4. In wildlife and laboratory studies, BPA induces alteration in steroid biosynthesis/ metabolism/excretion.
5. Wildlife residing in sediment is likely exposed to higher levels of BPA.

4.2.3. Issue 3: Laboratory animal research—human exposure connection

1. Human exposure is likely to be continuous, unlike exposure in most laboratory animal studies of BPA pharmacokinetics.

4.2.4. Issue 4: Life stage—relationship to exposure pharmacokinetics and health effects

1. Clearance of BPA in the fetus is reduced compared to other life stages. Different effects and metabolic clearance mechanisms are also observed in neonatal and adult animals. Conjugation (glucuronidation) and other mechanisms of metabolic clearance of BPA thus vary throughout life.
2. Exposure to BPA during different life stages differentially influences reproductive cancer etiology and progression, and exposure during sensitive periods in organogenesis may increase susceptibility to development of cancers in some organs, such as the prostate and mammary glands.
3. Early life exposure to environmentally relevant BPA doses may result in persistent adverse effects in humans.
4. The function of the immune system can be altered following adult exposure to BPA.
5. Effects on insulin metabolism occur following adult exposure.

4.3. Areas of uncertainty and suggestions for future research

4.3.1. Issue 1: In vitro mechanistic research—laboratory animal research connection

1. Since BPA can act as an agonist or an antagonist in different tissues and against different background physiological states, the specific co-regulators that mediated these different responses of BPA need to be elucidated based on *in vitro* mechanistic studies, which should be confirmed *in vivo*.
2. Research is needed on specific receptor sub-types (i.e., classical nuclear and non-classical membrane-associated estrogen receptors) in relation to the potency of BPA in different tissues.
3. The identification of multiple estrogen receptor genes and variants as well as different co-regulators with different activities reveals that different levels of potency of BPA could be obtained by complex interactions between these different components that would not be predicted in homogeneous recombinant systems.

4.3.2. Issue 2: Wildlife—laboratory animal research connection

1. To directly relate the effects seen in wildlife with BPA exposure, biomonitoring data are needed from wildlife. In addition to BPA levels, these studies should assay total estrogenic and antiandrogenic activity from other contaminants.
2. There is a need to examine sensitive endpoints in wildlife that have been identified in laboratory animals.
3. There are substantial amounts of plastic debris within marine and fresh water ecosystems, and studies are needed to examine the impact of BPA in the environment on aquatic organisms. Doses used in laboratory experiments involving wildlife should reflect environmental exposures.
4. More studies need to be done with BPA in invertebrates, and a fundamental understanding of estrogen action in invertebrates is required.
5. Studies should determine if amplification of BPA through the food chain occurs, particularly under anaerobic or hypoxic conditions due to the lack of microbial or photodegradation.
6. Future research emphasis should be placed on populations of aquatic animals exposed to landfill leachate and sewage effluent, as these are the primary point sources for BPA exposure.

4.3.3. Issue 3: Laboratory animal research—human exposure connection

1. Even though there have been attempts to estimate daily human intake of BPA, these estimates require many assumptions. The best measures we have to estimate whether humans may be affected by current exposures to BPA are levels in blood (not exposure levels), which can be related to blood levels in experimental animals after acute exposures. Known sources of human exposure to BPA do not appear sufficient to explain levels measured in human tissues and fluids.
2. While BPA is not persistent in the environment or in humans, biomonitoring surveys indicate that exposure is continuous. This is problematic because acute animal exposure studies are used to estimate daily human exposure to BPA, and at this time, we are not aware of any studies that have examined BPA pharmacokinetics in animal models following continuous low level exposures. Measurement of BPA levels in serum and other body fluids suggests that either BPA intake is much higher than accounted for, or that BPA can bioaccumulate in some conditions such as pregnancy, or both. Research using both animal models, as well as epidemiology studies, are needed to address these hypotheses, and this research needs to better mimic the apparent continuous exposure of humans to BPA.
3. More comprehensive exposure and biomonitoring studies are needed, especially in developing countries.
4. In both animal and human studies, internal exposure measures need to be related to health effects. In particular, there is a need for epidemiological studies relating health outcomes to BPA exposure, particularly during sensitive periods in development. These studies should be based on hypotheses from findings in experimental animals. This will require additional development of appropriate biomarkers in animal studies that can be used in epidemiological research.

4.3.4. Issue 4: Life stage—relationship to exposure pharmacokinetics and health effects

1. While there is a great need to continue studying prenatal and perinatal exposures in laboratory animal studies, many organs and endpoints continue developing at later stages (throughout puberty and adolescence). Additional studies are needed during these later periods of development.
2. Additional research is needed regarding exposure to BPA in adulthood to determine whether post-exposure effects are temporary or are permanent and associated with subsequent age-related diseases.
3. Because aging adults lose repair mechanisms, metabolic enzymes, and imprinted genes, the possibility that adult exposures (long-term, low level) can increase the risk of cancers and other conditions during aging should be addressed with additional human research and the development of appropriate animal models.
4. Epigenetics should be examined as a potential mechanism mediating developmental effects as well as the trans-generational effects of BPA and other contaminants. Potential effects of adult exposures also need to be examined in relation to disruption of epigenetic changes that occur normally during aging.
5. Trans- and multi-generational effects of BPA must be examined in laboratory animals and humans.
6. There is a need for studies that involve collection of human blood and urine from humans at several life stages, with specific emphasis on infants and young children and continued monitoring throughout adulthood. Additionally, there is a need to characterize the basis for the variability in BPA levels in studies examining both human urine and serum.
7. There is a need for research on the genetic basis for differences in susceptibility to BPA and other contaminants.
8. Studies are needed on comparative BPA pharmacokinetics in invertebrates and vertebrates (non-human primates included).
9. There is a need to measure total endocrine disrupter load in humans and wildlife. Therefore, biomarkers of endocrine disrupter exposure are necessary.
10. There is a need for more research directed at examining human exposure, pharmacokinetics and health effects of selected BPA precursors (i.e., BADGE, BISGMA, and BISDMA) and metabolites (e.g., halogenated BPAs).
11. There is a need for more studies focused on identification of other (non-estrogen-receptor mediated) mechanisms of action of BPA.
12. Effects of chemicals on the immune system are life stage dependent, and identifying the life stage dependency for BPA effects on the immune system is necessary. In addition, studies examining BPA effects on the immune system in wildlife are necessary.

5. Conclusions

The published scientific literature on human and animal exposure to low doses of BPA in relation to *in vitro* mechanistic studies reveals that human exposure to BPA is within the range that is predicted to be biologically active in over 95% of people sampled. The wide range of adverse effects of low doses of BPA in laboratory animals exposed both during development and in adulthood is a great cause for concern with regard to the potential for similar adverse effects in humans. Recent trends in human diseases relate to adverse effects observed in experimental animals exposed to low doses of BPA. Specific examples include: the increase in prostate and breast cancer, uro-genital abnormalities in male babies, a decline in semen quality in men, early onset of puberty in girls, metabolic disorders including insulin resistant (type 2) diabetes and obesity, and neurobehavioral problems such as attention deficit hyperactivity disorder (ADHD).

There is extensive evidence that outcomes may not become apparent until long after BPA exposure during development has occurred. The issue of a very long latency for effects *in utero* to be observed is referred to as the developmental origins of adult health and disease (DOHaD) hypothesis. These developmental effects are irreversible and can occur due to low dose exposure during brief sensitive periods in development, even though no BPA may be detected when the damage or disease is expressed. However, this does not diminish our concern for adult exposure, where many adverse outcomes are observed while exposure is occurring. Concern regarding exposure throughout life is based on evidence that there is chronic, low level exposure of virtually everyone in developed countries to BPA. These findings indicate that acute studies in animals, particularly traditional toxicological studies that only involve the use of high doses of BPA, do not reflect the situation in humans.

The fact that very few epidemiological studies have been conducted to address the issue of the potential for BPA to impact human health is a concern, and more research is clearly needed. This also applies to wildlife, both aquatic and terrestrial. The formulation of hypotheses for the epidemiological and ecological studies can be greatly facilitated by the extensive evidence from laboratory animal studies, particularly when common mechanisms that could plausibly mediate the responses are known to be very similar in the laboratory animal models, wildlife and humans.

Acknowledgements

Meeting support was provided by NIEHS and NIDCR, NIH/DHHS, the US-EPA and Commonweal. We thank Paul French for assistance with the meeting in web site and Albert Kingman for advice during preparation of the manuscript. This manuscript does not reflect US-EPA, USGS or NIH agency policy. FvS is supported by NIH grant ES11283.

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8 June 2007
Available online 27 July 2007

0090-9556/07/3510-1949–1955$20.00
DRUG METABOLISM AND DISPOSITION
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DMD 35:1949–1955, 2007

Vol. 35, No. 10
17327/3255799
Printed in U.S.A.

A Novel Mechanism for Polychlorinated Biphenyl-Induced Decrease in Serum Thyroxine Level in Rats

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Received June 19, 2007; accepted July 12, 2007

ABSTRACT:

We have previously suggested that the decrease in the levels of serum total thyroxine (T_4) and free T_4 by a single administration to rats of Kanechlor-500 (KC500) at a dose of 100 mg/kg is not necessarily dependent on the increase in hepatic T_4-UDP-glucuronosyltransferase (UDP-GT). In the present study, we determined whether or not a consecutive treatment with KC500 at a relatively low dose (10 mg/kg i.p., once daily for 10 days) results in a decrease in the level of serum total T_4 and further investigated an exact mechanism for the KC500-induced decrease in the T_4. At 4 days after final treatment with KC500, the serum total T_4 and free T_4 levels were markedly decreased in both Wistar and UGT1A-deficient Wistar (Gunn) rats, whereas significant increases in hepatic T_4-UDP-GT activity were observed in Wistar rats but not in Gunn rats. The level of serum thyroid-stimulating hormone was not significantly changed in either Wistar or Gunn rats. Clearance from serum of the [^{125}I]T_4 administered to the KC500-pretreated Wistar and Gunn rats was faster than that to the corresponding control (KC500-untreated) rats. The accumulated level of [^{125}I]T_4 was increased in several tissues, especially the liver, in the KC500-pretreated rats. The present findings demonstrated that a consecutive treatment with KC500 resulted in a significant decrease in the level of serum total T_4 in both Wistar and Gunn rats and further indicated that the KC500-induced decrease would occur through increase in accumulation of T_4 in several tissues, especially the liver, rather than increase in hepatic T_4-UDP-GT activity.

Most polychlorinated biphenyls (PCBs) are known to decrease the level of serum thyroid hormone and to increase the activity of hepatic drug-metabolizing enzymes in rats (Van Birgelen et al., 1995; Craft et al., 2002). As possible mechanisms for the PCB-induced decrease in the level of serum thyroid hormone, enhancement of thyroid hormone metabolism by PCB and displacement of the hormone from serum transport proteins, including transthyretin (TTR), by PCB and its ring-hydroxylated metabolites are considered (Barter and Klaassen, 1992a, 1994; Brouwer et al., 1998). In particular, the decrease in the level of serum thyroxine (T_4) by 3,3′,4,4′,5-pentachlorobiphenyl, Aroclor 1254, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rats is believed to occur mainly through induction of the UDP-glucuronosyltransferases (UDP-GTs), especially UGT1A subfamily enzymes, responsible for glucuronidation of T_4 (Barter and Klaassen, 1994; Van Birgelen et al., 1995). However, the magnitude of decrease in the level of serum total T_4 is not necessarily correlated with that of increase in T_4-UDP-GT activity (Craft et al., 2002; Hood et al., 2003). Furthermore, we have reported that in Kanechlor-500 (KC500)-treated mice, serum T_4 level decreased without an increase in T_4-UDP-GT activity (Kato et al., 2003) and that the decrease in serum total T_4 level by a single administration of either KC500 or 2,2′,4,5,5′-pentachlorobiphenyl occurred even in UGT1A-deficient Wistar (Gunn) rats (Kato et al., 2004). Thus, an exact mechanism for the PCB-induced decrease in the level of serum thyroid hormone remains unclear. To date, most studies on biological effects of PCB have been performed using experimental animals treated once at a high dose (more than 100 mg/kg body weight), and the effect of the consecutive treatment at a low dose has been little reported. Humans and wild animals are exposed to a wide variety of environmental chemicals, including PCB, at a low level over a long period of time. Therefore, a study on biological effects by consecutive treatment with PCB at a low dose would be very important.

In the present study, therefore, we examined whether or not a consecutive treatment with KC500 at a relatively low dose (10 mg/kg i.p., once daily for 10 days) results in decrease in the level of serum total T_4 and further discussed a mechanism underlying the PCB-induced decrease in the T_4.

