

# Effects of Endocrine Disrupting Chemicals on Amphibian Metamorphosis and Mitochondrial Membrane Permeability Transition

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The estrogenic chemical bisphenol A (BPA) has multiple hormonal activities, but its effects on thyroid hormone (TH) action are not fully understood. TH is required for the metamorphosis of tadpoles into frogs, and thus tadpole tail regression, one of the most spectacular events in metamorphosis, can be used for studying the effect of BPA and related compounds (BPAs) on TH action. We found that 3,3',5-triiodothyronine (T<sub>3</sub>)-induced tadpole tail regression in the wrinkled frog *Rana rugosa* (*R. rugosa*) is suppressed by BPA, tetrabromobisphenol A (TBBPA), tetrachlorobisphenol A (TCBPA), and tetramethylbisphenol A (TMBPA). BPAs also inhibited spontaneous metamorphosis in the tropical clawed frog *Silurana tropicalis* (*S. tropicalis*) controlled by endogenous circulating TH. These results indicate that BPAs are TH antagonists. For the African clawed frog *Xenopus laevis* (*X. laevis*), transgenic tadpoles carrying plasmid DNA containing TH response element (TRE) and 5'-upstream promoter region of the TH receptor (TR)  $\beta$  A1 gene linked to an enhanced green fluorescent protein (EGFP) gene, T<sub>3</sub> induced a strong EGFP expression in the hind limbs. This expression was suppressed by BPA, TBBPA, TCBPA and TMBPA, suggesting BPAs all act as antagonists to prevent the binding of T<sub>3</sub> to TR, resulting in inhibition of TR-mediated gene expression. Much work has been done in examining the apoptotic and other detrimental effects of polychlorinated biphenyls (PCBs), but the role of mitochondrial damage in such toxic action remains obscure. There is evidence however that mitochondrial membrane permeability transition (MPT) plays a crucial role in apoptosis. We investigated the effect of 4-hydroxy-3,3',4',5-tetrachlorobiphenyl (4-OH-TCB) on isolated rat liver mitochondria. In order to help clarify this, biochemical analysis revealed that 4-OH-TCB induced calcium release, reactive oxygen species (ROS) generation, depolarization, swelling and cytochrome *c* (Cyt. *c*) release in a time- and concentration-dependent manner. These 4-OH-TCB-induced changes in mitochondrial function were found to be inhibited by the presence of cyclosporin A (CsA), a specific inhibitor of MPT, suggesting a classic type of MPT. It is concluded that 4-OH-TCB induces MPT together with ROS generation, causing apoptosis in certain cells.

**Key words** — amphibian metamorphosis, bisphenol A related compound, polychlorinated biphenyl, membrane permeability transition, mitochondria, thyroid hormone inhibition

## INTRODUCTION

Endocrine disrupting chemicals (EDCs) are a

group of natural and synthetic compounds including among other things insecticides, bisphenol A (BPA), polychlorinated biphenyls (PCBs), and the heavy metals lead, mercury and cadmium,<sup>1–3)</sup> all of which interfere with the endocrine systems of humans and wildlife,<sup>4–6)</sup> and cause adverse effects in organisms or their offspring.<sup>7–10)</sup> The present studies focused on the actions of BPA and related com-

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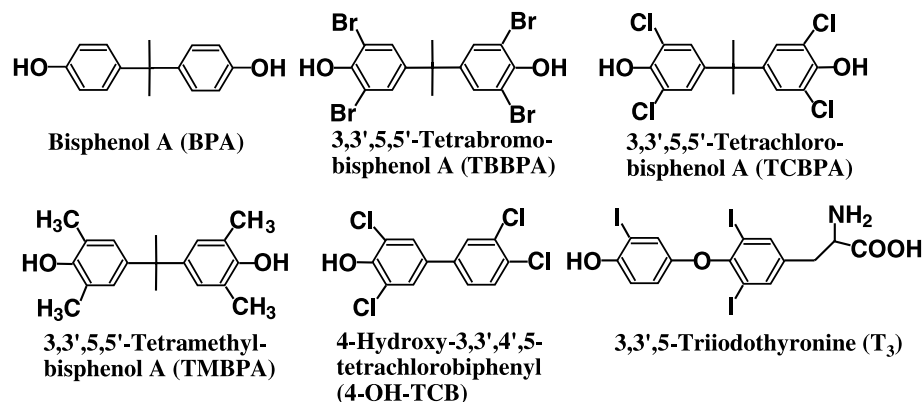


