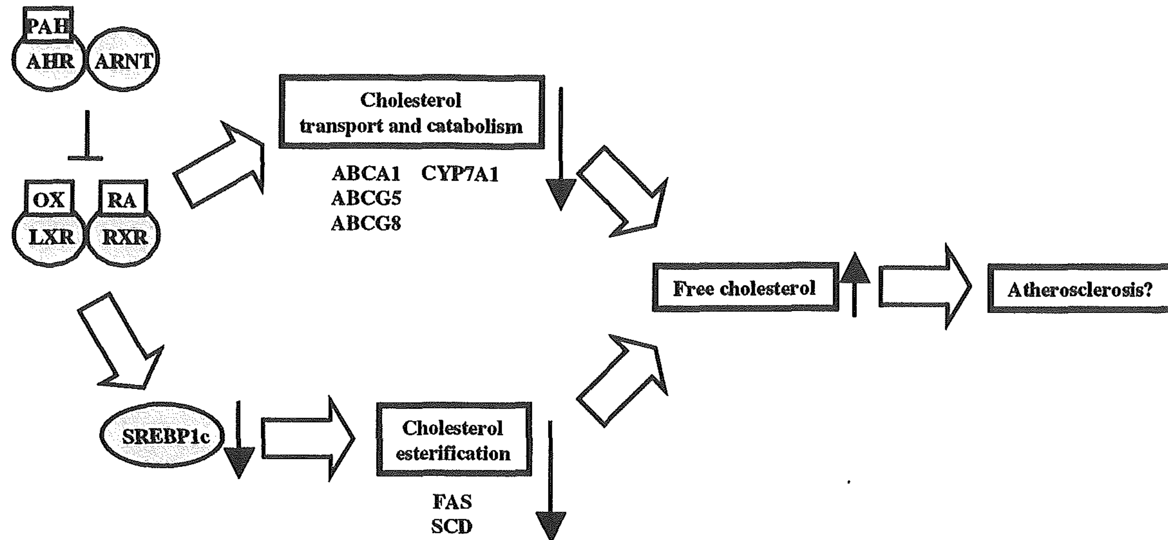

Fig. 5. Proposed molecular mechanism(s) for atherosclerosis induced by PAHs. OX, oxysterols; RA, retinoic acid.

Acknowledgments

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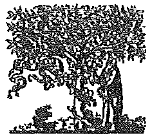

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Thyroid hormone-like and estrogenic activity of hydroxylated PCBs in cell culture

Shigeyuki Kitamura[a,*], Norimasa Jinno[a], Tomoharu Suzuki[a], Kazumi Sugihara[a], Shigeru Ohta[a], Hiroaki Kuroki[b], Nariaki Fujimoto[c]

[a] *Graduate School of Biomedical Sciences, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan*
[b] *Daiichi College of Pharmaceutical Sciences, Tamagawa 22-1, Minami-ku, Fukuoka 815-8511, Japan*
[c] *Research Institute for Radiation Biology and Medicine, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan*

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Abstract

The thyroid hormone-disrupting activity of hydroxylated PCBs was examined. 4-Hydroxy-2,2′,3,4′,5,5′-hexachlorobiphenyl (4-OH-2,2′,3,4′,5,5′-HxCB), 4-hydroxy-3,3′,4′,5-tetrachlorobiphenyl (4-OH-3,3′,4′,5-TCB) and 4,4′-dihydroxy-3,3′,5,5′-tetrachlorobiphenyl (4,4′-diOH-3,3′,5,5′-TCB), which have been detected as metabolites of PCBs in animals and humans, and six other 4-hydroxylated PCBs markedly inhibited the binding of triiodothyronine (1×10^{-10} M) to thyroid hormone receptor (TR) in the concentration range of 1×10^{-6} to 1×10^{-4} M. However, 4-hydroxy-2′,4′,6′-trichlorobiphenyl (4-OH-2′,4′,6′-TCB), 3-hydroxy-2,2′,5,5′-tetrachlorobiphenyl, 4-hydroxy-2,2′,5,5′-tetrachlorobiphenyl, 4-hydroxy-2,3,3′,4′-tetrachlorobiphenyl, 2,3′,5,5′-tetrachlorobiphenyl and 2,3′,4′,5,5′-pentachlorodiphenyl did not show affinity for TR. The thyroid hormonal activity of PCBs was also examined using rat pituitary cell line GH3 cells, which grow and release growth hormone in a thyroid hormone-dependent manner. 4-OH-2,2′,3,4′,5,5′-HxCB, 4,4′-diOH-3,3′,5,5′-TCB and 4-OH-3,3′,4′,5-TCB enhanced the proliferation of GH3 cells and stimulated their production of growth hormone in the concentration range of 1×10^{-7} to 1×10^{-4} M, while PCBs which had no affinity for thyroid hormone receptor were inactive. In contrast, only 4-OH-2′,4′,6′-TCB exhibited a significant estrogenic activity using estrogen-responsive reporter assay in MCF-7 cells. However, the 3,5-dichloro substitution of 4-hydroxylated PCBs markedly decreased the estrogenic activity. These results suggest that, at least for the 17 PCB congeners and hydroxylated metabolites tested, a 4-hydroxyl group in PCBs is essential for thyroid hormonal and estrogenic activities, and that 3,5-dichloro substitution favors thyroid hormonal activity, but not estrogenic activity.
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Keywords: Hydroxylated PCB; Thyroid hormonal activity; Thyroid hormone receptor; Estrogenic activity; Rat pituitary tumor cell line GH3; Human breast cancer cell-line MCF-7

1. Introduction

Polychlorinated biphenyls (PCBs) have been widely used throughout the world as industrial chemicals

* Corresponding author. Tel.: +81 82 257 5328; fax: +81 82 257 5329.
E-mail address: skitamu@hiroshima-u.ac.jp (S. Kitamura).

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for heat transfer and electrical insulation. However, they have contaminated almost every component of the global ecosystem, including wildlife and human adipose tissue, breast milk and serum, due to their lipophilic character (Safe, 1990, 1994). They have been reported to have a variety of toxic effects, including immunotoxicity, neurotoxicity, developmental toxicity, hepatotoxic effects, reproductive toxicity and carcinogenesis (Safe, 1984, 1990, 1994; Peterson et al., 1993; Seegal, 1996). They also induce biochemical responses, including induction of phases 1 and 2 drug-metabolizing enzymes (Safe, 1994; Byrne et al., 1987). Many of the PCB-induced toxic responses resemble those observed for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, which acts through the aryl hydrocarbon receptor (AhR) signaling pathway (Whitlock, 1993). These results are consistent with the identification of several PCB congeners, which bind to AhR and exhibit AhR agonistic actions (Safe, 1994). In recent years it has become evident that exposure to PCBs can also lead to thyroid hormone disturbances in wildlife and humans (Brouwer et al., 1998; Porterfield and Hendry, 1998). Decreased levels of circulating plasma thyroxin (T4) following PCB exposure have been shown in laboratory animals (Brouwer et al., 1998; Barter and Klaassen, 1994).

Many man-made chemicals, which have been widely released into the environment, are able to mimic or antagonize the biological activity of hormones and to disrupt the endocrine systems of humans and other animals (Colborn, 1995; Andersen et al., 1999). These chemicals, which include PCBs, are known as endocrine-disrupting chemicals. Interactions of estrogenic and anti-androgenic compounds with the respective hormone receptors have been demonstrated to account, among others, for endocrine-disrupting actions. PCBs are metabolized in vivo to hydroxylated PCBs, and these metabolites were identified in human serum, wildlife samples, and laboratory animals treated with PCBs (Jansson et al., 1975; Bergman et al., 1994; Morse et al., 1996; Darnerud et al., 1996; Sandau et al., 2000, 2002). Korach et al. (1988) reported that some hydroxy-PCBs such as 4,4 -dihydroxy-3,3 ,5,5 -tetrachlorobiphenyl competitively bind to the estrogen receptor (ER) and exhibit estrogenic activity in the mouse uterus. Other reports also show that hydroxylated PCBs are estrogenic or anti-estrogenic with respect to binding for ER (Moore et al., 1997; Ramamoorthy et al., 1997). Connor et al. (1997) demonstrated complex structure–estrogenicity/anti-estrogenicity relationships for hydroxylated PCBs.

It has also been reported that hydroxylated PCBs can disrupt thyroid hormone status. Hydroxylated PCBs showed high binding affinity for the serum thyroid hormone binding protein transthyretin, thereby displacing the natural ligand, T4 (Lans et al., 1993, 1994; Cheek et al., 1999). In vivo toxicity of hydroxylated PCBs on thyroid hormone homeostasis was also suggested on the basis of their high binding affinity to transthyretin (Darnerud et al., 1996; Meerts et al., 2002). However, the exact mechanisms of interference with thyroid hormonal action are not fully understood. Recently, we reported that tetrabromobisphenol A, a flame retardant, exhibits thyroid hormonal activity through its affinity to the thyroid hormone receptor (TR) (Kitamura et al., 2002). In the present report, we deal with the thyroid hormonal and anti-thyroid hormonal activities of fourteen hydroxylated PCBs and three non-hydroxylated PCBs as examined by means of binding assay with TR, as well as thyroid hormone-dependent growth assay and assay of production of growth hormone (GH) in pituitary cell line GH3 cells. The results were compared with the estrogenic activity observed in ERE-luciferase reporter assay. As substrates, we used 4-hydroxy-2,2 ,3,4 ,5,5 -hexachlorodiphenyl (4-OH-2 2 ,3,4 ,5,5 -HxCB), 4-hydroxy-3,3 ,4 ,5-tetrachlorobiphenyl (4-OH-3,3 ,4 ,5-TCB), 4,4 -dihydroxy-3,3 ,5, 5 -tetrachlorobiphenyl (4,4 -diOH-3,3 ,5,5 -TCB), 3-hydroxy-2,2 ,5,5 -tetrachlorobiphenyl (3-OH-2,2 ,5, 5 -TCB) and 4-hydroxy-2,2 ,5,5 -tetrachlorobiphenyl (4-OH-2,2 ,5,5 -TCB), which are metabolites of PCBs in animals and humans, as well as other hydroxylated PCBs (see Fig. 1).

