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Chapel Hill bisphenol A expert panel consensus statement: Integration of mechanisms, effects in animals and potential to impact human health at current levels of exposure

Keywords: Bisphenol A; *In vitro*; *In vivo*; Rat; Mouse; Aquatic animal; Cancer; Low dose; Non-monotonic dose–response curves; Developmental programming

1. Introduction

This document is a summary statement of the outcome from the meeting: “Bisphenol A: An Examination of the Relevance of Ecological, *In vitro* and Laboratory Animal Studies for Assessing Risks to Human Health” sponsored by both the NIEHS and NIDCR at NIH/DHHS, as well as the US-EPA and Commonwealth on the estrogenic environmental chemical bisphenol A (BPA, 2,2-bis(4-hydroxyphenyl)propane; CAS# 80-05-7). The meeting was held in Chapel Hill, NC, 28–30 November 2006 due to concerns about the potential for a relationship between BPA and negative trends in human health that have occurred in recent decades. Examples include increases in abnormal penile/urethra development in males, early sexual maturation in females, an increase in neurobehavioral problems such as attention deficit hyperactivity disorder (ADHD) and autism, an increase in childhood and adult obesity and type 2 diabetes, a regional decrease in sperm count, and an increase in hormonally mediated cancers, such as prostate and breast cancers. Concern has been elevated by published studies reporting a relationship between treatment with “low doses” of BPA and many of these negative health outcomes in experimental studies in laboratory animals as well as *in vitro* studies identifying plausible molecular mechanisms that could mediate such effects. Importantly, much evidence suggests that these adverse effects are occurring in animals within the range of exposure to BPA of the typical human living in a developed country, where virtually everyone has measurable blood, tissue and urine levels of BPA that exceed the levels produced by doses used in the “low dose” animal experiments.

Issues relating to BPA were extensively discussed by five panels of experts prior to and during the meeting, and are summarized in five reports included in this issue: (1) human exposure to bisphenol A (BPA) [1]; (2) *in vitro* molecular mechanisms of bisphenol A action [2]; (3) *in vivo* effects of bisphenol A in laboratory animals [3]; (4) an ecological assessment of bisphenol A: evidence from comparative biology [4]; (5) an evaluation

of evidence for the carcinogenic activity of bisphenol A [5]. Further discussion occurred at the meeting where participants from the panels were reorganized into four breakout groups. The consensus statements from the meeting are presented below.

The definition of “low dose” of BPA at this meeting used the same two criteria established at a prior NIH meeting concerning the low dose endocrine disruptor issue [6]: (1) for laboratory animal studies “low doses” involved administration of doses below those used in traditional toxicological studies conducted for risk assessment purposes. For BPA the lowest dose previously examined for risk assessment purposes was 50 mg (kg<sup>-1</sup> day<sup>-1</sup>) in studies with rats and mice. The 50 mg (kg<sup>-1</sup> day<sup>-1</sup>) dose is the currently accepted lowest adverse effect level (LOAEL) that was used to calculate the current US-EPA reference dose (the daily dose that EPA calculates is safe for humans over the lifetime) of 50 µg (kg<sup>-1</sup> day<sup>-1</sup>). The current reference dose is thus based on “high dose” experiments conducted in the 1980s [7]. (2) “Low dose” also refers to doses within the range of typical human exposure (excluding occupational exposures). For purposes of this meeting, the published literature that was reviewed met both of these criteria for being considered within the “low dose” range.

Hundreds of *in vitro* and *in vivo* studies regarding the mechanisms and effects of low doses of BPA, as well as studies of biomonitoring and sources of exposure, have been published in peer reviewed journals over the last 10 years, since the first “low dose” BPA *in vivo* studies were published [8–10]. The meeting was convened specifically to integrate this relatively new information. This task required the combined expertise of scientists from many different disciplines, and care was taken to ensure that participants covered these diverse areas.

BPA is a high-volume (>6 billion pounds per year) production chemical used to make resins and polycarbonate plastic [11]. Of particular concern is the use of BPA in food and beverage plastic storage and heating containers and to line metal cans. In addition, potential environmental sources of BPA contamination are due to use in dental fillings and sealants [12], losses at the production site [13], leaching from landfill [14,15], and presence in indoors air [16].

BPA has become a chemical of “high concern” only in recent years, even though BPA was shown to stimulate the reproductive

Abbreviations: ADHD, attention deficit hyperactivity disorder; BADGE, bisphenol A diglycidyl ether; BIS-DMA, bisphenol A dimethacrylate; BIS-GMA, bisphenol A glycerolate dimethacrylate; BPA, bisphenol A; ER, estrogen receptor

system in female rats and thus to be an “environmental estrogen” in 1936 [17], long before it was used as the monomer to synthesize polycarbonate plastic and resins in the early 1950s. However, more recent evidence has shown that BPA also exhibits other modes of endocrine disruption in addition to binding to estrogen receptors, such as alterations in endogenous hormone synthesis, hormone metabolism and hormone concentrations in blood. BPA also results in changes in tissue enzymes and hormone receptors, and interacts with other hormone-response systems, such as the androgen and thyroid hormone receptor signaling systems. While BPA was initially considered to be a “weak” estrogen based on a lower affinity for estrogen receptor alpha relative to estradiol [18], research shows that BPA is equipotent with estradiol in its ability to activate responses via recently discovered estrogen receptors associated with the cell membrane [19–22]. It is through these receptors that BPA stimulates rapid physiological responses at low picogram per ml (parts per trillion) concentrations.

## 2. Purpose and organization of the BPA meeting

### 2.1. Topic-focused expert panels

To address the strength of the evidence regarding the published BPA research, an organizing committee was formed, and five panels of experts from different disciplines were established. Each panel had a chair or co-chairs and included a scientist who agreed to be primarily responsible, along with the chair, for preparing a preliminary draft of the panel’s report. A web site was established on which all of the available electronic files of articles concerning BPA were posted, along with other pertinent information relating to the meeting. Prior to the meeting, the panel members began working on draft reports and communicated via electronic media and telephone conference calls. The resulting preliminary report from each panel was posted on the web site and distributed at the meeting for all participants to read. After the meeting, each panel completed a manuscript that is a part of this meeting report. These five panel reports were peer reviewed using the normal manuscript submission process to *Reproductive Toxicology*. The following specific concerns about BPA led to the five expert panels being established:

- (1) Leaching of BPA occurs from the resin lining of metal cans and from plastic food and beverage containers under conditions of normal use. BPA is also detected in water and air samples.
- (2) Parts per billion (ppb) levels of BPA that are unconjugated (not metabolized and thus biologically active) are detected in human blood and tissues in different countries, and these levels appear to be higher than blood levels that would be present in animals exposed to the US-EPA reference dose.
- (3) BPA causes a wide range of adverse effects at “low doses” that are below the US-EPA reference dose in animals, both terrestrial and aquatic.
- (4) There is evidence from *in vitro* mechanistic studies that indicates the potential for disruption of human and animal cell

function at concentrations of BPA far below unconjugated levels typically found in human blood and tissues.