Fig. 1. Structures of BPA, TBBPA, TCBPA, TMBPA, 4-OH-TCB and T<sub>3</sub>

pounds (BPAs) (Fig. 1) on amphibian metamorphosis, as well as PCBs on mitochondrial membrane permeability transition (MPT) in an attempt to clarify the mechanisms by which these chemicals exert their effects.

## BPA AND RELATED COMPOUNDS

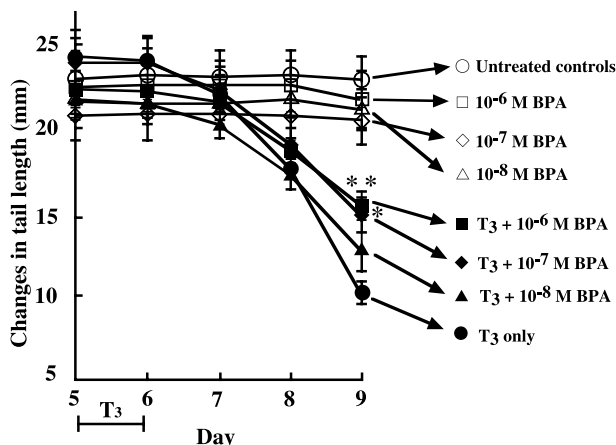
One of the most common EDCs, BPA is an industrial raw material for polycarbonate plastics and epoxy resins used in food containers, plastic baby bottles, dental fittings, *etc.*<sup>11–13</sup> BPA was reported to be found in the plasma and placenta of pregnant women, as well as in fetal plasma and amniotic fluid.<sup>14,15</sup> BPA is constantly released into the aquatic environment through various sources including the effluent from sewage treatment plants.<sup>16</sup> The brominated BPA analogue, tetrabromobisphenol A (TBBPA), is widely used as a flame retardant in building materials, paints, *etc.*<sup>17</sup> Thomsen *et al.*<sup>18</sup> reported that human serum concentrations of TBBPA and other brominated flame retardants increased from 0.44 ng/g lipids in 1977 to 3.3 ng/g lipids in 1999. For 0–4 year-old infants, however, the increase was 1.5 to 3.5 times as great. The chlorinated structural analogue tetrachlorobisphenol A (TCBPA) is found in the effluent from waste-paper recycling plants,<sup>19</sup> while the methylated structural analogue tetramethylbisphenol A (TMBPA) is used in polycarbonate resin.

A variety of effects have been reported for BPA and its analogues. BPA has been found to antagonize thyroid hormone (TH) action *in vitro* and *in vivo* assays.<sup>20–22</sup> TBBPA has been shown to be weakly estrogenic *in vitro*,<sup>2,23</sup> to bind TH recep-

tor (TR) *in vitro*,<sup>24,25</sup> and to antagonize the biological function of TH *in vivo*.<sup>22,25</sup> TBBPA inhibits larval development in *Xenopus laevis* (*X. laevis*).<sup>26</sup> Exposure to TBBPA, however, results in accelerated metamorphosis in Pacific tree frog *Pseudacris regilla* tadpoles, suggesting that this chemical acts as a TH agonist.<sup>27</sup> It is supposed that the effect of TBBPA on TH action will differ widely between species. Both TCBPA and TMBPA have been found to bind the TR.<sup>24,25</sup>

