Protective effects of vitamin E against 3,3',4,4',5-pentachlorobiphenyl (PCB126) induced toxicity in zebrafish embryos☆

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3,3′,4,4′,5-Pentachlorobiphenyl 126 (PCB126) is a global environmental contaminant that can induce cellular oxidative stress. We investigated whether vitamin E can protect against toxicity from PCB126 during zebrafish (Danio rerio) development. Zebrafish embryos were exposed to 100 nM PCB126 and compared with a second group that was co-exposed with 100 μM vitamin E until 5 days post fertilization. PCB126 induced pericardial sac edema, yolk sac edema, and growth retardation in zebrafish embryos. In contrast, vitamin E co-exposure group did not show any gross changes. Real-time PCR results showed that vitamin E co-exposure group were restored to control group for the expression levels of heat shock protein 70 Cognate, aryl hydrocarbon receptor type-2, cytochrome P450 1A, and superoxide dismutase-1. These data give insights into the use of vitamin E to reduce PCB126-mediated toxicity and into the use of zebrafish embryos for exploring mechanisms underlying the oxidative potential of AHR agonists.

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1. Introduction

Polychlorinated biphenyls (PCBs) are widespread persistent residual environmental contaminants, which have been widely used in various industrial applications (Ramadass et al., 2003). Exposure to PCBs produces oxidative stress in vertebrate cells, including those of rodents (Fadhel et al., 2002; Hassoun et al., 2002) and human hepatoma cells (Park et al., 1996). Oxidative stress caused by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and PCBs is associated with activation of aryl hydrocarbon receptor (AHR) followed by increased expression of cytochrome P450 1A (CYP1A) which results in increased reactive oxygen species (ROS) production in endothelial cells (Schlezinger et al., 1999). There is evidence that the cellular antioxidant defense, particularly the cellular level of vitamin E, is depressed after exposure to PCBs (Toborek et al., 1995; Hennig et al., 1999). These studies suggest that several antioxidant nutrients can down-regulate disease promotion from specific environmental contaminants (Ramadass et al., 2003).

There have been many reports about toxic mechanisms of PCBs (Toborek et al., 1995; Alcock et al., 1998; Slim et al., 1999; Hennig et al., 2000; Hennig et al., 2002; Ramadass et al., 2003; Yun et al., 2005; Arzuaga et al., 2006) and they demonstrated the relationship between oxidative stress and the site of the target organ, which is mainly the endothelium. Certain PCBs can compromise normal functions of vascular endothelial cells by activating oxidative stress-sensitive signaling pathways and subsequent proinflammatory events critical in the pathology of atherosclerosis and cardiovascular disease (Hennig et al., 2002). There were some reports about the protective effects of the antioxidant, vitamin E against PCBs in cell culture systems (Slim et al., 1999; Hennig et al., 2002; Teraoka et al., 2003). Likewise, Yun et al. showed that, in rats, vitamin E protects against neurotoxicity caused by Aroclor1254 (Yun et al., 2005).

From experimental evidence provided by others, we assumed that, in zebrafish, the antioxidant vitamin E would have a protective effect against the gross morphological changes by PCB126 exposure. As far as we know, this is the first time that zebrafish embryos have been used in research concerning vitamin E-mediated protection against PCB126 exposure. According to our previous screening, zebrafish embryos showed some typical morphological toxicities (data unpublished) in responding to pentachlorinated biphenyl 126 (PCB126), which contributes the most to the toxic equivalents (TEQ) of Aroclor (Alcock et al., 1998).
The sensitivity of zebrafish embryos to PCB126, and the abilities of molecular and genetic tools available for this model organism, make the zebrafish an attractive model for examining toxicity from PCB126 and the antioxidant protection of vitamin E against PCB126. First, we studied zebrafish embryo toxicity from PCB126 exposure, and then we investigated the protective effect of vitamin E, which is indicated by inhibition of pericardial edema, yolk sac edema, and growth retardation. To estimate the underlying mechanisms, we screened mRNA expression levels of zebrafish heat shock protein 70 cognate (zfHSC70), zebrafish CYP1A (zfCYP1A), and zebrafish aryl hydrocarbon receptor type-2 (zfAHR2), which are indicators of oxidative stress (Weber and Janz, 2001; Ramadass et al., 2003). In addition, zebrafish superoxide dismutase-1 (zfSOD1) and zebrafish superoxide dismutase-2 (zfSOD2) were used as indicators of antioxidant status of embryos (Gonzalez et al., 2006) exposed to PCB126.