- (5) There is evidence that at very low doses, BPA may be carcinogenic or increase susceptibility to cancer in animals.

The five panels each addressed a different topic related to their specific area of expertise with BPA and prepared a panel report that included documentation of the relevant published studies:

- Panel (1) Sources and amounts of human exposure to BPA as well as pharmacokinetics.
- Panel (2) *In vitro* studies related to the molecular mechanisms that mediate responses to BPA with an emphasis on studies using low doses.
- Panel (3) *In vivo* studies of BPA at “low doses” in laboratory animals.
- Panel (4) *In vivo* studies of BPA in aquatic wildlife and laboratory animals.
- Panel (5) Relationship of BPA to cancers.

The purpose of the 3-day meeting was to provide an opportunity for members of the different panels to interact with each other to integrate information from different disciplines concerning low dose effects of BPA after each panel of experts had prepared a report in its specific area. The agenda of the meeting was designed to allow the members of the five panels to have time to discuss the information in their panel reports and finalize statements about the strength of the evidence for the literature that the panel had reviewed.

### 2.2. Integration of information by breakout groups

For the second part of the meeting the focus was on integrating the information from each of the panel reports. This was accomplished by assigning panel members to one of four breakout groups. The four replicate breakout groups were established using the following criteria, such that each breakout group should have

- (1) At least two members from each of the five panels.
- (2) A person from each panel who had published on BPA.
- (3) A person with general knowledge of endocrine disruption research or endocrinology, but who had not necessarily published on BPA.
- (4) A person with experience in the process of reaching consensus.
- (5) A mixture of junior and senior investigators.

The charge to the replicate breakout groups was to individually integrate the information relating to the following four issues:

- Issue (1) Determine the degree to which the findings on BPA mechanisms of action identify mechanisms and bioactive doses that explain results of the studies reported by the panel on *in vivo* laboratory animal studies. Determine the strength of the evidence for plausible mechanisms mediating *in vivo* effects at low doses. In

addition, identify any *in vivo* findings that are unexpected based on the *in vitro* literature.

- Issue (2) Assess the degree to which ecological studies with wildlife are consistent with laboratory studies in similar and different species. For example, determine the similarity of exposure levels and types of responses seen in wildlife and laboratory animals.
- Issue (3) Discuss the degree to which the low doses of BPA used in laboratory animal studies relate to the levels detected in human serum and tissues (including urine).
- Issue (4) Assess the importance of life stage in the pharmacokinetics of BPA, levels of exposure to BPA, and the health effects of BPA in animals and humans.

### 3. Findings submitted by the four breakout groups

The reports from the breakout groups are presented below. The four breakout groups conducted a critical examination of the published research on BPA in relation to the four topics described above. Each of the breakout groups identified areas of knowledge and research gaps and made suggestions for future directions of research. In addition, each group identified which of the following two categories applied to specific outcomes:

- “We are confident of the following”: this category applied when there were findings reported in multiple papers from multiple labs that were in agreement. There should have been no papers reporting conflicting findings, unless there were flaws in those papers, in which case the flaw(s) should have been identified.
- “We believe the following to be likely but requiring confirmation”: This category applied when there were multiple consistent findings from one lab, or there may have been some conflicting reports along with reports of significant findings.

### 4. Levels of confidence for published BPA findings

The responses from the four different breakout groups were integrated together and organized based on levels of confidence. The criterion for a statement being included in a category was that there had to be consensus among all four of the breakout groups about the statement.

#### 4.1. Based on existing data we are confident of the following

##### 4.1.1. Issue 1: *In vitro* mechanistic research—laboratory animal research connection

1. *In vitro* studies have provided two routes of plausibility for low dose *in vivo* effects of BPA. These include binding to nuclear estrogen receptors that regulate transcription as well as estrogen receptors associated with the cell membrane that promote calcium mobilization and intracellular signaling. Receptors associated with the cell membrane are more sensitive to BPA than the nuclear receptors. Actions mediated by membrane associated receptor signaling may underlie much

of the low dose BPA phenomena (effects have been reported at doses as low as 1 pM or 0.23 ppt). This increases the plausibility of effects at low doses, which are within the range of environmentally relevant doses (human and wildlife levels of exposure).

2. *In vitro* mechanistic information has informed us that exposing tissues to only an extremely narrow range of doses of BPA may lead to erroneous conclusions. Non-monotonic dose–response curves are encountered frequently in basic endocrinological research, and numerous examples have been reported for BPA reviewed in Refs. [18,23,24]. Because of this animal experiments on unstudied systems must avoid narrow dose ranges, especially the use of only a few very high doses. Thus, testing one or two doses and concluding that there are no effects is inappropriate. At somewhat higher doses than are required for estrogen receptor (ER)-mediated responses, BPA also interacts with androgen and thyroid hormone receptors, making predictions of effects at different doses very complex.
3. *In vitro* studies can dissect mechanisms of complicated effects observed *in vivo*. The proposed potential mechanisms acting *in vitro* and *in vivo* are the same, involving estrogen receptor mediated (nuclear- and membrane-associated) actions. However, specific effects are dose and cell/tissue specific. In addition, there are *in vivo* processes that are not reflective of currently known mechanisms that have been identified *in vitro*. This is due to previously unknown mechanisms as well as the complexity (due to interactions among cell and tissue types) of *in vivo* systems.

