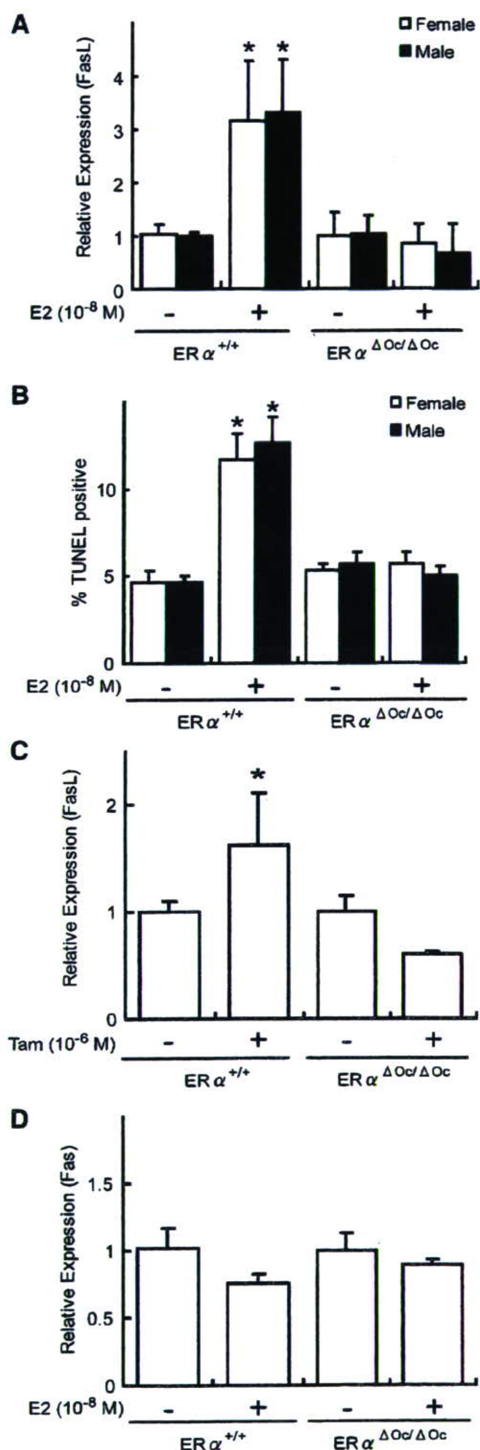

Figure 7. Estrogen-Induced *FasL* Expression and Apoptosis Required ERα in Cultured Osteoclasts

(A) Real-time RT-PCR analysis of *FasL* expression using total RNA obtained from in vitro primary cultured osteoclasts of each genotype at 3 days after RANKL stimulation, treated with or without E2 (10^{-8} M) for 4 hr (*$p < 0.05$ compared to the group treated without E2). Data are represented as mean ± SEM.

(B) Apoptotic cells were defined as those with TUNEL-positive nuclei among TRAP-positive multinucleated primary cultured osteoclasts treated with or without E2 (10^{-8} M) for 12 hr in 96-well plates (*$p < 0.05$ compared to the group treated without E2). Data are represented as mean ± SEM.

(C) *FasL* expression in each genotypic female osteoclastic cells treated with or without Tam (10^{-6} M) (*$p < 0.05$ compared to the group treated without Tam). Data are represented as mean ± SEM.

(D) Expression of *Fas* was measured as described in the legend of Figure 7A. Data are represented as mean ± SEM.

markedly elevated levels of testosterone in ERα KO females may be potent enough to maintain normal bone turnover (Syed and Khosla, 2005), it is likely that the activated AR might be functionally sufficient in male mice to compensate for the ERα deficiency in bone (Kawano et al., 2003). However, species differences in the osteoprotective action of sex steroid hormones still need to be carefully addressed.

Fas/FasL system-mediated apoptotic induction of osteoclasts by estrogen may well be a part of the mechanism for the antiresorptive action of estrogen and SERMs in trabecular bone areas (Delmas, 2002; Rodan and Martin, 2000; Simpson and Davis, 2001; Syed and Khosla, 2005; Tolar et al., 2004). Regulation of osteoclast differentiation is tightly coupled to osteoblastic function in terms of cytokine production and cell-cell contact (Karsenty and Wagner, 2002; Martin and Sims, 2005; Mundy and Elefteriou, 2006; Teitelbaum and Ross, 2003). Indeed, upregulation of osteoclastogenic cytokines by ovariectomy was unaffected in $ER\alpha^{\Delta Oc/\Delta Oc}$ females. Considering the observation that cortical bone mass is increased in ovariectomized $ER\alpha^{\Delta Oc/\Delta Oc}$ females during estrogen treatment, it is conceivable that the antiresorptive estrogen action in cortical bone is also mediated by osteoblastic ERα. In this regard, FasL induction by estrogen in osteoblasts may contribute to the osteoprotective estrogen action, and *FasL* gene induction by estrogen was in fact detected in primary cultured osteoblasts from female calvaria by us as well as another group (S. Krum and M. Brown, personal communication). Thus, similar experiments in which ERα is selectively ablated in osteoblasts are needed to define the role of ERα in these cells.