2. Materials and method

2.1. Animals

Fish were purchased from a local supplier. Wild-type adult zebrafish and fertilized eggs were raised according to the *The Zebrafish Book* (Westerfield, 1995). Ringer's solution (116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl$_2$, 5 mM HEPES, pH 7.2) containing chemicals were changed daily until the embryos were sacrificed. Care and treatment of the animals were in accord with guidelines established by the Seoul National University Institutional Animal Care and Use Committee (Approval no. SNU-050418-2).

2.2. Waterborne exposure of embryos to PCB126 and vitamin E

PCB126 of 99.8% purity was obtained from Neosyn Laboratories Inc. (CAS#57465-28-8) and solidified in DMSO. Vitamin E was purchased from Sigma (St. Louis, MO, USA). From approximately 2 h post-fertilization (2 hpf), embryos were maintained in Ringer's solution containing vehicle (0.1% DMSO) or 50, 100, 150, or 200 μM vitamin E in 5 ml glass scintillation vials. They were gently rocked for a period of 6 hours, then treated with PCB126 or co-treated with PCB126 and vitamin E for up to 5 days post-fertilization (5 dpf). Numbers of exposed embryos per vial were 20 in 2 ml of solutions. Solutions were changed daily with removal of chorion remnants and dead embryos for the duration of the experiment.

2.3. Experimental design

Three treatment groups were used: those treated with vehicle (0.1% DMSO), those treated with 100 nM PCB126 only, and those treated initially with 100 nM PCB126 and vitamin E together for a period of 5 dpf. For the assessment of edema and body length, five embryos were randomly selected in each treatment group at 5 dpf and analyzed at 5 dpf. For measuring the volume of the pericardial cavity, three treatment groups were used: those treated with vehicle (0.1% DMSO), those treated with 100 nM PCB126 for 5 days, and those treated initially with 100 nM PCB126 and vitamin E together for a period of 5 dpf followed by 28 cycles of denaturation (1 min, 95 °C), annealing (1 min, 50–60 °C), chain extension (2 min, 72 °C), and then 7 min of final extension at 72 °C after amplification. Each PCR product was visualized under ultraviolet light and band densities were measured with an analyzing program (Kodak Digital Science 1D, NEN Life Science Products, Boston, MA, USA), normalized by the density of zebrafish Wnt5A (Lin et al., 1994), and evaluated statistically.

2.4. Gross changes from embryo toxicity by PCB126 and image analysis

For image analysis of pericardial sac and yolk sac areas at 5 dpf after PCB126-exposure, lateral views of anesthetized embryos were imaged using a stereoscopic microscope (OLYMPUS Japan SZ-PT), and photographed using a Nikon COOLPIX 950 digital camera at the same magnification (Prasch et al., 2003). For body length, a straight line starting at the anterior-most point of the head and ending at the tail end was produced and measured. The outlines of the pericardial sac and yolk sac were traced, and the area within each tracing was determined by the discociation curve of the PCR product. This curve was obtained by following the SyberGreen fluorescence level during a gradual heating of the PCR products from 60 to 95 °C. Relative quantification of each gene expression level was normalized according to the Wnt5A gene expression (Lin et al., 1994).

2.5. Statistical analysis

Significant differences among groups were determined using Duncan's Multiple Range Test (SAS ver. 8.1, SAS Institute Inc., Cary, NC, USA). Values of *P* > 0.05 were considered as significant.

3. Results

Zebrafish embryos exposed to PCB126 from 8 hpf to 5 dpf showed several typical morphological defects (Fig. 1). At 5 dpf, embryos treated with 100 nM PCB126 showed short body length and, in some cases, incompletely differentiated tail ends (Fig. 1B). Heart curvature was unfolded and a dilated pericardial sac was observed, suggesting pericardial edema (Fig. 1C). Red blood cells accumulated in the anterior side of yolk due to circulation failure (Fig. 1C). Yolk consumption was slower than normal, suggesting growth retardation. Swim bladders were not inflated in embryos treated with PCB126. In addition, we observed vessel irregularity in the posterior caudal vein around the anal pore (Fig. 1E).