2. Materials and methods

2.1. Chemicals

L-3,5,3 -Triiodothyronine (T3; 98%) and 17 -estradiol (E2; >98%) were obtained from Sigma Chemical Co. (St. Louis, MO). ^{125}I-T3 (3,5,3 -^{125}I, radiochemical purity >95%, 28.8 TBq/mmol) was purchased from NEN Life Science Products, Boston, MA. 4,4 -DiOH-3,3 ,5,5 -TCB, 3-OH-2,2 ,5,5 -TCB, 2-hydroxy-3,3 ,

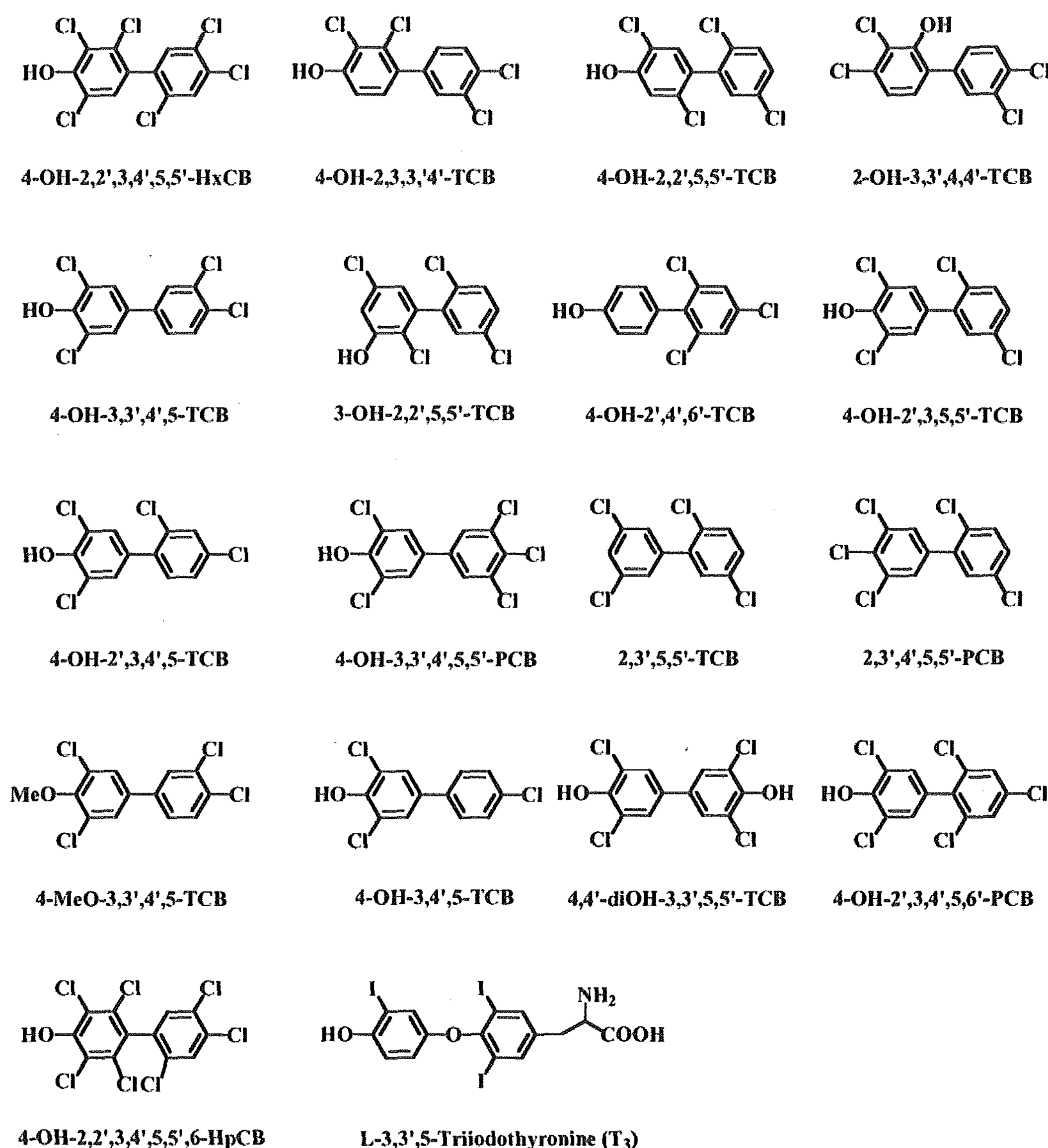

Fig. 1. Structures of PCBs and hydroxylated PCBs.

4 ,4 -tetrachlorobiphenyl (2-OH-3,3 ,4,4 -TCB), 4-methoxy-3,3 ,4 ,5-pentachlorobiphenyl (4-MeO-3,3 , 4 ,5-TCB) and eleven 4-hydroxylated PCBs, 4-OH-2, 2 ,3,4 ,5,5 -HCB, 4-OH-3,3 ,4 ,5-TCB, 4-OH-2,2 , 5,5 -TCB, 4-hydroxy-2 ,4 ,6 -trichlorobiphenyl (4-OH-2 ,4 ,6 -TCB), 4-hydroxy-3,4 ,5-trichlorobiphenyl (4-OH-3,4 ,5-TCB), 4-hydroxy-3,3 ,4 ,5,5 -pentachlorobiphenyl (4-OH-3,3 ,4 ,5,5 -PCB), 4-hydroxy-2 , 3, 5,5 -tetrachlorobiphenyl (4-OH-2 ,3,5,5 -TCB), 4-hydroxy-2,3 ,4 ,5,6 -pentachlorobiphenyl (4-OH-2 ,3,4 ,5,6 -PCB), 4-hydroxy-2,3,3 ,4 -tetrachlorobiphenyl (4-OH-2,3,3 ,4 -TCB), 4-hydroxy-2 ,3,4 ,5-tetrachlorobiphenyl (4-OH-2 ,3,4 ,5-TCB) and 4-hydroxy-2,2 ,3,4 ,5,5 ,6-heptachlorobiphenyl (4-OH-2,2 ,3,4 ,5,5 ,6-HpCB) were synthesized by the previously reported method (Bergman et al., 1994). 2,3 ,5,5 -Tetrachlorobiphenyl (2,3 ,5,5 -TCB) and 2,3 ,4 ,5,5 -pentachlorobiphenyl

(2,3 ,4 ,5,5 -PCB) were obtained from Accu Standard (New Haven, CT).

2.2. Cell culture

A rat pituitary cell line, GH3 and a human breast cancer cell line, MCF-7, were obtained from the Health Science Research Resources Bank (Osaka, Japan). The other rat pituitary cell line, MtT/E-2, was established in our laboratory (Fujimoto et al., 1999). GH3 and MtT/E-2 were maintained in DME/F12 mixed medium (Sigma Chemical Co.) containing penicillin and streptomycin with 15% horse serum (HS, Life Technologies) and 2.5% fetal bovine serum (FBS). Before cell growth assay and GH production assay, GH3 cells were maintained for 2–3 days in phenol-red free DEM/F12 (Sigma Chemical Co.) containing the same antibiotics along with dextran–charcoal treated HS and FBS. In the case of an estrogen-responsive human breast cancer cell-line MCF-7, cells were maintained in MEM (Sigma Chemical Co.) containing penicillin and streptomycin with 5% fetal bovine serum (Life Technologies, Rockville, MD).

2.3. Competitive binding assay to thyroid hormone receptor

Nuclear extracts of MtT/E-2 were used for the assay, since this cell line expresses high amounts of thyroid hormone receptor. The cells were homogenized in 0.32 M sucrose solution containing 3 mM $MgCl_2$ and 1 mM dithiothreitol and centrifuged at $700 \times g$ for 10 min. The pellets were resuspended in 2.4 M sucrose with $MgCl_2$ and centrifuged at $53{,}000 \times g$ for 45 min. The resulting nuclear pellets were resuspended in TMDS buffer (2 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 0.32 M sucrose, pH 7.4). Various concentrations of test chemicals and 3 nM ^{125}I-T3 were incubated in 0.2 ml of the nuclear suspension at 37 C for 40 min. After incubation, 0.25 ml of 2% Triton X-100 was added to terminate the reaction, and the mixture was centrifuged at $1000 \times g$ for 10 min. The pellets were washed twice with 1 ml of TMDS buffer and the supernatant was removed. Radioactivity of the pellets was counted with a gamma counter (Amersham Pharmacia Biotech, Uppsala, Sweden). IC_{50} values were calculated by fitting data to the logistic equation.

2.4. GH production assay in GH3 cells

The assay was performed as reported previously (Kitamura et al., 2002). Briefly, GH3 cells were seeded in 24-well plates at 1×10^4 cells/well and chemicals dissolved in 10 1 of ethanol were added the next day. Two days later, growth hormone in the culture medium was measured by radioimmunoassay with NIADDK reagents following the recommended protocol. The amount of growth hormone release at the maximum response (T3, 1×10^{-8} M) was 96.0 9.7 ng/10^5 cells/day.

2.5. Thyroid hormone-dependent growth assay in GH3 cells

The cells were seeded in 24-well plates at 1×10^4 cells/well, and chemicals were added on the following day. One week later, cell growth was measured with a modified MTT assay kit, which employs a newly developed tetrazolium salt, WST-1 (Dojindo Chemicals, Kumamoto, Japan). The details were previously reported (Kitamura et al., 2002).

2.6. Assay of estrogenic activity of hydroxylated PCBs

ERE-luciferase reporter assay using MCF-7 cells was performed according to the previously reported method (Kitamura et al., 2003). Briefly, transient transfections in MCF-7 cells were performed with Transfast^{TM} (Promega Co., Madison, WI), using the manufacturer's protocol. Transfections were done in 12-well plates at 1×10^5 cells/well with 1.9 g of p(ERE)$_3$-SV40-luc and 0.1 g of pRL/CMV (Promega Co.) as an internal standard. Twenty-four hours after addition of the sample (final concentration, 10^{-4} to 10^{-9} M), the assay was performed with a Dual Luciferase assay kit^{TM} (Promega Co.).

2.7. Statistics

Results are expressed as the mean S.D. Multiple comparison was made by ANOVA followed by Scheffe's test.

