each treatment, cells were rinsed with fresh serum-free culture medium and then added 0.5 ml of fresh serum-free culture medium supplemented with 1 μM E1 (Sigma, St. Louis, MO). After incubation for 4 h at 37 °C in an atmosphere of 5% CO_2, culture media were collected, and the total E2 content was determined by assay with a Correlate-EIA 17β-estradiol Enzyme Immunoassay kit (Assay Designs, Ann Arbor, MI).

2.4. *Quantitative reverse transcription-PCR (RT-PCR)*

JAr cells were treated with various tin compounds in regular culture medium supplemented with 5% charcoal-stripped FCS instead of 10% normal FCS, and then total RNA was extracted from the cells by using TRIzol reagent (Invitrogen). mRNA expression of 17β-HSD I in JAr cells was determined by quantitative RT-PCR. We reverse-transcribed 5 μg total RNA extracted from JAr cells in a total volume of 20 μl by using SuperScript III reagent (Invitrogen) and oligo-(dT) as primer and incubating for 1 h at 42 °C. After termination of cDNA synthesis, each reaction mixture was diluted with the addition of 80 μl TE buffer. Aliquots (2 μl) of diluted reverse-transcription products were amplified in a reaction mixture containing QuantiTect SYBR Green PCR reagent (Qiagen, Valencia, CA) and 0.5 μM of each primer using LightCycler (Roche Diagnostics, Mannheim, Germany). After preincubation of reaction mixtures at 95 °C for 15 min, PCR amplification was performed with 35–40 cycles of denaturation at 95 °C for 15 s, annealing at 65 °C for 30 s, and elongation at 72 °C for 10 s. Primers used were for human 17β-HSD I (HSD17B1), 5′-GGGCTGCCTTTCAATGACGTTT-3′ and 5′-ATCAGGCTCAAGTGGACCCCAA-3′, and human β-actin, 5′-CTACGAGCTGCCTGACGGC-3′ and 5′-GCCACAGGA-CTCCATGCCC-3′.

2.5. *Statistics*

Data were analyzed with Tukey's multiple comparisons test by using SPSS software (Chicago, IL). Control and treatment group data always were obtained from equal numbers of replicate experiments, and experiments were performed independently at least twice. Values at which P was <0.05 were considered statistically significant.

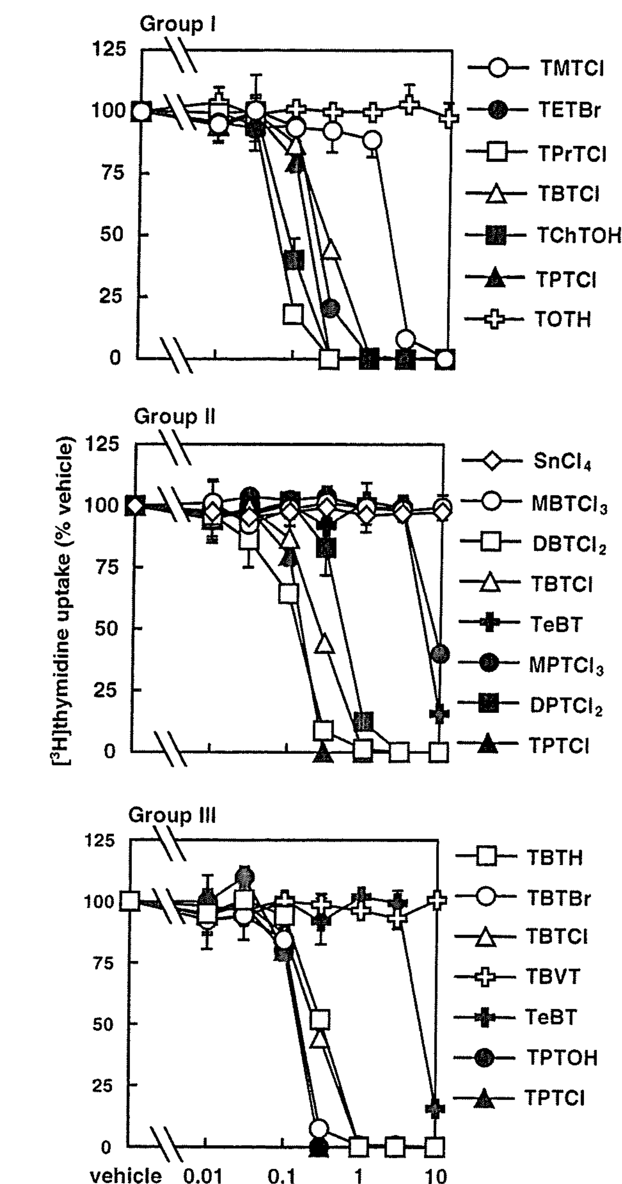

Fig. 1 – Effect of organotin compounds on DNA synthesis in JAr cells. Cells were treated with various concentrations of tin compounds for 48 h. Results are expressed as mean ± 1 S.D. of triplicate cultures. The radioactivity in vehicle-only cells, calculated from all experiments, was 111,709 ± 6182 cpm (*n* = 15). Group I, comparison of different structures of alkyl and aryl chains in trialkylated and triarylated tin compounds; Group II, comparison of different numbers of alkyl or aryl chains in butyltin and phenyltin compounds; and Group III, comparison of different fourth functional groups on the tin of TBT and TPT. The abbreviation for each compound used are indicated in Table 1.

3. Results

3.1. *Effects of organotin compounds on DNA synthesis in JAr cells*

To confirm the nontoxic concentration ranges of 17 tin compounds (Table 1) and to determine whether treatment with organotin compounds was associated with cytotoxic effects, we performed DNA synthesis assays. JAr cells were treated for 48 h with tin compounds at various concentrations, and DNA synthesis was evaluated by [^{3}H]thymidine incorporation. To help interpret the results, we classified these experiments into three groups as follows: Group I, comparison of different structures of alkyl and aryl chains in trialkylated and triarylated tin compounds; Group II, comparison of different numbers of alkyl or aryl chains in butyltin and phenyltin compounds; and Group III, comparison of different fourth functional groups on the tin of TBT and TPT (Fig. 1).

Many of the Group I trialkylated and triarylated tin compounds, which have ethyl, propyl, butyl, cyclohexyl, or phenyl groups on the tin atom, were highly toxic, and

exposure to >100–300 nM significantly inhibited $[^3H]$thymidine incorporation in JAr cells. Although TMTCl was one of the less toxic chemicals of Group I, a striking reduction of $[^3H]$thymidine incorporation to 5% of the control value occurred after treatment with >1 μM. TOTH had no significant effect on $[^3H]$thymidine incorporation at a concentration range of 10 nM to 10 μM. Among the Group II chemicals, the cytotoxicity of $DBTCl_2$ was nearly as high as that of the most highly toxic trialkyltins. $DPTCl_2$ was also toxic but less so than $DBTCl_2$. Although TeBT and $MPTCl_3$ were less toxic than other Group II compounds, they induced marked reduction of $[^3H]$thymidine incorporation at 10 μM. $SnCl_4$ and $MBTCl_3$ showed no effect, even at concentrations of 10 μM. Among the Group III chemicals, the TBT and TPT derivatives were similar in toxicity, and exposure to doses of 300 nM decreased $[^3H]$thymidine incorporation to <50% of control levels. TBVT showed no significant effect at the concentration range of 10 nM to 10 μM. Using these results as a guide, we established the maximal nontoxic concentration of each compound for use in investigating possible effects on the 17β-HSD I activity of JAr cells (Table 1).

3.2. *Effect of organotin compounds on 17β-HSD I activity in JAr cells*

At lethal concentrations, at which uptake of $[^3H]$thymidine was <10% of control levels, all organotin compounds abolished E2 production because of extinction of the cells. All tested TBT and TPT derivatives (Group III) were active and induced 17β-HSD I activity (Fig. 2): exposure to 100 nM of each of these organotin compounds caused statistically significant increases in 17β-HSD I activity in JAr cells. There were no significant differences in 17β-HSD I activity among the TBT and TPT derivatives (Group III), suggesting that the ligand on the trialkylated and triarylated tin compounds (as long as it is not an alkyl or aryl group) is relatively unimportant to stimulation of endocrine functions. However, the presence of a fourth alkyl group on the tin atom decreased the potency of the organotin compounds in inducing 17β-HSD I activity, because both TeBT and TBVT failed to stimulate this placental function at doses of <100 nM (Fig. 2, Group III). Among the other trialkyltin compounds (Group I), TETBr, TPrTCl, and TChTOH were significantly active. Metabolites of both TBTCl and TPTCl ($MBTCl_3$, $DBTCl_2$, $MPTCl_3$ and $DPTCl_2$; Group II) also altered 17β-HSD I activity, but the level of activation decreased in proportion to the dealkylation or dearylation of these organotin compounds (mono- < di- < tri-). These results suggest that the potency of the effects induced by organotin compounds is related to both the number and structure of the alkyl and aryl groups.