In osteoclastic cells, expression of the *FasL* gene, which leads to apoptosis, appears to be positive controlled by activated ERα. Not surprisingly, a direct binding site for ERα has been mapped in the *FasL* gene locus (S. Krum and M. Brown, personal communication). An osteoclast- and cell-differentiation stage-specific mechanism may underlie this gene induction in the *FasL* gene promoter. A recent study demonstrated that ERα recruitment to specific promoter sites of given ERα target genes was cell-type specific (Carroll et al., 2005). Thus, there is significant impetus to identify the osteoclastic factor that associates with ERα in the *FasL* gene promoter. Such identification will lead to a better understanding of the molecular basis of the osteoprotective estrogen action and provide a target against which to develop SERMs of greater effectiveness.

EXPERIMENTAL PROCEDURES

Ctsk-Cre Construction and Generation of the Knockin Mouse Lines

An RP23-422n18 BAC clone containing the mouse *Ctsk* gene was purchased from Invitrogen (Carlsbad, CA). The *FRT-Kan^r/Neo^r-FRT* and *nlsCre* fragments were obtained from plasmids pSK2/3-*FRT-Neo* and pIC-*Cre*. Two homologous arms of 500 bp from the *Ctsk* gene were inserted into both sides of the *nlsCre-FRT-Kan^r/Neo^r-FRT* cassette in the pSK2/3-*FRT-Neo* plasmid. The *nlsCre-FRT-Kan^r/Neo^r-FRT* cassette was introduced into the endogenous ATG start site of the *Ctsk* gene by recombineering approaches (Copeland et al., 2001). Targeted BAC was reduced in size from 189 kb to 26 kb and subcloned into the pMC1-DTpA vector by the gap-repair method. The targeted TT2 ES clones were selected after positive-negative selection with G418 and DT-A with Southern analysis, then aggregated with single eight-cell embryos from CD-1 mice (Yoshizawa et al., 1997). Chimeric mice were then crossed with a general deleter mouse line, *ACTB-Flpe* (Jackson Laboratory), to remove the *Kan^r/Neo^r* cassette. The *Ctsk-Cre* mice ($Ctsk^{Cre/+}$), originally on a hybrid C57BL/6 and CBA genetic background, were backcrossed for four generations into a C57BL/6J background. $FasL^{gld/gld}$ mice were also purchased from Jackson Laboratory.

Analysis of Cre Recombinase Activities

Expression of the Cre transcript was detected by RT-PCR. Southern analysis using a *Cre* cDNA probe was performed with total RNA extracted from 12-week-old mice. To evaluate the specificity and efficiency of Cre-mediated recombination, we mated the $Ctsk^{Cre/+}$ mice to *CAG-CAT-Z* reporter mice (kindly provided by J. Miyazaki) (Sakai and Miyazaki, 1997) and genotyped their offspring with *Cre*-specific primers. β-galactosidase activity of the expressed *LacZ* gene driven by the *CAG* promoter was expected to be detected in the given cells expressing functional Cre recombinase.

In Vitro Osteoclastogenesis and Ligand Application

Bone-marrow cells derived from 8-week-old mice were plated in culture dishes containing α-MEM (GIBCO-BRL) with 10% FBS (JRH) and 10 ng/ml M-CSF (Genzyme). After incubation for 48 hr, adherent cells were used as osteoclast precursor cells after washing out the nonadherent cells. Cells were cultured in the presence of 10 ng/ml M-CSF and 100 ng/ml RANKL (Peprotech) to generate osteoclast-like cells (Koga et al., 2004) for 3 days, so the total culture time was 5 days. Three days after RANKL stimulation, primary cultured osteoclasts were treated with 10^{-8} M of 17β-estradiol (E2) (Sigma-Aldrich Co.) or 10^{-6} M 4-hydroxytamoxifen (Tam) (Sigma-Aldrich Co.) in phenol-red free medium.

Generation of Osteoclast-Specific ERα KO Mice

The ERα conditional ($ER\alpha^{flox/flox}$) (Dupont et al., 2000) and null alleles with a C57BL/6J background have been previously described. $ER\alpha^{flox/flox}$ mice were crossed with $Ctsk^{Cre/+}$ mice to generate $Ctsk^{Cre/+}$; $ER\alpha^{flox/+}$ mice. $Ctsk^{Cre/+}$; $ER\alpha^{+/+}$ ($ER\alpha^{+/+}$) and $Ctsk^{Cre/+}$; $ER\alpha^{flox/flox}$ ($ER\alpha^{\Delta Oc/\Delta Oc}$) mice were obtained by crossing $Ctsk^{Cre/+}$; $ER\alpha^{flox/+}$ with $ER\alpha^{flox/+}$ mouse lines.