This work was supported in part by the Grant-in-Aid for Scientific Research (C) (no. 18510061; Y.K.) and for Scientific Research (B) (no. 19310042; K.H., Y.K.) from Japan Society for the Promotion of Science, and by a Health and Labour Sciences Research Grant for Research on Risk of Chemical Substances (H16-Kagaku-003; Y.K.) from the Ministry of Health, Labour and Welfare of Japan.

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.

doi:10.1124/dmd.107.017327.

ABBREVIATIONS: PCB, polychlorinated biphenyl; KC500, Kanechlor-500; T_3, triiodothyronine; T_4, thyroxine; TTR, transthyretin; TSH, thyroid-stimulating hormone; UDP-GT, UDP-glucuronosyltransferase.

Materials and Methods

Chemicals. Panacete 810 (medium-chain triglycerides) was purchased from Nippon Oils and Fats Co. Ltd. (Tokyo, Japan). The $[^{125}I]T_4$, radiolabeled at the 5′-position of the outer ring, was obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). The KC500 used in the present experiments contains 2,2′,5,5′-tetrachlorobiphenyl (5.6% of total PCBs), 2,2′,3,5′,6-pentachlorobiphenyl (6.5%), 2,2′,4,5,5′-pentachlorobiphenyl (10%), 2,3,3′,4′,6-pentachlorobiphenyl (7.4%), 2,3′,4,4′,5-pentachlorobiphenyl (7.7%), 2,2′,3,4,4′,5′-hexachlorobiphenyl (5.6%), and 2,2′,4,4′,5,5′-hexachlorobiphenyl (5.4%) as major PCB congeners (Haraguchi et al., 2005). All the other chemicals used herein were obtained commercially in appropriate grades of purity.

Animal Treatments. Male Wistar rats (160–200 g) and UGT1A-deficient Wistar rats (Gunn rats, 190–260 g) were obtained from Japan SLC., Inc. (Shizuoka, Japan). Male Wistar and Gunn rats were housed three or four per cage with free access to commercial chow and tap water, maintained on a 12-h dark/light cycle (8:00 AM to 8:00 PM light) in an air-controlled room (temperature, 24.5 ± 1°C; humidity, 55 ± 5%), and handled with human care under the guidelines of the University of Shizuoka (Shizuoka, Japan). Rats received consecutive intraperitoneal injections of KC500 (10 mg/kg) dissolved in Panacete 810 (5 ml/kg) at 24-h intervals for 10 days. Control animals were treated with vehicle alone (5 mg/kg).

In Vivo Study. Rats were killed by decapitation 4 days after the final administration of KC500. The liver was removed, and hepatic microsomes were prepared according to the method of Kato et al. (1995) and stored at −85°C until use. Blood was collected from each animal between 10:30 and 11:30 AM. After clotting at room temperature, serum was separated by centrifugation and stored at −50°C until use.

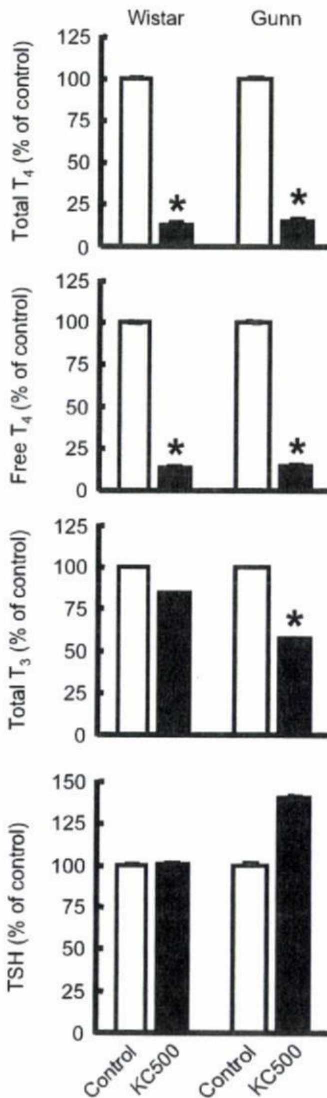

FIG. 1. Effects of KC500 on levels of serum total T_4, free T_4, total T_3, and TSH in Wistar and Gunn rats. Animals were killed 4 days after the final administration of KC500 (10 mg/kg i.p., once daily for 10 days), and levels of serum thyroid hormones were measured as described under *Materials and Methods*. Constitutive levels: total T_4, 4.29 ± 0.38 (Wistar, $n = 5$) and 5.80 ± 0.32 μg/dl (Gunn, $n = 5$); free T_4, 2.17 ± 0.16 (Wistar, $n = 5$) and 2.71 ± 0.17 ng/dl (Gunn, $n = 5$); total T_3, 0.34 ± 0.03 (Wistar, $n = 6$) and 0.96 ± 0.05 ng/ml (Gunn, $n = 4$); TSH, 4.89 ± 0.33 (Wistar, $n = 5$) and 7.48 ± 1.14 ng/ml (Gunn, $n = 5$). Each column represents the mean ± S.E. (vertical bars) for five to six animals. *, $P < 0.01$, significantly different from each control.

TABLE 1

Effects of KC500 on the activity of hepatic microsomal alkoxyresorufin O-dealkylases in Wistar and Gunn rats

Animals were killed at 4 days after the final administration of KC500 (10 mg/kg i.p., once daily for 10 days). The values shown are expressed as the mean ± S.E. for four to five animals.

Substrates	Wistar		Gunn	
	Control	KC500	Control	KC500
	nmol/mg protein/min		*nmol/mg protein/min*	
7-Benzyloxyresorufin	0.07 ± 0.01	3.34 ± 0.33*	0.03 ± 0.003	1.08 ± 0.27*
7-Pentoxyresorufin	0.03 ± 0.003	0.43 ± 0.05*	0.02 ± 0.003	0.22 ± 0.05*
7-Ethoxyresorufin	0.14 ± 0.01	9.02 ± 0.09*	0.21 ± 0.01	2.21 ± 0.29*

* $P < 0.05$, significantly different from each control.

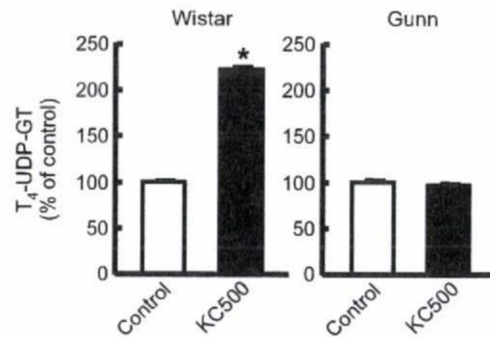

FIG. 2. Effects of KC500 on the activity of hepatic microsomal UDP-glucuronyltransferase in Wistar and Gunn rats. Each column represents the mean ± S.E. (vertical bars) for five to six animals. Constitutive levels: T_4-UDP-GT, 14.17 ± 1.11 pmol/mg protein/min (Wistar) and 6.36 ± 1.34 pmol/mg protein/min (Gunn). *, $P < 0.01$, significantly different from each control.

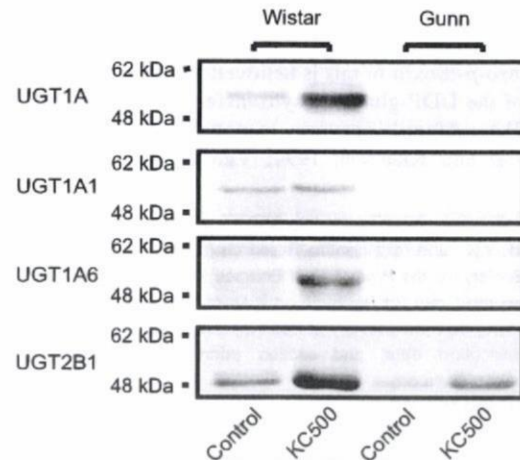

FIG. 3. Representive Western blot profiles for hepatic microsomal UGT isoforms in the KC500-treated Wistar and Gunn rats.

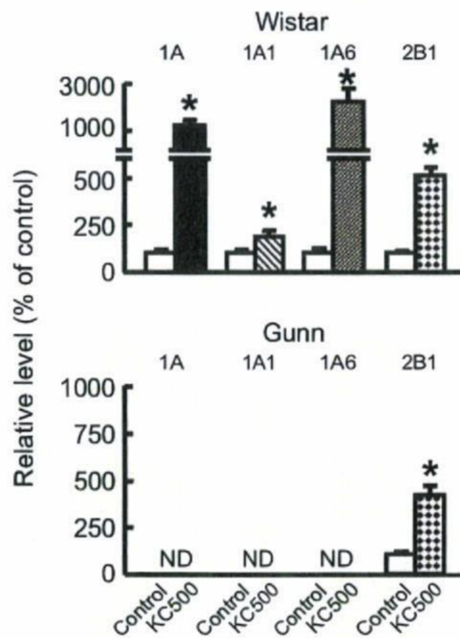

FIG. 4. Effects of KC500 on levels of hepatic microsomal UGT isoforms in Wistar and Gunn rats. The isolated bands responsible for UGT isoforms, which are shown in Fig. 3, were densitometrically quantified as described under *Materials and Methods*. The data are represented as the mean ± S.E. (vertical bars) for five to six animals. *, $P < 0.05$, significantly different from each control. ND, not detectable.

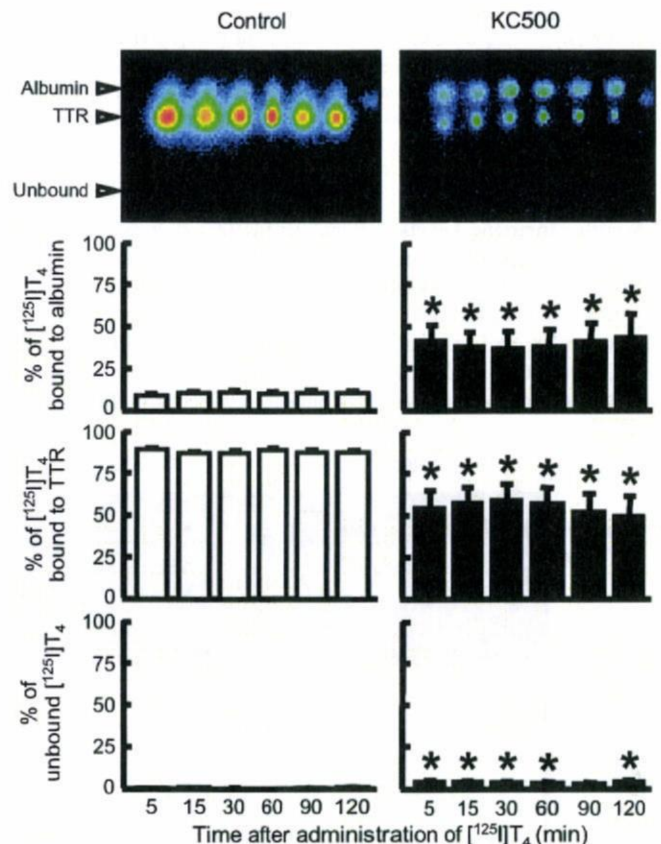

FIG. 5. Effects of KC500 on the binding of $[^{125}I]T_4$ to serum proteins in Wistar rats. Amounts of $[^{125}I]T_4$ bound to the serum proteins were assessed by the method described under *Materials and Methods*. Each column represents the mean ± S.E. (vertical bars) for three to six animals. *, $P < 0.05$, significantly different from each control.

Analysis of serum hormones. Levels of total T_4, free T_4, total triiodothyronine (T_3), and thyroid-stimulating hormone (TSH) were measured by radioimmunoassay using Total T4 and Free T4 kits (Diagnostic Products Corporation, Los Angels, CA), the Triiodothyronine kit GammaCoat T_3 II (DiaSorin Inc., Stillwater, MN), and the rTSH $[^{125}I]$ Biotrak assay system (GE Healthcare UK, Ltd., Little Chalfont, Buckinghamshire, UK), respectively.

Hepatic microsomal enzyme assays. Hepatic microsomal fraction was prepared according to the method described previously (Kato et al., 1995), and the amount of hepatic microsomal protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Microsomal *O*-dealkylase activities of 7-benzyloxy-, 7-ethoxy-, and 7-pentoxyresorufins were determined by the method of Burke et al. (1985).

Hepatic T_4-metabolizing enzyme assay. The activity of microsomal UDP-GT toward T_4 (T_4-UGT activity) was determined by the methods of Barter and Klaassen (1992b).