3.3. *Effect of organotin compounds on mRNA expression of 17β-HSD I (HSD17B1) in JAr cells*

We investigated the tin compound-induced mRNA expression of 17β-HSD I (*HSD17B1* gene) in JAr cells at either the concentration that elicited the greatest response in catalytic activity or the maximal nontoxic concentration. Except for

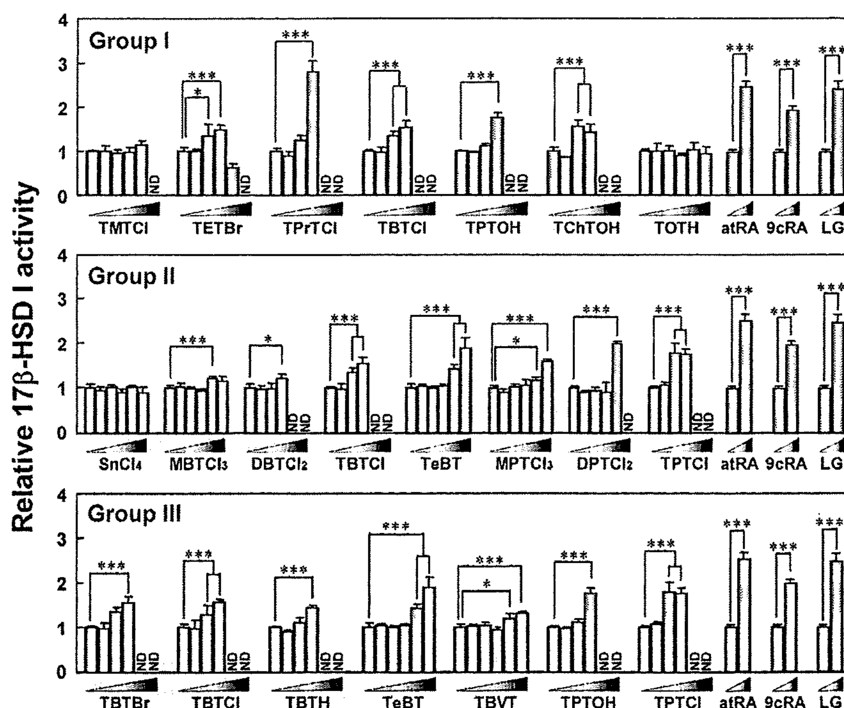

Fig. 2 – Effects of tin compounds on 17β-HSD I activity in JAr cells. Cells were treated for 48 h with tin compounds at various concentrations (0 nM, 1 nM, 10 nM, 100 nM, 1 μM, and 10 μM) or with 0 or 1 μM of atRA, 9cRA, or LG. Results are expressed as mean ± 1 S.D. of triplicate cultures. The 17β-HSD I activity (amount of converted E2) in vehicle-only cells, calculated from all experiments, was 4.44 ± 1.01 ng/well/4 h (n = 18). Groups I, II, and III correspond to the groups described in the legend for Fig. 1. *$P < 0.05$; **$P < 0.01$; and ***$P < 0.005$ indicate values significantly different from vehicle-control values. ND, not detectable.

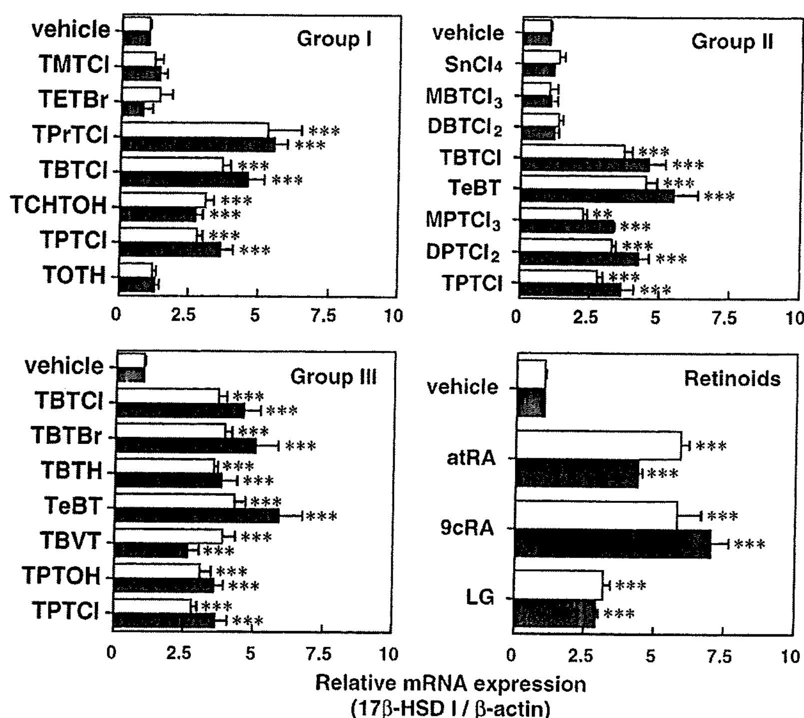

Fig. 3 – Effects of tin compounds on the mRNA expression of 17β-HSD I (HSD17B1) in JAr cells. Total RNA was isolated from JAr cells treated with tin compounds for 24 h (open bars) and 48 h (solid bars). The doses of each compound were: 100 nM of TETBr, TPrTCl, TBTCl, TChTOH, TPTOH, TPTCl, TBTH, TBTBr and $DBTCl_2$; 1 μM of TMTCl, $MBTCl_3$, $DPTCl_2$, 9cRA, atRA and LG; and 10 μM of TOTH, $SnCl_4$, $MPTCl_3$, TeBT and TBVT. The relative mRNA levels for each condition were determined by quantitative RT-PCR assays for each of the 3 independent cultures (see Section 2). Results are expressed as means ± 1 S.D. of three independent cultures. Groups I, II, and III correspond to the groups described in the legend for Fig. 1. $^{**}P < 0.01$; and $^{***}P < 0.005$ represents values significantly different from vehicle-control values.

TETBr, $MBTCl_3$, and $DBTCl_2$, the organotin compounds that significantly enhanced the catalytic activity of 17β-HSD I also significantly increased its mRNA expression. However, the mRNA effects were much more pronounced than the changes in catalytic activity (Figs. 2 and 3). Furthermore, atRA and 9cRA, which are known to enhance 17β-HSD I activity with mRNA expression, also showed their induction of mRNA expression more obviously than that of catalytic activity (Figs. 2 and 3).

In a recent study, we demonstrated that some organotin compounds function as agonists for retinoid X receptor (RXR), a nuclear receptor for retinoids, to stimulate the mRNA expression of human placental aromatase and human chorionic gonadotropin in human choriocarcinoma cells [14]. As shown in Figs. 2 and 3, organotin compounds that induced the transactivation function of RXR in our previous study (TBT and TPT derivatives, TPrTCl, TChTOH, TeBT, and TBVT) also enhanced 17β-HSD I mRNA transcription and enzyme activity. In addition, a synthetic RXR-specific ligand LG also enhanced 17β-HSD I enzyme activity and mRNA transcription (Figs. 2 and 3). These results suggest that these organotin compounds induce the expression of 17β-HSD I mRNA via RXR transactivation.

However, organotin compounds that did not induce the transactivation function of RXR in our previous study (TETBr, $MBTCl_3$, $DBTCl_2$, $MPTCl_3$ and $DPTCl_2$) also significantly enhanced 17β-HSD I enzyme activity. Interestingly, $MPTCl_3$ and $DPTCl_2$ significantly enhanced mRNA expression of 17β-HSD I, whereas TETBr, $MBTCl_3$, and $DBTCl_2$ had little effect on mRNA expression (Fig. 3). These results indicate that the observed organotin-induced alterations in 17β-HSD I activity are due not only to regulation at the mRNA level but also another mechanism.