Radiological Analysis

Bone radiographs of the femurs of 12-week-old $Ctsk^{Cre/+}$; $ER\alpha^{flox/flox}$ ($ER\alpha^{\Delta Oc/\Delta Oc}$) and $Ctsk^{Cre/+}$; $ER\alpha^{+/+}$ ($ER\alpha^{+/+}$) littermates were visualized with a soft X-ray apparatus (TRS-1005: SOFTRON). BMD was measured by DXA using a bone mineral analyzer (DCS-600EX: ALOKA). Micro Computed Tomography scanning of the femurs was performed using a composite X-ray analyzer (NX-CP-C80H-IL: Nittetsu ELEX Co.) (Kawano et al., 2003). Tomograms were obtained with a slice thickness of 10 μm and reconstructed at 12 × 12 pixels into a 3D image by the volume-rending method (VIP-Station; Teijin System Technology) using a computer.

Analysis of Skeletal Morphology

Twelve-week-old $Ctsk^{Cre/+}$; $ER\alpha^{flox/flox}$ ($ER\alpha^{\Delta Oc/\Delta Oc}$) and $Ctsk^{Cre/+}$; $ER\alpha^{+/+}$ ($ER\alpha^{+/+}$) littermates were double labeled with subcutaneous injections of 16 mg/kg of calcein (Sigma) at 4 and 2 days before sacrifice. Tibiae were removed from each mouse and fixed with 70% ethanol. They were stained with Villanueva bone stain for 7 days and embedded in methyl-methacrylate (Wako) (Yoshizawa et al., 1997). Frontal plane sections (5-μm thick) of the proximal tibia were cut using a Microtome (LEICA). The cancellous bone was measured in the secondary spongiosa located 500 μm from the epiphyseal growth plate and 160 μm from the endocortical surface (Kawano et al., 2003; Nakamichi et al., 2003). Bone histomorphometric measurements of the tibia were made using a semiautomatic image analyzing system (System Supply) and a fluorescent microscope (Optiphot; Nikon). Similar measurements of the lumbar vertebral bodies were done as previously reported (Takeda et al., 2002). Standard bone histomorphometrical nomenclatures, symbols, and units were used as described in the report of the ASBMR Histomophometry Nomenclature Committee.

Ovariectomy and Hormone Replacement

Female $Ctsk^{Cre/+}$; $ER\alpha^{flox/flox}$ ($ER\alpha^{\Delta Oc/\Delta Oc}$) and $Ctsk^{Cre/+}$; $ER\alpha^{+/+}$ ($ER\alpha^{+/+}$) littermates were ovariectomized or sham operated at 8–12 weeks of age for 2 weeks for all experiments, and slow releasing pellets of E2 (0.83 μg/day) or placebo (Innovative Research, Sarasota, FL) were implanted subcutaneously in the scapular region behind the neck (Sato et al., 2004; Shiina et al., 2006).

Immunohistochemistry

Twelve-week-old $Ctsk^{Cre/+}$; $ER\alpha^{flox/flox}$ ($ER\alpha^{\Delta Oc/\Delta Oc}$) and $Ctsk^{Cre/+}$; $ER\alpha^{+/+}$ ($ER\alpha^{+/+}$) littermates were fixed with 4% PFA by perfusion. Serial sections of the brain (20 μm thick) were divided into two groups and used for single labeling for the ERα or thionin to allow determination of the areas to be measured. Tibiae and femurs were decalcified in 10% EDTA for 2–4 weeks after fixation and then embedded in paraffin sections. Sections were incubated in L.A.B. solution (Polysciences) for 30 min to retrieve antigen. The cooled sections were incubated in 1% H_2O_2 for 30 min to quench endogenous peroxidase and then washed with 1% Triton X-100 in PBS for 10 min. To block nonspecific antibody binding, sections were incubated in blocking solution (DAKO) for 5 min. Sections were then incubated with anti-ERα (Santa Cruz, CA) and anti-FasL (Santa Cruz, CA) in blocking solution overnight at 4°C. Staining was then performed using the EnVision+ HRP System (Dako) and 3, 3′-diaminobendizine tetrahydrochloride substrate (Sigma), counterstained with TRAP, dehydrated through an ethanol series and xylene, before mounting (Sato et al., 2004).

ERα Overexpression

Two days after RANKL stimulation, an expression vector of mouse ERα was tranfected into immature osteoclastic cells from $ER\alpha^{\Delta Oc/\Delta Oc}$ mice using Superfect (QIAGEN) as manufacture's instruction.