Western blot analysis. The polyclonal anti-peptide antibodies against the common region of UGT1A isoforms and specific antibodies against UGT1A1, UGT1A6, and UGT2B1, which were established by Ikushiro et al. (1995, 1997), were used. Western blot analyses for microsomal UGT isoforms were performed by the method of Luquita et al. (2001). The bands corresponding to UGT1A1, UGT1A6, and UGT2B1 on a sheet were detected using chemical luminescence (ECL detection kit; GE Healthcare UK, Ltd.), and the level of each protein was determined densitometrically with LAS-1000 (Fuji Photo Film. Co., Ltd., Tokyo, Japan).

Ex Vivo Study. At 4 days after a consecutive 10-day treatment with KC500, the rats were anesthetized with a saline (2 ml/kg) containing sodium pentobarbital (25 mg/ml) and potassium iodide (1 mg/ml). The femoral artery was cannulated (polyethylene tube SP31; Natsume Inc., Tokyo, Japan) and primed with heparinized saline (33 units/ml), and then the animal's body was warmed to 37°C. Fifteen minutes later, the rats were given i.v. 1 ml of $[^{125}I]T_4$ (15 μCi /ml) dissolved in the saline containing 10 mM NaOH and 1% normal rat serum.

Clearance of $[^{125}I]T_4$ from serum. The study on the clearance of $[^{125}I]T_4$ from serum was performed according to the method of Oppenheimer et al. (1968). In brief, after the administration of $[^{125}I]T_4$, a portion (0.3 ml) of blood was sampled from the artery at the indicated times, and serum was prepared and stored at −50°C until use. Two aliquots (15 μl each) were taken from each serum sample for determining $[^{125}I]T_4$ level by a gamma counter (COBRA II AUTO-GAMMA 5002; PerkinElmer Life and Analytical Sciences).

Analysis of $[^{125}I]T_4$ bound to serum proteins. The levels of serum $[^{125}I]T_4$-albumin and $[^{125}I]T_4$-TTR complexes were determined according to the method of Davis et al. (1970). In brief, serum was diluted in 100 mM phosphate buffer (pH 7.4) containing 1 mM EDTA, 1 mM dithiothreitol, and 30% glycerol, and subjected to electrophoresis on 4 to 20% gradient native polyacrylamide gels PAG Mid "Daiichi" 4/20 (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). The electrophoresis was performed at 4°C for 11 h at 20 mA in the 0.025 M Tris buffer (pH 8.4) containing 0.192 M glycine. The human albumin and TTR, which were incubated with $[^{125}I]T_4$, were also applied on the gel as templates. After the electrophoresis, a gel was dried and radioautographed for 20 h at room temperature using Imaging Plate 2040 (Fuji Photo Film Co., Ltd.). The levels of $[^{125}I]T_4$-albumin and $[^{125}I]T_4$-TTR in serum were determined by counting the gel fractions identified from Bio Imaging Analyzer (BAS-2000II IP Reader; Fuji Photo Film Co., Ltd.).

Tissue distribution of $[^{125}I]T_4$. The study on the tissue distribution of $[^{125}I]T_4$ was performed according to the modified method of Oppenheimer et al. (1968). In brief, at 60 min after administration of $[^{125}I]T_4$ to KC500-pretreated rats, blood was sampled from abdominal aorta. Then, cerebrum, cerebellum, pituitary gland, thyroid gland, sublingual gland, submandibular gland, thymus, heart, lung, liver, kidney, adrenal gland, spleen, pancreas, testis, prostate gland, seminal vesicle, stomach, duodenum, jejunum, ileum, cecum, brown fat, skeletal muscle, bone marrow skin, spinal cord, and fat were removed and weighed. Radioactivities in serum and the tissues were determined by a gamma-counter (COBRA II AUTO-GAMMA5002; PerkinElmer Life and Analytical Sciences), and amounts of $[^{125}I]T_4$ in various tissues were shown as ratios of tissue to serum.

Statistics. The data obtained were statistically analyzed according to Stu-

dent's *t* test or Dunnett's test after analysis of variance. In addition, data of the clearance of $[^{125}I]T_4$ from serum and analysis of $[^{125}I]T_4$ bound to serum proteins were statistically analyzed according to the Newman-Keuls test after analysis of variance. The pharmacokinetic parameters of $[^{125}I]T_4$ were estimated with noncompartmental methods as described previously (Tabata et al., 1999).

Results

Serum Hormone Levels. Effects of KC500 on levels of serum thyroid hormones were examined in Wistar and Gunn rats (Fig. 1). In both Wistar and Gunn rats, KC500 treatment resulted in decreases of the serum total T_4 and free T_4, and the magnitude of the decrease in each serum thyroid hormone was almost the same in both strains of rats. On the other hand, a significant decrease in the level of serum total T_3 was observed in Gunn rats but not in Wistar rats. In addition, no significant change in TSH level was observed in either Wistar or Gunn rats.

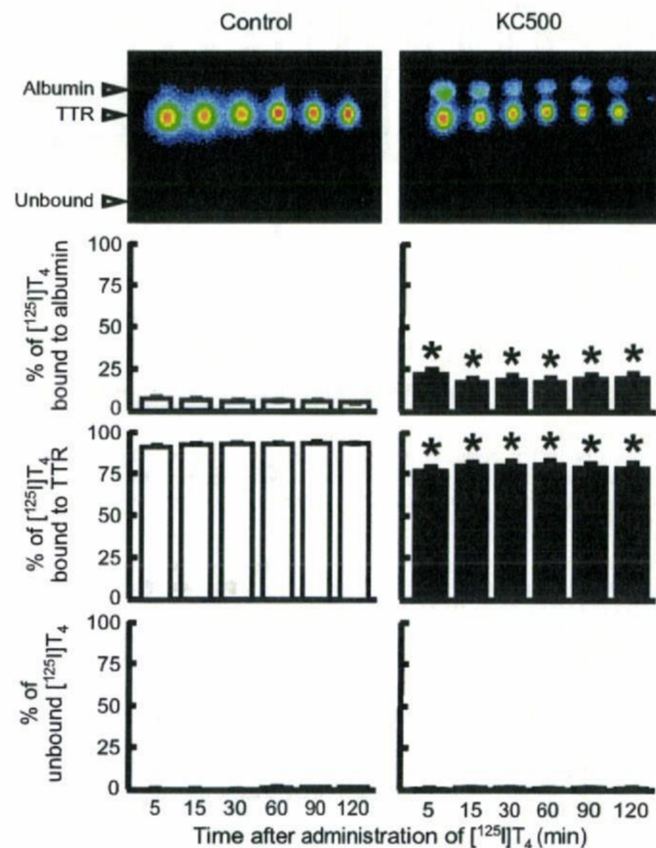

FIG. 6. Effects of KC500 on the binding of $[^{125}I]T_4$ to serum proteins in Gunn rats. Amounts of $[^{125}I]T_4$ bound to the serum proteins were assessed by the method described under *Materials and Methods*. Each column represents the mean ± S.E. (vertical bars) for four to five animals. *, $P < 0.05$, significantly different from each control.

Hepatic Drug-Metabolizing Enzymes. Effects of KC500 on hepatic microsomal activities of benzyloxyresorufin *O*-dealkylase (CYP2B1/2 and CYP3A1/2), pentoxyresorufin *O*-dealkylase (CYP2B1/2), and ethoxyresorufin *O*-dealkylase (CYP1A1/2) were examined in Wistar and Gunn rats. In both Wistar and Gunn rats, these enzyme activities were significantly increased by KC500 (Table 1), and the increase in each enzyme activity was much greater in Wistar rats than in Gunn rats.

Hepatic T_4-Metabolizing Enzyme Activities. T_4 glucuronidation is primarily mediated by hepatic T_4-UDP-GTs, such as UGT1A1 and UGT1A6, in the rat liver (Visser, 1996), and a chemical-mediated induction of the enzymes is considered to contribute to the decrease in the level of serum total T_4. Therefore, we examined effects of KC500 on hepatic microsomal T_4-UDP-GT activity in Wistar and Gunn rats. Constitutive activity of T_4-UDP-GT was approximately 2.2-fold higher in Wistar rats than in Gunn rats. Treatment with KC500 resulted in significant increase of T_4-UDP-GT activity in Wistar rats but not in Gunn rats (Fig. 2).

Western Blot Analysis for UGT1As. Levels of the proteins responsible for UGT1A enzymes, UGT1A1 and UGT1A6, were increased by KC500 treatment in Wistar rats but not in Gunn rats (Figs. 3 and 4). In addition, no expression of the UGT1A enzymes was confirmed in Gunn rats. On the other hand, the level of UGT2B1 was significantly increased by KC500 in both Wistar and Gunn rats, and magnitudes of the increase in both strains of rats were almost the same (Figs. 3 and 4).

Serum Proteins Bound to $[^{125}I]T_4$. The effects of KC500 on the binding of $[^{125}I]T_4$ to serum proteins, TTR, and albumin were examined in Wistar and Gunn rats (Figs. 5 and 6). In both Wistar and Gunn rats, pretreatment with KC500 resulted in a significant decrease in the level of $[^{125}I]T_4$-TTR complex, whereas it resulted in a significant increase in the level of $[^{125}I]T_4$ bound to albumin (Figs. 5 and 6).

Clearance of $[^{125}I]T_4$ from Serum. After an i.v. administration of $[^{125}I]T_4$ to the KC500-pretreated Wistar and Gunn rats, concentrations of $[^{125}I]T_4$ in sera were measured at the indicated times (Fig. 7). In both Wistar and Gunn rats, pretreatment with KC500 promoted the clearance of $[^{125}I]T_4$ from serum, and their serum $[^{125}I]T_4$ levels were decreased to approximately 40% of the initial level within 5 min. In the KC500-untreated Wistar and Gunn rats, serum $[^{125}I]T_4$ levels were gradually decreased to approximately 40% of the initial level at 120 min later. The serum pharmacokinetic parameters of the $[^{125}I]T_4$ estimated from these data (Fig. 7) were summarized in Table 2. The mean total body clearances (CL_{tb}) of $[^{125}I]T_4$ in the KC500-pretreated rats were 2.4 and 2.9 times, respectively, greater than those in the corresponding control rats. The steady-state volumes of distribution (Vd_{ss}) in the KC500-pretreated rats were 1.6 and 2.4 times, respectively, larger than those in the corresponding control rats.

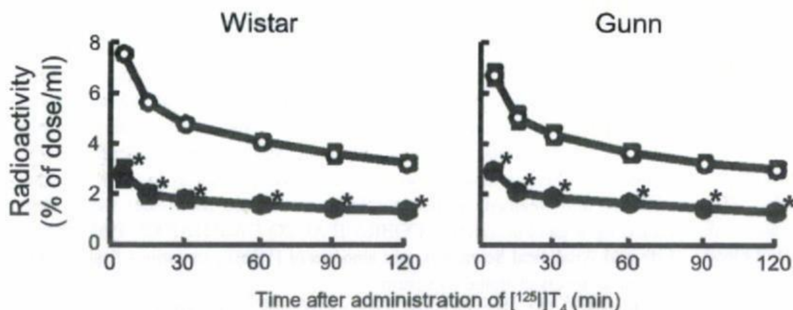

FIG. 7. Effects of KC500 on the clearance of $[^{125}I]T_4$ from serum in Wistar and Gunn rats. The amount of serum $[^{125}I]T_4$ was measured at the indicated times after the i.v. administration of $[^{125}I]T_4$. Each point represents the mean ± S.E. (vertical bars) for four to eight animals. *, $P < 0.001$, significantly different from each control. ○, control; ●, KC500.

Tissue Distribution of $[^{125}I]T_4$. The tissue-to-serum concentration ratio (K_p value) and distribution level of $[^{125}I]T_4$ in tissue after the administration of $[^{125}I]T_4$ to the KC500-pretreated Wistar and Gunn rats are shown in Figs. 8 and 9, respectively. K_p values of the thyroid gland and liver were the greatest among those of the tissues examined in either Wistar or Gunn rats (Fig. 8). In addition, K_p values in all the tissues examined, with the exception of the testis and ileum, were greater in KC500-pretreated Wistar rats than those in the corresponding control (KC500-untreated) rats. K_p values in the thyroid gland, liver, and jejunum in the KC500-pretreated Wistar and Gunn rats were 1.6 to 1.8, 3.3 to 3.8, and 4.7 to 11.5 times, respectively, higher than those in corresponding control rats (Fig. 8).

In the control Wistar and Gunn rats, the accumulation level of $[^{125}I]T_4$ was highest in the liver, among the tissues examined (Fig. 9). In both Wistar and Gunn rats, pretreatment with KC500 resulted in an increase in the accumulation level in the liver, and the levels increased to more than 40% of the $[^{125}I]T_4$ dosed (Fig. 9). Likewise, significant increase in accumulation of $[^{125}I]T_4$ was observed in the jejunum (Fig. 9). In addition, significant increases in the liver weight and accumulation level (per g liver) of $[^{125}I]T_4$ occurred in KC500-pretreated Wistar rats, but not in Gunn rats (Tables 3 and 4).