4. Discussion

Recently, organotin compounds have become recognized as endocrine-disrupting chemicals, because numerous marine organisms have been shown to exhibit sexual abnormalities after exposure to TBT or TPT. In gastropod mollusks, which are among the species most sensitive to organotin compounds, these chemicals have been demonstrated to induce the superimposition of male sex organs, such as a penis and/or a vas deferens, over female sex organs, a phenomenon known as imposex [10,11]. It has been theorized that TBT increases androgen levels through inhibition of aromatase activity or suppression of androgen excretion [15–17]; nevertheless this theory is not well founded. Although these organotin compounds also are reported to inhibit the catalytic activity of human steroidgenic enzymes, including aromatase [18,19], 3β-hydroxysteroid dehydrogenase type II, 5α-reductase type II, and 17β-HSD I and III [20,21], the concentrations effective for the inhibition of these enzymes were relatively high (>1 μM)

and generally toxic to mammalian cells [22–24], including human choriocarcinoma cells (Fig. 1). Therefore, in regard to effects on humans, we have to distinguish between the nonspecific cellular toxicity of organotins and their inhibition of steroidgenic enzymes. We previously demonstrated that nanomolar concentrations (i.e., 3–100 nM) of some organotin compounds, including TBT and TPT, enhance aromatase activity, which catalyzes the conversion androgen to estrogen, and that this increase in enzymatic activity occurs concurrently with increases in mRNA expression and E2 biosynthesis from androstenedione in human choriocarcinoma cells [14,22]. In addition, our current study showed that many of the same organotin compounds also enhance 17β-HSD I activity, which predominantly catalyzes the conversion of the weakly estrogenic chemical E1 into the strongly estrogenic compound E2 in JAr cells (Figs. 2 and 3). Our findings suggest that the endocrine-disrupting action of these organotin compounds in pregnant women may be to promote the biosynthesis of E2 in the placenta, an effect opposite to that theorized in gastropods.

Our current study has demonstrated that organotin compounds alter E2 biosynthesis in human placental cells in vitro. Although several reports have established the in vivo reproductive toxicity of organotin compounds in rodents [25–28], there are no reports on whether organotin-induced production of placental E2 is associated with teratogenetic effects. Accordingly, it remains unclear which endocrine-disrupting effects or malformations result, at least in part, from organtoin-induced local changes in E2 concentrations of the placenta in vivo. Further, the in vivo endocrine effects of environmental contaminants on the human placenta are difficult to estimate from animal studies, particularly those involving rodents, because the endocrine functions of the placenta vary considerably among different species. In particular, estrogen biosynthesis during pregnancy in humans is much different from that in rodents. In humans, ovarian function gradually declines after fertilization, as the placenta becomes the primary site of estrogen biosynthesis during pregnancy [29]. In contrast to the process in humans, the ovary (not the placenta) is the main source of estrogen during pregnancy in rodents, because the placenta of rodents expresses neither aromatase nor 17β-HSD I [30–32]. It has been suggested that rodents are therefore unsuitable for evaluating the effects of environmental contaminants on estrogen biosynthesis in the human placenta. The regulation of estrogen biosynthesis in placenta is very important for human embryo because altering placental function can cause permanent effects in the embryo. For example, the lack of placental aromatase causes female pseudohermaphroditism, as is seen in patients with aromatase deficiency [33]. Consequently, there is an urgent need to establish effective tools to evaluate the endocrine-disrupting effects and teratogenicity of environmental contaminants that induce changes in local estrogen concentrations of the placenta in vivo.

Several stimuli, such as cAMP analogues [34], the natural retinoic acid receptor (RAR) ligand atRA [35], and the natural RAR and RXR ligand 9cRA [36], induce the mRNA expression of 17β-HSD I, thereby increasing its activity. Organotin compounds, such as TBT and TPT, have no effect on intracellular cAMP production [22]. In a recent study, we demonstrated that some organotin compounds function as RXR agonists to stimulate the mRNA expression of human placental aromatase and human chorionic gonadotropin in human choriocarcinoma cells [14]. These chemicals bind directly to the ligand-binding domain of RXR and function as transcriptional activators. The RXR-agonistic organotin compounds also enhanced the expression of 17β-HSD I mRNA in the present study. The level of activation was nearly equal to the level of RXR activation induced by these compounds [14]. The RAR response element is located at −512 to −479 bp in the promoter region of 17β-HSD I [35], but the RXR response element has not yet been identified. However, Ro41, a specific RAR antagonist, fails to abrogate 9cRA-induced expression of 17β-HSD I mRNA [36]. In addition, we found that the RXR-specific ligand LG consistently enhanced 17β-HSD I enzyme activity and mRNA transcription (Figs. 2 and 3). Unlike 9cRA, these organotin compounds are RXR-specific and do not activate the RAR pathway [14]. In light of these findings, although transcriptional regulation in the 17β-HSD I promoter is not yet fully understood, we suggest that the *cis*-elements which have a response to the RXR-dependent signaling pathway may be located in the promoter region and that RXR-agonistic organotin compounds induce the expression of 17β-HSD I mRNA via RXR transactivation.

We assayed 17 tin compounds for their ability to induce both the catalytic activity and mRNA expression of 17β-HSD I in JAr cells. The concentrations needed to induce these two features of 17β-HSD I did not differ significantly among the TBT and TPT derivatives. Compared with those for the TBT and TPT derivatives, approximately 30- to 100-fold higher concentrations of tetraalkyltin compounds (e.g., TeBT and TBVT) were needed to affect 17β-HSD I activity or transcript levels. This observation may indicate that tetraalkyltin compounds are metabolically converted to trialkyltins, which are the active form, in the cells. This hypothesis is supported, in a broad sense, by previous results that show that organotin compounds undergo dealkylation by the microsomal monooxygenase system, which is dependent on cytochrome P450 in the liver and other organs [37–39]. In addition, our previous study suggested that it may be necessary for activation of RXR by these tetraalkyltin compounds to metabolically convert them into the active dealkylated form (e.g., TBT) in cells, because the presence of a fourth alkyl group on the tin atom interferes with the binding of alkyltin compounds to the receptor [14]. These findings support our speculation that these organotin compounds induce the expression of 17β-HSD I mRNA via RXR transactivation.

Tin compounds that failed to act as RXR agonists ($MBTCl_3$, $DBTCl_2$, $MPTCl_3$, $DPTCl_2$ and TETBr) [14] also significantly increased the catalytic activity of 17β-HSD I. Strangely enough, metabolites of TPT ($MPTCl_3$ and $DPTCl_2$) significantly enhanced mRNA expression of 17β-HSD I, whereas metabolites of TBT ($MBTCl_3$ and $DBTCl_2$) and TETBr had little effect on mRNA expression (Figs. 2 and 3). It remains unclear why these organotin compounds enhanced the activity of 17β-HSD I with or without altering its mRNA expression. At least, the induction appears due to a mechanism other than activation of RXRs.

To our knowledge, our study is the first to show that organotin compounds potentially promote estrogenic action to enhance 17β-HSD I activity in human placenta. However, the mRNA changes that the compounds induced were not comparable to the changes in catalytic activity. Consequently, we conclude that the observed organotin-induced alterations in JAr cells are due to other mechanisms in addition to regulation of 17β-HSD I mRNA levels. The toxic mechanisms of organotin compounds appear very intricate. For instance, organotin compounds function as inhibitors of steroidogenic enzymes [18–21] and RXR ligands [14] but also have been shown to enhance histone acetyltransferase activity [40]. Future studies need to clarify the precise mechanism of action of organotin compounds in human endocrine disruption in vitro and in vivo.

Acknowledgments

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Prenatal and neonatal exposure to low-dose of bisphenol-A enhance the morphine-induced hyperlocomotion and rewarding effect

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Abstract

Bisphenol-A has been extensively evaluated for toxicity in a variety of tests as the most common environmental endocrine disruptors. In the previous study, we reported that prenatal and neonatal exposure to high-dose of bisphenol-A affects the development of central dopaminergic system in the mouse limbic area. The present study was then undertaken to investigate whether prenatal and neonatal exposure to lower dose of bisphenol-A could change the morphine-induced several pharmacological actions such as rewarding effect and hyperlocomotion in mice. Prenatal and neonatal exposure to low-dose of bisphenol-A enhanced the morphine-induced hyperlocomotion and rewarding effect. Additionally, the treatment with bisphenol-A produced an up-regulation of dopamine receptor function to activate G-protein in the mouse limbic forebrain, which is thought to play a critical role for hyperlocomotion and rewarding effects by drugs of abuse. These findings suggest that prenatal and neonatal exposure to low-dose of bisphenol-A can potentiate the central dopamine receptor-dependent neurotransmission, resulting in the supersensitivity of the morphine-induced hyperlocomotion and rewarding effects in the mouse.
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Keywords: Bisphenol-A; Dopamine; Morphine; Rewarding effect; Hyperlocomotion; Endocrine disruptor

Bisphenol-A is an environmental endocrine-disrupting chemical that affects reproduction in wildlife [4,5]. Bisphenol-A is a monomer of polycarbonate plastics and a constituent of epoxy and polystyrene resins, which are used in the food cans and found as a contaminant not only in the liquid of the preserved foods, but also in the water autoclaved in the cans [1,7]. This chemical is also released from polycarbonate flasks during autoclaving [9]. Moreover, it has been reported that significant amounts of bisphenol-A are detected in the saliva of dental patients treated with fissure sealants [15].