Real-Time RT-PCR

One microgram of total RNA from each sample was reverse transcribed into first-strand cDNA with random hexamers using Superscript III reverse transcriptase (Invitrogen). Primer sets for all genes were purchased from Takara Bio. Inc. (Tokyo, Japan). Real-time RT-PCR was performed using SYBR Premix Ex Taq (Takara) with the ABI PRISM 7900HT (Applied Biosystems) according to the manufacturer's instructions. Experimental samples were matched to a standard curve generated by amplifying serially diluted products using the same PCR protocol. To correct for variability in RNA recovery and efficiency of reverse transcription, *Gapdh* cDNA was amplified and quantified in each cDNA preparation. Normalization and calculation steps were performed as reported previously (Takezawa et al., 2007).

TUNEL/TRAP Staining
The TUNEL method was performed using the ApopTag Fluorescein In Situ Apoptosis Detection Kit (CHEMICON international) according to the manufacturer's instructions with a slight modification. This was followed by TRAP staining as previously reported (Kobayashi et al., 2000).

Cytokine Assays
Bone marrow and blood were collected at 2 weeks after sham operation or ovariectomy. Bone-marrow cells were cultured for 3 days in DMEM. The levels of TNFα, IL-1α, and IL-6 in the culture media and serum RANKL were determined by ELISA (R&D Systems).

Western Blot
Osteoclast precursor cells were treated with or without 100 ng/ml of soluble RANKL. After 15 minutes, cell extracts were harvested from the cells using lysis buffer containing 100 mM Tris-HCl (pH 7.8), 150 mM NaCl, 0.1% Triton X-100, 5% protease inhibitor cocktail (Sigma), and 5% phosphatase inhibitor cocktail (Sigma). An equivalent amount of protein from each of the cell extracts and proteins of femoral bone extracted using ISOGEN was loaded for SDS-PAGE and transferred to PVDF membranes (Amersham Biosciences). The membranes were developed with enhanced chemiluminescence reagent (Amersham Biosciences) (Ohtake et al., 2003). Phosphorylation of p38 MAPK and IκB were evaluated using antibodies purchased from Cell Signaling Technology (Koga et al., 2004) and anti-FasL antibody was purchased from Santa Cruz Biotechnology (sc-834).

Actin-Ring Formation
Cells were fixed for 15 min in warm 4% paraformaldehyde (PFA). After fixation, cells were washed three times with PBS with 0.1% Triton X-100 (PBST) and incubated with 0.2 U/ml rhodamine phalloidin (Molecular Probes) for 30 min and washed again three times in PBST.

Statistical Analysis
Data were analyzed by two-tailed student's t test. For all graphs, data are represented as mean ± SEM.

Supplemental Data
Supplemental Data include Supplemental Experimental Procedures and four figures and can be found with this article online at http://www.cell.com/cgi/content/full/130/5/811/DC1/.

ACKNOWLEDGMENTS

We thank Drs. S. Krum and M. Brown to share with their unpublished results; Drs. K. Yoshimura, Y. Nakamichi, T. Watanabe, J. Miyamoto, H. Shiina, T. Fukuda, Ms. Y. Sato, and S. Tanaka for generation of the KO mice; Drs. T. Koga, H. Takagi, E. Ochiai, and N. Moriyama for technical help; Dr. J. Miyazaki for *CAG-CAT-Z* reporter mice, and H. Higuchi and K. Hiraga for manuscript preparation. This work was supported in part by priority areas from the Ministry of Education, Culture, Sports, Science and Technology (to S.K.) and the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN).

Received: February 23, 2007
Revised: May 21, 2007
Accepted: July 17, 2007
Published: September 6, 2007

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Accession Numbers

Microarray can be seen in Gene Expression Omnibus under accession number GSE7798.

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.

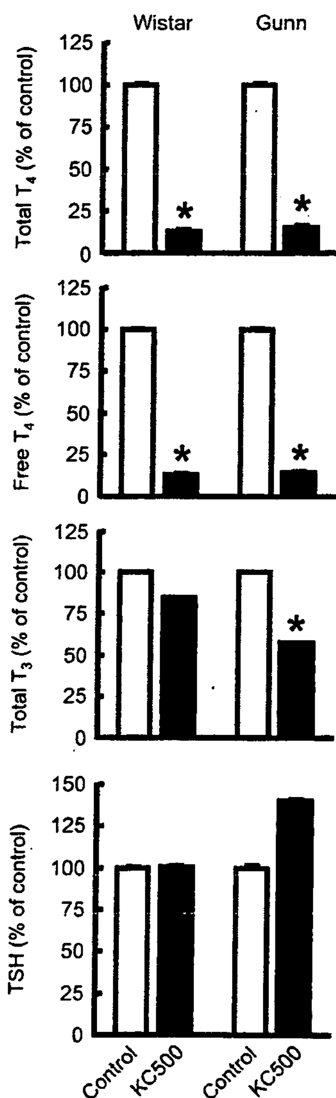

FIG. 1. Effects of KC500 on levels of serum total T_4, free T_4, total T_3, and TSH in Wistar and Gunn rats. Animals were killed 4 days after the final administration of KC500 (10 mg/kg i.p., once daily for 10 days), and levels of serum thyroid hormones were measured as described under *Materials and 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.