Discussion

In the present study, we found that consecutive treatment with KC500 (10 mg/kg i.p., once daily for 10 days; total dose, 100 mg/kg) promoted accumulation of T_4 in several tissues, especially the liver, and resulted in a drastic decrease in the levels of serum total T_4 and free T_4 in both Wistar and Gunn (UGT1A-deficient) rats. Thus, a decrease in the level of serum total T_4 is also observed in the Wistar and Gunn rats treated with KC500 (a single i.p. administration at a dose of 100 mg/kg) (Kato et al., 2004). In addition, constitutive levels of serum total T_4 and T_3 were higher in Gunn rats than in Wistar rats, and the results were identified with those as previously described by Benathan et al. (1983). The difference in constitutive level of serum thyroid hormone between Wistar and Gunn rats seems to be dependent on differences in the level and/or activity of T_4/T_3-UDP-GTs.

As a possible explanation for a chemical-induced decrease in serum thyroid hormones, a hepatic T_4-UDP-GT-dependent mechanism is generally considered, because T_4-UDP-GT inducers, including PCB, phenobarbital, 3-methylcholanthrene, pregnenolone-16α-carbonitrile, and clobazam, show strong activities for decreasing the level of serum total thyroid hormones, including T_4 and T_3 (Barter and Klaassen, 1994; Van Birgelen et al., 1995; Miyawaki et al., 2003). However, among the experimental animals treated with a T_4-UDP-GT inducer, the difference in magnitude of decrease in the level of serum total T_4 is not necessarily correlated with that of hepatic T_4-UDP-GT activity (Craft et al., 2002; Hood et al., 2003; Kato et al., 2003). Our present and previous results (Kato et al., 2004, 2005) using Wistar and Gunn rats support a hypothesis that significant decrease in the level of serum total thyroid hormones by either PCB or phenobarbital occurs primarily in a hepatic T_4-UDP-GT-independent pathway.

As a possible mechanism for the PCB-induced decrease in serum T_4 level, an increase in hepatic drug-metabolizing enzymes might be considered. However, these are induced to a greater extent in the Wistar rats than in the Gunn rats, whereas magnitudes of decrease in serum T_4 level in Wistar and Gunn rats were almost the same. Accordingly, the KC500-induced decrease in serum T_4 level is thought to be independent of the KC500-induced drug-metabolizing enzymes, including UDT-GTs and cytochromes P450.

As the factors regulating the level of serum total T_4, serum TSH, hepatic type I iodothyronine deiodinase, and TTR are known. However, no significant change in the level of serum TSH occurs in the PCB-treated rats (Liu et al., 1995; Hood et al., 1999; Hallgren et al., 2001; Kato et al., 2004). Hepatic type I iodothyronine deiodinase activity was significantly decreased in Wistar and Gunn rats by KC500 (Kato et al., 2004). On the other hand, a TTR-associated pathway might be considered as an explanation for the PCB-induced decrease in the level of serum total

TABLE 2

Pharmacokinetic parameters for $[^{125}I]T_4$ after the administration of $[^{125}I]T_4$ to the KC500-pretreated Wistar and Gunn rats

The experimental conditions were the same as those described in Fig. 7. The values shown are expressed as the mean ± S.E. for four to seven animals.

Animal	Treatment	Mean Total Body Clearance × 100	Distribution Volume
		ml/min	*ml*
Wistar	Control	7.82 ± 0.59	17.91 ± 0.52
	KC500	18.85 ± 3.49*	51.51 ± 6.34*
Gunn	Control	8.44 ± 0.22	20.21 ± 1.79
	KC500	13.84 ± 0.88*	48.91 ± 3.50*

* $P < 0.05$, significantly different from each control.

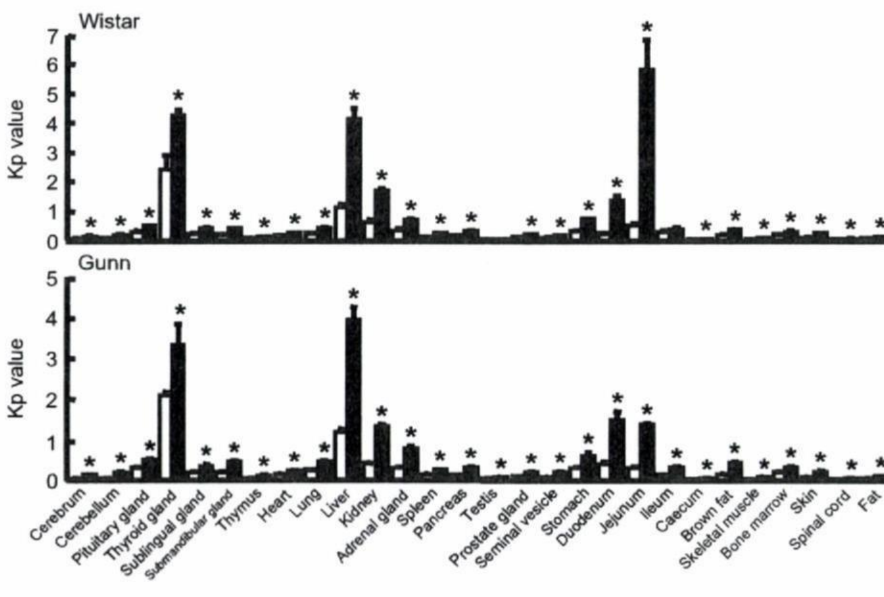

FIG. 8. Tissue-to-serum concentration ratio (K_p value) of $[^{125}I]T_4$ in various tissues after administration of $[^{125}I]T_4$ to the KC500-pretreated Wistar and Gunn rats. KC500 (10 mg/kg) was given i.p. to animals once daily for 10 days, and then, the animals were administered i.v. $[^{125}I]T_4$. At 60 min after administration of $[^{125}I]T_4$ the radioactivity in each tissue was measured. Each column represents the mean ± S.E. (vertical bars) for three to six animals. *, $P < 0.05$, significantly different from each control. □, control; ■, KC500.

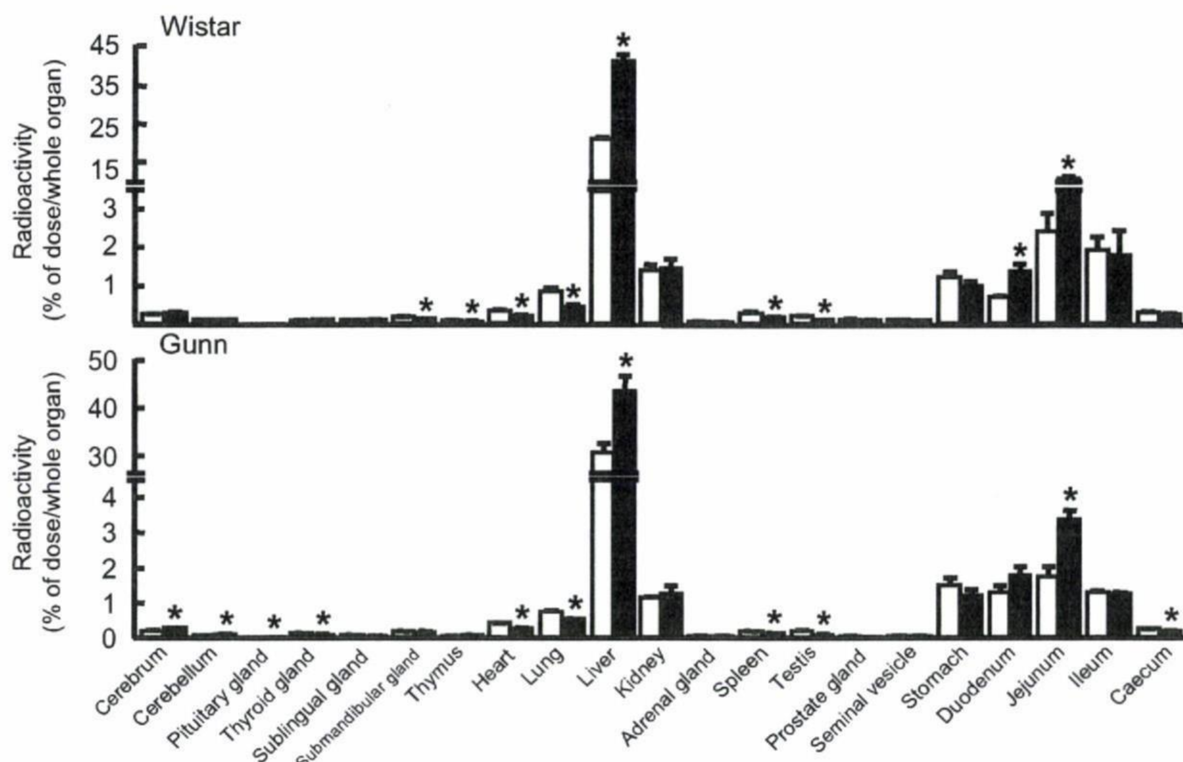

FIG. 9. Tissue distribution of $[^{125}I]T_4$ after administration of $[^{125}I]T_4$ to the KC500-pretreated Wistar and Gunn rats. The experimental conditions were the same as those described in Fig. 8. Each column represents the mean ± S.E. (vertical bars) for four to six animals. *, $P < 0.05$, significantly different from each control. □, control; ■, KC500.

TABLE 3

Liver weights after the administration of KC500 to Wistar and Gunn rats

Animals were killed at 4 days after the final administration of KC500 (10 mg/kg i.p., once daily for 10 days). The values shown are expressed as the mean ± S.E. for four to six animals.

Animal	Liver Weight	
	Control	KC500
	% of body weight	
Wistar	3.07 ± 0.04	3.81 ± 0.17*
Gunn	3.25 ± 0.08	3.38 ± 0.10

*$P < 0.01$, significantly different from each control.

TABLE 4

Accumulation of $[^{125}I]T_4$ in the KC500-pretreated Wistar and Gunn rat livers

The experimental conditions were the same as those described in Fig. 8. The values shown are expressed as the mean ± S.E. for four to six animals.

Animal	$[^{125}I]T_4$	
	Control	KC500
	% of dose/g liver	
Wistar	3.86 ± 0.18	6.01 ± 0.24*
Gunn	4.74 ± 0.43	6.33 ± 0.62

* $P < 0.001$, significantly different from each control.

T_4, because PCB and its ring-hydroxylated metabolites act as T_4 antagonists to TTR (Lans et al., 1993; Brouwer et al., 1998; Meerts et al., 2002; Kato et al., 2004). Thus, competitive inhibition by PCB and/or its metabolites would promote a decrease in the level of serum total T_4. In the present study, significant decrease in the level of $[^{125}I]T_4$ bound to serum TTR and increase in the level of $[^{125}I]T_4$ bound to serum albumin occurred in both KC500-pretreated Wistar and Gunn rats, suggesting that PCB and/or its metabolite(s) inhibit the formation of serum T_4-TTR complex.

Thus, inhibition of the T_4-TTR formation might lead to change in the tissue distribution of T_4. Therefore, to clarify this, we administered $[^{125}I]T_4$ to KC500-pretreated Wistar and Gunn rats and, thereafter, determined the levels of $[^{125}I]T_4$ in their tissues. In addition, since $[^{125}I]T_4$ in either plasma or tissues is known to be stable during 48 h (Oppenheimer et al., 1968), the radioactivity detected in the serum and tissues would be attributed to $[^{125}I]T_4$ in each tissue. Marked increases in the mean total body clearance of $[^{125}I]T_4$ and in the steady-state distribution volume of $[^{125}I]T_4$ were observed in the KC500-pretreated rats. A tissue-to-serum concentration ratio (K_p value) was greater in several tissues, especially the liver, of the KC500-pretreated Wistar and Gunn rats than in the corresponding control (KC500-untreated) rat tissues. In addition, in both KC500-pretreated Wistar and Gunn rats, more than 40% of the $[^{125}I]T_4$ dosed was accumulated in the liver.

In conclusion, the present findings confirmed that PCB-induced decrease in serum T_4 occurs not only in Wistar rats but also in Gunn (UGT1A-deficient) rats and further led to a hypothesis that the PCB-induced decrease occurs through increase in accumulation (transportation from serum to liver) of T_4 in the liver, rather than through induction of hepatic T_4-UDP-GT. In addition, the increased accumulation in the liver might be attributed to the PCB- and its metabolite(s)-mediated inhibition of formation of serum T_4-TTR complex.