On the endocrine-disrupting chemical problems, the low-dose actions of the endocrine-disrupting chemicals are serious problems. However, little is known about its action on the central nervous system induced by low-dose of bisphenol-A. The aim of the present study was then undertaken to investigate whether prenatal and neonatal exposure to low-dose of bisphenol-A in mice could affect the rewarding effect and locomotor-enhancing effects induced by morphine.

The present studies were conducted in accordance with the Guide for Care and Use of Laboratory Animals adopted by the Committee on Care and Use of Laboratory Animals of Hoshi University School of Pharmacy and Pharmaceutical Sciences, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

All experiments were performed using 7 weeks old male ddY mice (Tokyo Animal Science Co., Tokyo, Japan) that had been prenatally and neonatally exposed to bisphenol-A (Wako Pure Chemical Industries Ltd., Osaka, Japan). Adult female mice (10 weeks old) were chronically treated with bisphenol-A-admixed powder food containing 0 (control), 3×10^{-2}, 3×10^{-1}, 3, 5×10^{2}, 2×10^{3} μg bisphenol-A/g of food from mating to weaning. Their pups were prenatally and neonatally exposed to the respective concentration of bisphenol-A from their mothers. During the treatment with bisphenol-A, animals did not show weight loss and disruption of maternal behaviors.

The place conditioning paradigm has been known to the method to evaluate the motivational properties as the self-administration paradigm [13,18]. The apparatus was a shuttle box ($15 \times 30 \times 15$ cm: $w \times l \times h$) which was made of acrylic resin board and divided into two equal-sized compartments. One compartment is white with a textured floor, and the other is

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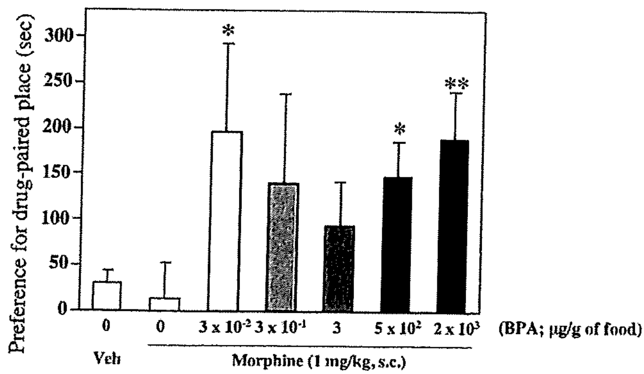

Fig. 1. Effect of prenatal and neonatal exposure to a wide range of concentrations of bisphenol-A on the morphine (1 mg/kg, s.c.) -induced rewarding effect in mice. Each column represents the mean conditioning score with S.E.M. of 6–14 mice. $^{*}p<0.05$, $^{**}p<0.01$ vs. morphine-treated control group.

black with a smooth floor to create equally preferred compartments. For conditioning, mice were confined to one compartment after drug injections and to the other compartment after saline injection. The order of the injection (drug or vehicle) and compartment (white or black) was counterbalanced across subjects. A day after these conditioning sessions, the animal is placed in the test apparatus without any confinements. At the dose of 1 mg/kg (s.c.), morphine produced neither place preference nor place aversion in control mice (Fig. 1). However, treatment with 1 mg/kg (s.c.) of morphine produced a significant place preference in the mice chronically treated with low- and high-dose of bisphenol-A, but not middle-dose of bisphenol-A, from mating to weaning (Fig. 1). The locomotor activity of mice was measured by an ambulometer as described previously [14]. Briefly, a mouse was placed in a tilting-type round activity cage 20 cm in diameter and 19 cm high. Any slight tilt of the activity cage, which was caused by horizontal movement of the mouse, was detected by three microswitches. Total activity counts were automatically recorded for 3 h following the injection of saline (10 ml/kg, s.c.) or morphine (10 mg/kg, s.c.). Total activity was counted for 3 h after the treatment. Treatment with 10 mg/kg (s.c.) of morphine produced a locomotor-enhancing effect in all groups (Fig. 2). In mice chronically treated with low- and high-dose of bisphenol-A, but not middle-dose of bisphenol-A, from mating to weaning, the hyperlocomotion induced by morphine was dramatically potentiated as compared to that in control (Fig. 2). These findings suggest that prenatal and neonatal exposure to low- and high-dose of bisphenol-A lead to the supersensitivity of morphine-induced pharmacological actions. It should be mentioned that prenatal and neonatal exposure to bisphenol-A shows the biphasic effect on the supersensitivity of morphine-induced pharmacological actions. Recently, several investigations have provided evidence that the treatment of adult animals with bisphenol-A could not affect the reproductive function and social behaviors [2,3]. We have already confirmed that acute administration of bisphenol-A with adult mice could not affect the dopamine-related behaviors (data not shown). On the other hand, the behavioral abnormalities are induced by prenatal and neonatal exposure to bisphenol-A [11,12,19,20]. These findings indicate that prenatal and neonatal exposure to bisphenol-A may cause the neuronal toxicity specifically in the developmental process. We next investigated the influence of prenatal and neonatal exposure to bisphenol-A in the development of central dopaminergic function using [^{35}S]GTPγS binding assay as described previously [20]. In the membrane preparation, mice were killed by decapitation and the limbic forebrain including the nucleus accumbens was then dissected as described previously [17]. The limbic forebrain was rapidly excised at 4 °C, and the tissues were homogenized using a Potter-Elvehjem tissue grinder with a Teflon pestle in 20 volumes (w/v) of ice-cold Tris–Mg^{2+} buffer containing 50 mM Tris–HCl (pH 7.4), $MgCl_2$ and 1 mM EGTA for the [^{35}S]GTPγS binding assay. The homogenate was centrifuged at 4 °C for 10 min at 48,000 × *g*. The pellet was resuspended in ice-cold Tris buffer of [^{35}S]GTPγS binding assay buffer containing 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA, and 100 mM NaCl and centrifuged at 4 °C for 10 min at 48,000 × *g*. The resultant pellet was resuspended in ice-cold Tris buffer or [^{35}S]GTPγS binding assay buffer and stored at −70 °C until used. The membrane homogenate (3–8 µg protein/assay) was incubated at 25 °C for 2 h in 1 ml of assay buffer with 10 µM dopamine, 30 µM guanosine-5′-diphosphate

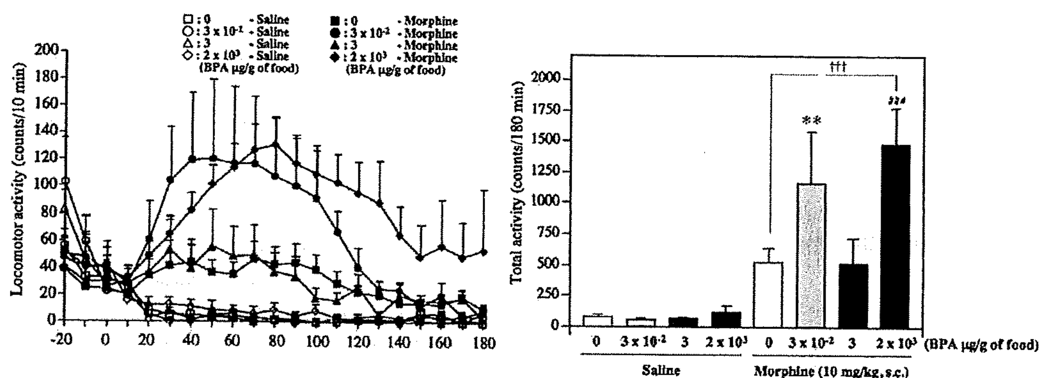

Fig. 2. Effect of prenatal and neonatal exposure to a wide range of concentrations of bisphenol-A on the morphine-induced hyperlocomotion in mice. (A) Time course changes in the morphine-induced hyperlocomotion in mice. Each point represents the mean activity counts for 10 min with S.E.M. of 5–15 mice. (B) Total locomotor activity of morphine-induced locomotor enhancing effect in mice. Each column represent the mean activity for 180 min with S.E.M. of 5–15 mice. $^{**}p<.0.01$ vs. saline-treated 3×10^{-2} BPA µg/g of food-treated group. $^{\#\#\#}p<0.001$ vs. saline-treated 2×10^{3} BPA µg/g of food-treated group. $^{\dagger\dagger\dagger}p<0.001$ vs. morphine-treated control group.