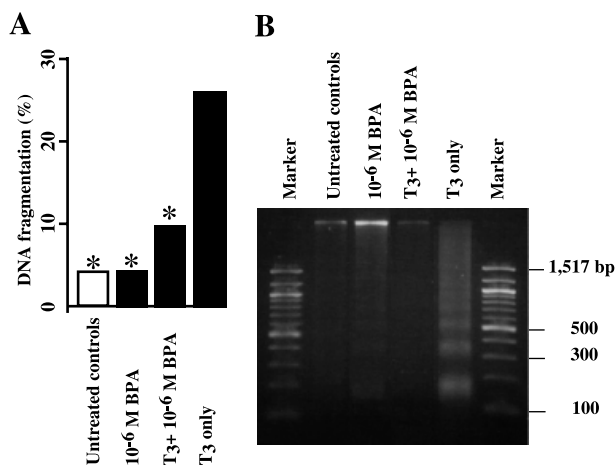
## EFFECTS OF BPA AND RELATED COMPOUNDS ON TH

THs, such as 3,3',5-triiodothyronine (T<sub>3</sub>) and thyroxine (T<sub>4</sub>), play important roles in the growth and development of organisms. Amphibian tadpole metamorphosis is a TH-triggered and controlled developmental process.<sup>28–31</sup> TH action is mediated by the interaction with TR. TR forms a heterodimer with a retinoid X receptor (RXR), thereby binding to the TH response element (TRE) in the regulatory region of target genes, followed by binding to T<sub>3</sub> to enhance or repress gene transcription.<sup>31</sup> Tadpole tail shortening during anuran metamorphosis is a dramatic change resulting from apoptosis, or programmed cell death,<sup>32</sup> and is triggered and controlled by TH.<sup>31</sup> In our investigation,<sup>22</sup> we found that T<sub>3</sub>-treated *Rana rugosa* (*R. rugosa*) tadpole tails display marked apoptotic features, including DNA fragmentation and ladder-like profiles (Figs. 2 and 3), as opposed to essentially little or no fragmentation or ladder formation for such tails further treated with BPA, TBBPA, TCBPA and TMBPA. Addition of THs to culture



**Fig. 2.** Effect of BPA on Tail Shortening in T<sub>3</sub>-Treated and Untreated *R. rugosa* Tadpoles<sup>22)</sup>

\*Significantly greater ( $p < 0.05$ ) than corresponding values for T<sub>3</sub>-treated tadpoles. \*\*Significantly greater ( $p < 0.01$ ) than corresponding values for T<sub>3</sub>-treated tadpoles.



**Fig. 3.** Effect of BPA on DNA Fragmentation and Ladder Formation in Tails of T<sub>3</sub>-Treated and Untreated *R. rugosa* Tadpoles<sup>22)</sup>

A, DNA fragmentation; B, Ladder formation. Each experiment was carried out using three tadpoles. Values given represent the mean value of three individuals. \*Significantly less ( $p < 0.01$ ) than T<sub>3</sub>-treated value.

medium (Cl-free water) of premetamorphic stage tadpoles results in an enhanced metamorphic process, and such TH-induced enhancement has proven to be a useful tool for investigating the effects of *N*-monomethyl-L-arginine (NMMA), *N*-tosyl-L-phenylalanyl chloromethyl ketone (TPCK), cyclosporin A (CsA) and other substances on the thyroid system and growth and development.<sup>33)</sup> We<sup>22)</sup> made use of TH-induced enhancement to examine the disruptive effects of BPAs on T<sub>3</sub> activity and tail apoptosis in three amphibian species.

Several *in vitro* and *in vivo* studies have reported the adverse effects of BPAs on TH action.<sup>20, 21, 34, 35)</sup> Using nuclear fraction of GH3 cells, Kitamura *et al.*<sup>24, 25)</sup> found that TBBPA, TCBPA and TMBPA markedly inhibit T<sub>3</sub> binding to TR. Recently Iwamuro *et al.*<sup>36)</sup> also reported that BPA acts as a T<sub>3</sub> antagonist through suppression of TR  $\alpha$  and TR  $\beta$  gene expression in *Xenopus* tail in culture.