Image analysis data showed that vitamin E protected the morphological changes by PCB126. When zebrafish embryos were exposed to 100 nM PCB126 for 5 days, the volume of the pericardial cavity increased nearly two times normal size, although there was some variability between individuals (Fig. 2).
embryos were administered a pretreatment of vitamin E, we observed no pericardial and yolk sac edema. The size of yolk sac area in the group that received PCB126 treatment alone was about four times that of the control group. However, vitamin E clearly protected zebrafish embryos against PCB126. At 5 dpf, zebrafish embryos under PCB126 exposure showed a shortening of the body length of 10% or more compared with the control but no growth arrest occurred when vitamin E was also administered.

In semi-quantitative RT-PCR for zfCYP1A (Fig. 3A), the PCB126-treated group showed significantly higher gene expression levels at 2 and 4 dpf as compared with control larvae, and these changes began at 2 dpf. However, when 100 μM vitamin E was co-administered, the zfCYP1A gene expression level was found to be lower than that of the control group. zfHSC70 expression levels of the PCB126-treated group were low compared to those of the control embryos until 3 dpf and increased steeply at 4 dpf (Fig. 3B). The curve for the 100 μM vitamin E co-exposure group was higher than the control curve for the entire 5 days. In particular, the expression level of 2 dpf was four times as high as the control.

We carried out quantitative real-time PCR to investigate possible mechanisms of vitamin E in protecting against toxicity from PCB126 exposure in zebrafish embryos at the second day after chemical exposure. Interestingly, the PCB126-treated group showed 80% lower zfHSC70 expression levels compared to control group (Fig. 4A) and vitamin E recovered this inhibition up to normal ranges. zfAHTR2 and zfCYP1A showed the same tendency as shown in Fig. 4B. The PCB126-treated group expressed higher levels of zfAHTR2 and zfCYP1A than normal, and the quantity of zfAHTR2 was over three times that of zfCYP1A expression when larvae were treated with PCB126 alone. Co-exposure with 100 μM vitamin E decreased the zfAHTR2 and zfCYP1A gene expression level to 37% and 24% of the PCB126 treatment group, respectively. This expression level was still higher than control but it nevertheless seemed effective to protect against the morphological changes from PCB126. Real-time PCR data for zfSOD1 and zfSOD2 showed conflicting results (Fig. 4C). zfSOD1 expression increased by nearly 5-fold in embryos exposed to PCB126 alone. However, vitamin E lowered the expression level of zfSOD1 to 34% of the PCB126 alone. In contrast, in the case of zfSOD2, which is considered to be a more potent antioxidant, there was a slight increase with no significant difference under PCB126 exposure, but a 2.6-fold increase during vitamin E co-exposure.
4. Discussion

As expected, the antioxidant vitamin E has a protective effect against the gross morphological changes induced by PCB126 exposure in zebrafish embryos. Zebrafish embryos respond to PCB126 sensitively, allowing us to measure gradations of morphological disruption, particularly pericardial sac edema, yolk sac edema, and shortening of the body axis. Gross changes from PCB126 are edema, vessel irregularity, and blood circulation failure, all of which are related to endothelial damage (Cantrell et al., 1996; Dong et al., 2002; Hennig et al., 2002).