(GDP) and 50 pM [^{35}S]GTPγS (specific activity, 1000 Ci/mmol; Amersham, Arlington Heights, IL). The reaction was terminated by filtration using a Brandle cell harvester and Whatman GF/B glass filters presoaked in 50 mM Tris–HCl (pH 7.4) and 5 mM $MgCl_2$ at 4 °C for 2 h. Filters were then washed three times with 5 ml of an ice-cold Tris–HCl buffer (pH 7.4), transferred to scintillation counting vials containing 0.5 ml of Soluene-350 and 4 ml of Hionic Fluor, equilibrated for 12 h and the radioactivity in the samples was determined with a liquid scintillation analyser. Non-specific binding was measured in the presence of 10 μM unlabeled GTPγS. Comparable results were obtained from at least three independent sets of experiments. Dopamine (10 μM) produced an increase in [^{35}S]GTPγS binding to membranes from the limbic forebrain including the nucleus accumbens of control mice. Under these conditions, the stimulation of [^{35}S]GTPγS binding induced by dopamine was potentiated in mice chronically treated with wide range of concentrations of bisphenol-A from mating to weaning. Especially, the enhancement of the stimulation of [^{35}S]GTPγS binding induced by dopamine in mice chronically treated with low- and high dose of bisphenol-A was noted, witch suggests that bisphenol-A shows the biphasic effect on the regulation of dopamine receptor function in the limbic forebrain (Fig. 3). Taken together, these findings suggest that the supersensitivity of morphine-induced pharmacological actions following prenatal and neonatal exposure to especially low- and high dose of bisphenol-A may result from a drastic up-regulation of dopamine receptor function in the limbic forebrain (see Fig. 4).

All of data represent the mean counts with S.E.M. Statistical analyses were performed using one-way ANOVA with Bonferroni/Dunnett's test.

As mentioned above, humans might be orally exposed to bisphenol-A in daily life. In the previous study, we chronically treated female mice with bisphenol-A-admixed powder food containing 2×10^3 μg of bisphenol-A/g of food, and this

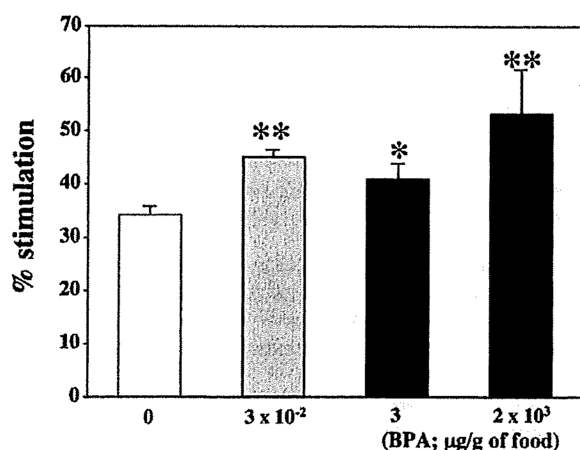

Fig. 3. Comparison of the stimulation of [^{35}S]GTPγS binding to membranes from the limbic forebrain by dopamine between control and a wide range of concentrations of bisphenol-A-treated mice. Membranes were incubated with [^{35}S]GTPγS (50 pM) and GDP (30 μM) with dopamine. The data are shown as the percentage of basal [^{35}S]GTPγS binding measured in the presence of GDP and absence of dopamine. Each column represents the mean with S.E.M. of three samples. $^{**}p < 0.01$ vs. control group.

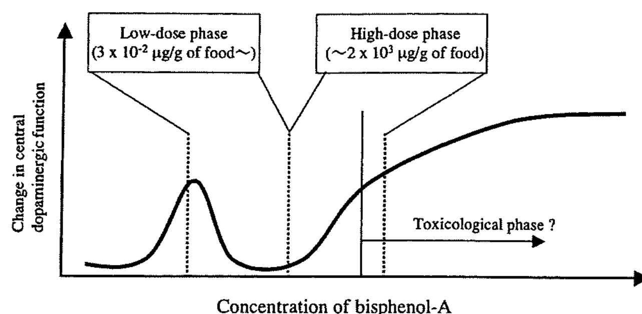

Fig. 4. A schematic drawing of the biphasic effect on the central dopaminergic function by prenatal and neonatal exposure to bisphenol-A. In the previous study, we reported that prenatal and neonatal exposure to high dose (2–2×10^3 μg/g of food) of bisphenol-A change the central dopaminergic function. Here, our findings suggest that supersensitivity of morphine-induced pharmcological actions following prenatal and neonatal exposure to low dose (3×10^{-2}–3 μg/g of food) of bisphenol-A may result from an up-regulation of dopamine receptor function in the limbic forebrain.

enhanced the rewarding effect induced by drugs of abuse in their pups [11,12,20]. Under these conditions, the blood level of bisphenol-A in their pups was approximately 10 ng/ml, which is considered to be more than 30 times higher than the level for healthy human exposure [20]. On the other hand, vom Saal et al. estimated that humans are exposed to bisphenol-A at a dose of 2–20 μg/kg/day [21]. Based on these reports, we here ascertained the effects of low dose of exposure to bisphenol-A. Adult female mice were chronically treated with bisphenol-A-admixed powder food containing 0 (control), 3×10^{-2}, 3×10^{-1}, 3×10^2, 5×10^2, 2×10^3 μg bisphenol-A/g of food from mating to weaning.

Bisphenol-A and alkylphenols have been reported to have estrogenic activity [8]. Recent molecular studies have suggested the transcriptional activation of the human dopamine D_1 receptor gene by estrogen [10]. Since bisphenol-A has only very weak estrogenic effects [23], it does not seem likely that low dose of bisphenol-A is accompanied by a classical estrogenic activity. It was reported that the binding of bisphenol-A to the non-classical membrane-bound estrogen receptor activates a guanylyl cyclase, protein kinase G and closing K_{ATP} channels [16]. Additionally, low dose of bisphenol-A can activate the transcription factor, cAMP-responsive element binding protein (CREB). Phosphorylated CREB has been shown to be increased after only a 5 min application of bisphenol-A in a calcium-dependent manner [16]. Therefore, the supersensitivity of morphine-induced pharmacological actions caused by prenatal and neonatal exposure to low dose of bisphenol-A may be mediated by non-classical membrane-bound estrogen receptors.

On the other hand, the animal model for hyperactivity was produced by Shaywitz et al., who demonstrated that rat pups treated with 6-hydroxydopamine (6-OHDA) via intracisternal administration at 5 days of age developed increased motor activity caused by the reduction of tyrosine hydroxylase (TH)-sensitive dopamine, leading to cognitive difficulties in shuttle-box learning between 2 and 4 weeks of age [17]. Additionally, Ishido et al. have reported that high dose of bisphenol-A affects the central dopaminergic system, resulting in hyperactivity due most likely to a large reduction TH activity in the midbrain

[6]. Based on these reports, we hypothesize that prenatal and neonatal exposure to high dose of bisphenol-A may cause the dopamine depletion for the limited time period and in turn induce the long-lasting supersensitivity of dopamine receptor-related action following chronic treatment with morphine.

It is very difficult to explain the fact that the prenatal and neonatal exposure to middle-dose of bisphenol-A have only weak effect on the disruption of functional changes in the dopaminergic transmission. Although the mechanisms of the weak effect by the prenatal and neonatal exposure to middle-dose of bisphenol-A remain unclear, one possibility is that the potentiation of the central dopaminergic transmission caused by the prenatal and neonatal exposure to low dose of bisphenol-A can be offset by the middle-dose of bisphenol-A through the negative feedback regulation. It is also likely that prenatal and neonatal exposure to high dose of bisphenol-A may potentiate the dopamine receptor function following a dramatic deletion of TH-sensitive dopamine and/or dysfunction of negative feedback mechanism against dopamine receptor function following the overshooting of its negative feedback.