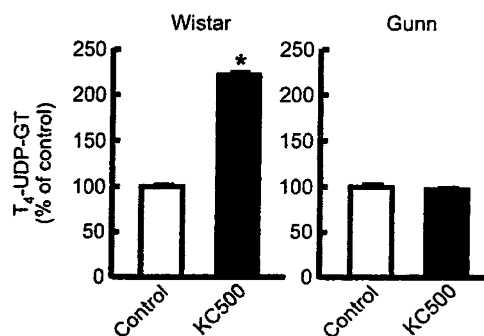

FIG. 2. Effects of KC500 on the activity of hepatic microsomal UDP-glucuronyltransferase in Wistar and Gunn rats. Each column represents the mean ± S.E. (vertical bars) for five to six animals. Constitutive levels: T_4-UDP-GT, 14.17 ± 1.11 pmol/mg protein/min (Wistar) and 6.36 ± 1.34 pmol/mg protein/min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

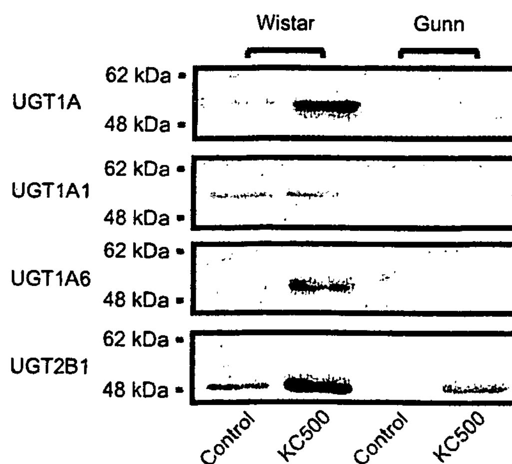

FIG. 3. Representive Western blot profiles for hepatic microsomal UGT isoforms in the KC500-treated Wistar and Gunn rats.

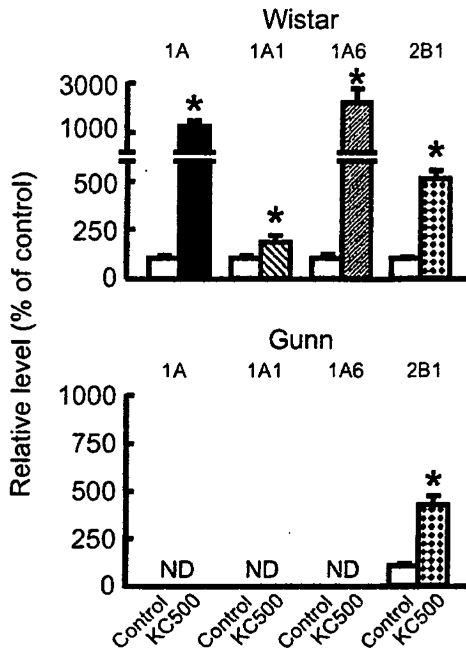

FIG. 4. Effects of KC500 on levels of hepatic microsomal UGT isoforms in Wistar and Gunn rats. The isolated bands responsible for UGT isoforms, which are shown in Fig. 3, were densitometrically quantified as described under *Materials and Methods*. The data are represented as the mean ± S.E. (vertical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

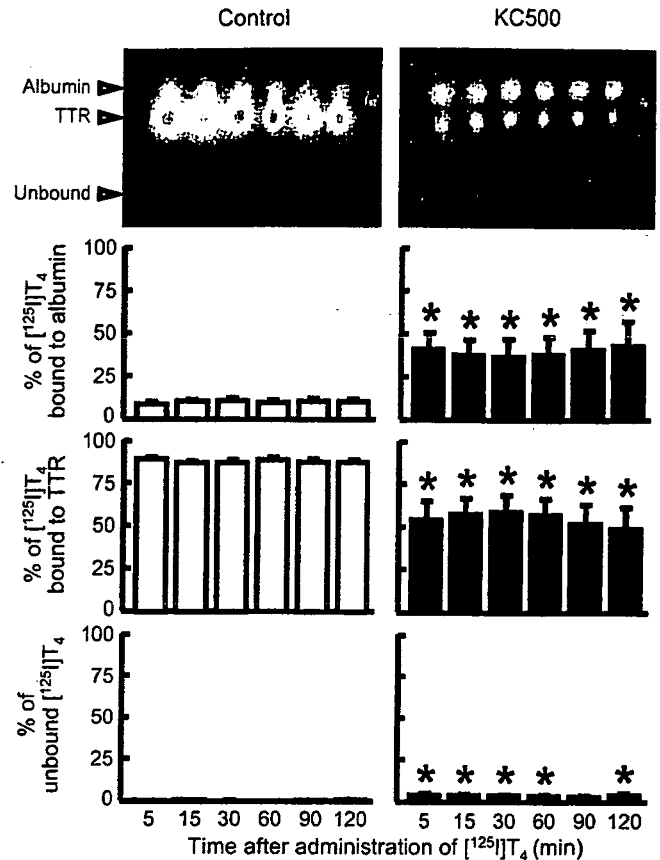

FIG. 5. Effects of KC500 on the binding of $[^{125}I]T_4$ to serum proteins in Wistar rats. Amounts of $[^{125}I]T_4$ bound to the serum proteins were assessed by the method described under *Materials and Methods*. Each column represents the mean ± S.E. (vertical bars) for three to six animals. *, $P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