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Review

In vitro molecular mechanisms of bisphenol A action ☆

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Received 12 April 2007; accepted 18 May 2007
Available online 29 May 2007

Abstract

Bisphenol A (BPA, 2,2-bis (4-hydroxyphenyl) propane; CAS# 80-05-7) is a chemical used primarily in the manufacture of polycarbonate plastic, epoxy resins and as a non-polymer additive to other plastics. Recent evidence has demonstrated that human and wildlife populations are exposed to levels of BPA which cause adverse reproductive and developmental effects in a number of different wildlife species and laboratory animal models. However, there are major uncertainties surrounding the spectrum of BPA's mechanisms of action, the tissue-specific impacts of exposures, and the critical windows of susceptibility during which target tissues are sensitive to BPA exposures. As a foundation to address some of those uncertainties, this review was prepared by the "*In vitro*" expert sub-panel assembled during the "*Bisphenol A: An Examination of the Relevance of Ecological, In vitro and Laboratory Animal Studies for Assessing Risks to Human Health*" workshop held in Chapel Hill, NC, Nov 28-29, 2006. The specific charge of this expert panel was to review and assess the strength of the published literature pertaining to the mechanisms of BPA action.

Abbreviations: 4-*tert*-OP, 4-tertiary octylphenol; 17α-EE, 17alpha-ethinyl estradiol; AR, androgen receptor; ARE, androgen response element; BADGE, bisphenol A diglycidyl ether; BFDGE, bisphenol F diglycidyl ether; BP-3, *p,p'*-dihydroxybenzphenone; BP-4, 2,2-bis(*m*-methyl-*p*-hydroxyphenyl)propane; BP-5, 2,2-bis(*p*-hydroxyphenyl) perfluoropropane; BPA, 2,2-bis(4-hydroxyphenyl) propane; BPA-OMe, 2,2-bis(4-methoxyphenyl)propane; BPE, 1,1-bis(4-hydroxyphenyl)ethane; BPF, 4,4'-methylenebisphenol; BPM, 4,4'-(1,3-phenylenedisopropylidene)bisphenol; BPP, 4,4'-(1,4-phenylenedisopropylidene)phenol; BPS, 4,4'-sulfonyldiphenol; BPZ, 4,4'-cyclohexylidenebisphenol; DDE, *o,p'*-dichlorodiphenylethylene; *o,p'*-DDT, 1,1,1-trichloro-2-[*o*-chlorophenyl]-2-[*p*-chlorophenyl]ethane; DEHP, bis(2-ethylhexyl)phthalate; DES, diethylstilbesterol; DHT, dihydrotestosterone; DMSO, dimethyl sulfoxide; E2, 17beta-estradiol; EDC, endocrine disrupting chemical; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; ERE, estrogen response element; ETOH, ethanol; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; GH, growth hormone; HBSS, Hank's buffered saline solutions; h, hour; HRP, horseradish peroxidase; inos, inducible nitric oxide synthase; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide; min, minute; NO, nitric oxide; NP, nonylphenol; OP, octylphenol; PCB, polychlorinated biphenyl; PMA, phorbol 12-myristate 13-acetate; PR, progesterone receptor; PSA, prostrate specific antigen; RIA, radio-immuno assay; RT-PCR, reverse transcription-polymerase chain reaction; RXR, retinoid X receptor; s, second; TCDD, 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin; T3, L-3,3',5-triiodothyronine; T4, 3,3',5,5'-tetraiodo-L-thyronine; TR, thyroid hormone receptor.

☆ SMB is supported by the NIH grants RO1-ES015145 and the University of Cincinnati Center for Environmental Genetics (P30-ES06096). YBW is supported by the NIEHS Kirschstein-National Research Service Award (T32 ES07069-26).

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0890-6238/$ – see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.reprotox.2007.05.010

The resulting document is a detailed review of published studies that have focused on the mechanistic basis of BPA action in diverse experimental models and an assessment of the strength of the evidence regarding the published BPA research.
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Keywords: Bisphenol A (BPA, 2,2-bis(4-hydroxyphenyl) propane; Endocrine disruption; Endocrine disrupting chemical (EDC); Rapid signaling; Estrogen receptor; Androgen receptor; Thyroid receptor; Thyroid hormone; GPR30; Non-classical membrane estrogen receptor; Immune system; Allergic resonse; *In vitro* mechanisms; Cell specificity; Expert panel review

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1. Introduction

Bisphenol A (BPA, 2,2-bis(4-hydroxyphenyl) propane; CAS# 80-05-7) is a chemical used primarily in the manufacture of polycarbonate plastic, epoxy resins and as a non-polymer additive to other plastics. Because of BPA's extensive use in the manufacture of consumer goods and products, including polycarbonate food containers and utensils, dental sealants, protective coatings, some flame retardants, and water supply pipes, there is a widespread and well-documented human exposure to BPA [1,2]. Recent discoveries regarding the environmental distribution and presence of BPA in humans and wildlife have generated persistent scientific, regulatory, and public interest in assessing the potential health risks associated with BPA exposure. The detection of adverse health effects in a number of laboratory animal models upon exposure to environmentally relevant doses of BPA, as well as potential effects on human reproduction and development, have fueled additional concern. While the exposure of wildlife species and humans to BPA has been increasingly reported in the literature, there are research gaps regarding its molecular mechanisms of action, the tissue-specific impacts of exposure, and knowledge of the critical windows of susceptibility, during which target tissues are especially sensitive to BPA.

The lack of an integrated and systemic understanding of BPA's endocrine disruptive actions has considerably complicated risk assessment efforts and safety recommendations by regulatory agencies. Current limitations in understanding the global consequences of BPA exposures include incomplete understanding of the cell/tissue specific actions and effects, a limited understanding of the receptor systems and signaling cascades through which BPA acts, and understanding when these receptors and signaling systems are present and active in target cells at different critical times of sensitivity during the entire life-span.

2. Overview

This white paper was prepared for the *in vitro* expert subpanel assembled during the "Bisphenol A: An Examination of the Relevance of Ecological, *In vitro* and Laboratory Animal Studies for Assessing Risks to Human Health" workshop at the NIEHS. The specific charge of this expert panel was to review and assess the strength of the published literature pertaining to the mechanisms of BPA action. Particular attention was paid to studies employing *in vitro* models and the results, findings and conclusions from those studies were integrated into the broader biological/physiologic context of BPA action. Thus, the goal of

the white paper was to review in detail peer-reviewed published studies that have focused on the mechanistic basis of BPA action in diverse experimental models.

3. Definition of endocrine disruptor

The U.S.-Environmental Protraction Agency (EPA) has defined an environmental endocrine disruptor or endocrine disrupting chemical (EDC) as "an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" [3]. This definition is not limited to endocrine disrupting effects exclusive of the estrogen system. Rather, endocrine disruption encompasses effects on other endocrine systems including effects mediated by androgens, thyroid hormone, prolactin, and insulin, among others.

4. Definition of "low dose"

The primary focus of this review is on *in vitro* studies that investigate the molecular mechanisms of BPA action. However, full consideration of BPA's mode of action cannot rely solely on *in vitro* studies performed at a range of concentrations. It is critically important to consider experimental findings from numerous *in vivo* studies investigating BPA actions in animals at a physiologically relevant exposure dose, also known as a "low-dose". Thus, a low-dose effect was determined based on the U.S.-EPA criterion for low-dose effects of EDCs as effects observed at concentrations below those used for traditional toxicological studies. Levels below the current lowest observed effect level (LOAEL) of 50 μg/kg/day were considered as low dose for *in vivo* studies. For *in vitro* cell or organ culture studies, estimates of circulating levels of BPA at the LOAEL cut-off have defined an equivalent low dose concentration as <50 ng/mL or $<2.19 \times 10^{-7}$ M [4]. Based on experimental designs of most studies, we elected to consider an alternative and more conservative definition of low-dose for *in vitro* BPA effects as $\leq 1 \times 10^{-7}$ M.

5. Definition of estrogen

When considering the mechanisms of BPA action, it was considered critical to determine whether the molecular and physiological EDC effects of BPA could be fully explained by its ability to act as an agonist or antagonist of endogenous estrogens in target cells, or whether the molecular and physiological EDC effects of BPA extend beyond the molecular, subcellular and/or cellular mechanism of estrogens' actions. According to the Merriam-Webster Medical Dictionary of the U.S. National Library of Medicine, (http://www.nlm.nih.gov/medlineplus/mplusdictionary.html) estrogen is defined as: any of various natural steroids (as estradiol) that are formed from androgen precursors, that are secreted chiefly by the ovaries, placenta, adipose tissue, and testes, and that stimulate the development of female secondary sex characteristics and promote the growth and maintenance of the female reproductive system; also: any of various synthetic or semisynthetic steroids (as ethinyl estradiol) that mimic the physiological effect of natural estrogens.

In this review, "estrogen" is used as a class name for endogenous estrogenic compounds, and "estradiol" or "17β-estradiol" will be used more specifically in reference to 1,3,5(10)-estratrien-3,17β-diol.

6. Mechanisms of BPA action

Diverse biological effects have been attributed to low-dose environmental BPA exposure in a variety of tissues. Molecular studies employing an array of *in vitro* models have revealed a variety of molecular pathways through which BPA may elicit cellular responses. As a prototypical non-steroidal estrogen, BPA interferes with the activity of endogenous estrogens (e.g. 17β-estradiol) by disrupting the proper activity of the estrogen nuclear hormone receptors in a diverse set of target tissues [5–21]. However, the primary endocrine disrupting activities of BPA extend beyond its ability to mimic, enhance or inhibit the activity of endogenous estrogens and/or disrupt estrogen nuclear hormone receptor action, and include the following: effects upon the androgen systems [22–29]; disruption of thyroid hormone function [30–40]; diverse influences on development, differentiation and function of the central nervous system [40–46]; and influences on the immune system [21,47–65]. There is additional clear evidence for BPA to directly impact intracellular signal transduction pathways through mechanisms independent of the transactivational activity of nuclear hormone receptors [43,46,66–79]. Secondary metabolic and pharmacokinetic actions of BPA that impact its bioavailability and bioavailability of endogenous steroid hormones have also been described. Those secondary effects include modification of cytochrome P450 enzyme expression and activity [80–83] and alterations of serum hormone binding protein expression and interactions [84–87]. Recent studies have also begun to highlight the potential for transmission of early exposures to physiological expression later in life and across generations via epigenetic mechanisms such as methylation-meditated promoter silencing [88–90]. Table 1 summarizes the results from published studies that were used in the preparation of this review. Clearly, BPA is an endocrine disrupting compound or EDC in the broad sense of the definition. However, because of the pleiotropic mechanisms of BPA action, narrowly defining BPA as a selective estrogen receptor modulator [SERM; a class of pharmacological compounds (such as raloxifene or tamoxifen) that bind nuclear estrogen receptors and act as estrogen agonists in some tissues and estrogen antagonists in other tissues], or exclusively as an environmental estrogen, is inaccurate. The use of these self-limiting terms only partially describes the extent of BPA's endocrine disrupting and other biological activities.

