

# Liver Proteins that are Sensitive to a Dioxin-Like Toxic Compound, Coplanar Polychlorinated Biphenyl, 3,3',4,4',5-Pentachlorobiphenyl

Yuji Ishii\*,<sup>a</sup> and Kazuta Oguri<sup>b</sup>

<sup>a</sup>Department of Environmental Medicine, Institute of Community Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan and <sup>b</sup>Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

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This review deals with the reducing ability of a dioxin-like toxic compound, coplanar polychlorinated biphenyl, 3,3',4,4',5-pentachlorobiphenyl (PCB126) in protein levels of rat liver aldolase B, carbonic anhydrase III, class I alcohol dehydrogenase (ADH), glucose regulated protein 78 (GRP78), GRP94, calreticulin, and calnexin as well as the inducing ability in hepatic cytosolic 54 K protein (tentative selenium binding protein/acetaminophen binding protein homologue), heat shock protein 70 (HSP70) and HSP90. These data could provide new aspects of dioxin's toxicity *via* marked changes in the levels of proteins with important functions.

**Key words** — dioxin, suppression, wasting syndrome, alcohol dehydrogenase, carbonic anhydrase, aldolase

## INTRODUCTION

In 1968, a serious outbreak of subacute intoxication by polychlorinated aromatic hydrocarbons, so-called Yusho, occurred in the southwest part of Japan.<sup>1,2</sup> The coplanar polychlorinated biphenyls (PCBs) are well known as one of the causal agents of Yusho<sup>3,4</sup> and are also widespread environmental pollutants.<sup>5</sup> 3,3',4,4',5-Pentachlorobiphenyl (PenCB, PCB126), which is one of the coplanar PCBs and the most toxic among the 209 PCB congeners, was used in this study.<sup>6</sup> PCB126 exhibits similar toxicity to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and the toxic equivalency factor (TEF) is defined as 0.1.<sup>7</sup> In 1999, the Japanese government decided to include coplanar PCBs in the category of dioxins following the consultation report of the World Health Organization (WHO) and set up a tolerable daily intake (TDI) of 4 pgTEQ/kg/day.<sup>8</sup> Dioxins have been recognized as carcinogens and teratogens and more recently as endocrine dis-

rupters.<sup>9</sup> The low dose effects of dioxins on the endocrine and reproductive systems have provoked long and heated discussions. It is important to develop a strategy to minimize such effects, however, herein we primarily discuss high dose toxic effects on rats.

Arylhydrocarbon (Ah)-receptor (AhR), a PAS-family transcription factor, is present in cytosol and is associated with two molecules of heat shock protein 90 (HSP90).<sup>10,11</sup> After binding to the AhR ligands such as TCDD or PCB126, AhR translocates to the nucleus and forms a heterodimer with Arnt (AhR nuclear translocator); thereby the complex binds to xenobiotic responsive elements (XREs) which are found in 5'-regulatory elements of a TCDD-inducible gene such as CYP1A1.<sup>10,11</sup> Many toxicological studies including those using AhR knockout mice support that there is no doubt that AhR is important in the toxicity caused by dioxins.<sup>12-14</sup> However, the toxic effect of TCDD on human and experimental animals is pleiotropic and the crucial mechanism of toxic manifestations like severe suppression of body weight gain and wasting syndrome has not been fully elucidated.

Thus, we hypothesized that "the levels of important proteins involved in the PCB126 toxicity are significantly induced or suppressed when PCB126

\*To whom correspondence should be addressed: Department of Environmental Medicine, Institute of Community Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan. Tel.: +81-298-53-3297; Fax: +81-298-53-3039; E-mail: ishiiug@md.tsukuba.ac.jp

elicits the toxicity.” According to our hypothesis, rats were treated with PCB126 and the hepatic proteins were screened using SDS-PAGE. Further, the proteins were analyzed by two-dimensional gel electrophoresis of non-equilibrated pH gradient gel electrophoresis (NEPHEGE) and SDS-PAGE. Several proteins sensitive to PCB126-treatment were found after sequencing of internal peptides obtained by *in situ* peptide mapping with  $V_8$ -protease.