3. Results

3.1. *Competitive binding assay for thyroid hormone-like compounds*

The inhibitory effects of PCBs on binding of T3 to TR were examined using nuclear fraction of MtT/E-2 cells. T3 competitively inhibited the binding of ^{125}I-T3 (1×10^{-10} M) to TR in the range of 1×10^{-9} to 1×10^{-8} M. 4-OH-2,2 ,3,4 ,5,5 -HxCB, 4-OH-3,3 ,4 ,5-TCB and 4,4 -diOH-3,3 ,5,5 -TCB, which have been detected as metabolites of PCBs in humans and other animals, also markedly inhibited the binding of ^{125}I-T3 to the receptor in the concentration range of 1×10^{-5} to 1×10^{-4} M (Fig. 2A and B). Furthermore, some 4-hydroxylated PCBs, 4-OH-2 ,3,5,5 -TCB, 4-OH-3,3 ,4 ,5,5 -PCB, 4-OH-2,2 ,3,4 ,5,5 ,6-HpCB, 4-OH-3,4 ,5-TCB, 4-OH-2 ,3,4 ,5,6 -PCB and 4-OH-2 ,3,4 ,5-TCB, examined in this study showed binding affinity to TR (Fig. 2A–C). 2,3 ,5,5 -TCB, 2,3 ,4 ,5,5 -PCB, 4-OH-2 ,4 ,6 -TCB, 2-OH-3,3 ,4,4 -TCB, 3-OH-2,2 ,5,5 -TCB, 4-OH-2,3,3 ,4 -TCB and 4-OH-2,2 ,5,5 -TCB showed little or no affinity (data not shown). As evidenced by the IC_{50} values for TR binding of PCBs (Table 1), elevated binding affinity was observed for 4-hydroxylated PCBs with chlorine substitution at both the 3- and 5-positions of the phenyl group, including 4,4 -diOH-3,3 ,5,5 -TCB. Among these compounds, 4-OH-2,2 ,3,4 ,5,5 ,6-HpCB exhibited the greatest activity, followed by 4-OH-2,3,4 ,5,6 -PCB, 4-OH-3,3 ,4,5,5 -PCB, 4-OH-3,3 ,4 ,5-TCB. 4-Hydroxy-PCBs chlorinated at the 3- or 5-position, but not both, showed little activity. PCBs with a hydroxyl group at the 3- or 2-position of the phenyl ring, and 4-methoxy-3,3 ,4 ,5-TCB also showed little affinity.

Table 1
IC_{50} values of PCBs for binding to thyroid hormone receptor

Compound	IC_{50} (M)
T3	0.0033
4-OH-2,2 ,3,4 ,5,5 ,6-HpCB	4.6
4-OH-2 ,3,4 ,5,6 -PCB	9.1
4-OH-3,3 ,4 ,5,5 -PCB	9.3
4-OH-3,3 ,4 ,5-TCB	9.9
4-OH-2 ,3,4 ,5-TCB	16
4-OH-3,4 ,5-TCB	22
4-OH-2,2 ,3,4 ,5,5 -HxCB	34
4,4 -diOH-3,3 ,5,5 -TCB	42
4-OH-2 ,3,5,5 -TCB	53
2,3 ,5,5 -TCB	>1000
2,3 ,4 ,5,5 -PCB	>1000
4-OH-2,2 ,5,5 -TCB	>1000
3-OH-2,2 ,5,5 -TCB	>1000
4-OH-2,3,3 ,4 -TCB	>1000
2-OH-3,3 ,4,4 -TCB	>1000
4-OH-2 ,4 ,6 -TCB	>1000
4-Methoxy-3,3 ,4 ,5-TCB	>1000

3.2. *Thyroid hormonal activity of hydroxylated PCBs evaluated by assay of GH production by GH3 cells*

The thyroid hormonal activities of hydroxylated PCBs were examined by measuring the ability of these compounds to induce the production of GH by GH3 cells using 4-hydroxylated PCBs detected as metabolites in humans and other animals. GH release activity was observed with T3 in the range of 1×10^{-10} to 1×10^{-8} M. When GH release from GH3 cells was measured after the addition of 4-OH-2,2 ,3,4 ,5,5 -HxCB and 4-OH-3,3 ,4 ,5-TCB, an increase was observed in the concentration range of 1×10^{-6} to 1×10^{-5} M (Fig. 3A). Positive result was also observed in 4,4 -diOH-3,3 ,5,5 -TCB at 1×10^{-5} M (Fig. 3B). However, 2,3 ,5,5 -TCB, 3-OH-2,2 ,5,5 -TCB and 4-OH-2,2 ,5,5 -TCB showed no significant effect (Fig. 3A and B). The inhibitory effects of PCBs on the hormonal activity of T3 on GH3 cells were examined. These compounds at 1×10^{-5} and 1×10^{-4} M did not show antagonistic action towards GH production induced by 1×10^{-10} and 1×10^{-9} M T3 (data not shown). These results suggest that some hydroxylated PCBs tested in this study act as thyroid hormone agonists, but not antagonists.

3.3. *Thyroid hormonal activity of hydroxylated PCBs evaluated by growth assay of GH3 cells*

The thyroid hormonal activity of hydroxylated PCBs was also examined by assay of thyroid hormone-dependent growth of GH3 cells. The growth-inducing effect of T3 on the cells was observed over the concentration range of 1×10^{-10} to 1×10^{-8} M. When 4-OH-2,2 ,3,4 ,5,5 -HxCB, 4-OH-3,3 ,4 ,5-TCB and 4,4 -diOH-3,3 ,5,5 -TCB were added to the cells, growth was also stimulated at 1×10^{-6} to 1×10^{-5} M. 4-OH-

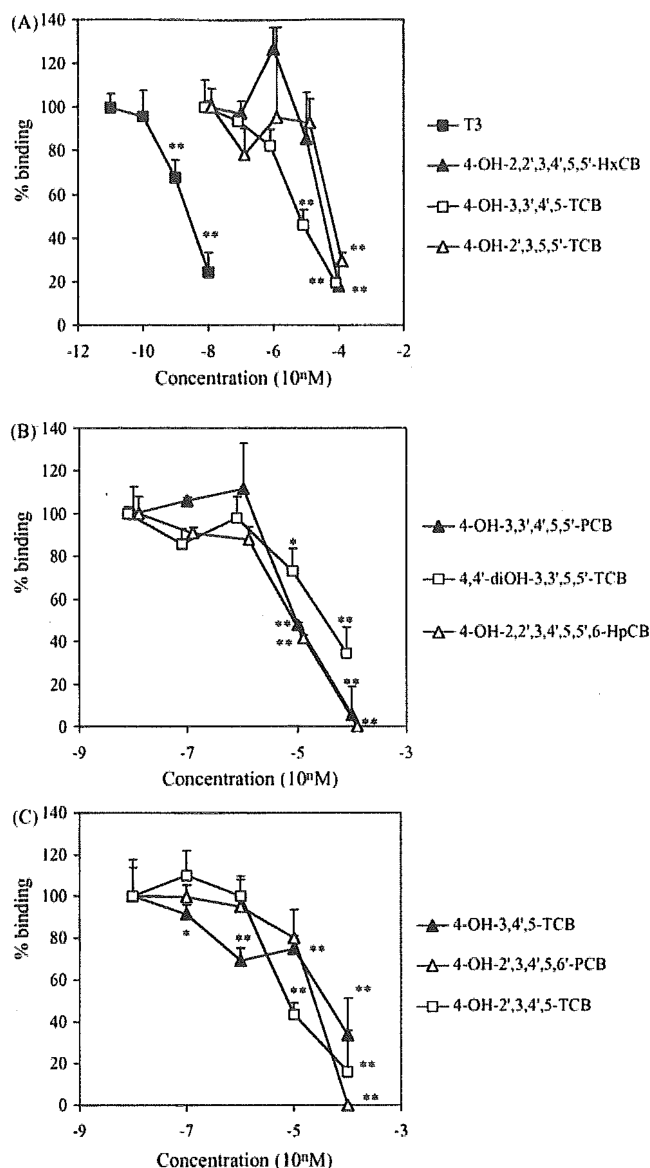

Fig. 2. Binding assay of thyroid hormonal compounds to thyroid hormone receptor. The nine positive hydroxylated PCBs are divided into three groups (A–C) simply for clarity. Each value represents the mean S.D. of four experiments. Activity is expressed relative to the control with no added test compound. T3; L-3,5,3 -triiodothyronine. $^{*}p<0.05$, $^{**}p<0.01$ compared with control.

2,2 ,3,4 ,5,5 -HxCB showed the highest activity, followed by 4-OH-3,3 ,4 ,5-TCB and 4,4 -diOH-3,3 ,5,5 -TCB (Fig. 4). However, 2,3 ,5,5 -TCB, 3-OH-2,2 ,5,5 -TCB and 4-OH-2,2 ,5,5 -TCB showed no significant effect. No cytotoxic effect of these positive hydroxylated PCBs towards GH3 cells was observed in this concentration range. These compounds at concentrations of 1×10^{-5} and 1×10^{-4} M did not inhibit the induction of GH3 cell growth by 1×10^{-10} and 1×10^{-9} M T3 (data not shown). These results confirmed that the hydroxylated PCBs examined in this assay, 4-OH-2,2 ,3,4 ,5,5 -HxCB, 4-OH-3,3 ,4 ,5-TCB

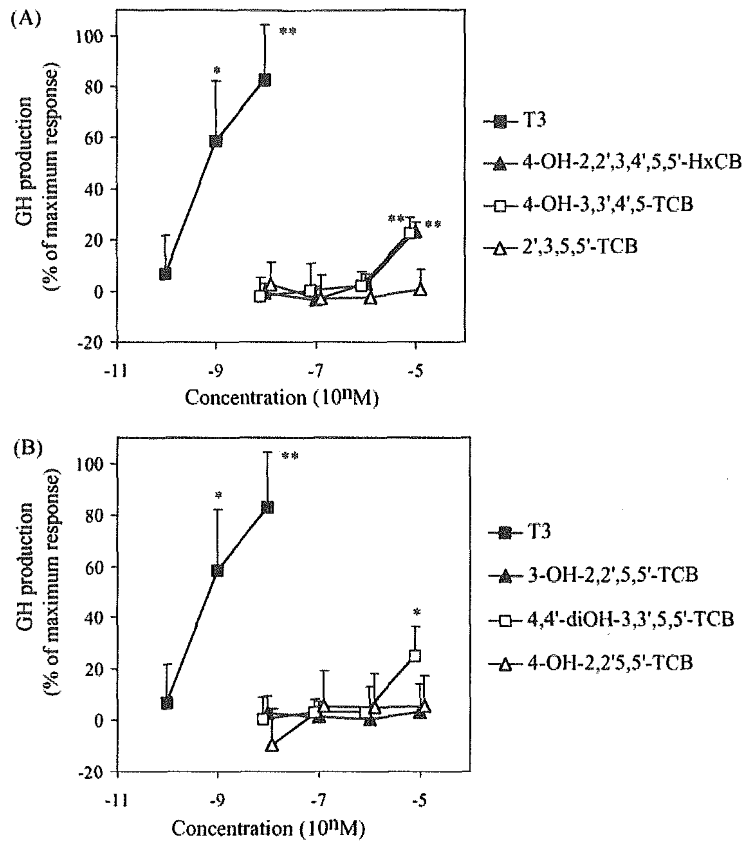

Fig. 3. Growth hormone (GH) release from GH3 cells by hydroxylated PCBs. Three positive compounds and three negative compounds are shown in two panels simply for clarity. Each bar represents the mean S.D. of four experiments. Activity is expressed relative to the control untreated GH3 cells. The amount of growth hormone release at the maximum response (T3, 1×10^{-8} M) was 96.0 9.7 ng/10^5 cells/day. T3; L-3,5,3 -triiodothyronine. $^*p < 0.05$, $^{**}p < 0.01$ compared with control.