7. Nuclear estrogen receptors, coregulators, and the estrogen-response element (ERE)

It is well established that BPA can exert some of its effects by binding at the nuclear steroid receptors ERα and ERβ to induce estrogenic signals that modify estrogen-responsive gene expression. Mechanisms for ER-mediated gene regulation are

Table 1
Published papers reporting physiological effects of BPA *in vitro*

Reference	Species	Model	Endpoints	Exposure: vehicle	Exposure time	LOEC (M) (*$P<0.05$)	Doses tested (M) (*$P<0.05$)	Inhibitors	Other chemicals tested
Adipose models									
Masuno et al. 2005 [167]	Mice	3T3-L1 (preadipocyte)	Adipocyte differentiation	Culture medium, Not indicated	6 days	2×10^{-5}*	4×10^{-6}, 2×10^{-5}*, 4×10^{-5}*, 8×10^{-5}*	LY294002	BPA-derivatives; NP; OP
Masuno et al. 2002 [168]	Mice	3T3-L1 (preadipocyte)	Adipocyte differentiation	Culture medium, Not indicated	11 days	2×10^{-6}*	2×10^{-6}*, 10^{-5}*, 2×10^{-5}*		BPA-derivatives
Bone models									
Kanno et al. 2004 [169]	Mice	MC3T3-E1 (C57BL/6) osteoblast-like	Cell proliferation, Differentiation (ALP activity), Ca^{2+} content	Culture medium, ETOH	3–4 days	10^{-4}*, 10^{-6}*, 10^{-6}*	10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}*, 10^{-5}*, 10^{-4}*		E2; coumestrol; genistein; daidzein; NP; DEHP
Breast cancer models									
Buterin et al. 2006 [101]	Human	T47D/Luc, MCF7	ERE-promoter activation, Cell proliferation	Culture medium, DMSO	3–24 h, 24 h	10^{-7} (24 h)	10^{-13} to 10^{-5}, 10^{-5}		E2; genistein; PCB54; PCB126
Cappelletti et al. 2003 [170]	Human	T47D, BT20	Cell growth, ER, PR mRNA (RT-PCR)	Culture medium, Not indicated	6 days, 10, 24, 48 h	10^{-6}*, 10^{-8}*	10^{-8}, 10^{-7}, 10^{-6}*, 10^{-5}*		E2; 4-*tert*-OP; 2-OH-biphenol
Inadera et al. 2000 [60]	Human	MCF7	IL-1 induced MCP-1 expression	Culture media, <0.1% ETOH	24 h	5×10^{-7}*	10^{-7}, 5×10^{-7}*, 10^{-6}*	ICI 182,780	E2; NP
Iso et al. 2006 [171]	Human	MCF7, MDA-MB-231	DNA damage (comet tail length assay)	Culture medium, <0.1% ETOH	1, 3, 24 h	10^{-6}*	10^{-8}, 10^{-6}*, 10^{-4}*	ICI 182,780	E2
Olsen et al. 2003 [15]	Human	MCF7	Cell proliferation, Estrogen-responsive gene expression	Culture medium, ≤0.2% ETOH	6 days, 3 days	5×10^{-7}*	10^{-8}, 10^{-7}, 5×10^{-7}*, 10^{-6}*, 10^{-5}*	4-OHTamoxifen; ICI 182,780	E2; tetra-Cl-BPA; tetra-Br-BPA; T3; T4; 4-OH-biphenol; 4,4-OH biphenol
Recchia et al. 2004 [9]	Human	MCF7, T47D	Cell proliferation, ERα-expression & nuclear localization, ERE-luciferase reporter	Culture medium, Not indicated	6 days, 2 h	10^{-6}*	10^{-6}*	4-OH Tamoxifen	4-NP
Samuelsen et al. 2001 [142]	Human	MCF7, T47D	Cell proliferation, PR, pS2-expression	Culture medium, ≤0.1% ETOH	6 days	10^{-7}	10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}		E2; Br-BPA derivatives
Singleton et al. 2006 [98]	Human, *S. cerevisiae*	MCF7-C4-12 (HA-ERα)	Differential mRNA expression, ERE-LacZ	Culture media, ETOH	3 h, 24 h	10^{-6}, 10^{-7}	10^{-7}, 3×10^{-7}, 10^{-6}, 3×10^{-6}		E2
Vivacqua et al. 2003 [10]	Human	Ishikawa (endometrial) MCF7, MCF7SH	ERE-luciferase, ERα, pS2 expression, Cell growth	Culture medium, Not indicated	20–24, 6 days	10^{-9}	10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}*, 10^{-6}, 10^{-5}	4-OH Tamoxifen	E2; 4-NP
Walsh et al. 2005 [74]	Human	MCF7, MDA-MB-231, SKBR-3	Intracellular Ca^{2+}	Kreb's solution, ≤0.001% methanol	0–15 min	10^{-10}*	10^{-10}*, 10^{-9}*, 10^{-8}*, 10^{-7}*	ICI 182,780	*o,p'*-DDT; DES; 4-*tert*-OP
Embryonic/development models									
Iwamuro et al. 2003 [146]	*Xenopus laevis*	Tadpole tail organ culture	Tail length regression (also see *in vivo*)	Culture medium, ETOH	1–4 days	10^{-6}*	10^{-6}*, 10^{-5}*, (±10^{-7}T3)		E2
Iwamuro et al. 2006 [32]	*Xenopus laevis*	Tadpole tail organ culture	Tail length regression, TRα, TRβ, RXRγ, mRNA expression (RT-PCR)	Culture medium, ETOH	1–5 days	10^{-7}*	10^{-7}*, 10^{-6}*, 10^{-5}*, (±10^{-7} T3)		
Tsutsui et al. 1998 [172]	Hamster (Syrian)	Primary embryo cells	Cell growth, morphological chromosomal aneuploidy, DNA adduct formation	Culture medium, 0.6% DMSO	24, 48, 72 h	5×10^{-5}*	5×10^{-5}*, 10^{-4}*, 2×10^{-5}*		
Tsutsui et al. 2000 [173]	Hamster (Syrian)	Primary embryo cells	Cell growth, morphological, DNA adduct formation, aneuploidy	Culture medium, 0.6% DMSO	24, 48, 72 h	10^{-4}*	5×10^{-5}*, 10^{-4}*, 2×10^{-5}*		BPF; BP-3; BP-4; BP5
Endothelium models									
Lee et al. 2003 [25]	Human	Female primary endothelial cells	Cell proliferation	Culture medium, Not indicated	24, 48, 72 h	10^{-6}*	10^{-8}, 10^{-6}*, 10^{-4}*		Aroclor 1254
Female reproductive tissue models									
Jin et al. 2005 [174]	Human	BeWo (b30) placental trophoblast	Calcein AM efflux	HBSS 25 mM glucose, ETOH 0.1%	48 h	10^{-7}*	10^{-7}*, 10^{-5}*, 5×10^{-5}*		E2
Xu et al. 2002 [112]	Mice B6C3F1	Primary granulosa cells	Cell viability/apoptosis	Culture medium, 0.1% ETOH	72 h	10^{-10}*	10^{-13}, 10^{-10}*, 10^{-7}*, 10^{-4}*		
Immune system models									
Alizadeh et al. 2006 [47]	Mice BALB/c	Primary splenocytes; Sex not specified	Cytokine production, (IL-4:-12; IFN-γ ELISA)	*In vivo* IP-injection, corn oil	8 days	100 mg/kg every other days	100 mg/kg for IL-4*; IFN-γ*		
Canesi et al. 2004 [75]	Mussels	Primary hemocytes	Lysosomal membrane stability, ERK2, p38, STAT3, STAT5 phosphorylation	Artificial sea water/serum, ETOH	15–110 min, 5–30 min	2.5×10^{-5}*, 2.5×10^{-5}*	2.5×10^{-5} 2.5×10^{-4}, 2.5×10^{-5}*	4-OHTamoxifen; SB203580; PD98059; wortmannin; GF109203X	DES; NP

Table 1 (*Continued*)

Reference	Species	Model	Endpoints	Exposure; vehicle	Exposure time	LOEC (M) (* $P<0.05$)	Doses tested (M) (* $P<0.05$)	Inhibitors	Other chemicals tested
Goto et al. 2004 [64]	Mice BALB/c	Female primary splenocytes	Cell proliferation	Culture medium, 0.156% DMSO	72 h	2×10^{-5}*	2.5×10^{-6}, 5×10^{-6}, 1×10^{-5}, 2×10^{-5}*		
Han et al. 2002 [63]	Mice BALB/c	Male primary splenocytes	Immunoglobulin production (ELISA)	Culture medium, <0.1% ETOH	7 days	10^{-8} (IgE; IgM)	10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-3}		E2; tamoxifen; DES; diadzein genistein quercetin; luteolin
Hong et al. 2004 [62]	Mice BALB/c	RAW 264 (macrophage), Female Abdominal macrophages	LPS-induced TNF-α production, NO-production	Culture medium DMSO, DMSO/corn oil	4 h	nd	4.4×10^{-5}* (NO only), NS		Various EDCs
Kim et al. 2003 [48]	Mice	RAW 264.7 (macrophage), Female Abdominal macrophages	TNFα; NO production (total nitrite), iNOS expression (RT-PCR)	Culture medium (± LPS), <0.1% DMSO	6, 24 h	10^{-5}*	10^{-6}, 10^{-5}*, 5×10^{-5}*, * vs. LPS control	ICI 182,780	E2
Lee et al. 2003 [61]	Mice BALB/c	Female primary lymph node cells, hybridoma cells (3.155, GK1.5, EL4)	PMA/KLH-primed IL4-production	Culture medium, Not indicated	2, 4 days	10^{-5}*	10^{-7}, 10^{-6}, 10^{-5}*, 5×10^{-5}*, 10^{-4}*	Nitrendipine; BATA-AM; thapsigargin; FK506	NP
Ndebele et al. 2004 [49]	Human	CD4+ Jurkat T cell line	IL-2 production (ELISA; RT-PCR)	Culture medium	48 h	ns	10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}		Coumesterol; TCDD; DDT
Reistad et al. 2005 [160]	Human	Primary neutrophil granulocytes	Reactive oxygen species, Intracellular Ca^{2+} (secondary endpoints)	HEPES-buffered salt solution, ≤0.1% ETOH	60 min, 250 s	2×10^{-6}*	2×10^{-6}*, 6×10^{-6}*, 1.2×10^{-5}*, 2×10^{-5}*		4-Br-BPA
Sakabe et al. 1999 [50]	Mice	Primary thymus epithelial cells	Thymosin-α1 suppression (ELISA; HPLC)	Culture medium, Not indicated	7 days	3×10^{-6}*	3×10^{-8}, 3×10^{-7}, 3×10^{-6}*, 3×10^{-5}*3×10^{4}*		E2; genistein; coumesterol; α-zeralanol; progesterone; cholesterol
Sawai et al. 2003 [51]	Mice C57BL/6;	Primary splenic mononuclear cells	ConA induced IFN-γ secretion (ELISA)	Culture medium, Not indicated	24 h	5×10^{-8}*	5×10^{-10}, 5×10^{-8}*, 5×10^{-6}*		
Watanabe et al. 2003 [53]	Human	HL-60	Neutrophilic differentiation, O_2^- production, CD18 expression	Culture medium, DMSO	6 days	10^{-10}*	10^{-12}, 10^{-10}, 10^{-9}*, 10^{-8}	4-OH Tamoxifen	
Liver models									
Jeong et al. 2000 [83]	Mice	Hepa-1c1c7 (hepatoma)	Cyp1a-1 activity, Cyp1a-1 mRNA expression (RT-PCR)	Culture medium, DMSO <0.2%	18 h, 6 h	10^{-5}*	10^{-6}, 10^{-5}*, 5×10^{-5}*	4-OH Tamoxifen	TCDD
Kubo et al. 2004 [78]	Human	Hep3B (hepatoma)	Inhibition of hypoxic response, Erythropoietin, HIF-1a expression	Culture medium, Not indicated	6 h	10^{-4}	5×10^{-5}, 10^{-4}, 5×10^{-4}	CoCl; Mg132	BPA-OMe; BPE; BPF
Male reproductive tissue models									
Fiorini et al. 2004 [136]	Rat	SerW3 (Sertoli cells testis)	Qualitative junctional protein expression	Culture medium, DMSO <0.1%	24 h	4.5×10^{-5}	4.5×10^{-5}		BPA-methacrylate; o,p'-DDT
Iida et al. 2003 [175]	Rat Wistar	Primary Sertoli cells	Viability/apoptosis	Culture media	<12–48 h	1.5×10^{-4}*	5×10^{-5}, 10^{-4}, 1.5×10^{-4}*, 2×10^{-4}*, 3×10^{-4}*		
Lee et al. 2003 [29]	*S. cerevisiae*, Human, Mice	Hela, HepG2, 15p-1 (Sertoli cell line)	2-hybrid AR/ASC1, GFP-AR translocation, Inhibition of ARE-transcription (luciferase)	Culture medium, ETOH	3 h, 24 h, 24 h	10^{-6}	10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}		E2; NP; cyproterone
Nikula et al. 1999 [135]	Mice	mLTC-1 (Leydig tumor cell line)	cAMP, progesterone production	Culture medium, ETOH	48 h	10^{-7}*	10^{-7}, 10^{-6}*, 10^{-5}*, 10^{-4}*		E2; *tert*-OP; OP; DES
Song et al. 2002 [137]	Mice	K28 (Leydig tumor cell line)	Nur77 expression S.	Culture media, 0.1% ETOH	30 min – 24 h	10^{-8}	10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}	H89; PD98059; GFX; wortmannin	
Tabuchi et al. 2006 [176]	Mice	TTE3 Sertoli cells (testis)	Steroidogenic function mRNA expression	Culture media, 0.1% DMSO	3, 6, 12 h	2×10^{-4}*	2×10^{-4}*		BPE; BPF; BPM; BPP; BPS; BPZ
Wetherill et al. 2002 [26]	Human	LNCaP (prostate)	Cell proliferation, AR-translocation	Culture medium, 0.1% ETOH	72 h, 2, 7 h	10^{-9}*, 10^{-10}*	10^{-10}, 10^{-9}*, 10^{-8}, 10^{-7}	Bicalutamide	DHT
Wetherill et al. 2005 [28]	Human	LNCaP (prostate)	PSA, AR expression, AR transactivation	Culture medium, 0.1% ETOH	48 h	10^{-9}	10^{-9}, 10^{-5}	Bicalutamide	DHT
Satoh et al. 2004 [12]	Hamster	CHO-K1 (ARE—luciferase)	AR/ARE—luc expression (± DHT), Cell proliferation	Culture medium, DMSO, ≤0.1% ETOH	16–24 h	10^{-6}, 10^{-7}	10^{-11}, 10^{-10}, 10^{-9}, 10^{-7}, 10^{-7}, 10^{-5}		BADGE; BADGE-2H_2O; BADGE-2HCl; BPF; BFDGE; BFDGE-2H_2O; BFDGE-2HCl; Cyproterone, Mibolerone
Nervous system models									
Miyatake et al. 2006 [44]	Mice ICR	Primary culture midbrain astrocytes & neuron/glia	GFAP/Neu-N immunoreactivity, $[Ca^{2+}]i$	Culture medium, Not indicated	24 h	10^{-13}*	10^{-14}, 10^{-13}*, 10^{-12}*, 10^{-11}*, 10^{-10}, 10^{-10}, 10^{-10}, 10^{-7}*, 10^{-6}*	ICI 182,780; mifepristone; flutamide	E2