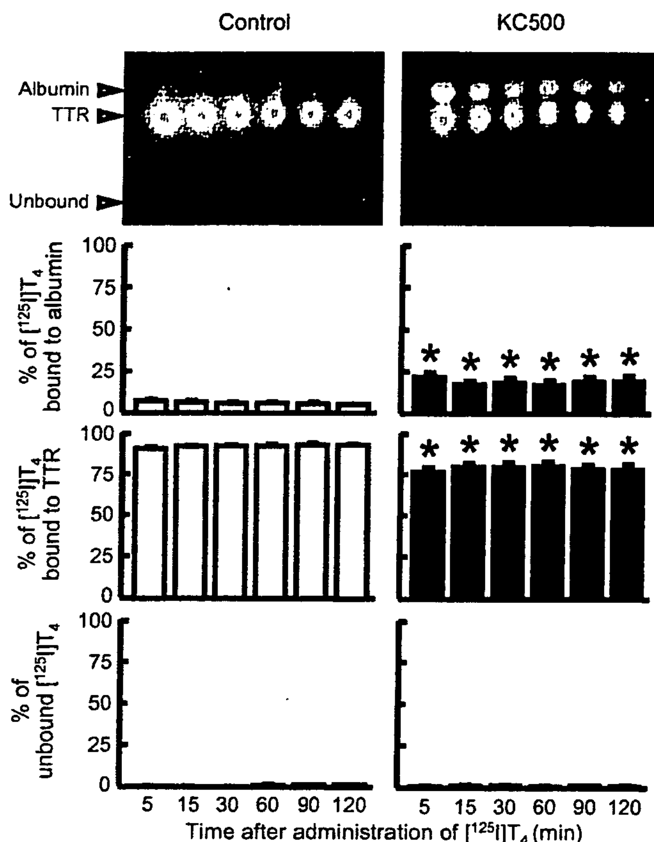

FIG. 6. Effects of KC500 on the binding of $[^{125}I]T_4$ to serum proteins in Gunn rats. Amounts of $[^{125}I]T_4$ bound to the serum proteins were assessed by the method described under *Materials and Methods*. Each column represents the mean ± S.E. (vertical bars) for four to five animals. *, $P 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

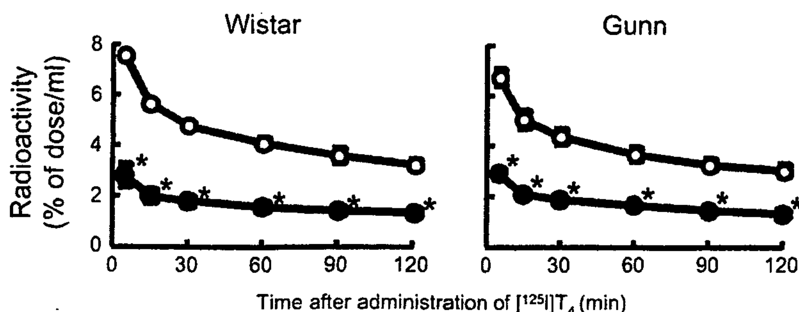

FIG. 7. Effects of KC500 on the clearance of $[^{125}I]T_4$ from serum in Wistar and Gunn rats. The amount of serum $[^{125}I]T_4$ was measured at the indicated times after the i.v. administration of $[^{125}I]T_4$. Each point represents the mean ± S.E. (vertical bars) for four to eight animals. *, $P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