On the endocrine-disrupting chemical problems, the low dose actions are serious problems. As well as described in the present study, it was recently reported that there were effects caused by exposure to low doses of bisphenol-A on rate of growth and sexual maturation, hormone levels in blood, reproductive organ function, fertility, immune function, enzyme activity, and brain structure, brain chemistry and behavior [22]. Therefore, our findings warn that prenatal and postnatal exposure to low- and high doses of bisphenol-A may dramatically change the neuronal transmission including dopaminergic transmission in the adult brain. This phenomenon could explain the aggravation of the development of dependence on drugs of abuse.

Acknowledgments

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Quantitative analysis of benzo[*a*]pyrene biotransformation and adduct formation in Ahr knockout mice

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Abstract

Benzo[*a*]pyrene (BP) is an ubiquitous environmental pollutant with potent mutagenic and carcinogenic properties. The Ah receptor (Ahr) is involved in the metabolic activation of BP and is therefore important in the induction of chemical carcinogenesis. In this study, the relationship between Ahr genotype and biotransformation of BP in internal organs was investigated in Ahr (+/+), Ahr (+/−) and Ahr (−/−) mice. The mice were treated with BP (100 mg/kg) by gavage. Gene expression was measured after 24 h by real-time RT-PCR and showed induction of Cyp1a1 in liver and lung, and Cyp1b1 in lung in both Ahr (+/+) and Ahr (+/−). No induction of the Cyp genes was observed in the Ahr (−/−). There was a significant basal expression of Cyp1b1 in the liver of all genotypes, and this expression was independent of the BP exposure. Analyzed by HPLC-fluorescence, there were increased levels of protein and DNA adducts, metabolites, conjugates and unmetabolized BP in the internal organs of Ahr (−/−) as compared to Ahr (+/+) and Ahr (+/−) mice. This may be partly explained by a delayed bioactivation of BP in the Ahr deficient mice. The BP metabolism observed in the Ahr (−/−) mice is also evidence of an Ahr independent biotransformation of BP.
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Keywords: Aryl hydrocarbon receptor; Knockout mice; Benzo[*a*]pyrene; Benzo[*a*]pyrene adducts; Benzo[*a*]pyrene metabolites; Cytochrome P450

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) constitute a large class of compounds formed during incomplete combustion of organic matter and fossil fuels in industrial processes, automobile exhaust, cigarette smoke and charbroiled food (IARC Monographs, 1983a,b). Exposure to PAHs is high in certain occupational environments. Several of the PAH congeners are classified as carcinogens. Benzo[*a*]pyrene (BP) is a well-studied member of the PAH family and has served as a model for the biotransformation and carcinogenic effects of PAHs (Conney, 1982; Dipple, 1995; Harvey and Geacintov, 1988; Hogan et al., 1981; Stowers and Anderson, 1985). BP and other PAHs are primarily activated by P450 enzymes regulated by the aryl hydrocarbon receptor (Ahr) pathway (Whitlock, 1999). The Ahr also plays an important role in the regulation of cell growth and differentiation. The discovery of the Ahr originated from studies with Ah responsive/non-responsive mouse models (Nebert, 1989). The importance of the Ahr in the activation of PAH has then led to several Ahr and cytochrome P450 knockout mouse models (Kondraganti et al., 2003; McFadyen et al., 2003; Nakatsuru et

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al., 2004; Shimizu et al., 2000; Uno et al., 2004, 2006).

BP acts as a ligand and binds to the Ahr in the cytoplasm. The liganded Ahr is then translocated to the nucleus where it forms a heterodimer with the Ahr-nuclear translocator (Arnt). The Ahr/Arnt heterodimer recognize and binds to xenobiotic responsive element (XRE) sequences located in the promoter region of several genes such as cytochrome P450 (Cyp)1a1, Cyp1a2, Cyp1b1, glutathione S-transferases (Gst), and UDP-glucoronosyl-transferases (Ugt) (Nebert et al., 2000; Whitlock, 1999). The binding results in transcriptional activation of the genes and induction of phases I and II metabolizing enzymes as well as phase III transporter proteins (Klaassen, 2002; Xu et al., 2005). The encoded cytochrome P450 enzymes will then transform PAH to hydroxyl containing metabolites that are rapidly conjugated to glucoronides and sulphates by phase II enzymes. The bioactivation of BP goes through reactive intermediates, like epoxides, that may produce DNA and protein adducts (Fig. 1). The formation of covalent DNA adducts is an important first step in the initiation of PAH induced carcinogenesis (Hogan et al., 1981; Stowers and Anderson, 1985), and it has been suggested that increased adduct levels may be predictive of cancer risk (Veglia et al., 2003).

Shimizu et al. (2000) found that BP carcinogenicity was lost in mice lacking the Ahr. The mice received topical application and subcutaneous injection of the PAH, and only the Ahr (+/+) and Ahr (+/−) mice developed

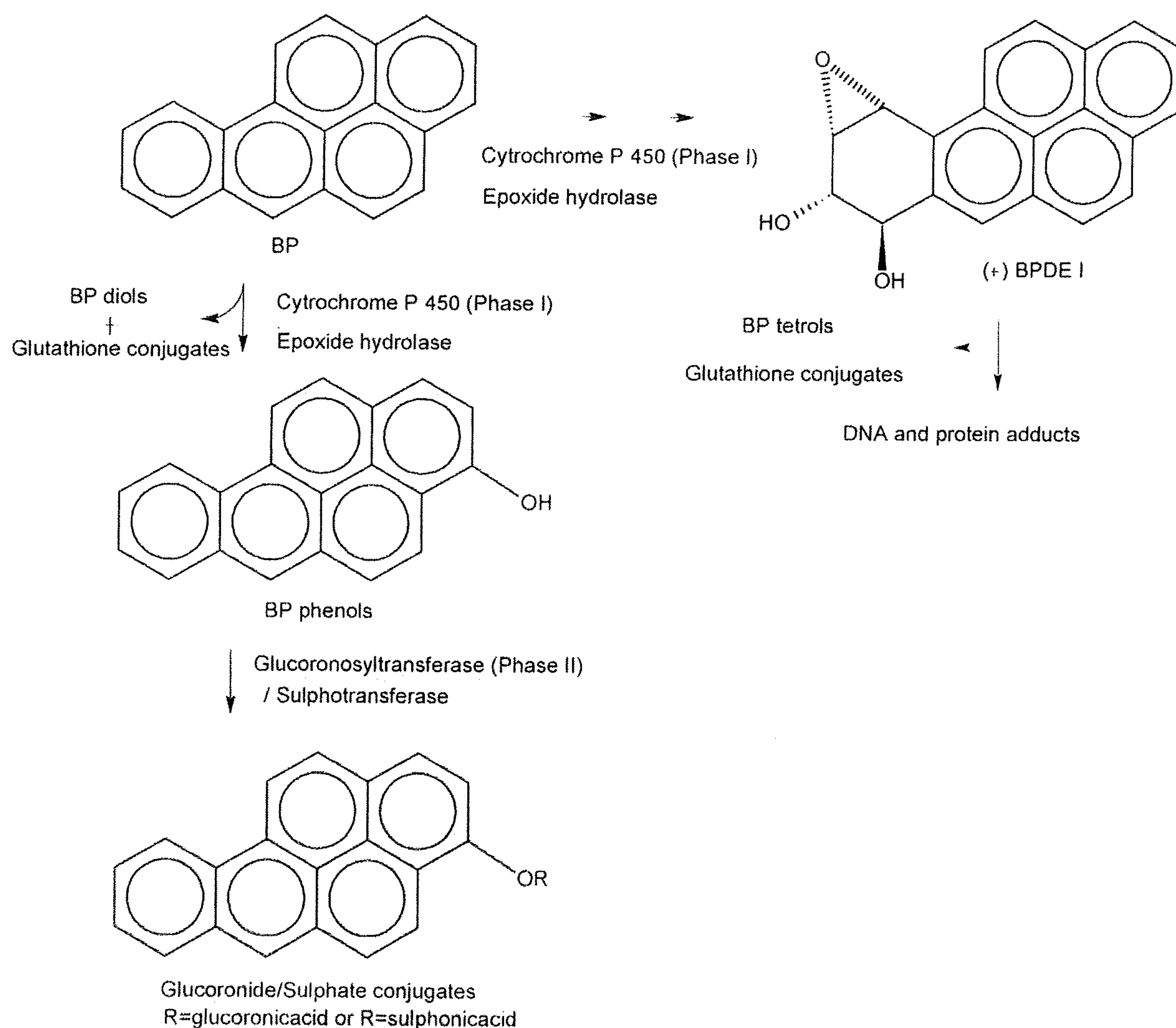

Fig. 1. Metabolism of BP by the cytochrome P450 (phase I) that initially transform BP to hydroxyl containing metabolites. Reactive epoxides are rapidly hydrolysed by epoxide hydrolase or conjugated by glutathione transferase. The horizontal reaction illustrates the formation of the ultimate carcinogenic metabolite, the 7,8-diol-9,10-epoxide (BPDE I) that may form DNA and protein adducts. The vertical reaction illustrates the detoxifying pathway of metabolites by phase II conjugation.

tumors. Kondraganti et al. (2003) found that total hepatic BP-DNA adduct levels were almost equal in Ahr (−/−) and Ahr (+/+) mice after a single i.p. dose of BP. In the knockout studies by Uno et al. (2004, 2006), it was shown that BP-DNA adducts and genotoxicity increased in the absence of the Cyp 1a1 and Cyp 1b1 genes.