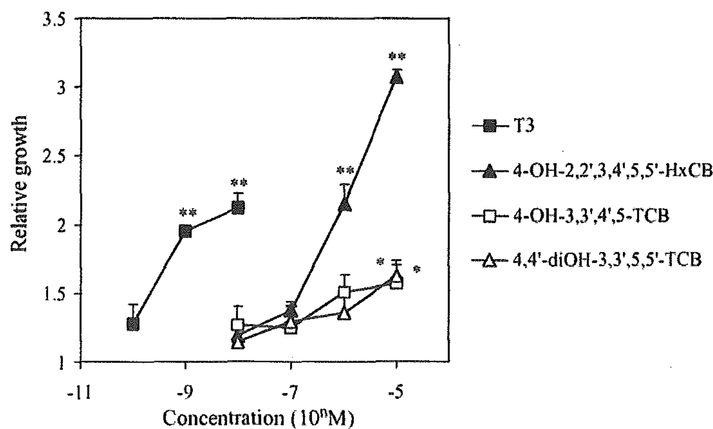

Fig. 4. Growth of GH3 cells by hydroxylated PCBs. Each bar represents the mean S.D. of four experiments. Activity is expressed relative to the control untreated GH3 cells. T3; L-3,5,3 -triiodothyronine. $^*p < 0.05$, $^{**}p < 0.01$ compared with control.

and 4,4 -diOH-3,3 ,5,5 -TCB, act as thyroid hormone-disruptors by exhibiting agonistic activity.

3.4. Estrogenic activity of hydroxylated PCBs

The estrogenic activities of hydroxylated PCBs were examined using ERE-luciferase reporter assay in MCF-7 cells. When 4-OH-2 ,4 ,6 -TCB was added to the cells in the concentration range of 1×10^{-7} to 1×10^{-5} M, estrogenic activity was apparent in the estrogen screening assay (Fig. 5A). 4-OH-2,3,3 ,4 -TCB, 2-OH-3,3 ,4,4 -TCB, 4-OH-2,2 ,5,5 -TCB, 4-OH-3,3 ,4 ,5-TCB and 3-OH-2,2 ,5,5 -TCB exhibited low activity in the concentration of 1×10^{-5} M. These hydroxylated PCBs were not cytotoxic to MCF-7 cells at concentrations below 1×10^{-5} M (Fig. 5A and B). However, no estrogenic activity of 4-OH-2,2 ,3,4 ,5,5 -HxCB, 4,4 -diOH-3,3 ,5,5 -TCB, 4-OH-2,2 ,3,4 ,5,5 -HxCB, 4-OH-3,3 ,4 ,5-TCB, 3-OH-2,2 ,5,5 -TCB, 4-OH-2,2 ,5,5 -TCB, 2,3 ,5,5 -TCB or 2,3 ,4 ,5,5 -PCB was observed (data not shown). These experiments indicate that a hydroxyl group of PCBs is essential for estrogenic activity, but 4-hydroxyl PCBs with an *ortho*-chlorine substituent show decreased estrogenic activity, though thyroid hormonal activity is unaffected.

4. Discussion

Hydroxylated PCBs have been detected in wildlife and humans as metabolites of PCBs (Jansson et al., 1975; Bergman et al., 1994; Sandau et al., 2000). Here, we present the first evidence that some hydroxylated PCBs, which have been detected as metabolites of PCBs, exhibit thyroid hormonal activity through interaction with TR. There are at least another three mechanisms through which environmental contaminants interact with the thyroid hormone system. These are: (1) direct toxicity at the thyroid gland, which can lead to decreased synthesis of thyroid hormones (Collins and Capen, 1980), (2) disturbance of thyroid hormone metabolism, such as glucuronidation, sulfation or deiodination (Van Birgelen et al., 1995; Brouwer et al., 1998), and (3) interaction with thyroid hormone transport proteins, leading to a reduced plasma T4 level

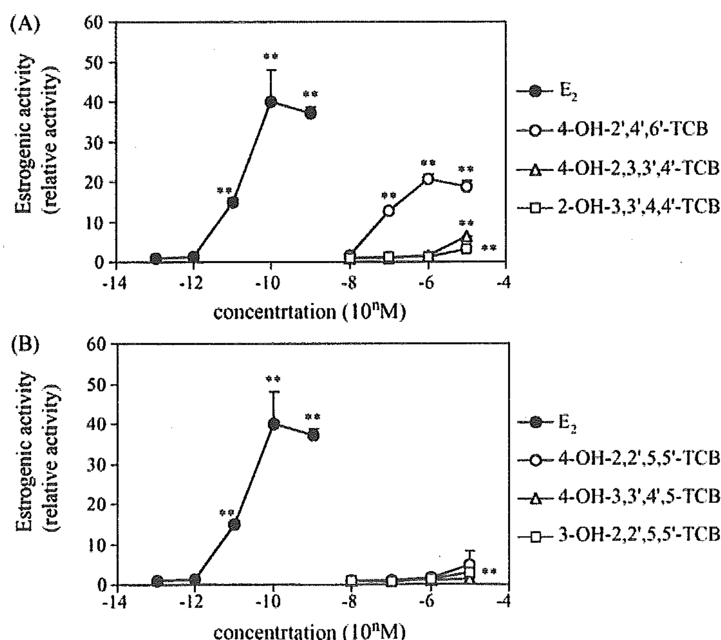

Fig. 5. Estrogenic activity of hydroxylated PCBs using estrogen-reporter assay of MCF-7 cells. Results for 6 hydroxylated PCBs are shown in two panels simply for clarity. Each value represents the mean S.D. of four experiments. Estrogenic activity is expressed as fold change of ERE-luciferase activity over the vehicle control. E2; 17 -estradiol. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with control.

(Lans et al., 1993, 1994; Morse et al., 1996). Various chemicals have been reported to bind to transthyretin, one of the thyroid hormone-binding transport proteins in plasma of vertebrate species. PCBs bind to human transthyretin and thyroid-binding globulin in vitro, competing with thyroid hormone binding (Cheek et al., 1999; Lans et al., 1994; Meerts et al., 2002). In the current study, it was shown that nine hydroxylated PCBs interact with thyroid hormonal receptor. These results suggest that hydroxylated PCBs have the potential to disrupt thyroid hormonal activity in vivo by interaction with TR, besides binding with transthyretin.

We demonstrated that of the molecules tested only hydroxylated PCBs, but not PCBs themselves, have agonistic activity for thyroid hormone using GH3 cells, which respond to thyroid hormone. In other words, hydroxylated PCBs are active metabolites of PCBs from the viewpoint of disrupting thyroid hormonal activity at the cellular level. The levels of these active metabolites in the body would be critical in determining whether these compounds actually show activity in vivo. Sandau et al. (2000, 2002) reported that the amount of total hydroxylated PCBs in normal human populations is of the order of 0.15–0.46 ng/g wet weight of blood, but in highly exposed populations, such as the Inuits, the concentration can reach 11.6 ng/g wet weight of blood at a maximum level. The average values for Inuit men and women are 1.73 and 1.01 ng/g blood, respectively. The concentration of hydroxylated PCBs in humans is thus two or three orders of magnitude less than the concentration shown to cause thyroid hormonal activity in this in vitro study. Therefore, these effects may not occur in the real world of humans and other animals world because of the PCB concentration. High levels of hydroxylated PCBs in marine mammals may be expected (Hoekstra et al., 2003). These compounds may be accumulated in the animal body, especially marine mammals, through the generations and by bioconcentration in the food chain. Hydroxylated PCBs may exhibit thyroid hormonal activity as mixtures of congeners or in combination with other environmental contaminants, such as TCDD. The apparent affinity of these xenobiotics for the thyroid hormone receptor is lower than that of endogenous thyroid hormones. Nevertheless, serum thyroid hormone levels are tightly regulated through the hypothalamus–pituitary thyroid axis in vivo, and this may not be the case for these chemicals, which may act directly on the endocrine organs. In contrast to the present results, it was reported that hydroxylated PCBs disrupt the central nervous system through antagonistic action against thyroid hormones (Iwasaki et al., 2002; Miyazaki et al., 2004). If hydroxylated PCBs are taken up into fetuses, these compounds may induce thyroid hormone-disrupting effects through this action in combination with the agonistic mechanism shown in the present study. Darnerud et al. (1996) also reported that hydroxylated PCBs bind with fetal transthyretin, and this is accompanied with a decrease in fetal plasma thyroxin levels. Further work is necessary to assess the in vivo endocrine-disrupting action of hydroxylated PCBs, taking into account the other thyroid hormone-disrupting actions of these compounds.

In this study, the potency of hydroxylated PCBs to compete for binding with TR was tested, in view of their structural resemblance to the thyroid hormones. Recently, thyroid hormone-disrupting action of some hydroxy-PCBs has been discussed. These hydroxy-PCBs were reported to have binding capability to TR, as well as the serum transport protein transthyretin (Cheek et al., 1999; Connor et al., 1997). Iwasaki et al. (2002) reported that a synthesized hydroxylated PCB, 4-hydroxy-2 ,3,3 ,4 ,5 -pentachlorobiphenyl, acts as an antagonist by suppression of the interaction of TR and a coactivator. In the current study using GH3 cells, we found thyroid hormone agonistic activities of hydroxylated PCBs that have been detected as metabolites in the body. A rat pituitary cell line, GH3, isolated from a rat pituitary tumor has been widely used as a standard pituitary cell model. Cell proliferation, as well as growth hormone secretion, has been shown to depend on thyroid hormones, but only slightly on estrogen (Perrone et al., 1980; Kitagawa et al., 1987). The reason for the difference between the agonistic and antagonistic actions of hydroxy-PCBs may be due to the different responses of the cells used.