Table 1 (*Continued*)

Reference	Species	Model	Endpoints	Exposure: vehicle	Exposure time	LOEC (M) (*$P<0.05$)	Doses tested (M) (*$P<0.05$)	Inhibitors	Other chemicals tested
Seiwa et al. 2004 [177]	Mice ICR	Primary oligodendrocyte precursor cells	T3-induced differentiation	Culture media. Not indicated	2 days	10^{-5}	10^{-9}, 10^{-7}, 10^{-6}, 0^{-5}		
Sato et al. 2002 [178]	Rat Wistar	Hippocampus organotypic primary culture	Glutamate-induced cell death (CA3)	Culture medium	24 h	10^{-12}*	10^{-12}*	ICI 182,780;	E2; 17α-EE; DES; p-NP
			NMDA-receptor expression. Spine & mossy fiber density	≤0.1% ETOH			10^{-9}* 10^{-6}* 10^{-4}*	4-OH Tamoxifen	
Yamaguchi et al. 2006 [40]	Mice	SFME cells	LIF/BMP-2 induced astrocyte differentiation	Culture medium, Not indicated	48 h	4.4×10^{-12}*	4.4×10^{-13}, 4.4×10^{-12}*, 4.4×10^{-11}*, 4.4×10^{-10}*, 4.4×10^{-9}, 4.4×10^{-8}, 4.4×10^{-7}, 4.4×10^{-6}	4-OH Tamoxifen	
Yoneda et al. 2003 [76]	Rat	PC12 (adrenal)	Dopamine secretion	Culture medium, ≤0.1% DMSO	10 min	2.5×10^{-6}*	2.5×10^{-6}*, 5×10^{-6}*, 1×10^{-5}*, 1.5×10^{-5}*	Diltiazem; clorgyin; pargyline; ω-agatoxin; ω-conotoxin; H7; H-89; ryanodine; ruthenium red; Rp-cAMPS; GDPβs,	
Zsarnovszky et al. 2005 [46]	Rat Sprague-Dawley	Developing cerebellar neurons (male & female)	ERK1/2 phosphorylation	Culture medium, artificial cerebrospinal fluid, DMSO	5 min	10^{-12}*	10^{-12}*, 10^{-11}*, 10^{-10}*, 10^{-9}, 10^{-8}, 10^{-7}*, 10^{-6}*, 10^{-4}	U0128	E2
Pancreatic models									
Adachi et al. 2005 [21]	Rat Wistar	Male; primary pancreatic islets	Insulin release-RIA	Krebs-Ringer bicarbonate buffer –16.7 mM glucose	60 min, 24 h	nd, 4.4×10^{-8}*	4.4×10^{-10}, 4.4×10^{-9}, 4.4×10^{-8}*, 4.4×10^{-8}*, 4.4×10^{-6}	Actinomycin-D; ICI 182,780	E2; NP
Alonso-Magdalena et al. 2005 [66]	Mice Swiss albino OF1	Male; primary pancreatic islets and isolated islet cells	Intracellular Ca^{2+}	Modified Ringers—5 or 0.5 mM glucose, DMSO	5 min	10^{-9}*	10^{-9}*	ICI 182,780; KT-5823; pertussis toxin; ODQ; L-NAME	E2; E2-HRP; DES; DDT
Alonso-Magdalena et al. 2006 [67]	Mice Swiss albino OF1	Male; primary pancreatic islets and isolated islet cells	Insulin release-RIA; insulin content	*In vivo* sc injection; tocopherol-stripped corn oil		10 μg/kg/day	10; 100 μg/kg/day	ICI 182,780	E2
Nadal et al. 2000 [70]	Mice Swiss albino OF1	Male; primary pancreatic islets and isolated islet cells	Fluo-3 AM $[Ca^{2+}]$i	Perfusion buffer –8 mM glucose	4–8 min	10^{-10}	10^{-10}, 10^{-11}, 10^{-9}, 10^{-7}		E2; E2-HRP; DES; *o,p'*-DDT
Quesada et al. 2002 [73]	Mice Swiss albino OF1	Male; primary pancreatic islets and isolated islet cells	Intracellular Ca^{2+}, Phospho-CREB-IR	Profusion buffer	0–15 min	10^{-9}	10^{-9}	ICI 182,780	E2; E2-HRP
Pituitary models									
Bulayeva et al. 2004 [69]	Rat	GH3/B6/F10 (ERα-positive)	ERK1/2 phosphorylation immunoreactivy	Culture medium; ETOH	3–30 min	nd	10^{-9}		E2; NP; endosulfan; DDE; dieldrin; coumesterol
Ghisari et al. 2005 [30]	Rat	GH3	Cell proliferation (T-screen)	Culture medium	6 days	10^{-7}*	10^{-8}, 10^{-7}*, 10^{-6}*, 10^{-5}*	ICI 182,780	E2; T3; BPA-methacrylate; 4-OP; 4-n-NP; iprodion; prochloraz; chlorpyrifos; 4-OH-PCB 106; 4-OH-PCB 69; 4-OH-PCB121
Kitamura et al. 2002 [33]	Rat	GH3	Cell proliferation	Culture medium, ETOH	7 days	10^{-6}*	10^{-7}, 10^{-6}*10^{-5}*	4-OH Tamoxifen	T3; E2; TBBPA; TCBPA
Kitamura et al. 2005 [35]	Human, Mice, Rat	MCF7, NIH3T3, GH3	ERE_3-luciferase, ARE-luciferase, GH-induction	Culture medium, ETOH	24 h, 24 h, 2 days	10^{-7}*, 10^{-6}*, NS	10^{-8}, 10^{-7}*, 10^{-6}*, 10^{-5}*, 10^{-4}*	4-OHTamoxifen	E2; various BPA-related compounds
Steinmetz et al. 1997 [157]	Rat	GH3	Prolactin release, Cell proliferation, Prolactin-luciferase	Culture media, ≤0.001% ETOH	1–8 days, 8, 24 h	10^{-6}, 10^{-9}	10^{-6}, 10^{-9}		E2
Watson et al. 2005 [72]	Rat	GH3/B6/F10 GH3/B6/D9	Intracellular Ca^{2+}, Prolactin release	Ringer's solution, Culture medium	1, 3 min	10^{-12}	10^{-12}, 10^{-10}*, 10^{-9}*, 10^{-8}*, 10^{-7}		Coumesterol; DDE; DES; dieldrin; endosulfan; *p*-NP
Wozniak et al. 2005 [77]	Rat	GH3/B6/F10 GH3/B6/D9	Intracellular Ca^{2+}, Prolactin release	Ringer's solution, Culture medium	0–20 min, 1–15 min	10^{-12}*	10^{-12}*, 10^{-10}*, 10^{-9}*, 10^{-8}*, 10^{-12}*, 10^{-11}*, 10^{-10}, 10^{-9}, 10^{-8}*	Nifedipine	Coumesterol; DDE; DES; dieldrin; endosulfan; *p*-NP

complex, and depend on the recruitment of tissue-specific co-regulatory factors that differentially affect the interaction of ERs with EREs of different target genes [91,92]. BPA selectively binds to ERα and ERβ, and has a higher affinity for ERβ in target cells [6–8]. Kuiper et al. reported a Ki for BPA of 195 and 35 nM at ERα and ERβ, respectively [6]. It was also found that the binding affinity relative to 17β-estradiol for BPA at ERβ was 6.6-fold higher than at ERα; 0.33 and 0.05, respectively [6]. Satoh et al. also showed that BPA could bind to human ERα and reported an IC_{50} (the concentration of chemical required to reduce specific 17β-estradiol binding by 50%) of 7.8 μM [12]. Gould et al. demonstrated that as an endocrine disruptor BPA is not merely a weak estrogen mimic, but it exhibits characteristics of a distinct molecular mechanism of action at ERα, with BPA interacting differently within the ligand-binding domain [5]. These findings reveal that the interactions of BPA and 17β-estradiol with an estrogen receptor can induce differential molecular effects, thus ultimately impacting the physiological response of sensitive cells.

In addition to the differential ER binding properties, there are major differences in the effects of BPA on co-activator recruitment by ERα and ERβ [8]. Specifically, the BPA/ERβ receptor/ligand complex showed over 500-fold greater potency than the BPA/ERα complex in recruiting the co-activator TIF2. Moreover, the enhanced (relative to ERα) ability of ERβ to recruit co-activators in the presence of BPA was consistent with a greater capacity of ERβ to potentiate reporter gene activity in transiently transfected HeLa cells expressing ERβ and TIF2 over a concentration range of 1–100 μM [8]. A higher relative binding affinity (RBA) for BPA at ERβ was also observed (estradiol RBA for BPA at ERβ was 0.75 versus 0.073 for ERα). Based on those data it was concluded that ligand-dependent differences in the ability of ERα and ERβ to recruit co-activator proteins would contribute to the complex tissue-dependent agonistic or antagonistic responses observed with exposure to endocrine disruptors such as BPA.

Numerous isoforms of ERα and ERβ have been identified and they likely play unique roles as mediators of the actions of endogenous estrogens and BPA. For example, Ramsey et al. used different ERE-sequence containing reporter-constructs to demonstrate that expression of different isoforms of ERβ differentially impacted ERE-regulated gene expression in a ligand dependent manner [93]. These results suggest that the cell-type specific expression of different ER isoforms together with availability of a specific ligand, contributes to the cellular complexity of ERE-regulated estrogen responsive target gene expression. Moreover, a study by Masuyama et al. showed that compared to 17β-estradiol, BPA blocks the ubiquitination and degradation of ERβ, suggesting that BPA may affect ERβ-mediated transcription of target genes by differential degradation of nuclear receptors [94].

8. Differential responsiveness of sensitive cells and tissues

Regarding cell-specific differences in response to BPA, it was proposed that the nature of the estrogen-responsive promoter would also influence the activity of endocrine disrupting chemicals through ERs, similar to the way in which SERMs manifest their gene-specific activities. The idea that ligands play an active role in ER function was proposed by McDonnell and others who demonstrated that different ligands induce distinct conformational changes in the ER, correlating with the observed diversity in transcriptional and biological responses [95–97].

Pennie et al. showed that the nature of the ERE, along with the ratio of ERα and ERβ in a particular cell or tissue could influence ER-responsive genes in the presence of estrogenic compounds such as BPA [14]. In those studies, the effects of BPA at doses from 1 to 100 μM and 17β-estradiol (1 nM to 1 μM) in COS-1 cells transiently transfected with either vitellogenin ERE or LHβ ERE reporters were characterized. A follow-up study by Hall et al. confirmed these findings by demonstrating that the transcriptional activities of EDCs are mediated in a promoter-specific manner, and that estrogen-responsive promoters communicate differentially with ERα and ERβ by influencing the receptor structures, thus resulting in a diversity of transcriptional responses [13]. Specifically, HepG2 (human hepatoma), Ishikawa (human endometrial carcinoma) or UCI107 (human ovarian carcinoma) cells were co-transfected with ERα or ERβ expression plasmids together with different firefly luciferase reporter constructs. The reporter constructs contained either three copies of the vitellogenin ERE, the estrogen-responsive promoters from pS2, lactoferrin, or complement 3 (C3). Notably, BPA action through ERβ was significantly influenced by the nature of the promoter utilized. In particular, BPA displayed remarkably high potency on pS2 promoter through ERβ, with higher ERα-mediated activity at vitellogenin and lactoferrin promoters [13]. The authors concluded that different estrogen-responsive promoters may influence the ability of the ER to form a transcriptionally active conformation, providing a mechanism for the promoter-specific activities observed. These studies are in agreement with Kurosawa et al. who demonstrated that BPA exhibits estradiol-like agonist activity via ERβ, and has mixed agonist and antagonist activity in some types of cells via ERα [16]. Thus, for a given cell type, the activity of BPA likely depends in part on the levels of different ER isoforms and variants expressed in the sensitive cell or tissue.