##### 4.1.2. Issue 2: Wildlife—laboratory animal research connection

1. BPA is found in the environment: aquatic, terrestrial and air.
2. Studies of wildlife demonstrate estrogenic responses that are similar to responses seen in laboratory animals. Specifically, reductions in spermatogenesis are seen in wildlife at ecological concentrations of BPA, and these effects are also seen in controlled laboratory studies with BPA. In addition, vitellogenin response is a common biomarker in non-mammalian wildlife and laboratory species for BPA-induced estrogen receptor activation as well as activation by other estrogens.
3. BPA exposure induces similar effects in reproductive systems in wildlife and experimental animal model systems, but concentrations used in experiments involving wildlife species are often higher than environmental exposures. There are conditions in the environment, such as landfill leachates and effluent outflow that cause episodic exposure of field populations to elevated doses of BPA.
4. Responses in a variety of vertebrate wildlife species are qualitatively consistent with controlled laboratory studies with BPA. Thus, animals in the wild show evidence of harm, and controlled laboratory studies with model aquatic animals (i.e., medaka, zebrafish, and fathead minnows) are consistent with observations made in wildlife species. Low dose effects of BPA (low ppb range) have been observed in many of these animals.

5. The similar effects observed in wildlife and laboratory animals exposed to BPA predict that similar effects are also occurring in humans.

#### 4.1.3. Issue 3: Laboratory animal research—human exposure connection

1. Human exposure to BPA is widespread.
2. Human exposure to BPA is variable, and exposure levels cover a broad range [central tendency for unconjugated BPA: 0.3–4.4 ng ml<sup>-1</sup> (ppb)] in tissues and fluids in fetuses, children and adults.
3. Because the current published literature states that there is a linear relationship between administered dose and circulating levels of BPA in animal studies, this allows circulating levels at lower administered doses to be predicted in experimental animals based on the results from studies in which higher doses were administered.
4. All of the currently published metabolic studies in rats predict circulating BPA levels after acute low dose oral exposures at blood levels less than or equal to 2 ng ml<sup>-1</sup> (ppb), which is the approximate median and mean unconjugated circulating BPA level in humans. Therefore, the commonly reported circulating levels in humans exceed the circulating levels extrapolated from acute exposure studies in laboratory animals.
5. BPA levels in the fetal mouse exposed to BPA by maternal delivery of 25 µg kg<sup>-1</sup>, a dose that has produced adverse effects in multiple experiments, are well within the range of unconjugated BPA levels observed in human fetal blood.

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

1. Sensitivity to endocrine disruptors, including BPA, varies extensively with life stage, indicating that there are specific windows of increased sensitivity at multiple life stages. Therefore, it is essential to assess the impact of life stage on the response to BPA in studies involving wildlife, laboratory animals, and humans.
2. Developmental windows of susceptibility are comparable in vertebrate wildlife species and laboratory animals.
3. BPA alters “epigenetic programming” of genes in experimental animals and wildlife that results in persistent effects that are expressed later in life [25]. These organizational effects (functional and structural) in response to exposure to low doses of BPA during organogenesis persist into adulthood, long after the period of exposure has ended. Specifically, prenatal and/or neonatal exposure to low doses of BPA results in organizational changes in the prostate, breast, testis, mammary glands, body size, brain structure and chemistry, and behavior of laboratory animals.
4. There are effects due to exposure in adulthood that occurs at low doses of BPA. Substantial neurobehavioral effects and reproductive effects in both males and females have been observed during adult exposures in laboratory animals.
5. Adult exposure studies cannot be presumed to predict the results of exposure during development.

6. Life stage impacts the pharmacokinetics of BPA.

#### 4.2. We believe the following to be likely but require confirmation

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

1. BPA metabolism occurs in cell culture systems, and although there are differences between cell types, there is less variability than in the entire animal. Metabolism is an important issue for humans and wildlife field populations with large genetic variability. Individual differences in BPA pharmacokinetics allow for underlying variability within a population, and may allow for the identification of sensitive and insensitive subpopulations.
2. The activity of various enzymes involved in drug, chemical, and hormone metabolism, as well as protection against oxidative stress, are programmed by hormone levels during sensitive periods in development. Developmental alterations in hormonal programming (activation or inhibition) may thus affect metabolism of BPA and other hormones and chemicals. Direct interaction of BPA with enzymes in cells has only been reported at higher doses than expected for human exposures.
3. The set of genes regulated by BPA is expected to differ among doses. Therefore, different doses of BPA do not produce different effects only due to a quantitative difference in the expression of the same set of genes.
4. Differential expression of estrogen receptor subtypes ( $\alpha/\beta$ ; variant isoforms), and protein–protein interactions (estrogen receptor homo- and hetero-dimer formation, co-regulators, etc) modulate the cellular response to BPA. Direct actions of BPA on intracellular signal transduction modulate some cellular responses, which are similarly dependent on differential expression and protein–protein interactions.
5. Bioactive doses can be mathematically modeled, but further model refinement and experimental confirmation is required.
6. Other mechanisms (androgen receptors, thyroid hormone receptors) may be relevant for BPA action, but at higher doses than for estrogen responsive mechanisms.

##### 4.2.2. Issue 2: Wildlife—laboratory animal research connection

1. The effects observed in laboratory animals could be present in wildlife, because the low doses being studied in laboratory animals are now relevant to environmental exposure levels of wildlife. The similarities in mechanisms that have been observed between different species suggest that field populations will respond to the same low levels.
2. Measurements of vitellogenin production in fish have established that there are exogenous estrogenic signals in their environment. BPA may be contributing to this phenomenon as it enters natural water systems after leaching from landfills and due to plastic debris in water.
3. Delayed spawning is seen in male and female fish, which may relate to observed changes in estrous cyclicity in mammals in laboratory experiments.

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.

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

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### 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).

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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  $T_4$ .

**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 [<sup>125</sup>I]T<sub>4</sub>, 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.

TABLE I

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
	<i>nmol/mg protein/min</i>		<i>nmol/mg protein/min</i>	
7-Benzyloxyresorufin	0.07 ± 0.01	3.34 ± 0.33*	0.03 ± 0.003	1.08 ± 0.27*
7-Pentoxoresorufin	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.

