyp1a1*expression with 1nM TCDD treatment up to 24 hour treatment and 24 hour treatment resulted the maximal stimulation.

Cobalt chloride inhibition on the TCDD induced luciferase activity.

pm*Cyp1a1*-Luc transfected Hepa I cells were treated with various concentrations (1 μM, 10 μM, 100 μM) of cobalt chloride for 17 hours before the treatment of 1 nM TCDD for 24 hours. The treatment with 1 nM TCDD resulted in 4170-fold induction of luciferase activity, whereas 1 μM or 10 μM or 100 μM cobalt chloride treatment decreased the TCDD stimulated luciferase activity with dose dependent manner when it was pretreated. pm*Cyp1a1*-Luc transfected Hepa I cells were treated with 100 μM cobalt chloride for various time and followed by either 0.1% DMSO treatment for control or 1 nM TCDD treatment for 24 hours. One hour pretreatment with 100 μM cobalt chloride showed 34% inhibition of 1 nM TCDD induced luciferase activity and as increased the time of the pretreatment of cobalt chloride, the luciferase activity was inhibited. 17 Hour pretreatment with 100 μM cobalt chloride showed 89% inhibition of 1 nM TCDD induced luciferase activity, thus for the dose response study with cobalt chloride, 17 hour pretreatment was applied. These data show that cobalt chloride inhibits the TCDD induced luciferase activity and strongly suggest that iron chelating agent also known as hypoxic agent, such as cobalt chloride inhibits the TCDD induction of *Cyp1a1* expression.

Desferrioxamine inhibition on the TCDD induced luciferase activity.
pm*Cyp1a1*-Luc transfected Hepa I cells were treated with various concentrations (1 μM, 10 μM, 100 μM) of desferrioxamine for 17 hours before the treatment of 1 nM TCDD for 24 hours. The treatment with 1 nM TCDD resulted in 7250-fold induction of luciferase activity, which was decreased with dose dependent manner when 1 μM or 10 μM or 100 μM desferrioxamine was pretreated for 17 hours. pm*Cyp1a1*-Luc transfected Hepa I cells were treated with 100 μM desferrioxamine for various time and followed by either 0.1% DMSO treatment for control or 1 nM TCDD treatment for 24 hours. As increased the time of desferrioxamine pretreatment, the luciferase activity was inhibited and 8 hour pretreatment with 100 μM desferrioxamine showed 35% inhibition and 17 hour pretreatment showed 94% inhibition of 1 nM TCDD induced luciferase activity. These data demonstrate that desferrioxamine inhibits the TCDD induced luciferase activity and strongly suggest that hypoxic agent, such as desferrioxamine inhibits the TCDD induction of *Cyp1a1* expression.

Picolinic acid inhibition on the TCDD induced luciferase activity.
pm*Cyp1a1*-Luc transfected Hepa I cells were treated with various concentrations (1 μM, 10 μM, 100 μM) of picolinic acid for 17 hours before the treatment of 1nM TCDD for 24 hours. The treatment of 1 nM TCDD resulted in 2680-fold induction of luciferase activity, which was decreased with 1 μM or 10 μM or 100 μM picolinic acid pretreatment to 85%, 37%, 17% that of 1 nM TCDD treated luciferase activity respectively. These data show picolinic acid that one of hypoxic gents inhibits the TCDD induced luciferase activity with dose dependent manner and suggest that picolinic acid can inhibit the TCDD induced *Cyp1a1* gene expression.

The effect of ferrous sulfate on luciferase activity inhibited by either desferrioxamine or picolinic acid treatment.
Various concentrations of desferrioxamine (1 μM, 10 μM, 100 μM) in the presence or absence of 150 μM ferrous sulfate were administered into Hepa I cells that were transfected with pm*Cyp1a1*-Luc for 17 hours before the treatment with either 0.1% DMSO or 1 nM TCDD for 24 hours. As shown in Fig 8, desferrioxamine inhibited the TCDD induced luciferase activity with dose dependent manner, and this inhibition was mostly removed when cells were treated with the ferrous sulfate concomitantly. This result shows iron chelating agent inhibits TCDD induced *Cyp1a1* gene expression. Likewise, in the case of picolinic acid treatment, the inhibition of luciferase activity by picolinic acid was also mostly recovered with ferrous sulfate treatment. This data strongly suggests that iron chelation inhibits TCDD induced *Cyp1a1* gene expression.

Acknowlwdgements
The present study was supported by the grant # 1998-019-F0074 from ministry of education in korea.

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