Although altered ER-regulated gene expression through the ERE is a major mechanism of BPA action, recently it was shown by Singleton et al., that high concentrations of BPA could regulate expression of a distinct set of genes involved in growth and development, such as HOXC1 and C6, Wnt5A, Frizzled, TGFβ-2, and STAT inhibitor 2 in MCF-7 breast cancer cells stably expressing HA-tagged ERα [98]. When HOXC6 mRNA expression was examined by quantitative RT-PCR, three independent ERα-HA cell cultures showed down-regulation (2.2–2.8-fold) following 3 h treatment with 1 μM BPA. HOX genes encode transcription factors containing the highly conserved 183-base-pair homeodomain sequence and are critically important for embryonic development [99]. Although biological functions of the HOXC6 gene product are not well defined, targeted disruption of this gene in mice has indicated a role in mammary ductal development [100]. In contrast to Singeleton's study, Buterin et al. demonstrated that exposure of MCF-7 and T47D breast

cancer cells to pharmacological concentrations of BPA (10 μM) resulted in identical transcriptional effects as induced by physiological concentrations of estradiol [101]. These authors suggest that endocrine disrupting compounds may act in conjunction with endogenous steroid hormones to induce additive effects in target tissues. Another study demonstrated that exposure to low BPA doses (ranging from 0.1 nM to 25 μM) increased HOXA10 protein expression in the uterus [102]. Thus, the results from these studies reveal novel non-ERE-regulated effects on gene expression by BPA, suggesting a role for additional transcriptional mechanisms of BPA action in reproductive and mammary gland development.

In order to identify tissues and cells that contain functionally active ERs, and to define their ability to respond to different ligands, Nagel et al. developed ER action indicator (ERIN) transgenic mice (strain C57BL/6-SJL). This model system integrates the upstream requirements of ER action, including the receptor, ligand, and accessory comodulators with activation of the ER, resulting in expression of the enzyme β-galactosidase (β-gal), which allows for enzymatic amplification of the estrogenic signal and histological localization of its activity [103]. In these studies, BPA elicited a distinct cellular profile of gene expression, similar to SERMs. BPA stimulated ER transcriptional activity at a dose of 800 μg/kg (40% efficacy as compared to DES-maximum) and exhibited 20% of DES-induced maximum response at a low dose of 25 μg/kg; however, BPA had little uterotropic activity in this system [103]. In contrast, in the same model, the well-established SERM tamoxifen significantly increased uterine weight at 25 mg/kg, while minimally inducing ER reporter activity in this tissue [103]. The authors suggest that these differential actions of BPA may be due to this ligand's selective ER modulatory activities, resulting in tissue-selective ER activity.

In agreement with Nagel and colleagues, a recent study by Hong et al., also demonstrated distinct expression patterns of responsive genes following exposure to either estradiol or endocrine disrupting compounds, including BPA, thus further implicating differential effects of estradiol versus BPA in reproductive tissues [104]. Based on the studies described above, it is likely that a BPA-induced ER conformation results in recruitment of cell- and tissue-specific co-modulatory factors responsible for the differential responses to this ligand.

9. BPA effects on cells and tissues of the reproductive system

The findings described above raise further questions about the differential roles of endocrine disruptors in human development and reproduction. Based on recent molecular studies, it was proposed by Welshons et al., that BPA is a SERM [4]. An investigation into SERMs/endocrine disruptors confirmed that BPA effects in uterus, vagina and the bone are not purely estrogenic. Utilizing low doses of BPA (37 and also 370 μg/kg/body weight) in Sprague–Dawley rats, Seidlova-Wuttke et al. found no significant effect on uterine weight, but a clear increase in epithelial thickness, which was contrasted by a reduction in the myometrial thickness [11]. Following 3 months treatment with 37 μg/kg body weight/day BPA, a modest reduction in endometrial thickness was observed. These effects were at much lower doses than used in previous experiments [105]. Hence, the uterotrophic assay in this study revealed subtle, low-dose effects of BPA that cannot be defined as "classical" estrogenic effects. Seidlova-Wuttke et al. also used quantitative RT-PCR analysis to characterize the effects of BPA on ERα, ERβ and C3 transcripts in the uterus. It was found that estradiol had no effect on ERα mRNA, decreased ERβ mRNA, and greatly increased C3 mRNA expression. Interestingly, these effects were not mimicked by BPA. In fact, BPA exposure increased ERα, did not effect ERβ, and decreased C3 mRNA expression [11]. Treatment with both 37 and 370 μg/kg BPA also resulted in significant reductions of bone mineral density, which was in stark contrast to estradiol, whose action is essential for maintenance of normal bone structure. The authors suggest that BPA-induced effects on bone metabolism were through mechanisms other than those regulated by ERα or ERβ. It is known that the bone expresses the aryl hydrocarbon receptor (AhR) [106,107], but whether BPA serves as a ligand for the AhR still needs to be established. While the rapid signaling effects of BPA on bone cells have not been investigated, sex-specific rapid effects of estradiol on intracellular signal transduction in osteoblasts are well documented [108], and the signaling network for the rapid actions of the phytoestrogen diadzein has also been described [109]. Thus, it is likely that BPA acts via membrane ER isoforms, contributing to regulation of bone physiology. Additionally, BPA may modulate the available co-regulatory factors (enhancers or repressors of ER) in the uterus and bone. Clearly, further investigations are needed to decipher BPA's molecular mode of action in these tissues.

Based on studies described in this section, it is evident that some actions of BPA are compatible with its characterization as a SERM. However, mounting research has demonstrated that BPA cannot be defined exclusively as a SERM. The actions of BPA extend beyond its ability to mimic, enhance, or inhibit the activity of endogenous estrogens at the estrogen receptor: as described below, BPA is not ER "selective," because it also binds and affects other nuclear receptors, including the androgen and thyroid receptors.

10. BPA effects related to fertility

Numerous studies have implicated BPA and other EDCs in reduced female fertility and the role of estrogens in oocyte development is a topic of considerable research interest [110]. It is known that theca cells of the late-stage follicle express both the ERα and ERβ, whereas the granulosa cells express predominantly ERβ. Accordingly, BPA has the potential to affect both theca and granulosa cells, however the degree to which the differential expression of ERα and ERβ, and the differences in receptor affinity for BPA might impact these ovarian cells is unknown [6–8,11].

In studies using a primary culture system of human endometrial cells, inhibition of cell growth was observed following exposure to 1 μM BPA [25]. Granulosa cells express ERs and are essential for ovarian follicullogenesis and steroidogenesis (reviewed in [111]). The impact of BPA, at low 0.1 nM dose,

on murine ovarian granulosa cells was investigated by Xu et al., who found that this low and physiologically relevant dose of BPA decreased granulosa cell viability by inducing apoptosis and G2-to-M cell cycle arrest, suggesting that granulosa cells are highly sensitive to BPA [112].

In contrast, others have reported that pharmacological concentrations of 17β-estradiol (1 μg/mL) exert antiapoptotic effect on ovarian granulosa cells [113,114]. Based on previous findings demonstrating that BPA could partially antagonize the effects of 17β-estradiol at ERα, but not ERβ [115], Xu et al. (2002) suggested that their results can be explained by BPA's ability to antagonize antiapoptotic effect of intrinsic estrogens produced by granulosa cells. While these researchers showed that BPA increased the expression of Bax and concomitantly decreased the expression of Bcl2 at both protein and mRNA levels, the precise mechanism of BPA action to induce apoptosis in granulosa cells still remains to be determined.

An *in vivo* study by Hunt et al., found that chronic low-dose exposure to BPA causes meiotic aneuploidy in mouse oocytes, thus implicating BPA in disruption of female meiosis. Specifically, this study demonstrated that low oral BPA exposure to BPA at 20, 40, or 100 μg/kg body weight/day for 6–8 days preceding the analysis, adversely affects oocyte chromosome segregation [116]. The molecular mechanism(s) through which BPA may exert effects on meiosis is an important research topic that is in need of further investigation.

11. BPA actions at the androgen receptor

Several studies using *in vitro* yeast-based assays revealed that BPA exhibits strong anti-androgenic activity [23,29]. Using ligand competition assays, it was demonstrated that BPA could compete with 5α-dihydrotestosterone (DHT) for binding at the androgen receptor (AR) with an IC_{50} (the concentration of chemical required to reduce the specific DHT binding by 50%) value of 2.14 μM [24,29]. By utilizing an elegant yeast two-hybrid system, Lee et al. demonstrated that BPA affects AR interaction with its co-regulator ASC1, to subsequently modulate AR-transactivation. In addition, analysis of GFP-AR fusion protein in the presence of BPA showed inhibition of AR nuclear localization [29]. These findings suggest that BPA can affect multiple steps of the activation and function of AR, including modulation of AR interaction with its co-regulator ASC1 and inhibition of the binding of native androgens to the AR, resulting in antagonistic action of BPA on the AR.

A recent study by Xu et al. has confirmed that BPA can act as an anti-androgen in a mammalian system [27]. In this study, a human androgen receptor (hAR) reporter gene assay using African monkey kidney cell line CV-1 transiently transfected with the reporter gene plasmid pMMTV-CAT was utilized. BPA showed significant inhibitory effects on the transcriptional activity induced by DHT with an IC_{50} value of ~0.8 μM; the highest anti-androgenic activity of any endocrine-disrupting compounds tested [27].

Using two stably transfected Chinese hamster ovarian cell lines (CHO-K1 cells) as an AR-EcoScreen for androgenic activity and c-luc for cell toxicity evaluation, Satoh et al. investigated *in vitro* hormonal activity of BPA and its derivates bisphenol A diglycidyl ether (BADGE) and bisphenol F diglycidyl ether (BFDGE) [12]. These BPA derivatives are used as interior coating for food cans and components of some dental sealants. One of the CHO-K1 derived cell lines stably expressed androgen-inducible luciferase, while the other stably expressed luciferase independent of androgen induction. It was found that BPA, BADGE and BFDGE exhibited anti-androgenic activity, suggesting that chlorohydroxy compounds such as BADGE and BFDGE act as androgen antagonist through binding to the AR. BPA had a potent binding affinity for AR, and inhibited specific testosterone binding to the receptor in a concentration-dependent manner. The IC_{50} value for AR was 7.9 μM, and complete inhibition appeared at 19 μM [12]. A follow-up study by Roy et al., using CHO-K1 stably expressing hAR and a mouse mammary tumor virus-neomycin-luciferase reporter gene, confirmed that BPA is an anti-androgenic compound with an IC_{50} = 19.6 μM [22]. However, a study by Gaido et al., failed to demonstrate antiandrogenic activities of BPA in HepG2 cells [117].

Thus, research results show that BPA can act as AR antagonist in some cell systems. Once bound to AR, the AR/BPA complex may prevent endogenous androgens from regulating androgen-dependent transcription. The specificity of AR–ligand interaction may be critical in eliciting adverse effects on male reproductive system. However, discrepancies between different research groups on antiandrogenic activities of BPA exist – this area of research requires, further investigation.

Similar to endogenous estrogens, several endocrine disrupting compounds have been hypothesized to alter androgen action or prostate growth and development [118]. In fact, developmental (*in utero* or neonatal) exposure to low dose BPA and other compounds, including polychlorinated bisphenols (PCBs), hexachlorobenzene (HCB) and diethylstilbestrol (DES), has been reported to increase prostate size and weight, suggesting that these agents influence early prostate growth patterns [119–125]. Although the mechanism of BPA action in the prostate is yet to be determined, recent studies have shown its ability to influence AR function and activity.

Given the importance of the AR for prostate development and tumorigenesis, several studies monitored effects of BPA exposure on AR expression and function *in vivo*. While two studies demonstrated that both BPA (25 or 250 μg/kg/day) and estradiol (0.02–2 μg/kg) increased AR expression in dorsolateral and ventral prostate lobes [123,126], a report by Ramos et al. observed no change in the ventral prostate upon BPA exposure [122]. However, the differentiation pattern of periductal stromal cells of the ventral prostate was found altered by prenatal exposure to BPA at 25 or 250 μg/kg/day [122]. Together, the mitogenic effect of BPA on prostate cells often correlates with changes in the AR status, although future studies are needed to elucidate the relationship between BPA and AR in the developing prostate.

The impact of BPA on AR regulation in prostate cancer cells and the response to androgen-deprivation therapy (ADT) has also been examined. Because of the androgen dependence of prostatic adenocarcinomas, the mainline therapeutic intervention in advanced prostate cancer is ADT. Although ADT