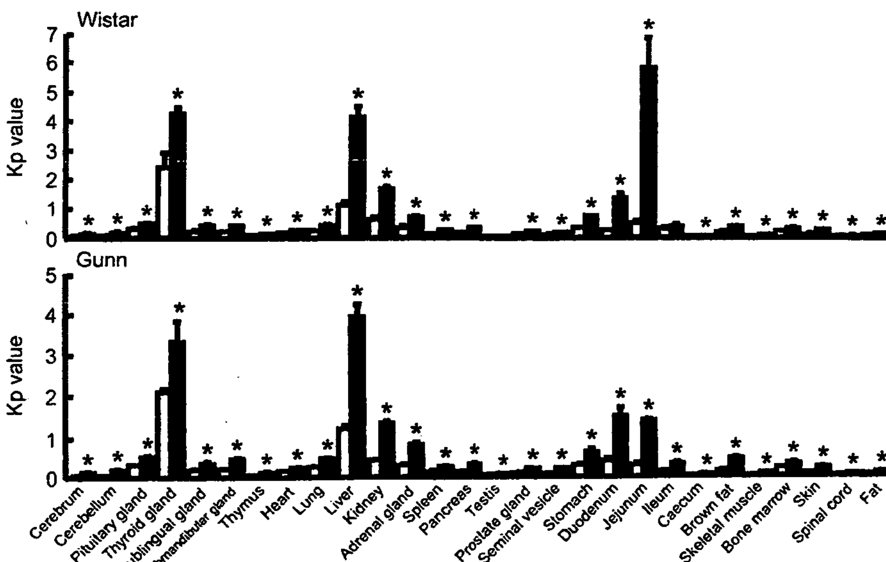

FIG. 8. Tissue-to-serum concentration ratio (K_p value) of $[^{125}I]T_4$ in various tissues after administration of $[^{125}I]T_4$ to the KC500-pretreated Wistar and Gunn rats. KC500 (10 mg/kg) was given i.p. to animals once daily for 10 days, and then, the animals were administered i.v. $[^{125}I]T_4$. 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.

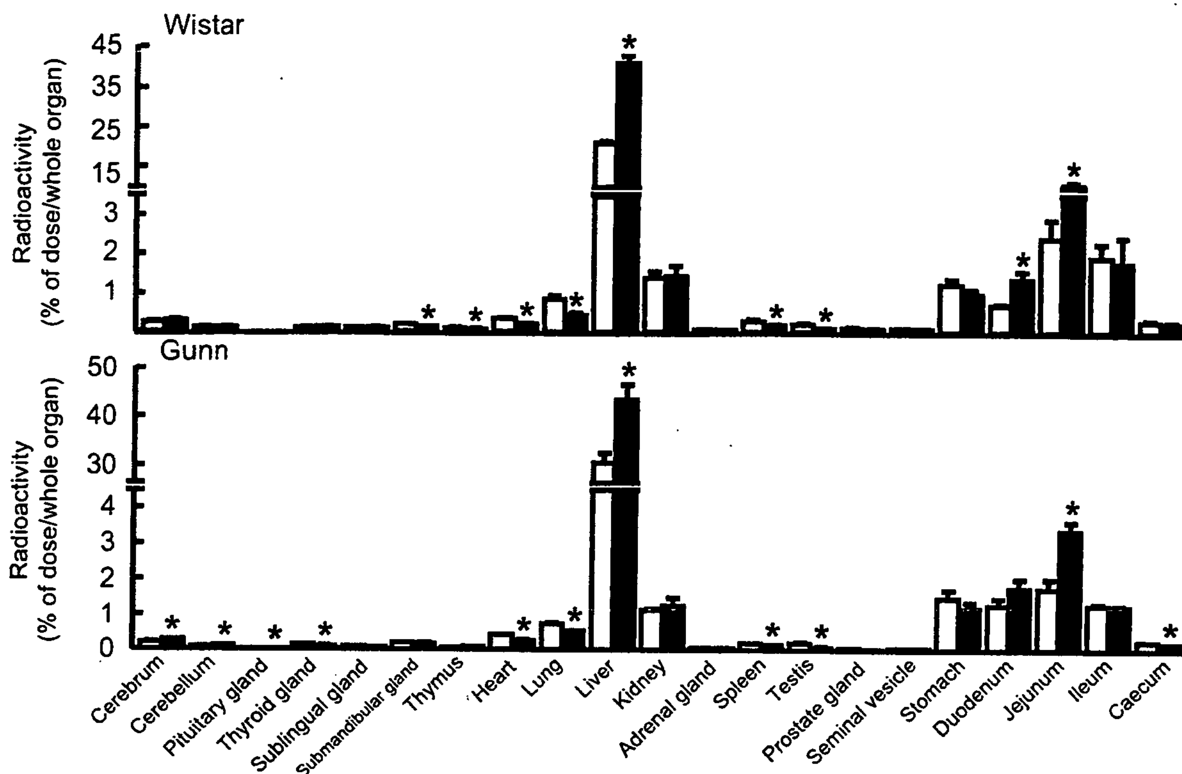

FIG. 9. Tissue distribution of $[^{125}I]T_4$ after administration of $[^{125}I]T_4$ to the KC500-pretreated Wistar and Gunn rats. The experimental conditions were the same as those described in Fig. 8. Each column represents the mean ± S.E. (vertical bars) for four to six animals. *, $P < 0.05$, significantly 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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Reproductive Toxicology 24 (2007) 131–138

Reproductive Toxicology

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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 Commonweal 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 theses 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^{-1} day^{-1}) in studies with rats and mice. The 50 mg (kg^{-1} day^{-1}) 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^{-1} day^{-1}). 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

0890-6238/$ – see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.reprotox.2007.07.005

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^{-1} (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^{-1} (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 $\mu g\,kg^{-1}$, 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 (α/ß; 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 the 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.