We found that PCB congeners substituted with two chlorine atoms adjacent to the hydroxyl group on an aromatic ring show thyroid hormonal activity, though other PCBs do not. It is important to understand the structural requirements for thyroid hormonal activity in xenobiotics. A 4-hydroxyl group and adjacent 3,5-chlorine substituents on the phenyl group seem to be essential structural factors for binding to TR. In this respect, they apparently resem-

ble T4, rather than T3. However, the atomic size of chlorine is much smaller than that of iodine. In view of the affinity of hydroxy-PCBs for TR, 3,5-dichloro substituents may be favorable for binding to TR. We also recognized a similar structural requirement for TR affinity in a halogenated bisphenol A, tetrabromobisphenol A, a flame retardant (Kitamura et al., 2002). Another chlorinated phenyl ring substituted at the 1-position of the phenyl ring bearing the 4-hydroxyl group also seems to be necessary for thyroid hormonal activity, because 4-hydroxy-3,5-dichlorobenzene lacks the activity (data not shown). 2,2 ,6 or 6 -Chloro substitution on aromatic rings did not influence the activity. In other words, there is not a difference in thyroid hormonal activity between coplanar and non-coplanar PCBs. We also tested whether PCBs, including hydroxylated PCBs, exhibit estrogenic activity. We found that 4-OH-2 ,4 ,6 -TCB, a 4-hydroxy-PCB without 3,5-chlorine atoms, is estrogenic by means of an estrogen-responsive reporter test in a human breast cancer cell line MCF-7. However, 4-hydroxy-3,5-dichlorinated PCBs exhibited little estrogenic activity. These results are consistent with the estrogenic activity of hydroxylated PCBs reported elsewhere (Korach et al., 1988; Ramamoorthy et al., 1997). It is interesting that when 4-hydroxy-PCBs have 3,5-dichloro substitution, their estrogenic activity is markedly decreased, whereas their thyroid hormonal activity is not. The results suggest that hydroxylated PCBs exhibit endocrine-disrupting action via effects on at least two different hormonal activities in vivo. In conclusion, the structural requirements of hydroxylated PCBs for thyroid hormonal activity are a 4-hydroxyl group and 3,5-dichloro atoms substituted adjacent to the hydroxyl group. The requirement for estrogenic activity is a 4-hydroxyl group, but 3,5-dichloro substitution of a 4-hydroxy-PCB reduces the estrogenic activity.

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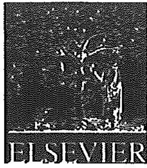

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Estrogenic and antiandrogenic activities of 17 benzophenone derivatives used as UV stabilizers and sunscreens

Tomoharu Suzuki[a], Shigeyuki Kitamura[a,*], Ryuki Khota[a], Kazumi Sugihara[a], Nariaki Fujimoto[b], Shigeru Ohta[a]

[a]*Graduate School of Biomedical Sciences, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima, 734-8551, Japan*
[b]*Research Institute for Radiation Biology and Medicine, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima, 734-8551, Japan*

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Abstract

Estrogenic and antiandrogenic activities of benzophenone and 16 of its derivatives, which are used as UV stabilizers, were comparatively examined with hormone-responsive reporter assay in various cell lines. Hydroxylated benzophenones exhibited estrogenic activity in human breast cancer cell line MCF-7, but their activities varied markedly. The highest activity was observed with 2,4,4′-trihydroxybenzophenone (2.4.4′-triOH-BP), followed by 2,3′,4,4′-tetrahydroxybenzophenone, 4,4′-dihydroxybenzophenone, 2,2′,4,4′-tetrahydroxybenzophenone, 4-hydroxybenzophenone and 2,4-dihydroxybenzophenone. Benzophenone itself showed little activity in the assay. In contrast, benzophenone and some related compounds showed significant inhibitory effects on the androgenic activity of dihydrotestosterone in rat fibroblast cell line NIH3T3. The highest activity was observed with 2,4,4′-triOH-BP, followed by 2,3′,4,4′-tetrahydroxybenzophenone, 2,2′,4,4′-tetrahydroxybenzophenone, 3-hydroxybenzophenone and 2,2′-dihydroxybenzophenone. However, 2,3,4,4′-tetrahydroxybenzophenone and 2,3,4-trihydroxybenzophenone showed little activity. 2,4-Dihydroxybenzophenone, 2,4,4′-triOH-BP and benzophenone gave positive responses in uterotrophic assay using ovariectomized rats, and 2,4,4′-triOH-BP was positive in the Hershberger assay using castrated rats. These results suggest that a 4-hydroxyl group on the phenyl ring of benzophenone derivatives is essential for high hormonal activities, and the presence of other hydroxyl groups markedly alters these activities.
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Keywords: Benzophenones; Estrogenic activity; Antiandrogenic activity; Human breast cancer cell line MCF-7; Rat cancer cell line NIH3T3

Introduction

Hydroxybenzophenones such as 2,4-dihydroxybenzophenone (2,4-di-OH-BP; benzophenone-1) and 2,2′,4,4′-tetrahydroxybenzophenone (2,2′,4,4′-tetra-OH-BP; benzophenone-2) have the ability to absorb and to dissipate ultraviolet (UV) light A (400–315 nm) (Klein, 1992). Consequently, they are used as UV stabilizers in plastic surface coatings on food packaging to prevent polymer degradation and loss of quality of the packed food owing to UV light irradiation. These compounds may be transferred from the packaging to the contents, and subsequently ingested by humans. However, they are natural components of plants such as mango and muscat grape, and are also used as flavorings. It has been reported that some derivatives have cytotoxic effects (Matsumoto et al., 2003). Hydroxybenzo-

Abbreviations: (BP), Benzophenone; (2-OH-BP), 2-hydroxybenzophenone; (3-OH-BP), 3-hydroxybenzophenone; (4-OH-BP), 4-hydroxybenzophenone; (2,2′-diOH-BP), 2,2′-dihydroxybenzophenone; (2,4-diOH-BP), 2,4-dihydroxybenzophenone; (4,4′-diOH-BP), 4,4′-dihydroxybenzophenone; (2,3,4-triOH-BP), 2,3,4-trihydroxybenzophenone; (2,4,4′-triOH-BP), 2,4,4′-trihydroxybenzophenone; (2,2′,4,4′-tetraOH-BP), 2,2′,4,4′-tetrahydroxybenzophenone; (2,3,4,4′-tetraOH-BP), 2,3,4,4′-tetrahydroxybenzophenone; (2,3′,4,4′-tetraOH-BP), 2,3′,4,4′-tetrahydroxybenzophenone; (2-OH-4-MeO-BP), 2-hydroxy-4-methoxybenzophenone; (2,2′-diOH-4,4′-diMeO-BP), 2,2′-dihydroxy-4,4′-dimethoxybenzophenone; (4-OH-2′,4′-diMeO-BP), 4-hydroxy-2′,4′-dimethoxybenzophenone; (2-OH-4-MeO-BP-5-sulfonic acid), 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid; (2,2′-diOH-4-MeO-BP), 2,2′-dihydroxy-4-methoxybenzophenone; (DHT), dihydrotestosterone; (E2), 17β-estradiol.

* Corresponding author. Fax: +81 82 257 5329.
E-mail address: skitamu@hiroshima-u.ac.jp (S. Kitamura).

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phenones and their derivatives are also used as sunscreens for humans. These compounds are reported to be absorbed through human skin, and bioaccumulation may occur in wildlife and humans (Felix et al., 1998; Hagedorn-Leweke and Lippold, 1995; Hayden et al., 1997; Jiang et al., 1999). In spite of some toxic effects of benzophenone, such as hepatotoxicity, hydroxylated benzophenones are safe for topical application to humans. Acute and subchronic systemic toxicity of these compounds after dermal application is considered to be rather low (Burdock et al., 1991; Dutta et al., 1993; Okereke et al., 1995). However, some problems have arisen with photoallergic reactions in patients with suspected clinical photosensitivity (Schauder and Ippen, 1997).

Endocrine-active chemicals arise from many different sources, including pesticides, industrial chemicals, pharmaceuticals, and phytochemicals. These chemicals are widely distributed in the environment and are able to mimic the biological activities of hormones. Chlorinated insecticides, such as kepone, *o,p′*-DDT, dieldrin and methoxychlor, and compounds used in the plastics and detergent industries, such as alkylphenols and bisphenol A, are known to have estrogenic activity (Andersen et al., 1999; Gaido et al., 1997; Soto et al., 1994). *p,p′*-DDE, a metabolite of p,p'-DDT, vinclozolin, an antifungal agent, and fenitrothion and fenthion, insecticides, are known to have anti-androgenic activity (Chen et al., 1997; Gray et al., 1999; Kelce et al., 1995; Kitamura et al., 2003; Kupfer and Bulger, 1987; Tamura et al., 2001). Interactions of estrogenic and anti-androgenic compounds with the respective hormone receptors have been demonstrated to account for the endocrine-disrupting actions of the compounds. These chemicals can alter reproductive development in mammals. Some hydroxylated benzophenones also show estrogenic activity in the yeast two hybrid system with estrogen receptor and coactivators (Kawamura et al., 2003). It was reported that 2-hydroxy-4-methoxybenzophenone (2-OH-4-MeO-BP) and 4-hydroxybenzophenone (4-OH-BP) are weakly positive in the uterotrophic assay using immature rats (Nakagawa and Tayama, 2001; Schlumpf et al., 2001). Androgenic and anti-androgenic properties of hydroxylated benzophenones are also an important problem. Yamasaki et al. (2003) reported that some hydroxybenzophenones do not act as antiandrogens in the Hershberger assay. Satoh et al. (2001) showed that benzophenone has no affinity for androgen receptor. Ma et al. (2003) reported that 2-OH-4-MeO-BP is a weak antagonist for androgen receptor. However, the activities in reporter assays have not been fully examined, and the relationship between the structure and activity of benzophenone derivatives is not yet understood.

In this report, endocrine-disrupting activity, that is, estrogenic, androgenic, and anti-androgenic activities, of hydroxybenzophenones and related compounds was examined using hormone-responsive reporter assays in human breast cancer cell-line MCF-7 for estrogenic activity and rat fibroblast cell line NIH3T3 for androgenic activity. Benzophenone derivatives tested in this study are listed in Fig. 1. We found that hydroxybenzophenones and some related compounds exhibit estrogenic and anti-androgenic activities. The structure–activity relationship of hydroxylated benzophenones is discussed.