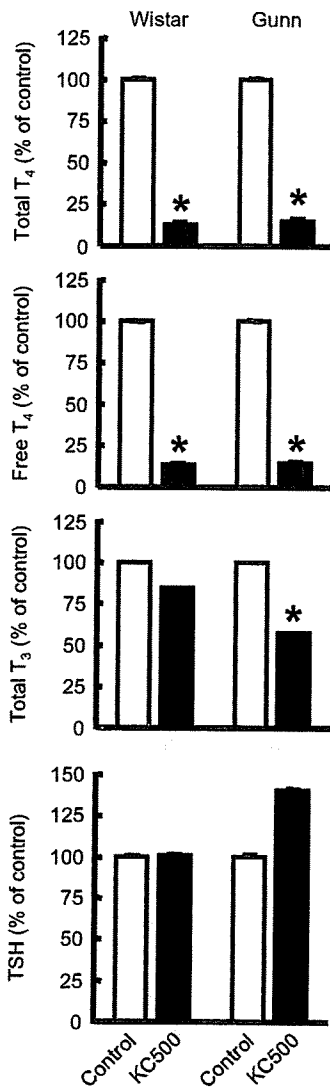


FIG. 1. Effects of KC500 on levels of serum total T<sub>4</sub>, free T<sub>4</sub>, total T<sub>3</sub>, and TSH in Wistar and Gunn rats. Animals were killed 4 days after the final administration of KC500 (10 mg/kg i.p., once daily for 10 days), and levels of serum thyroid hormones were measured as described under *Materials and Methods*. Constitutive levels: total T<sub>4</sub>, 4.29 ± 0.38 (Wistar, n = 5) and 5.80 ± 0.32 µg/dl (Gunn, n = 5); free T<sub>4</sub>, 2.17 ± 0.16 (Wistar, n = 5) and 2.71 ± 0.17 ng/dl (Gunn, n = 5); total T<sub>3</sub>, 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.

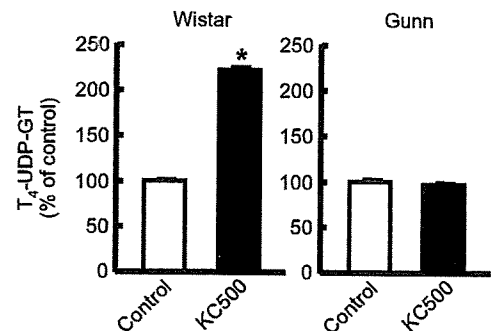


FIG. 2. Effects of KC500 on the activity of hepatic microsomal UDP-glucuronyltransferase in Wistar and Gunn rats. Each column represents the mean ± S.E. (vertical bars) for five to six animals. Constitutive levels: T<sub>4</sub>-UDP-GT, 14.17 ± 1.11 pmol/mg protein/min (Wistar) and 6.36 ± 1.34 pmol/mg protein/min (Gunn). \*, P < 0.01, significantly different from each control.

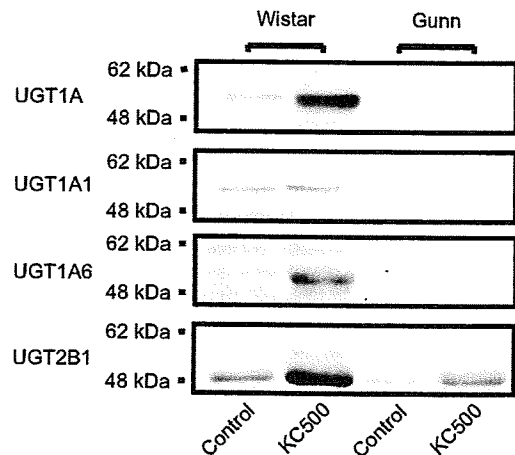


FIG. 3. Representative Western blot profiles for hepatic microsomal UGT isoforms in the KC500-treated Wistar and Gunn rats.

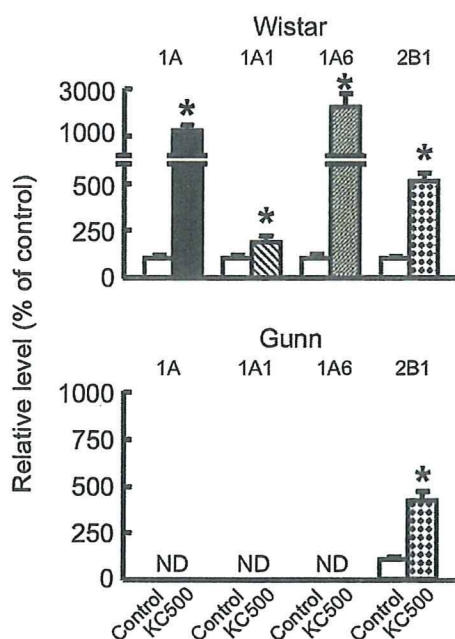


FIG. 4. Effects of KC500 on levels of hepatic microsomal UGT isoforms in Wistar and Gunn rats. The isolated bands responsible for UGT isoforms, which are shown in Fig. 3, were densitometrically quantified as described under *Materials and Methods*. The data are represented as the mean  $\pm$  S.E. (vertical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

**Analysis of serum hormones.** Levels of total T<sub>4</sub>, free T<sub>4</sub>, total triiodothyronine (T<sub>3</sub>), and thyroid-stimulating hormone (TSH) were measured by radioimmunoassay using Total T<sub>4</sub> and Free T<sub>4</sub> kits (Diagnostic Products Corporation, Los Angeles, CA), the Triiodothyronine kit GammaCoat T<sub>3</sub> II (DiaSorin Inc., Stillwater, MN), and the rTSH [<sup>125</sup>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-pentoxoresorufins were determined by the method of Burke et al. (1985).

**Hepatic T<sub>4</sub>-metabolizing enzyme assay.** The activity of microsomal UDP-GT toward T<sub>4</sub> (T<sub>4</sub>-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 [<sup>125</sup>I]T<sub>4</sub> (15  $\mu$ Ci/ml) dissolved in the saline containing 10 mM NaOH and 1% normal rat serum.