To gain further insight in the role of the Ahr in the metabolic activation and detoxication of PAH, we have studied the relationship between Ahr genotype and bioactivation and biotransformation of BP in internal organs. In contrast to previous studies we have treated the animals with a single dose of BP by gavage. In the present report, we have quantitated protein and DNA adducts and metabolites. By the use of a highly specific HPLC-fluorescence method, we find increased levels of protein and DNA adducts, metabolites and unmetabolized BP in the Ahr (−/−) as compared to Ahr (+/+) mice. Gene expression has been measured by quantitative real-time RT-PCR.

2. Materials and methods

2.1. Chemicals and standards

(±)-Benzo[*a*]pyrene-r-7,t-8,t-9,c-10-tetrahydrotetrol (BP-tetrol I-1), (±)-benzo[*a*]pyrene-r-7,t-8,t-9,10-tetrahydrotetrol (BP-tetrol I-2), (±)-benzo[*a*]pyrene-r-7,t-8,c-9,t-10-tetrahydrotetrol (BP-tetrol II-1), (±)-benzo[*a*]pyrene-r-7,t-8,c-9,c-10-tetrahydrotetrol (BP-tetrol II-2), benzo[*a*]pyrene-4,5-dihydrodiol, benzo[*a*]pyrene-7,8-dihydrodiol, benzo[*a*]-pyrene-9,10-dihydrodiol, benzo[*a*]pyrene-3-phenol and benzo[*a*]pyrene-9-phenol were purchased from the National Cancer Institute (NCI), Chemical Carcinogen Repository (Midwest Research Institute, Kansas City, MO, USA). The two remaining tetrols, BP-tetrol III-1 and BP-tetrol III-2 were prepared in our laboratory as described earlier (Sagredo et al., 2006). Benzo[*a*]pyrene was purchased from Sigma (St. Louis, MO, USA). HPLC grade methanol was obtained from Fluka (Buchs, Switzerland). Water was obtained from a Milli-Q ultrapure water purification system (Milipore, Bedford, MA, USA). Benzo[*a*]pyrene glucoronide and sulphate conjugates were prepared in our laboratory.

2.2. Animals and treatment

The Ahr heterozygote model (C57BL6) has been described previously (Shimizu et al., 2000). The animals were aclimatized after arrival in a germ free facility using air-filtered controlled environment. The Ahr (+/−) were interbred to generate Ahr (+/+), Ahr (+/−) and Ahr (−/−) mice. Genotyping was carried out as described previously (Shimizu et al., 2000). The formation of the knockout offspring did not follow the Mendelian law, since repeatedly only about 10–15% of the offspring had the Ahr (−/−) genotype. Real-time RT-PCR measurement of AHR expression was carried out on lung tissue samples at the end of the experiments to verify genotypes. There were no observed differences in growth rate and appearance between the different genotypes. The animals were fed standard diet (B&K Universal A/S, Norway) and water ad libitum. BP was solubilized in corn oil (10 mg/ml) and the animals were treated with a single dose of BP (100 mg/kg) by gavage. Three females and 3 males of each genotype were included, in total 18 animals. The control group of three animals, one of each genotype, received pure corn oil. After 24 h, the animals were sacrificed and lung, liver, spleen, kidney and heart were removed and blood samples collected. The experiments were repeated with similar result (data not shown). All animal handling and experimental procedures were conducted in conformity with laws and regulations controlling experiments on live animals in Norway and the European Convention for the Protection of Vertebrate Animals used in Experimental and Other Scientific Purposes.

2.3. Protein and DNA isolation

Harvested organs were homogenized in a phosphate buffer solution using a mixer mill followed by a brief centrifugation. The pellet was used for DNA isolation (liver) and the supernatant for protein and metabolite quantification. Protein concentrations were determined by the Lowry method (Lowry et al., 1951). Blood from the animals was collected with Na-heparin, and plasma separated by centrifugation at 1200 × *g* for 15 min. The plasma and the supernatant were withdrawn and proteins were precipitated with the addition of two volumes of cold acetone. Samples were left for 30 min followed by centrifugation at 1200 × *g*. The precipitated proteins were washed twice with 4 ml acetone:ethylacetate (1:1) to remove unbound BP metabolites. The washing fractions were pooled and stored for HPLC analysis. The precipitate was air dried at room temperature and solubilized in 900 μl of 10 mM Tris–HCl/1 mM EDTA pH 8.0. DNA was isolated as described by Beach and Gupta (1992). DNA concentration was quantitated spectrophotometrically and by fluorescence measurements with a Hoechst 33258 instrument.

2.4. Adduct purification

BP-protein and DNA adducts were measured as released BP-tetrols after acid hydrolysis. The ultimate carcinogenic diolepoxide, BPDE-I, gives rise to the two tetrols BP-tetrol-I-1 and BP-tetrol-I-2. The less carcinogenic diolepoxide, BPDE-II, gives rise to the two tetrols BP-tetrol-II-1 and BP-tetrol-II-2. In addition, two BP-tetrols were detected after DNA and protein hydrolysis, originating from the non-bay region diolepoxide, BPDE III (Sagredo et al., 2006).

The 900 μl protein solution was added 100 μl of 1 M HCl, and this solution was incubated at 70 °C for 3 h. Water and methanol were added to a final volume of 5 ml with 10% methanol. This solution was applied to preconditioned Sep-Pak C_{18} cartridges (Millipore, Milford, MA) followed by 10 ml washing with water and the tetrols were eluted with 5 ml methanol. The eluate was evaporated at 45 °C under a nitro-

gen stream and resolubilized in 500 μl of 10% methanol. The samples were stored at −20 °C.

2.5. HPLC-fluorescence quantitation

The analysis was performed on an Agilent 1100 LC system using a Hypersil ODS, 5 μm, 3.9 mm × 150 mm column (Agilent) equipped with an Agilent 1100 fluorescence detector. The column temperature was 40 °C and the flow rate was set at 1 ml/min. The injection volume was typically 20 μl and the samples were separated by a linear gradient of water and methanol by increasing the methanol content from 30% to 100% in 40 min. The excitation and emission wavelengths were 341 and 381 nm, respectively. The benzo[*a*]pyrene glucoronide and sulphate conjugates were analyzed directly without any previous hydrolysis. Separation was achieved using a 10 mM amoniumphosphate buffer (pH 6) at a flow rate of 0.8 ml/min. The linear gradient of buffer and methanol was increased from 20% to 98% methanol in 55 min.

2.6. Gene expression

Gene expression measurements were carried out by quantitative real-time RT-PCR on an ABI PRISM 7900 (Applied Biosystems) as described previously (Berge et al., 2004). In brief, total RNA was reverse transcribed by the aid of random primers. Primers for β-actin (Actb), Cyp1a1 and Cyp1b1 were as in Berge et al. (2004). Primer for Ahr were designed by the PrimerExpress 2.0.0 software (Applied Biosystems) and the sequences were: Ahr upper: 5′-CAG TCC AAT GCA CGC TTG ATT-3′, Ahr lower: 5′-ACA GCC TCT CCG GTA GCA AA-3′ (146 bp). The amount of target cDNA in each sample was established by determining a fractional PCR threshold cycle number (C_t-value). Specific gene expression levels were normalized to the expression of β-actin and calculated by the formula: $2^{-(C_t\text{gene}-C_t\beta\text{-actin})}$.

2.7. Statistical analysis

For the analysis of gene expression, protein adducts and metabolites, means were compared by the independent-samples *t*-test. Due to significant variations in standard deviation Welch correction was applied.