Kitamura *et al.*<sup>25)</sup> reported that TBBPA and TCBPA exhibit significant anti-thyroid hormone effects on T<sub>3</sub> activity *in vitro*. They also showed that TBBPA suppresses *R. rugosa* tadpole tails undergoing regression during T<sub>3</sub> enhancement. Goto *et al.*<sup>22)</sup> reported that BPAs inhibit the regression of *R. rugosa* tadpole tail induced by exogenously added T<sub>3</sub>, as well as suppressing spontaneous *Silurana tropicalis* (*S. tropicalis*) metamorphosis in a dose-dependent manner, TMBPA showing the highest activity, followed by TCBPA, TBBPA and BPA respectively, suggesting that BPAs act as TH antagonists. As such, *in vivo* *S. tropicalis* and *R. rugosa* metamorphosis assays are useful in screening for chemicals with TH antagonist or agonist activity. Moreover they also examined the ability of BPAs to suppress TR-mediated gene expression using transgenic *X. laevis* tadpoles expressing enhanced green fluorescent protein (EGFP) driven by TR  $\beta$  A1 promoter region containing TRE. They found that T<sub>3</sub> induces a marked expression of EGFP gene expression in the hind limbs, while the addition of BPA, TBBPA, TCBPA or TMBPA inhibits this EGFP expression in a dose-dependent manner. It seems probable that by binding to TR, these chemicals compete with T<sub>3</sub>, resulting in suppression of TR  $\beta$ -mediated gene transcription. Such transgenic lines overexpressing TH enhanced gene sets have proven to be a useful bioassay for detecting chemical compounds possessing TH antagonism or agonism.

## PCB

Among the most widespread pollutants in the global environment, PCBs are highly lipophilic and stable, leading to their bioaccumulation in animals and humans.<sup>37, 38)</sup> Although PCBs have been found to affect various biological processes resulting in such adverse changes as cell toxicity and mutation in animals, the cellular mechanism remains poorly understood. Apoptosis is a genetically regulated mechanism for controlling cell number by elimi-

nating unwanted cells and plays a major role during normal developmental and homeostatic maintenance of multi-cellular organisms.<sup>32,39,40</sup> Apoptosis can be induced in various types of cells by exposing them to UV irradiation, cytotoxic drugs, growth factor deficiency, heat shock and viral infection and other such stimuli.<sup>41</sup>

Exposure to PCBs decreases viability and increases apoptosis in certain cells by disrupting mitochondrial function.<sup>42–47</sup> Several investigations have suggested that the release of cytochrome *c* (Cyt. *c*) from mitochondria into the cytosol through a MPT-pore plays an important role in the process of apoptosis,<sup>48–51</sup> and thus it is expected that MPT is also involved in PCB-induced apoptosis.<sup>52</sup> It has been well documented that MPT occurs when critical thiol groups of inner membrane proteins such as adenine nucleotide translocator (ANT) are oxidized to induce conformational changes that form large non-selective proteins,<sup>53</sup> further inducing the release of Cyt. *c* from mitochondria. We<sup>54</sup> investigated the effect of the PCB compound, 4-hydroxy-3,3',4',5-tetrachlorobiphenyl (4-OH-TCB) on isolated rat liver mitochondria to help clarify the effect of PCBs on MPT. We found that 4-OH-TCB enhanced the uncoupling oxidative phosphorylation, the generation of reactive oxygen species (ROS), Ca<sup>2+</sup> release, membrane depolarization, swelling and Cyt. *c* release, and that the presence of CsA inhibited this enhancing effect.

Ca<sup>2+</sup> induces a typical classic type of MPT in mitochondria, which occurs with the release of accumulated Ca<sup>2+</sup>, membrane depolarization, high sensitivity to CsA, *etc.*<sup>55,56</sup> In the presence of inorganic phosphate and respiratory substrate, Ca<sup>2+</sup> also stimulates the generation of ROS.<sup>53,57</sup> From the findings from previous investigations, the following causal sequence of changes might explain the Ca<sup>2+</sup>-induced MPT mechanism: structural change of inner membrane by binding of Ca<sup>2+</sup> to cardiolipin, modulation of electron transport system, generation of ROS, oxidation of protein thiol groups and membrane lipids in an MPT inhibitor CsA-sensitive mechanism.<sup>52,53,58</sup> These findings indicate that CsA might affect not only pore opening of ANT by binding to cyclophilin D, but also affect the initial steps of MPT, including Ca<sup>2+</sup> transport.<sup>52,59</sup>