In this review, we introduce the PCB126-suppressive hepatic proteins aldolase B,<sup>15)</sup> class I alcohol dehydrogenase (ADH),<sup>16)</sup> carbonic anhydrase III (CAIII),<sup>17)</sup> glucose regulated protein (GRP) 78 (GRP78, BiP),<sup>18,19)</sup> GRP94,<sup>18,19)</sup> calreticulin<sup>19)</sup> and calnexin<sup>19)</sup> as well as the PCB126-inducible hepatic proteins 54 K protein [tentative selenium binding protein (SeBP)/acetaminophen binding protein (APBP) homologue],<sup>20)</sup> HSP70<sup>21)</sup> and HSP90.<sup>21)</sup>

### Induction of 54 K Protein

PCB126 (25 mg/kg, i.p.)-treatment to Wistar rats significantly induces 54 K and 58 K protein in liver cytosol.<sup>20)</sup> The 58 K protein is identified as a class 3 aldehyde dehydrogenase known to TCDD-inducible enzyme.<sup>22,23)</sup> The 54 K protein is highly homologous to mouse SeBP<sup>24)</sup> and APBP.<sup>25)</sup> The 54 K protein was also induced by another AhR ligand, 3-methylcholanthrene (MC)-treatment,<sup>20)</sup> whereas induction of the 58 K protein was not significant by this treatment. Taken together, PCB126-treatment induces the homologue(s) of SeBP and/or APBP. The induction of the 54 K protein was confirmed to be PCB126-inducible by immunoblotting using the antibody raised against MC-inducible 54 K protein purified by electrophoretically using Rotofore Cell™ (BioRad).<sup>26)</sup> In addition, the 54 K protein is inducible by butylated hydroxytoluene (BHT) as well as MC in a dose dependent manner. The induction mode was significantly correlated to that of NAD(P)H:quinone oxidoreductase (NQO) activity<sup>27)</sup> which is up regulated with AhR-ligands and an antioxidant.<sup>28)</sup> In recent years, NFE2-related factor 2 (Nrf2) has been shown to be involved in antioxidant response.<sup>29)</sup> Taken together, the 54 K protein is possibly up-regulated by a phenolic antioxidant through Nrf2<sup>29)</sup> as well as by AhR ligands. The selenium binding protein that does not possess selenocystein in its primary structure,<sup>24)</sup> binds to selenium in a manner that has not been clarified. The 54 K protein purified by an improved method which did not necessitate electrophoresis was subjected to selenium content analysis, and the content was much lower than that of se-

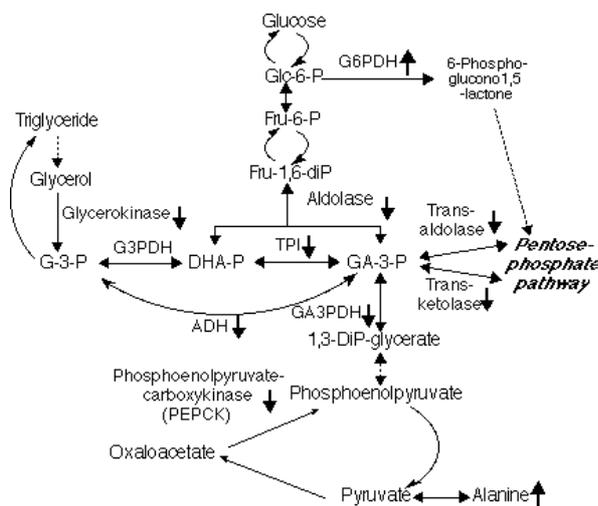
lenium dependent glutathione peroxidase.<sup>30)</sup> Recently, Porat *et al.*, reported that SeBP participates in intra-Golgi protein transport.<sup>31)</sup> There could be another function for SeBP because mouse SeBP,<sup>23)</sup> mouse APBP<sup>25)</sup> and human SeBP<sup>32)</sup> possess two bis(cysteiny) sequence motifs which are found in the proteins involved in redox-regulation such as thioredoxin.<sup>33)</sup> Further, such motifs are also found in Ah-receptor ligands-inducible SeBP of rat of which the cDNA has recently been isolated.<sup>34)</sup> The deduced amino acid sequence of rat SeBP<sup>34)</sup> exhibited homologies with mouse SeBP,<sup>24)</sup> mouse APBP<sup>25)</sup> and human SeBP<sup>32)</sup> of 92%, 93% and 87%, respectively.