**Clearance of [<sup>125</sup>I]T<sub>4</sub> from serum.** The study on the clearance of [<sup>125</sup>I]T<sub>4</sub> from serum was performed according to the method of Oppenheimer et al. (1968). In brief, after the administration of [<sup>125</sup>I]T<sub>4</sub>, 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  $\mu$ l each) were taken from each

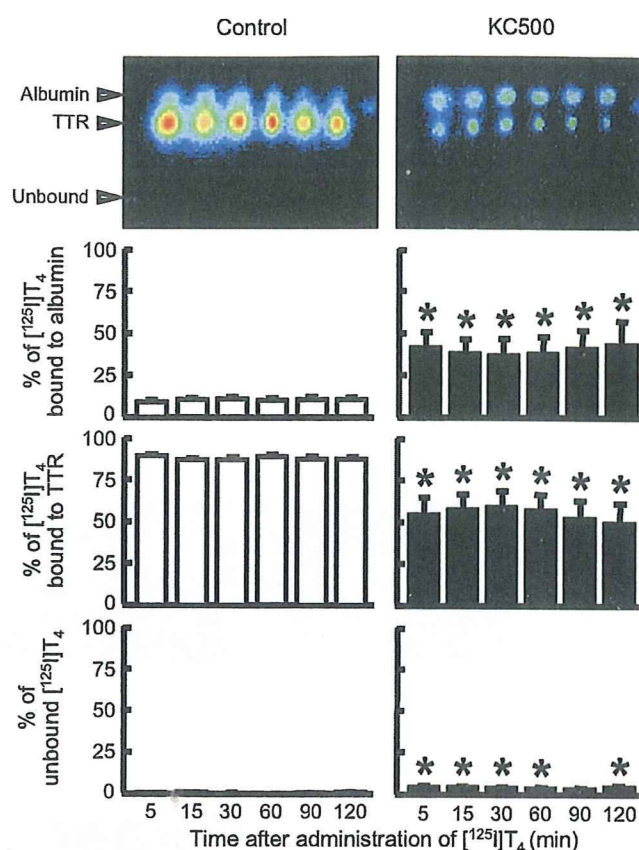


FIG. 5. Effects of KC500 on the binding of [<sup>125</sup>I]T<sub>4</sub> to serum proteins in Wistar rats. Amounts of [<sup>125</sup>I]T<sub>4</sub> bound to the serum proteins were assessed by the method described under *Materials and Methods*. Each column represents the mean  $\pm$  S.E. (vertical bars) for three to six animals. \*,  $P < 0.05$ , significantly different from each control.

serum sample for determining [<sup>125</sup>I]T<sub>4</sub> level by a gamma counter (COBRA II AUTO-GAMMA 5002; PerkinElmer Life and Analytical Sciences).

**Analysis of [<sup>125</sup>I]T<sub>4</sub> bound to serum proteins.** The levels of serum [<sup>125</sup>I]T<sub>4</sub>-albumin and [<sup>125</sup>I]T<sub>4</sub>-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 [<sup>125</sup>I]T<sub>4</sub>, 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 [<sup>125</sup>I]T<sub>4</sub>-albumin and [<sup>125</sup>I]T<sub>4</sub>-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 [<sup>125</sup>I]T<sub>4</sub>.** The study on the tissue distribution of [<sup>125</sup>I]T<sub>4</sub> was performed according to the modified method of Oppenheimer et al. (1968). In brief, at 60 min after administration of [<sup>125</sup>I]T<sub>4</sub> 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 [<sup>125</sup>I]T<sub>4</sub> 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,

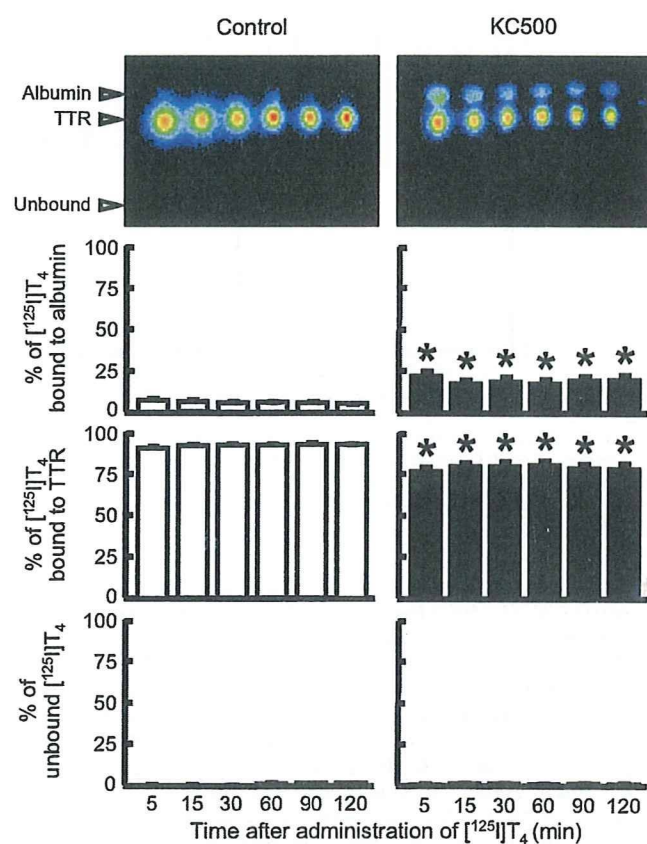


Fig. 6. Effects of KC500 on the binding of [ $^{125}$ I]T $_4$  to serum proteins in Gunn rats. Amounts of [ $^{125}$ I]T $_4$  bound to the serum proteins were assessed by the method described under *Materials and Methods*. Each column represents the mean  $\pm$  S.E. (vertical bars) for four to five animals. \*,  $P < 0.05$ , significantly different from each control.

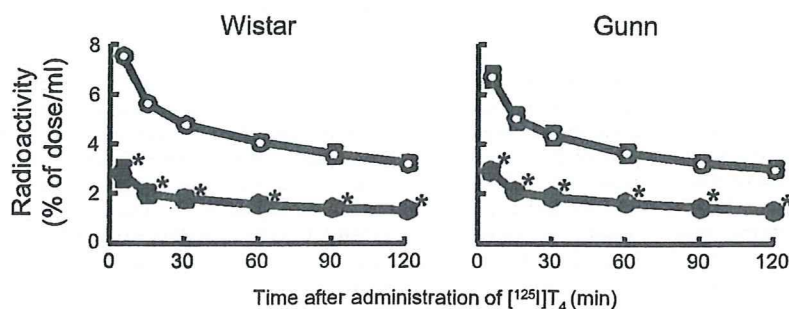


Fig. 7. Effects of KC500 on the clearance of [ $^{125}$ I]T $_4$  from serum in Wistar and Gunn rats. The amount of serum [ $^{125}$ I]T $_4$  was measured at the indicated times after the i.v. administration of [ $^{125}$ I]T $_4$ . Each point represents the mean  $\pm$  S.E. (vertical bars) for four to eight animals. \*,  $P < 0.001$ , significantly different from each control.  $\circ$ , control;  $\bullet$ , KC500.

no significant change in TSH level was observed in either Wistar or Gunn rats.