In our investigation,<sup>54</sup> we observed that 4-OH-TCB disrupts mitochondrial functions as follows: in the presence of Pi and succinate, state 4 respiration increased with increasing concentration of 4-OH-TCB, while the respiratory control and oxida-

tive phosphorylation activity ratios decreased; CsA prevented the effect of 4-OH-TCB on respiration. These results indicate that 4-OH-TCB possibly generates ROS by modulating the electron transport chain in the inner membrane of the mitochondria.

According to Kanno *et al.*<sup>53</sup>) and Krumschnabel *et al.*,<sup>58</sup>) mitochondrial Ca<sup>2+</sup> loading triggers the release of Cyt. *c* from mitochondria through MPT, which induces the apoptosis cascade, ultimately leading to apoptosis. Our results therefore support the idea that a pathway involving MPT, Cyt. *c* release and caspase activation might be involved in the mechanism of PCB-induced apoptosis.<sup>44,60–62</sup> Since PCBs affect mitochondrial functions in different ways depending on their chemical structure, the exact mechanism of PCB-induced apoptosis is thought to differ according to cell types and PCB chemical structure.<sup>63</sup> Polychlorinated biphenyl mixtures (Aroclors) induce apoptosis *via* Bcl-2, Bax and caspase-3 proteins in neuronal cell cultures.<sup>43</sup> However, a recent study revealed that Bax does not directly participate in the Ca<sup>2+</sup>-induced MPT.<sup>57</sup> These results further support the idea that PCBs induce apoptosis by different mechanisms depending on cell types and PCB chemical structure. Previously we reported that T<sub>3</sub> induced MPT in isolated rat liver mitochondria,<sup>64</sup> and that CsA suppressed T<sub>3</sub>-induced tadpole tail shortening.<sup>65</sup> Furthermore, preliminary experiments have shown that 4-OH-TCB enhances T<sub>3</sub>-induced tadpole tail shortening in a concentration dependent manner, suggesting the agonistic action of 4-OH-TCB, and that CsA mitigates the enhancing effect of 4-OH-TCB. Together these results suggest that 4-OH-TCB enhances apoptosis in some cells through the process of classic type of MPT.

## CONCLUSION

The present paper is a survey of recent investigations into endocrine disruption and the mechanisms of action of BPAs and 4-OH-TCB. BPAs have been shown to suppress spontaneous and TH-induced tail apoptosis and inhibit TR-mediated gene expression. Further investigation is needed however to clarify whether 4-OH-TCB can act as a TR agonist *in vivo*. Kashiwagi *et al.*<sup>64,66</sup> Hanada *et al.*<sup>65,67</sup> and Inoue *et al.*<sup>68</sup>) have shown that MPT plays an important role in the mechanism of T<sub>3</sub>-induced tadpole tail shortening, and that tail muscle apoptosis is regulated by *Bax* gene in a CsA

sensitive mechanism. PCB-related toxic syndrome in humans and animals is characterized by severe loss of body mass, carcinogenicity, immunotoxicity, hepatotoxicity, neurotoxicity, reproductive toxicity, and endocrine disruption including thyroid toxicity.<sup>69–72</sup> In this context, we found that 4-OH-TCB induced a classic type of MPT in isolated rat liver mitochondria, suggesting that toxic syndrome produced by PCBs might be mediated by mitochondrial MPT, and that it is possible to use CsA for therapy of PCB-induced toxic syndrome. From our results we conclude that 4-OH-TCB induces classic MPT, thereby helping to clarify the involvement of mitochondrial MPT in the process of PCB-induced apoptosis. TBBPA has been found to cause cytotoxicity in isolated rat liver mitochondria as the result of dysfunction related to oxidative phosphorylation and lipid peroxidation.<sup>73</sup> Further studies are needed to clarify the precise mechanism by which BPAs and 4-OH-TCB induce MPT, especially how CsA suppresses the generation of ROS.

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