The 54 K protein is also highly homologous to APBP which is important during intoxication of overdosed acetaminophen.<sup>35,36)</sup> The binding of APBP to a reactive acetaminophen metabolite, NAPQI may be the trigger reaction of acetaminophen intoxication.<sup>35,36)</sup> On the contrary, there is a report that APBP prevents oxidative stress caused by NAPQI.<sup>37)</sup> Possible homologues of SeBP/APBP have been found in *Caenorhabditis elegans* and *Arabidopsis thaliana* on the basis of sequence similarity on the GenBank data base. It is not yet clear whether induction of the 54 K protein contributes to provoke toxicity; the induction is possibly a response to reduce the toxicity. It is likely that SeBP is important throughout evolution, so that further investigation is needed to understand the physiological significance of the induction of the 54 K protein.

### Suppression of Aldolase B

Reduced gluconeogenesis was observed in rats receiving TCDD and PCB126 accompanied by the suppression of phosphoenolpyruvate carboxykinase.<sup>38,39)</sup> Aldolase is an important enzyme in both glycolysis and gluconeogenesis. Fructose-1,6-diphosphate (FDP) aldolase (EC 4.1.2.13), a glycolytic enzyme, catalyzes the reversible conversion of FDP to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. Three isoforms of aldolase, termed aldolase A, B and C in mammals, are different gene products<sup>40-42)</sup> and their tissue distributions are also different. Aldolase B (Ald B, liver-type) is found in the liver, kidney, and small intestine and its catalytic activity is different from those of aldolase A (muscle-type) and aldolase C (brain-type).<sup>40-42)</sup> Ald B is also capable of catalyzing the conversion of fructose 1-phosphate (F1P) to dihydroxyacetone phosphate and glyceraldehyde, while the other two isoforms have little capacity.<sup>40-42)</sup> PCB126-treatment

(25 mg/kg, i.p.) to Wistar rats significantly reduces aldolase activity towards both FDP and F1P.<sup>15)</sup> The immunoblot after SDS-PAGE and two-dimensional PAGE of NEPHGE and SDS-PAGE supports that PCB126 elicits the marked suppression of aldolase B.<sup>15)</sup> Since aldolase catalyzes the reaction in both glycolysis and gluconeogenesis, the suppression could be one of the causes of the wasting syndrome by PCB126. Thus, we examined the effect of PCB126 on triose phosphate metabolism.<sup>43)</sup> The activities of transaldolase, transketolase, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase and glycerokinase were significantly reduced by PCB126-treatment. Glycerol-3-phosphate forming activities from dihydroxyacetone-phosphate and glyceraldehyde-3-phosphate were also significantly reduced. Triose phosphate contents in PCB126-treated and pair-fed control groups were markedly decreased compared to free-fed controls. Giving nutritional supplements to TCDD-treated rats failed to cure the wasting syndrome caused by TCDD.<sup>44)</sup> Figure 1 summarizes the effects of PCB126 on triose phosphate metabolism in rat liver. It is likely that the suppressed triose phosphate metabolism in PCB126-treated rats affects the utility of energy sources. In combination with these observations, there was a suppressive response to the en-