We surmised that vitamin E can show protective effects against these irreversible toxicities of PCB126, and thus, we treated embryos with vitamin E before PCB126 exposure. Pre-treatment of vitamin E blocked some of the molecular pathways that can have connections with irreversible damage to endothelial cells (Fig. 3). We selected zfCYP1A and zfHSC70 as indicators for oxidative stress (Cantrell et al., 1996; Weber and Janz, 2001) in our semi-quantitative RT-PCR experiments. Because zfCYP1A expression levels increased steeply at 2 dpf by PCB126 (Fig. 3A) and that for the vitamin E co-exposure group were much lower than the PCB126-exposure group in our quantitative RT-PCR data, we infer that vitamin E blocked the toxic effects of zfCYP1A over-expression in zebrafish embryos by reducing the expression level of zfCYP1A. We focused our ‘2 dpf’ because most angiogenesis of early vessels in zebrafish occurs by 1.5 dpf (Vogel and Weinstein, 2000), also the cardiovascular system has been shown to be a site of strong induction of CYP1A (Stegeman et al., 1989). Cantrell et al. demonstrated the embryonic vasculature is a physiological target for TCDD-induced DNA damage and the antioxidant N-acetylcysteine also provided significant protection against the embryotoxicity of TCDD in Medaka (Cantrell et al., 1996). Thus, extensive zfCYP1A induction at 2 dpf by PCB126 seems to be related to gross changes including edema (endothelial disruption). There was a decrease of blood flow to the mesencephalic vein at 50 hpf by TCDD exposure in zebrafish embryos, and simultaneously, CYP1A mRNA was enhanced at 50 hpf in the mesencephalic vein and heart (Dong et al., 2002). Therefore, zfCYP1A induction at 2 dpf can be said to be related to the possible consequences of endothelial cell damage. We also noticed that vitamin E increased the zfHSC70 expression level dramatically at 2 dpf (Fig. 3B). Although we found some different expression levels of zfHSC70 between Figs. 3B and 4A at day 2 time point, it can be explained as due to lower precision of size-based discrimination from semi-quantitative RT-PCR (Chen et al., 1999). Expression tendency of zfHSC70 from Figs. 3B and 4A is similar in that they both showed more increased zfHSC70 expression level in vitamin E co-exposure group than that of PCB-126 treatment group. HSC70, a new molecular target of PCB toxicity, is a member of the heat-shock protein 70 family; the members of this family function as molecular chaperones (Kreiling et al., 2007). Like Kreiling et al. (2007), we confirmed...
that early embryonic exposure to PCB126 induced reduction in expression of HSC70. Interestingly, Rajagopalan suggested that heat shock treatment in rat hepatocytes attenuates the negative effects triggered by the addition of the toxic inducers and possibly stabilizes the levels of cytochrome P-450 proteins (Rajagopalan et al., 2005). Together with this study, we also showed that HSC70 possibly have some protective effect against the negative gross changes in zebrafish embryos.

For further analysis of the molecular changes brought about at 2 dpf by PCB126 and vitamin E, we performed quantitative real-time PCR using zfAHR2, zfCYP1A, and zfHSC70 as indicators for oxidative stress (Weber and Janz, 2001; Ramadass et al., 2003) and zfSOD1 and zfSOD2 as indicators for the antioxidant status of embryos (Gonzalez et al., 2006). Two divergent AHRs, AHR1, and AHR2, have been identified and characterized in zebrafish. The reason for choosing zfAHR2 is that zfAHR2 s are the functional members of the AHR pathway in zebrafish (Andreasen et al., 2002). As expected, zfAHR2 increased over 30-fold in the PCB126-treated group compared with control, and vitamin E co-exposure reduced the excessive increase to one third that of PCB126-treated group. This expression level is still higher than that of control, but it seems to be enough to reduce zfCYP1A expression to normal ranges. Vitamin E-mediated alleviating oxidative stress would reduce zfCYP1A activity (Slim et al., 1999) that can block positive feedback to induce AHR2 expression. In connection with this reduced oxidative stress, we also screened zfSODs. SODs represent a family of cellular enzymes involved in converting superoxide into peroxide, which can then be converted into water by catalase. SOD1 codes for cytosolic SOD, and SOD2 (mitochondrial SOD) is thought to be a more physiologically important antioxidant than SOD1 (Erker et al., 2006). A different tendency was shown in the vitamin E co-exposure group corresponding to zfSOD1 and zfSOD2. PCB126 increased zfSOD1 over five-fold more than that of control, although we do not know the exact mechanism of this increase. Vitamin E enhanced zfSOD2 about two-fold compared with the PCB126 treatment group, and this is meaningful because superoxide levels can be decreased by ROS-dependent down-regulation of the AHR pathway (Barouki and Morel, 2001). Thus, reduced zfAHR expression levels in the vitamin E co-exposure group may have some relation with zfSOD2. We still do not know whether vitamin E directly acts on ROS production by PCB126 or whether it inhibits the activity of ROS by upregulating zfSOD2 and zfHSC70 while downregulating zfAHR2 and zfCYP1A.
However exploratory, this study may offer some insight into how vitamin E improves the oxidative status of PCB126-treated zebrafish larvae for the better, and some related gene expressions like zfHSC70, zfAHR2, zfCYP1A, zfSOD1, zfSOD2 produce those protective effects of vitamin E.

5. Conclusion

We investigated the protective effects of vitamin E against PCB126 in zebrafish embryos for the first time. Our results demonstrate that PCB126 induces toxicities in the zebrafish embryo and vitamin E can protect zebrafish embryos against PCB126 by increasing zfHSC70 and zfSOD2 expression and inhibiting zfAHR2 and zfCYP1A expression. We conclude that zebrafish embryos are valuable laboratory animals for exploring mechanisms underlying the oxidative potential of AhR agonists.

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References