Materials and methods

Chemicals. Benzophenone (BP), 2-hydroxybenzophenone (2-OH-BP), 3-hydroxybenzophenone (3-OH-BP), 4-OH-BP, 2,2′-dihydroxybenzophenone (2,2′-diOH-BP), 2,4-diOH-BP, 4,4′-dihydroxybenzophenone (4,4′-diOH-BP), 2,3,4-trihydroxybenzophenone (2,3,4-triOH-BP), 2,4,4′-trihydroxybenzophenone (2,4,4′-triOH-BP), 2,2′,4,4′-tetraOH-BP, 2,3,4,4′-tetrahydroxybenzophenone (2,3,4,4′-tetraOH-BP), 2,3′,4,4′-tetrahydroxybenzophenone (2,3′,4,4′-tetraOH-BP), 2-OH-4-MeO-BP, 2,2′-dihydroxy-4,4′-dimethoxybenzophenone (2,2′-diOH-4,4′-diMeO-BP), 4-hydroxy-2,′4′-dimethoxybenzophenone (4-OH-2′,4′-diMeO-BP), 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid (2-OH-4-MeO-BP-5-sulfonic acid), 2,2′-dihydroxy-4-methoxybenzophenone (2,2′-diOH-4-MeO-BP), 4-hydroxyacetophenone, 5α-dihydrotestosterone (DHT), and flutamide were obtained from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan), 17β-estradiol (E2) was from Sigma (St. Louis, MO).

Animals. Rats were housed at 22 °C and a relative humidity of 55% with a 12-h light/dark cycle, with free access to tap water and a standard pellet diet MM-3 (Funabashi Farm, Funabashi, Japan). All experiments were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" of Hiroshima University.

Cell culture. NIH3T3 and MCF-7 cells were maintained in DMEM (Sigma) plus 5% calf serum (Gibco/Invitrogen Corp., Carlsbad, CA) and DMEM plus 5% fetal bovine serum (Gibco/Invitrogen), respectively, containing penicillin and streptomycin. For assay, the medium was changed to phenol red-free DMEM (Sigma) containing the same antibiotics along with dextran-charcoal-treated calf serum or fetal bovine serum for a week.

Plasmids. The p(ARE)2-luc plasmid was constructed with pGL3-basic (Promega Co., Madison, WI) by inserting a synthetic double-strand oligo-DNA containing the consensus androgen-responsive element 5′-CATAGTACGT-GATGTTCTAGGCCTAGTACGTGATGTTCTCTATAC (with *SacI*/*XhoI* ends). For pSG5-hAR, human androgen receptor cDNA was amplified from human prostate cDNA by PCR with LA-Taq (Takara Bio Inc., Otsu, Japan) and introduced into pCR2.1 with a TA Cloning Kit (Invitrogen). The *Eco*RI fragment was then inserted into the *Eco*RI site in the pSG5 plasmid. The p(ERE)$_3$-SV40-luc plasmid, which contains three tandem copies of the

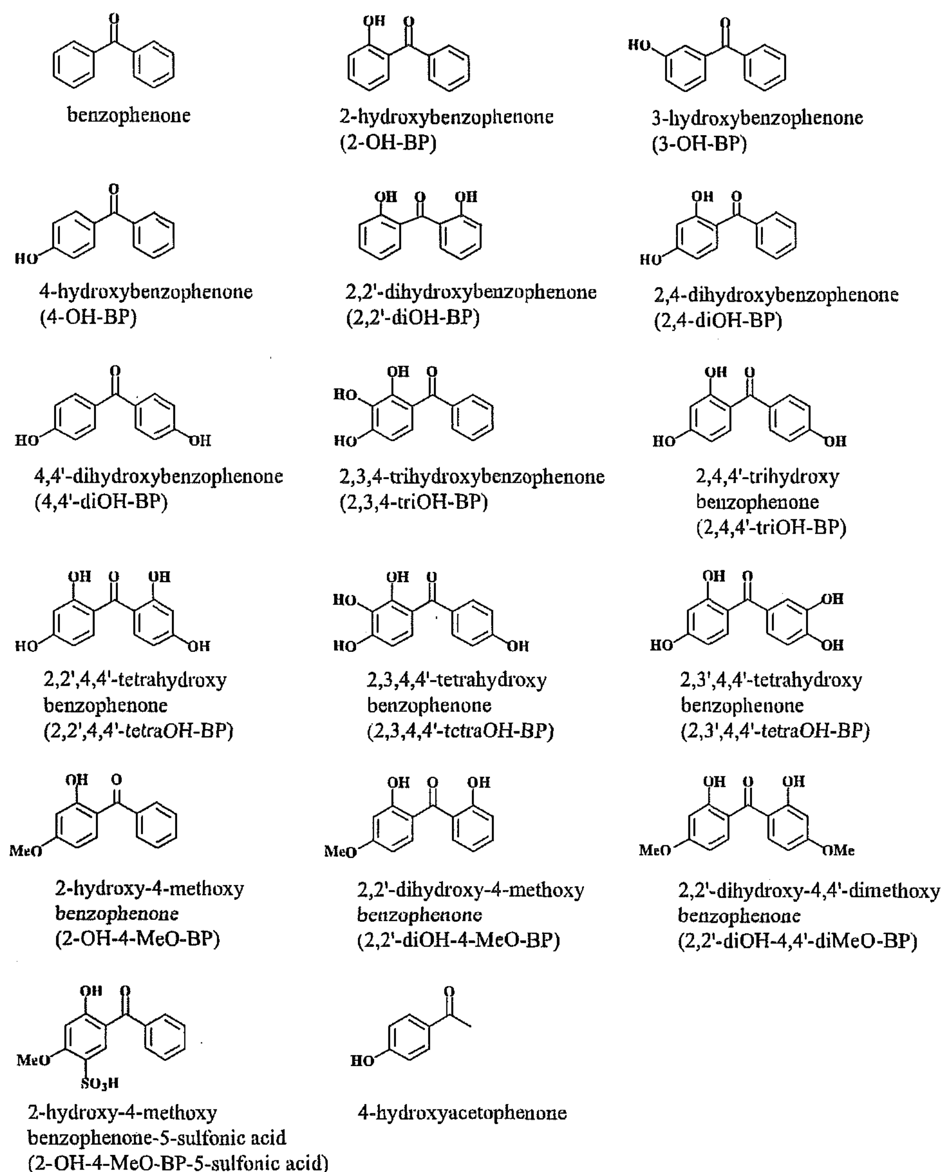


Fig. 1. Structures of benzophenone and related compounds tested in this study.

consensus estrogen-responsive element, was a gift from Dr. M. Kudoh, Yamanouchi Pharmaceutical Co., Tsukuba, Japan. PRL-CMV (Promega Co.) was used as the internal control.

Assay of estrogenic activity of benzophenone and related compounds. ERE-luciferase reporter assay using MCF-7 cells was performed according to the previously reported method (Sugihara et al., 2000). Briefly, transient transfections in MCF-7 cells were performed using Transfast (Promega Co.), according to the manufacturer's protocol. Transfections were done in 48-well plates at 2 10^{4} cells/well with 0.3 μg of $p(ERE)_3$-SV40-luc and 2 ng of phRL/CMV (Promega Co.) as an internal standard. Twenty-four hours after addition of the sample (final concentration, 10^{4} to 10^{8} M), the assay was performed with a Dual Luciferase assay kit™ (Promega Co.).

Assay of androgenic and antiandrogenic activities of benzophenone and related compounds. Assay of androgens was performed by ARE-luciferase reporter assay using NIH3T3 cells according to the previously reported method (Kitamura et al., 2003). Briefly, the culture medium was changed to phenol red-free DMEM (Sigma) containing penicillin, streptomycin, and dextran-charcoal-treated fetal bovine serum for 2–3 days. Transient transfections of NIH3T3 cells were performed using Transfast™ (Promega Co.), according to the manufacturer's protocol. Transfections were done in 48-well plates at 2 10^{4} cells/well with 0.3 μg of $p(ERE)_3$-SV40-luc and 2 ng of phRL/CMV (Promega Co.) as an internal standard. Twenty-four hours after addition of the sample (final concentration, 10^{5} to 10^{8} M) dissolved in 4 μl of ethanol, the assay was performed with a Dual Luciferase assay kit™ (Promega Co.) according to the manufacturer's protocol. For the assay of anti-

androgens, the inhibitory effect of benzophenone derivatives on the androgenic activity of DHT at the concentration of 1 10^{10} M was examined.

Assay of estrogenic activity in vivo (uterotrophic assay). F344 female rats obtained from Charles River Co. (Kanagawa, Japan) were used. They were surgically ovariectomized at 6 weeks old. At the age of 9 weeks, they were divided into eight groups each consisting of five animals. The rats were treated once a day for 3 days with intraperitoneal doses of 0.2 ml of vehicle (Panacete 810, Nippon Oils and Fats Co., Ltd., Tokyo, Japan), 17β-estradiol (50 μg/kg/day), 2,4,4′-triOH-BP, 2,4-diOH-BP, 2-OH-4-MeO-BP or BP (20–500 mg/kg/day). Animals were sacrificed under anesthesia and the uterus was dissected and weighed.

Assay of androgenic and antiandrogenic activities in vivo (Hershberger assay). F344 male rats were surgically castrated at 4 weeks old. At the age of 7 weeks, they were divided into five groups each consisting of 6 animals. The rats were treated once a day for 10 days with subcutaneous doses of 0.3 ml of vehicle (dimethyl sulfoxide), testosterone propionate (500 μg/kg/day), testosterone propionate plus flutamide (6 mg/kg/day) or testosterone plus 2,4,4′-triOH-BP (100 or 300 mg/kg/day). Animals were sacrificed under anesthesia and the prostate and seminal vesicles were dissected and weighed.