**Fig. 1.** Summary of Effects of PCB126 on Intermediary Metabolism in Rat Liver<sup>15,16,39,43,45)</sup>

Arrows represent the tendencies of the marked response to PCB126-treatment compared to controls. Abbreviations: Glc-6-P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; Fru-6-P, fructose-6-phosphate; Fru-1,6-diP, fructose-1,6-diphosphate; G-3-P, glycerol-3-phosphate; G3PDH, glycerol-3-phosphate dehydrogenase; DHA-P, dihydroxyacetone phosphate; GA-3-P, glyceraldehyde-3-phosphate; GA3PDH, glyceraldehyde-3-phosphate dehydrogenase; TPI, triosephosphate isomerase; 1,3-diP-glycerate, 1,3-diphosphoglycerate.

zymes of glycolysis and gluconeogenesis pathways, which might influence the energy utilization.

### Suppression of Alcohol Dehydrogenase

ADH is an important enzyme in alcohol metabolism, especially, class I ADH possesses the lowest  $K_m$  for ethanol among the isoforms.<sup>46)</sup> ADH activity in hepatic cytosol is significantly decreased by PCB126-treatment (25 mg/kg, i.p.).<sup>43)</sup> Although an endogenous substrate for ADH has not been clarified, at least retinol is one candidate<sup>47)</sup>; triosephosphates were also suggested to be substrates.<sup>48)</sup> When glycerol-3-phosphate formation activities from dihydroxyacetone-phosphate and glyceraldehyde-3-phosphate in hepatic cytosol were examined in the presence or absence of an ADH inhibitor, 10 mM of pyrazol, the pyrazol-sensitive activities were significantly suppressed.<sup>43)</sup> In addition, the result of immunoblot proved that class I ADH protein level in hepatic cytosol is remarkably suppressed by the PCB126-treatment (Fig. 2).<sup>16)</sup> On the contrary, TCDD elevates class I ADH $\alpha$  in human hepatoma, HepG2 cells.<sup>49)</sup> The class I ADH is a dimeric protein found predominantly in liver.<sup>50)</sup> Rats have only one class I ADH,<sup>51)</sup> whereas class I ADHs in human are composed of homo- and hetero-dimers of three very closely related subunits,  $\alpha$ ,  $\beta$  and  $\gamma$ .<sup>50)</sup> There is room for further investigation about the involvement of such environmental pollutants as a negative factor in ethanol metabolism.

### Suppression of Carbonic Anhydrase III

PCB126-treatment (25 mg/kg, i.p.) to rats elicits a reduced level of hepatic CAIII (Fig. 2). The suppression of CAIII by PCB126 was dose-dependent.<sup>17)</sup> The carbonic anhydrases catalyze the reversible reaction of carbon dioxide and water to form



**Fig. 2.** Effect of PCB126-Treatment to Rats on the Protein Level of Hepatic CAIII and Class I ADH

Liver was removed 5 days after PCB126 (25 mg/kg, i.p.)-treatment. Liver cytosolic protein (3  $\mu$ g) from free-fed control, pair-fed control and PCB126-treated rats was subjected to SDS-PAGE. Immunoblot analyses were performed using anti-alcohol dehydrogenase antibody<sup>16)</sup> and anti-CAIII antibody.<sup>52)</sup>