**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.

**Tissue Distribution of [<sup>125</sup>I]T<sub>4</sub>.** The tissue-to-serum concentration ratio ( $K_p$  value) and distribution level of [<sup>125</sup>I]T<sub>4</sub> in tissue after the administration of [<sup>125</sup>I]T<sub>4</sub> 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 [<sup>125</sup>I]T<sub>4</sub> 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 [<sup>125</sup>I]T<sub>4</sub> dosed (Fig. 9). Likewise, significant increase in accumulation of [<sup>125</sup>I]T<sub>4</sub> was observed in the jejunum (Fig. 9). In addition, significant increases in the liver weight and accumulation level (per g liver) of [<sup>125</sup>I]T<sub>4</sub> 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<sub>4</sub> in several tissues, especially the liver, and resulted in a drastic decrease in the levels of serum total T<sub>4</sub> and

free T<sub>4</sub> in both Wistar and Gunn (UGT1A-deficient) rats. Thus, a decrease in the level of serum total T<sub>4</sub> 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<sub>4</sub> and T<sub>3</sub> 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<sub>4</sub>/T<sub>3</sub>-UDP-GTs.

As a possible explanation for a chemical-induced decrease in serum thyroid hormones, a hepatic T<sub>4</sub>-UDP-GT-dependent mechanism is generally considered, because T<sub>4</sub>-UDP-GT inducers, including PCB, phenobarbital, 3-methylcholanthrene, pregnenolone-16 $\alpha$ -carbonitrile, and clobazam, show strong activities for decreasing the level of serum total thyroid hormones, including T<sub>4</sub> and T<sub>3</sub> (Barter and Klaassen, 1994; Van Birgelen et al., 1995; Miyawaki et al., 2003). However, among the experimental animals treated with a T<sub>4</sub>-UDP-GT inducer, the difference in magnitude of decrease in the level of serum total T<sub>4</sub> is not necessarily correlated with that of hepatic T<sub>4</sub>-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<sub>4</sub>-UDP-GT-independent pathway.

As a possible mechanism for the PCB-induced decrease in serum T<sub>4</sub> 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<sub>4</sub> level in Wistar and Gunn rats were almost the same. Accordingly, the KC500-induced decrease in serum T<sub>4</sub> 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<sub>4</sub>, 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 [<sup>125</sup>I]T<sub>4</sub> after the administration of [<sup>125</sup>I]T<sub>4</sub> 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  $\pm$  S.E. for four to seven animals.

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

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

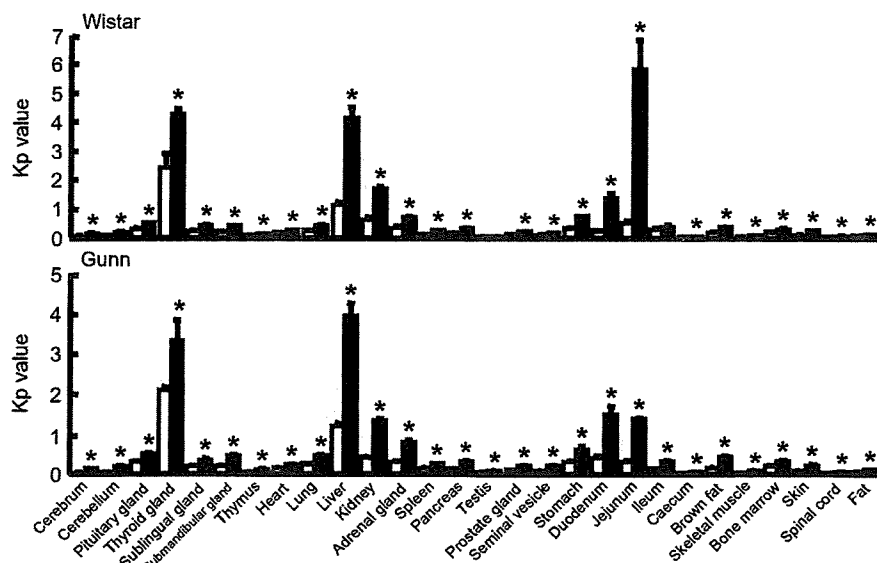


FIG. 8. Tissue-to-serum concentration ratio ( $K_p$  value) of [<sup>125</sup>I]T<sub>4</sub> in various tissues after administration of [<sup>125</sup>I]T<sub>4</sub> to the KC500-pretreated Wistar and Gunn rats. KC500 (10 mg/kg) was given i.p. to animals once daily for 10 days, and then, the animals were administered i.v. [<sup>125</sup>I]T<sub>4</sub>. At 60 min after administration of [<sup>125</sup>I]T<sub>4</sub>, the radioactivity in each tissue was measured. Each column represents the mean  $\pm$  S.E. (vertical bars) for three to six animals. \*,  $P < 0.05$ , significantly different from each control. □, control; ■, KC500.