Results

Estrogenic activity of BP and related compounds

Estrogenic activity of benzophenone and its derivatives was examined using ERE-luciferase reporter assay in MCF-7 cells. Hydroxybenzophenones exhibited estrogenic activity in the estrogen screening assay, but their activities showed a wide range. Some tetra- and trihydroxybenzophenones showed significant estrogenic activity in the concentration range of 1 10^{7} – 1 10^{4} M. Di- and monohydroxybenzophenones showed estrogenic activity at 1 10^{6}– 1 10^{4} M. However, 2,3,4-trihydroxybenzophenones, methoxybenzophenones, and BP showed little activity. The active compounds were cytotoxic to MCF-7 cells at concentrations above 1 10^{5} M or 1 10^{4} M, so their apparent activities decreased above this concentration (Fig. 2). When ICI 182,780, a pure estrogen receptor antagonist, was added at the concentration of 1 10^{8} M, the estrogenic activities of the above compounds were markedly inhibited (data not shown). The EC_{50} values of the estrogenic activities are shown in Table 1. The highest activity was observed with 2,4,4′-triOH-BP, followed by 2,3′,4,4′-tetraOH-BP, 4,4′-diOH-BP, 2,2′,4,4′-tetraOH-BP, 4-OH-BP, 2,4-diOH-BP, 3-OH-BP, 2-OH-BP, BP, 2,3,4-triOH-BP, 2-OH-4-MeO-BP and 2,3,4,4′-tetraOH-BP. However, 4-hydroxyacetophenone, 2,2′-diOH-BP, 2-OH-4-MeO-BP-5-sulfonic acid, 2,2′-diOH-4-MeO-BP and 2,2′-diOH-4,4′-diMeO-BP were negative in the estrogen screening assay. Thus, 2,4,4′-trihydroxyl derivatives of benzophenone exhibited the highest activity. The activity was abolished by substitution with a methoxyl group. A 2,3,4-triol moiety on benzophenone markedly decreased the estrogenic activity. 4-Hydroxyacetophenone, lacking one phenyl ring of BP, showed little activity. Thus, hydroxyl group(s) on BP derivatives are essential for estrogenic activity. Both phenyl groups are also necessary.

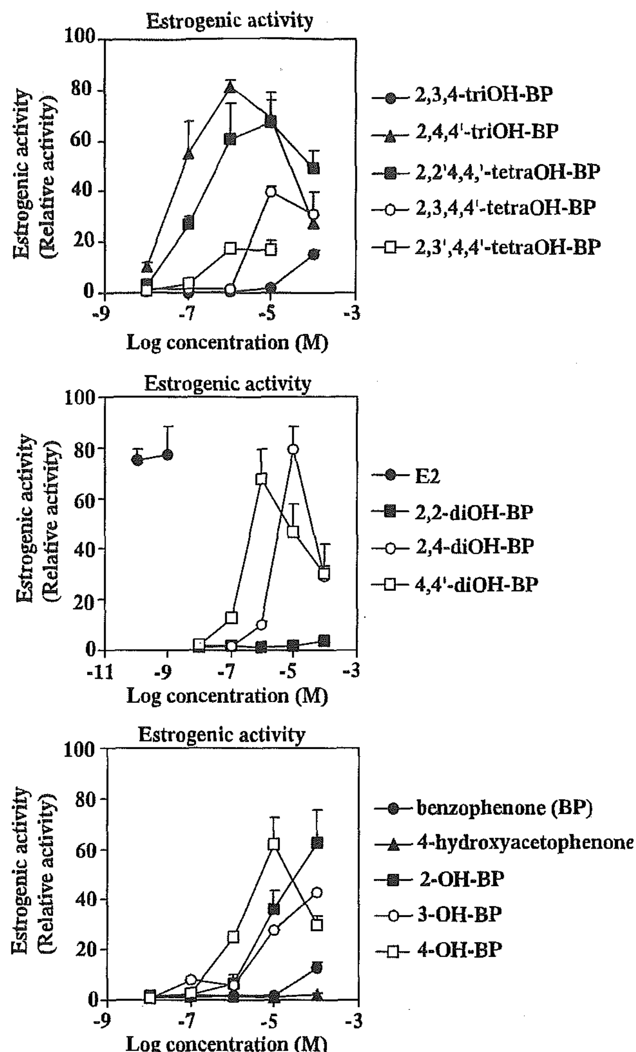
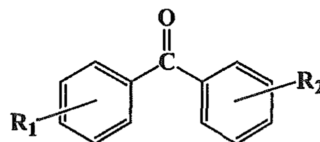


Fig. 2. Estrogenic activity of benzophenone (BP) and related compounds using ERE-luciferase reporter assay in MCF-7 cells. Each value represents the mean ± SD of four experiments. Estrogenic activity of benzophenones was expressed as a relative activity with respect to the control using MCF-7 cells. *$p < 0.05$, **$p < 0.01$ compared with control.

Androgenic and antiandrogenic activities of BP and related compounds

Androgenic activity of benzophenone and related compounds was examined using NIH3T3 cells transfected with

Table 1
Estrogenic activity (EC_{50}) and antiandrogenic activity (IC_{50}) of benzophenone derivatives



R1	R2	Estrogenic EC_{50} (μM)	Antiandrogenic IC_{50} (μM)
2,4-OH	4′-OH	0.10	0.30
2,4-OH	3′,4′-OH	0.14	0.97
4-OH	4′-OH	0.14	4.78
2,4-OH	2′,4′-OH	0.30	1.53
4-OH	H	0.72	5.01
2,4-OH	H	1.26	10.0
3-OH	H	1.54	3.02
2-OH	H	4.47	3.81
H	H	8.13	11.9
2,3,4-OH	H	11.8	>100
2-OH-4-MeO	H	19.5	>100
2,3,4-OH	4′-OH	31.3	26.5
4-hydroxyacetophenone		>100	>100
2-OH	2′-OH	>100	3.20
2-OH-4-MeO	2′-OH	>100	>100
2-OH-4-MeO	2′-OH-4′-MeO	>100	13.0
2-OH-4-MeO-5-SO_3H	H	>100	>100

a luciferase reporter gene. DHT exhibited marked androgenic activity toward NIH3T3 cells at 1 10 ^{11} – 1 10 ^{8} M. However, no androgenic activity of BP or the derivatives examined in this study was observed in the concentration range of 10 ^{7} to 10 ^{4} M (data not shown).

When BP and its derivatives were added to the DHT assay system in the concentration range of 1 10 ^{8} to 1 10 ^{5} M, the activity of 1 10 ^{10} M DHT was inhibited concentration-dependently (Fig. 3). Table 1 shows the IC_{50} values of these compounds against the androgenic activity of 1 10 ^{10} M DHT. The highest inhibitory effect among the test compounds was observed with 2,4,4′-triOH-BP, followed by 2,3′,4,4′-tetraOH-BP, 2,2′,4,4′-tetraOH-BP, 3-OH-BP, 2,2′-diOH-BP, 2-OH-BP, 4,4′-diOH-BP, 4-OH-BP, 2,4-diOH-BP, 2,3,4,4′-tetraOH-BP, BP and 2,2′-diOH-4,4′-diMeO-BP. However, 2,3,4-triOH-BP, 2,2′-diOH-4-MeO-BP, 2-OH-4-MeO-BP, 2-OH-4-MeO-BP-5-sulfonic acid and 4-hydroxyacetophenone showed little activity. These results suggest that some benzophenone derivatives have antiandrogenic as well as estrogenic activity, and hydroxyl groups are essential for this activity.

Estrogenic and antiandrogenic activities of BP derivatives in vivo

Estrogenic potential of hydroxylated benzophenones in vivo was further investigated in the uterotrophic assay using ovariectomized rats. Body weight of the rats administered 2,4,4′-triOH-BP, 2,4-diOH-BP, 2-OH-4-MeO-BP, or BP was not decreased compared with other groups. The uterine weight in the group dosed 2,4,4′-triOH-BP, 2,4-diOH-BP or BP was weakly increased compared with the group given vehicle only, but that in the 2-OH-4-MeO-BP group was not changed (Fig. 4A). Thus, the estrogenic effect of hydroxylated benzophenones was confirmed in vivo.

The in vivo antiandrogenic potential of 2,4,4′-triOH-BP, which showed the highest antiandrogenic activity in the in vitro reporter assay, was also investigated by the Hershberger assay using F344 rats (Hershberger et al., 1953). Body weight in the group administered 2,4,4′-triOH-BP (300 mg/kg/day) was decreased by 9% compared with the group given testosterone, while there were no significant differences among other groups. Testosterone treatment significantly increased the weights of the prostate and seminal vesicles, and these increases were completely blocked by flutamide administration. 2,4,4′-triOH-BP also significantly suppressed the effect of testosterone on the weight of both glands (Fig. 4B). Thus, the antiandrogenic effect of 2,4,4′-triOH-BP was confirmed in vivo.

Discussion

Hydroxylated benzophenones are used in sunscreens as skin-protecting agents and as UV stabilizers for plastic packaging. In this study, we examined the relationship between the structure of BP derivatives and their estrogenic and antiandrogenic activities. For estrogen receptor ligand activity, xenobiotics require an unhindered hydroxyl group on an aryl ring and a hydrophobic group attached *para* to the hydroxyl group (Blair et al., 2002; Fang et al., 2000;

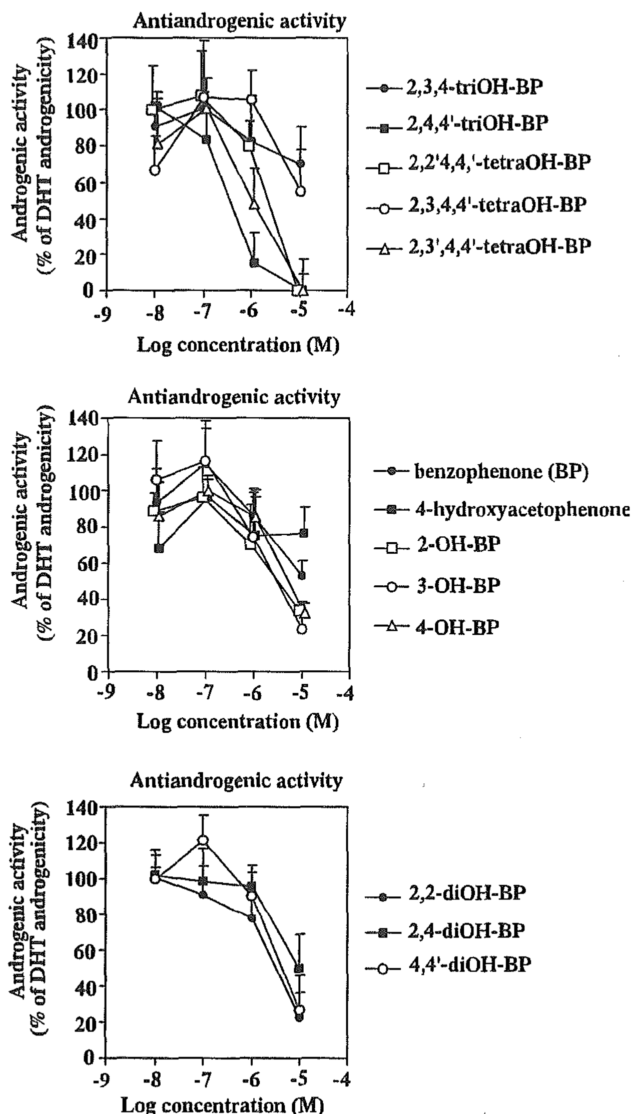


Fig. 3. Antiandrogenic activity of benzophenone (BP) and its derivatives against the androgenic activity of dihydrotestosterone (DHT) using ARE-luciferase reporter assay in NIH3T3 cells. Antiandrogenic activity of benzophenones was expressed as percent values of ARE-luciferase activity relative to the level induced by DHT (1 10^{10} M) using NIH3T3 cells. Each value represents the mean ± SD of four experiments. $*p < 0.05$ and $**p < 0.01$ indicate significant differences from control experiments in which only DHT was added.