carbonic acid<sup>53)</sup> and several isozymes are known.<sup>53-55)</sup> CA III can be distinguished from the other isozymes by several characteristics, particularly its lower specific activity and resistance to acetazolamide, which inhibits isozymes I and II.<sup>55,56)</sup> CAIII possesses carbonic anhydrase activity that is quite low compared to CAI and CAII;<sup>56)</sup> however CAIII is unique among the carbonic anhydrases since it also possesses tyrosine phosphatase activity.<sup>57)</sup> In addition, Räsänen *et al.*, showed that CAIII was an antioxidant that prevents H<sub>2</sub>O<sub>2</sub>-inducible apoptosis,<sup>58)</sup> thus, PCB126-treatment may cause reduced response to oxidative stress and affect signal transduction. Further, current work in our laboratory has shown that PCB126 provoked a reduced activity of catalase, glutathione reductase, selenium dependent glutathione peroxidase and a decrease in glutathione content.<sup>59-61)</sup> Taken together, these responses could raise the oxidative stress caused by PCB126. Since CAIII is abundantly expressed in liver and muscle of rats,<sup>62)</sup> we examined the effect of PCB126 on the level of muscle CAIII. The mRNA level of CAIII in rat liver was dramatically decreased by PCB126,<sup>17)</sup> whereas that of muscle CAIII was not decreased by the treatment.<sup>63)</sup> Thus, PCB126 suppresses the CAIII in a liver specific fashion.

### Effects on Molecular Chaperones

Glucose regulated proteins (GRP) 78 (GRP78, BiP) and GRP94 which are stress proteins in the endoplasmic reticulum play an important role in the quality control of the proteins.<sup>64,65)</sup> PCB126-treatment (0.1, 1, 10 and 25 mg/kg, i.p.) to Wistar rats resulted in markedly reduced protein levels of GRP78 and GRP94 in hepatic microsomes.<sup>18,19)</sup> Further, PCB126-treatment (10 mg/kg, i.p.) to rats suppresses the protein levels of calreticulin and calnexin<sup>19)</sup> which are also known as molecular chaperones in endoplasmic reticulum,<sup>66,67)</sup> and thereby it might cause impaired stress response. Interestingly, the mRNA level of GRP78 was not significantly affected by PCB126-treatment.<sup>19)</sup> Taken together, the mechanism of the suppression of GRP78 is probably different from that of CAIII.<sup>17)</sup> On the contrary, TCDD elevates the GRP78 level in testicular cell lines of mouse Leydig TM3 and Sertoli TM4.<sup>68)</sup> It is unclear why the effect of dioxins on GRP78 appears to have different profiles in liver and testis.

PCB126-treatment to Wistar rats causes an increased level of heat shock protein 70 (HSP70) and HSP90<sup>21)</sup> which are molecular chaperones in cytosol;<sup>69)</sup> these rises were in a dose responsive manner.

Interestingly, both HSP70 and HSP90 are also inducible in the rat testis Leydig cells LC540 by 10 pM PCB126 which is assumable as that in the environmental level.<sup>70)</sup> Elevation of HSP70 and HSP90 may account for hormone disruption because both are included in the steroid hormone receptor complex such as estrogen receptor and androgen receptor.<sup>71,72)</sup> Thus, the molecular chaperone levels in liver cytosol and microsomes are affected by PCB126 with opposite direction.

### General Discussion

The liver proteins that are sensitive to PCB126-treatment found in this study are summarized in Table 1. Not all of them seem to be related to the PCB126 toxicity but they gave us new insight in understanding the toxicity including wasting syndrome and endocrine disruption.