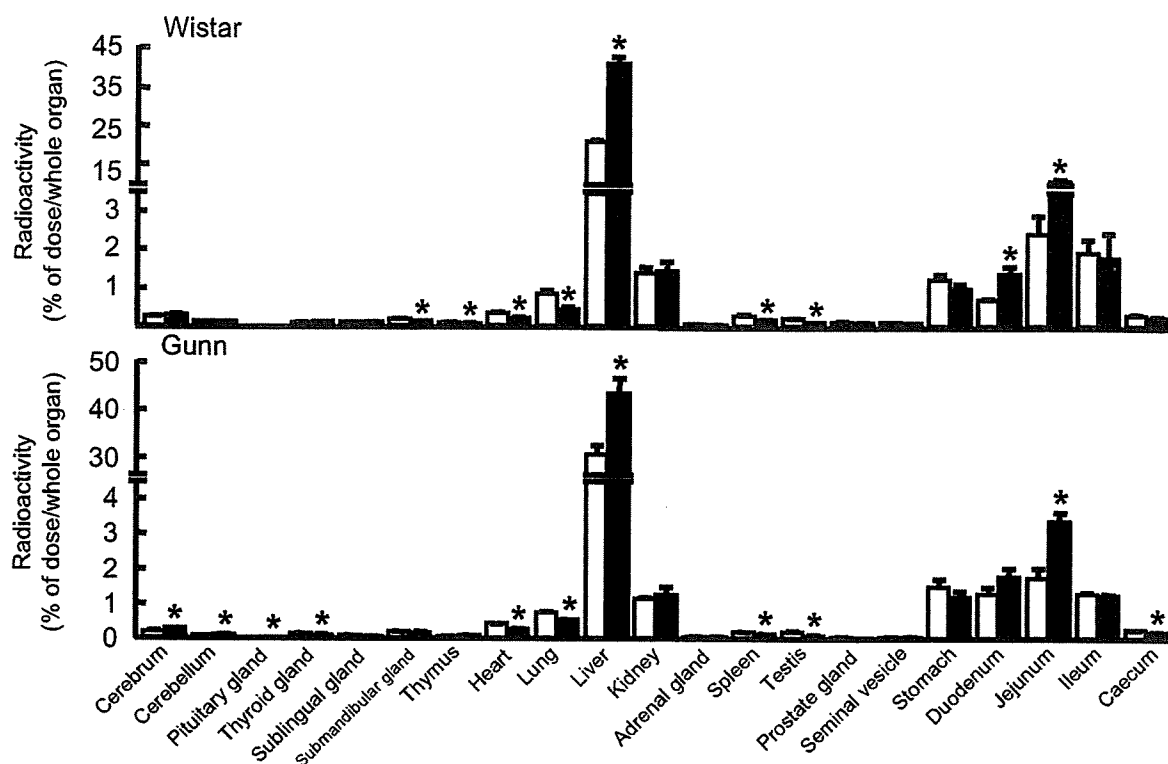


FIG. 9. Tissue distribution of [ $^{125}\text{I}$ ]T $_4$  after administration of [ $^{125}\text{I}$ ]T $_4$  to the KC500-pretreated Wistar and Gunn rats. The experimental conditions were the same as those described in Fig. 8. Each column represents the mean  $\pm$  S.E. (vertical bars) for four to six animals. \*,  $P < 0.05$ , significantly different from each control.  $\square$ , control;  $\blacksquare$ , 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  $\pm$  S.E. for four to six animals.

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

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

TABLE 4

*Accumulation of [ $^{125}\text{I}$ ]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  $\pm$  S.E. for four to six animals.

Animal	[ $^{125}\text{I}$ ]T $_4$	
	Control	KC500
	% of dose/g liver	
Wistar	3.86 $\pm$ 0.18	6.01 $\pm$ 0.24*
Gunn	4.74 $\pm$ 0.43	6.33 $\pm$ 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 [ $^{125}\text{I}$ ]T $_4$  bound to serum TTR and increase in the level of [ $^{125}\text{I}$ ]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 [ $^{125}\text{I}$ ]T $_4$  to KC500-pretreated Wistar and Gunn rats and, thereafter, determined the levels of [ $^{125}\text{I}$ ]T $_4$  in their tissues. In addition, since [ $^{125}\text{I}$ ]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 [ $^{125}\text{I}$ ]T $_4$  in each tissue. Marked increases in the mean total body clearance of [ $^{125}\text{I}$ ]T $_4$  and in the steady-state distribution volume of [ $^{125}\text{I}$ ]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 [ $^{125}\text{I}$ ]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<sup>☆</sup>

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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; 17 $\alpha$ -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.