Hong et al., 2002). The key structural requirement for estrogenic activity of BP derivatives is a phenolic hydroxyl group. The number and position of hydroxyl substituents markedly influences the activity. High activity was observed with tetra- and trihydroxybenzophenones. Benzophenones hydroxylated at the 2-, 4- and 4′-positions showed the highest activity. The 4-hydroxyl group is presumably necessary for binding with the estrogen receptor, like 3-OH in 17β-estradiol, and the 4′-hydroxyl group is also required as an H-bond donor. The 2-hydroxyl group forms a hydrogen bond with the carbonyl group, contributing to the hydrophobicity. Monohydroxybenzophenones exhibited activity, in the order of 4-hydroxyl>3-hydroxyl>2-hydroxyl. Schultz et al. (2000) also reported weak estrogenic activities of 4-OH-BP and 3-OH-BP, but detected no activity of 2-OH-BP. Benzophenones with three adjacent hydroxyl groups, such as 2,3,4,4′-tetraOH-BP and 2,3,4-triOH-BP, showed very low activity, presumably because of steric hindrance. Nakagawa and Suzuki (2002) reported that 2,4-diOH-BP and 2,3,4-triOH-BP showed estrogenic activity in proliferation assay using MCF-7 cells, and the activity of 2,3,4-triOH-BP was lower than that of 2,4-diOH-BP. Kawamura et al. (2003) reported that symmetrically hydroxylated benzophenones showed lower activity than asymmetrical derivatives in the yeast two-hybrid assay. This tendency was not observed in our study using reporter assay in MCF-7 cells.

Yamasaki et al. (2003) reported that some hydroxylated benzophenones did not show antiandrogenic activity in Hershberger assay. In this study, we found that 2,4,4′-triOH-BP was positive in the Hershberger assay. This positive result may have been due to subcutaneous dosing of 2,4,4′-triOH-BP; Yamasaki et al. used oral administration. Furthermore, we demonstrated that benzophenone and some of its derivatives are potent in vitro antiandrogens, though the activity varies markedly depending on the structure. The reasons for this are not clear, but hydroxyl groups may be a factor for the antiandrogenic activity, as in the case of estrogenic activity. Tri- and tetrahydroxyl substitution on the phenyl rings seems to increase the activity. Benzophenones substituted with hydroxyl groups at the 2,4,4′-positions showed the highest activity, as found for estrogenic activity. The order of activity is also the same, that is, 2,4,4′-triOH-BP>2,3′,4,4′-tetraOH-BP>2,2′,4,4′-tetra-BP. Benzophenones substituted with 2,3,4-trihydroxyl groups showed little inhibitory effect on the androgenic activity of DHT. Further study is needed to understand the relationship of structure to anti-androgenic activity of environmental chemicals, including BP derivatives.

The in vivo estrogenic activity of 2,4,4′-triOH-BP, 2,4-diOH-BP and BP was confirmed in uterotrophic assay, that is, the weight of the uterus in ovariectomized rats dosed with these BP derivatives was increased compared to that in rats dosed with vehicle alone. Yamasaki et al. (2003) also examined the estrogenic activities of some hydroxylated benzophenones in uterotrophic assay using immature rats. 2,2′,4,4′-TetraOH-BP and 2,4,4′-triOH-BP increased the weight of the uterus, while 4-OH-BP and 4,4′-diOH-BP showed weak activity. The activity of hydroxylated benzophenones may be decreased by reduction of the carbonyl moiety or conjugation with glucuronic acid or sulfuric acid at a hydroxyl group. However, Okereke et al. (1993) reported that 2-OH-4-MeO-BP is mainly converted to the desmethylated metabolite in rats in vivo. Thus, desmethylated metabolites of methoxybenzophenone derivatives, which are commonly used in sunscreens, may contribute to estrogenic activity. Metabolic activation of this group is possible, as observed with methoxychlor (Stresser and Kupfer, 1998). In contrast, Nakagawa and Tayama (2001)

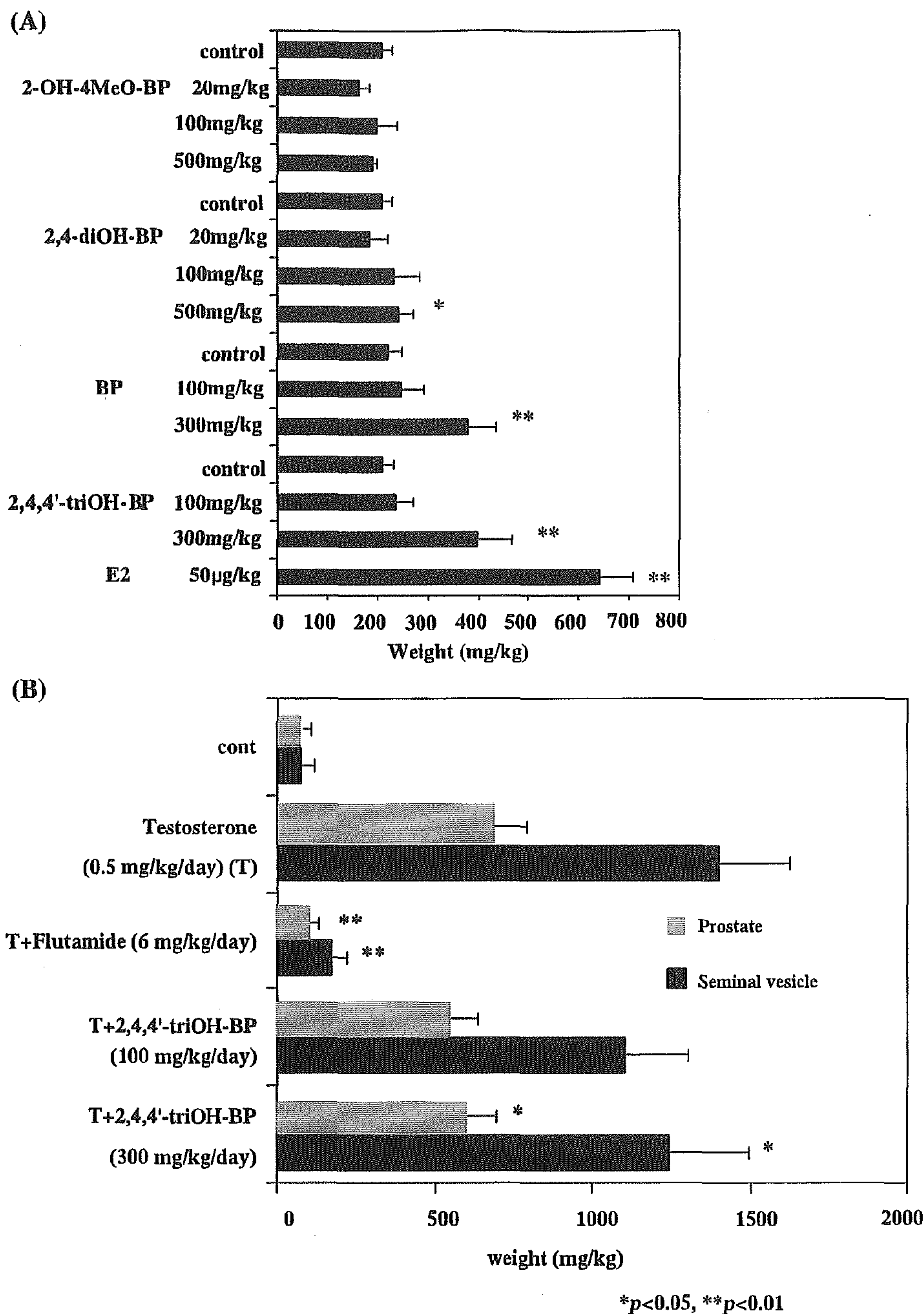


Fig. 4. Estrogenic and antiandrogenic activities of benzophenone derivatives in vivo. (A) The uterotrophic assay of benzophenone derivatives. Weights of the uteri of rats are shown. Ovariectomized female F344 rats were treated with 17β-estradiol (E2; 50 mg/kg/day), 2-OH-4-MeO-BP (20, 100, or 500 mg/kg/day), 2,4,4′-triOH-BP (100 or 300 mg/kg/day), benzophenone (100 or 300 mg/kg/day) or 2,4-diOH (20, 100, or 500 mg/kg/day) for a week. Each group consisted of six animals. (B) The Hershberger assay of benzophenone derivatives. Weights of the prostate gland and seminal vesicle of rats are shown. Castrated male F344 rats were treated with testosterone propionate (T; 500 mg/kg/day) and/or 2,4,4′-triOH-BP (100 or 300 mg/kg/day) or flutamide (6 mg/kg/day) for a week. Each group consisted of six animals. Each bar indicates mean ± SD of six rats. Asterisks indicate significant differences from testosterone-dosed rats at $*p < 0.05$ and $**p < 0.01$.

reported that 4-OH-BP, a metabolite of benzophenone, showed a positive result in uterotrophic assay using juvenile female rats, but benzophenone did not show such in vivo estrogenic activity. We observed estrogenic activity of benzophenone in vivo in this study. Benzophenone can be activated to the 4-hydroxyl derivative in the body, and so the estrogenic activity might have been due to the active metabolite. However, we did not find estrogenic activity of 2-OH-4-MeO-BP in vivo. However, Schlumpf et al. (2001) observed a weak activity of the compound in uterotrophic assay. Further study is necessary to evaluate the influence of metabolic modification of hydroxybenzophenones and methoxybenzophenones on the hormonal toxicity.