The induction mechanism of proteins by dioxins is well documented as an Ah-battery system, *e.g.* CYP1A1.<sup>11)</sup> We also dealt with the induction of the 54 K protein, HSP70 and HSP90 in liver cytosol by PCB126,<sup>20,21,70,73)</sup> although the involvement of AhR in that induction remains to be elucidated. In contrast, we know relatively little about the suppression mechanism of particular proteins. The suppression of epidermal growth hormone receptor, estrogen receptor, prolactin receptor, and a class I major histocompatibility complex Q1<sup>b</sup> (MHC Q1<sup>b</sup>) has been reported and the involvement of AhR has been suggested.<sup>74-78)</sup> In addition, antiestrogenic effects of TCDD are mediated by direct transcriptional interference with the liganded estrogen receptor through cross-talk between aryl hydrocarbon- and estrogen-mediated signaling.<sup>79,80)</sup> AhR may be involved in the suppression of CAIII because it was observed to take place in a manner which was PCB126-dose responsive.<sup>17)</sup> We have found that PCB126-treatment to Wistar rats reduces the hepatic protein levels of aldolase B, class I ADH, CAIII, GRP78, GRP94, calreticulin and calnexin.<sup>15-19)</sup> Reduced level of CAIII protein in liver cytosol by PCB126 was due to remarkable suppression of the mRNA level. However the suppression of GRP78 was not accompanied by a significant change in the level of the mRNA. The mechanism of the suppression of GRP78 is probably different from that of CAIII. AU-rich elements which are known to be a destabilizing motif<sup>81)</sup> were found in a 3'-untranslated region of CAIII mRNA; such elements were also found in TCDD-suppressible MHC Q1<sup>b</sup>.<sup>78)</sup> The authors discussed the possible involvement of both destabilization and transcrip-

**Table 1.** Summary of Rat Liver Proteins that Are Sensitive to a Dioxin-like Toxic Compound Coplanar Polychlorinated Biphenyl, 3,3',4,4',5-pentachlorobiphenyl-Treatment

| Proteins                                       | Subcellular localization | Ref.   |
|--|--------------------------|--------|
| Induction                                      |                          |        |
| 54K protein<br>(tentative SeBP/APBP homologue) | cytosol                  | 20     |
| HSP70  | cytosol                  | 21     |
| HSP90  | cytosol                  | 21     |
| Suppression                                    |                          |        |
| Aldolase B                                     | cytosol                  | 15     |
| ADH (class I)                                  | cytosol                  | 16     |
| CAIII  | cytosol                  | 17     |
| GRP78 (BiP)                                    | microsomes               | 18, 19 |
| GRP94  | microsomes               | 18, 19 |
| Calreticulin                                   | microsomes               | 19     |
| Calnexin                                       | microsomes               | 19     |

tional regulation. Interestingly, suppression of CAIII by PCB126 was observed in a liver specific fashion.<sup>63)</sup> Further study on the liver specific suppression could help to understand the suppression mechanism of particular proteins by PCB126.

In recent years, structure and function of AhR have been thought to be important for responsiveness to dioxins. In fact, AhR from the responder C57 BL mouse shows higher affinity to aryl hydrocarbon than that from non-responder DBA mouse.<sup>82)</sup> Responsiveness of human to dioxins was also discussed with the functional similarity of the AhR to that of DBA mouse.<sup>82)</sup> Recently, guinea pig AhR was cloned and it is rather similar to human AhR.<sup>83)</sup>

According to advanced technology, a microarray system is available for screening of gene expression pattern.<sup>49)</sup> Further, the two-dimensional gel electrophoresis system has been improved and adapted to TOF/MS system for analyzing proteome.<sup>68)</sup> Finding of specific AhR ligand(s)<sup>84)</sup> and inhibitors<sup>85,86)</sup> could also help the interpretation of data. Taken together, it is likely that biochemical approaches which are based on the endpoint of the toxicity caused by dioxins are also important in understanding the pleiotropic effects.

We have learned that Yusho patients have suffered from dioxins, and environmental pollution by dioxins has been found worldwide. This disaster is an end result of the 20th century paradigm<sup>87)</sup> which we pursued only for the convenience and comfort of we humans while neglecting the overall eco-cycle. We should abandon such egotism, and exert all our wisdom toward solving this problem. We believe

that, for this issue, the solution will not come only from the government or authorities, but from individuals' determination to stop producing dioxins through waste disposal. In order to attain the reduction of toxic waste, we individuals have to change our life style, even giving up some of our conveniences and comforts. It is essential that we change ourselves, mentally and behaviorally, so that we can accept responsibility for the entire ecosystem.

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