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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 response; *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 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 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 Protection 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/plusdictionary.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-mediated 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
Masuno et al. 2005 [167]	Mice	3T3-L1 (preadipocyte)	Adipocyte differentiation	Culture medium. Not indicated	6 days	2 × 10 <sup>-5</sup> * 2 × 10 <sup>-6</sup> *	4 × 10 <sup>-6</sup> , 2 × 10 <sup>-5</sup> , 4 × 10 <sup>-5</sup> , 8 × 10 <sup>-5</sup> , 2 × 10 <sup>-6</sup> , 10 <sup>-5</sup> , 2 × 10 <sup>-5</sup> *	LY294002	BPA-derivatives; NP; OP BPA-derivatives
Masuno et al. 2002 [168]	Mice	3T3-L1 (preadipocyte)	Adipocyte differentiation	Culture medium. Not indicated	11 days	2 × 10 <sup>-6</sup> *	10 <sup>-9</sup> , 10 <sup>-8</sup> , 10 <sup>-7</sup> , 10 <sup>-6</sup> , 10 <sup>-5</sup> , 10 <sup>-4</sup>		E2; coumestrol; genistein; daidzein; NP; DEHP
Bone models Kanno et al. 2004 [169]	Mice	MC3T3-E1 (C57BL/6) osteoblast-like	Cell proliferation, Differentiation (ALP activity), Ca <sup>2+</sup> content	Culture medium, ETOH	3–4 days	10 <sup>-13</sup> , 10 <sup>-10</sup> , 10 <sup>-8</sup> *	10 <sup>-9</sup> , 10 <sup>-8</sup> , 10 <sup>-7</sup> , 10 <sup>-6</sup> , 10 <sup>-5</sup> , 10 <sup>-4</sup>		
Breast cancer models Buerin et al. 2006 [101]	Human	T-47D/Luc, MCF7	ERE-promoter activation, Cell proliferation	Culture medium, DMSO	3–24 h, 24 h	10 <sup>-7</sup> (24 h)	10 <sup>-13</sup> to 10 <sup>-5</sup> , 10 <sup>-5</sup>		E2; genistein; PCB34; PCB126
Cappelletti et al. 2003 [170]	Human	T-47D, BT20	Cell growth, ER, PR mRNA (RT-PCR)	Culture medium, Not indicated	6 days, 10, 24, 48 h	10 <sup>-6</sup> , 10 <sup>-8</sup> *	10 <sup>-8</sup> , 10 <sup>-7</sup> , 10 <sup>-6</sup> , 10 <sup>-5</sup> *		E2; 4-tert-OP; 2-OH-biphenyl
Inadera et al. 2000 [60]	Human	MCF7	IL-1 induced MCP-1 expression	Culture media	24 h	5 × 10 <sup>-7</sup> *	10 <sup>-7</sup> , 5 × 10 <sup>-7</sup> , 10 <sup>-6</sup> *	ICI 182,780	E2; NP
Iso et al. 2006 [171]	Human	MCF7, MDA-MB-231	DNA damage (comet tail length assay)	<0.1% ETOH Culture medium	1, 3, 24 h	10 <sup>-6</sup> *	10 <sup>-8</sup> , 10 <sup>-6</sup> , 10 <sup>-4</sup> *	ICI 182,780	E2
Olsen et al. 2003 [15]	Human	MCF7	Cell proliferation, Estrogen-responsive gene expression	<0.1% ETOH Culture medium, ≤0.2% ETOH	6 days, 3 days	5 × 10 <sup>-7</sup> *	10 <sup>-8</sup> , 10 <sup>-7</sup> , 5 × 10 <sup>-7</sup> , 10 <sup>-6</sup> , 10 <sup>-5</sup> *	4-OHTamoxifen; ICI 182,780	E2; tetra-C1-BPA; tetra-B1-BPA, T3; T4; 4-OH-biphenyl; 4,4-OH biphenyl +NP
Recchia et al. 2004 [9]	Human	MCF7, T47D	Cell proliferation, ERE-expression & nuclear localization, ERE-luciferase reporter	Culture medium, Not indicated	6 days, 2 h	10 <sup>-6</sup> *	10 <sup>-6</sup> *	4-OH Tamoxifen	
Samuelsen et al. 2001 [142]	Human	MCF7, T47D	Cell proliferation, PR, pS2-expression	Culture medium, ≤0.1% ETOH	6 days	10 <sup>-7</sup>	10 <sup>-8</sup> , 10 <sup>-7</sup> , 10 <sup>-6</sup> , 10 <sup>-5</sup> , 10 <sup>-4</sup>		E2; B1-BPA derivatives
Singleton et al. 2006 [98]	Human, <i>S. cerevisiae</i>	MCF7, C4-12 (HA-ERα) Ishikawa (endometrial)	Differential mRNA expression, ERE-LacZ	Culture media, ETOH	3 h, 24 h	10 <sup>-6</sup> , 10 <sup>-7</sup>	10 <sup>-7</sup> , 3 × 10 <sup>-7</sup> , 10 <sup>-6</sup> , 3 × 10 <sup>-6</sup>		E2
Vivacqua et al. 2003 [10]	Human	MCF7, MCF7SH	ERE-luciferase, ERα, pS2 expression, Cell growth	Culture medium, Not indicated	20–24, 6 days	10 <sup>-9</sup>	10 <sup>-10</sup> , 10 <sup>-9</sup> , 10 <sup>-8</sup> , 10 <sup>-7</sup> , 10 <sup>-6</sup> , 10 <sup>-5</sup>	4-OH Tamoxifen	E2; 4-NP
Walsh et al. 2005 [74]	Human	MCF7, MDA-MB-231, SKBR-3	Intracellular Ca <sup>2+</sup>	Not indicated, ≤0.001% methanol	0–15 min	10 <sup>-10</sup> *	10 <sup>-10</sup> , 10 <sup>-9</sup> , 10 <sup>-8</sup> , 10 <sup>-7</sup> *	ICI 182,780	<i>o,p'</i> -DDT, DES, 4-tert-OP
Embryonic/development models Iwamura et al. 2003 [146]	<i>Xenopus laevis</i>	Tadpole tail organ culture	Tail length regression (also see <i>in vitro</i> )	Culture medium, ETOH	1–4 days	10 <sup>-6</sup> *	10 <sup>-6</sup> , 10 <sup>-5</sup> , (±10 <sup>-7</sup> T3)		E2
Iwamura et al. 2006 [32]	<i>Xenopus laevis</i>	Tadpole tail organ culture	Tail length regression, TRα, TRβ, RXRγ, mRNA	Culture medium, ETOH	1–5 days	10 <sup>-7</sup> *	10 <sup>-7</sup> , 10 <sup>-6</sup> , 10 <sup>-5</sup> , (±10 <sup>-7</sup> T3)		
Tsutsui et al. 1998 [172]	Hamster (Syrian)	Primary embryo cells	expression (RT-PCR)	Culture medium, 0.6% DMSO	24, 48, 72 h	5 × 10 <sup>-5</sup> *	5 × 10 <sup>-5</sup> , 10 <sup>-4</sup> , 2 × 10 <sup>-4</sup> *		BPF; BP-3; BP-4; BPS
Tsutsui et al. 2000 [173]	Hamster (Syrian)	Primary embryo cells	Cell growth, morphological chromosomal aneuploidy, DNA adduct formation	Culture medium, 0.6% DMSO	24, 48, 72 h	10 <sup>-4</sup> *	5 × 10 <sup>-5</sup> , 10 <sup>-4</sup> , 2 × 10 <sup>-4</sup> *		
Endothelium models Lee et al. 2003 [25]	Human	Female primary endothelial cells	Cell proliferation	Culture medium, Not indicated	24, 48, 72 h	10 <sup>-6</sup> *	10 <sup>-8</sup> , 10 <sup>-6</sup> , 10 <sup>-4</sup> , 2 × 10 <sup>-4</sup> *		Atoclor 1254
Female reproductive tissue models Jin et al. 2005 [174]	Human	BeWo (h30) placental trophoblast	Calcein AM efflux	HBSS 25 mM glucose, ETOH 0.1% Culture medium, 0.1% ETOH	48 h	10 <sup>-7</sup> *	10 <sup>-7</sup> , 10 <sup>-5</sup> , 5 × 10 <sup>-5</sup> *		E2
Xu et al. 2002 [112]	Mice B6C3F1	Primary granulosa cells	Cell viability/apoptosis	Culture medium, 0.1% ETOH	72 h	10 <sup>-10</sup> *	10 <sup>-13</sup> , 10 <sup>-10</sup> , 10 <sup>-7</sup> , 10 <sup>-4</sup> *		
Immune system models Alizadeh et al. 2006 [47]	Mice BALB/c	Primary splenocytes; Sex not specified	Cytokine production, (IL-4; IL-12; IFN-γ ELISA)	<i>In vitro</i> IP-injection, corn oil	8 days	100 mg/kg every other days	100 mg/kg every other days	4-OHTamoxifen; SB203580; wortmannin; GF109203X	DES; NP
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 <sup>-4</sup> , 2.5 × 10 <sup>-5</sup> *	2.5 × 10 <sup>-4</sup> , 2.5 × 10 <sup>-5</sup> *		