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

Development 134, 1561-1569 (2007) doi:10.1242/dev.000836

The negative regulation of Mesp2 by mouse Ripply2 is required to establish the rostro-caudal patterning within a somite

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The Mesp2 transcription factor plays essential roles in segmental border formation and in the establishment of rostro-caudal patterning within a somite. A possible Mesp2 target gene, *Ripply2*, was identified by microarray as being downregulated in the *Mesp2*-null mouse. *Ripply2* encodes a putative transcriptional co-repressor containing a WRPW motif. We find that Mesp2 binds to the *Ripply2* gene enhancer, indicating that *Ripply2* is a direct target of Mesp2. We then examined whether Ripply2 is responsible for the repression of genes under the control of Mesp2 by generating a *Ripply2*-knockout mouse. Unexpectedly, *Ripply2*-null embryos show a rostralized phenotype, in contrast to *Mesp2*-null mice. Gene expression studies together with genetic analyses further revealed that Ripply2 is a negative regulator of Mesp2 and that the loss of the *Ripply2* gene results in the prolonged expression of Mesp2, leading to a rostralized phenotype via the suppression of Notch signaling. Our study demonstrates that a Ripply2-Mesp2 negative-feedback loop is essential for the periodic generation of the rostro-caudal polarity within a somite.

KEY WORDS: Somitogenesis, Notch signaling, Presomitic mesoderm, Segmentation

INTRODUCTION

Somites are generated by sequential segregation of cell masses from the anterior part of the unsegmented presomitic mesoderm (PSM), in both a spatially and temporally coordinated manner every two hours (Iulianella et al., 2003; Pourquie, 2003; Saga and Takeda, 2001). The somites provide the basic axial structures that underlie the segmental architecture of not only the vertebra, ribs and muscles, which are all somite derivatives, but also of the vascular and nervous systems (Borycki and Emerson, 2000; Brand-Saberi and Christ, 2000; Monsoro-Burq and Le Douarin, 2000). Periodicity is generated by Notch signal oscillations linked to the segmentation clock (Bessho et al., 2001; Huppert et al., 2005; Morimoto et al., 2005; Rida et al., 2004). The temporal information that results from this is translated into spatial patterns in the anterior PSM, which is defined by the so-called determination front (Dubrulle and Pourquie, 2004).

The Mesp2 transcription factor plays important roles during somitogenesis (Saga et al., 1997), and its expression is periodically activated by cyclic Notch signaling and Tbx6 at the anterior PSM in the determination front (Yasuhiko et al., 2006). Mesp2 demarcates the next segmental boundary and defines the rostro-caudal identity of somites (Takahashi et al., 2000). It has been shown that *Mesp2*-null embryos fail to segment and that the resulting non-segmented somites show caudalized properties (Saga et al., 1997). Previously, we have shown that Mesp2 suppresses Notch activity via the activation of *Lfng*, which might function as a negative regulator of Notch signaling (Morimoto et al., 2005). In addition, Mesp2 acts as the transcriptional activator of *Epha4* in the anterior PSM (Nakajima et al., 2006). Mesp2 is also known to be a strong suppressor of genes such as *Dll1* and *Uncx4.1* that confer caudal properties upon the somitic cells via Notch signaling (Takahashi et al., 2000). However, the manner in which the caudal genes are suppressed is currently unknown. In our current study, which aimed to elucidate the molecular mechanisms underlying the regulation of somitogenesis by Mesp2, we have compared the gene expression patterns of $Mesp2^{+/-}$ and $Mesp2^{-/-}$ embryos, and found that several genes are affected by the *Mesp2* knockout. Among the downregulated genes that we identified in the *Mesp2*-null embryo, we focused on a putative transcriptional repressor. This gene turned out to be *Ripply2*, which was recently reported as a mouse homolog of zebrafish *ripply1* (Kawamura et al., 2005). Morpholino-mediated knockdown analysis revealed that *ripply1* is required for the proper transition from the PSM to somites. We generated a *Ripply2*-knockout mouse and now show that *Ripply2* is activated by Mesp2, but also functions negatively toward Mesp2 to regulate the levels of Notch signaling in the anterior PSM. This negative regulation is required for the periodic generation of the rostro-caudal patterning within a somite.

MATERIALS AND METHODS

GeneChip analysis

Total RNA was purified from cells corresponding to the S–1 to S2 somites and PSM of wild-type, *Mesp2-GFP* knock-in heterozygous and homozygous embryos at E10.5 using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. First-strand cDNAs were synthesized by incubating 5 μg of total RNA with 200 U SuperScript II reverse transcriptase (Invitrogen) and 100 pmol T7-$(dT)_{24}$ primer [5′-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$-3′]. After second-strand synthesis, the double-stranded cDNAs were purified using a GeneChip Sample Cleanup Module (Affymetrix), according to the manufacturer's instructions. Our detailed methods for the labeling of the

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Accepted 14 February 2007

DEVELOPMENT