3. Result

In animals exposed to BP, real-time RT-PCR analysis showed induction of Cyp1a1 in liver and lung in both Ahr (+/+) and Ahr (+/−) but no induction in the Ahr (−/−) (Fig. 2A). There was also an induction of Cyp1b1

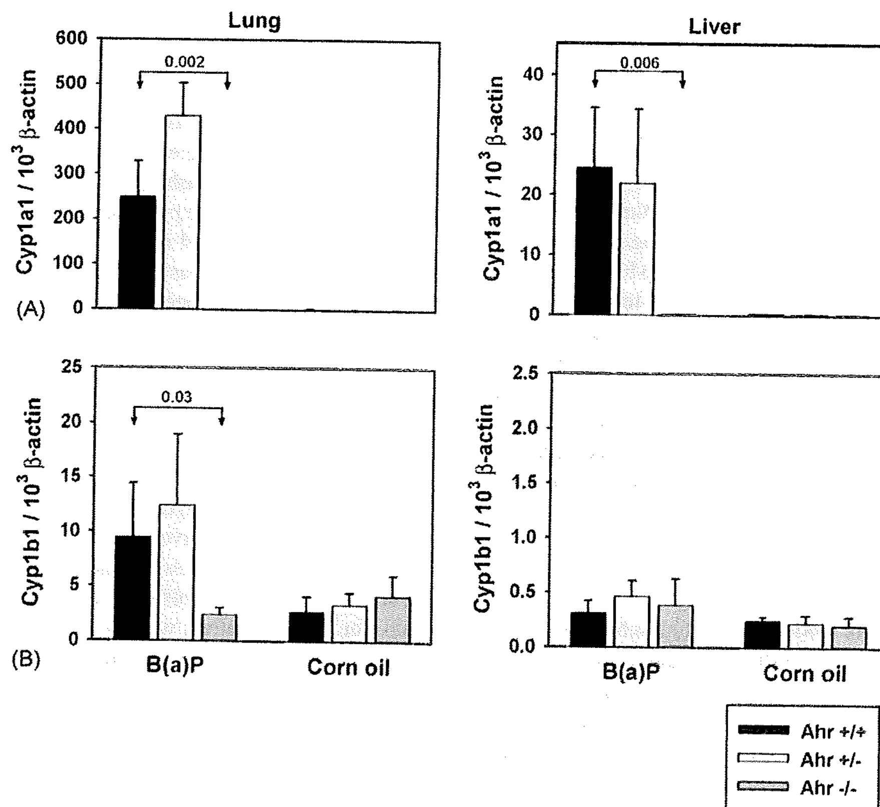

Fig. 2. (A) Cyp1a1 and (B) Cyp1b1 levels in lung and liver tissues were measured by real-time RT-PCR and normalized to the expression of β-actin. Mice were given a single oral dose with BP (100 mg/kg) in corn oil ($n=6$) for 24 h and pure corn oil for the control group ($n=3$). Means were compared by the independent samples *t*-test with Welch correction.

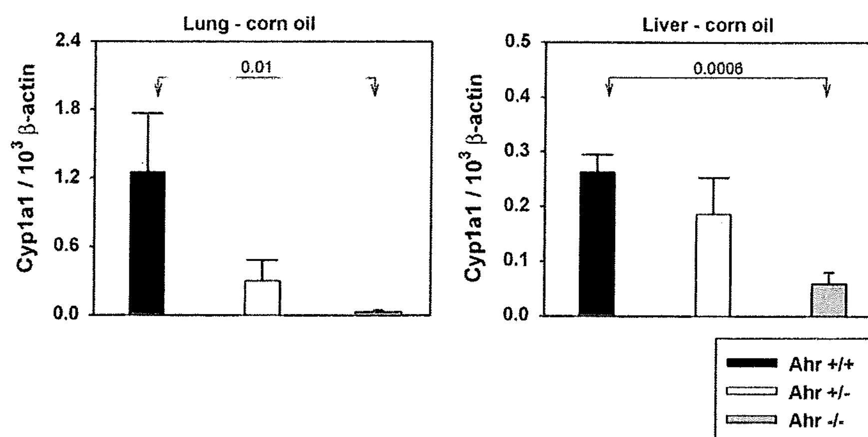

Fig. 3. Basal expression of Cyp1a1 in lung and liver of Ahr (+/+), Ahr (+/−) and Ahr (−/−) mice (n = 3) exposed to corn oil. Cyp1a1 expression was normalized to the expression of β-actin. Means were compared by the independent samples t-test with Welch correction.

in the lung of both Ahr (+/+) and Ahr (+/−), but no induction in the Ahr (−/−) (Fig. 2B). There was a significant basal expression of Cyp1b1 in the liver of all genotypes, and this expression was independent of the BP exposure. Constitutive Cyp1a1 expression level showed an Ahr gene–dose relationship in both the liver and lung, where Ahr (+/+) > Ahr (+/−) > Ahr (−/−) (Fig. 3). This was not observed for Cyp1b1.

HPLC-fluorescence analysis of the BP-protein adduct hydrolysis showed that the total BP-tetrol levels (the sum of the BP-tetrol I, BP-tetrol II and BP-tetrol III) were significantly higher in the Ahr (−/−) group compared to Ahr (+/+) and Ahr (+/−), in all of the tissues (Fig. 4; Table 1). In general, the total levels of BP-tetrols were,

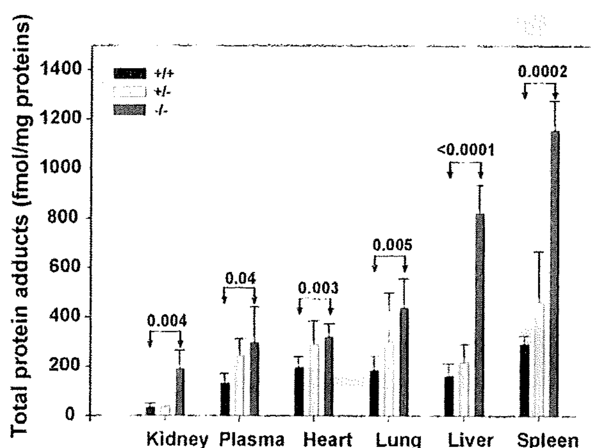

Fig. 4. Total sum (fmol/mg proteins) of the protein adduct hydrolysis products in kidney, plasma, heart, lung, liver and spleen from BP exposed mice. The tetrols were measured by HPLC-fluorescence as described in Section 2. The values are the sum of the BP-tetrol I, BP-tetrol II and BP-tetrol III. The BP-tetrol values are the means ± S.D. of data from six animals of each genotype. Means were compared by the independent samples t-test with Welch correction.

in descending order, spleen, liver, lung, heart, plasma and kidney. When examining specific tetrol levels, the BP-tetrol-I-1 and BP-tetrol-II-2 showed an inverse relationship compared to the Ahr gene–dose, with Ahr (−/−) > Ahr (+/−) > Ahr (+/+). In liver an increased ratio of BP-tetrol-II-2 to BP-tetrol-I-1 was found in Ahr (−/−) mice. There were significantly higher levels of the BP-tetrol I-1 and BP-tetrol II-2 in all the tissues in Ahr (−/−) group compared to the wild type animals (Fig. 5). In liver, BP-DNA adducts were found to parallel the levels of BP-protein adducts (Fig. 6).

The levels of unbound BP-tetrols were substantially higher in the Ahr (−/−) mice (Table 2). The lung and spleen displayed the highest levels while the heart and liver contained the lowest levels of free metabolites. The levels of unmetabolized BP were also significantly higher in the Ahr (−/−) mice as compared to the wild type mice. The Ahr (−/−) mice showed the highest levels of BP in the distal organs like lung and spleen, and the lowest levels in the liver. The BP content was 300 times higher in Ahr (−/−) lung and 190 times higher in Ahr (−/−) spleen compared to the corresponding levels in lung and spleen in the Ahr (+/+) (Table 2).

The formation of sulphate and glucoronide conjugates in the lung, spleen and heart were significantly higher in the Ahr (−/−) as compared to the Ahr (+/+). The highest levels of conjugates were found in kidney in both Ahr (−/−) and Ahr (+/+). The conjugate levels in kidney were higher in the Ahr (−/−) as compared to Ahr (+/+), but the difference was not statistically significant. Unconjugated phenolic compounds were detected in kidney and liver in both Ahr (+/+) and Ahr (−/−) mice, although Ahr (−/−) mice displayed the highest levels. These compounds were otherwise not found in the other organs (Table 2).

Table 1
BP-tetrol levels in internal organs and plasma from Ahr (+/+) and Ahr (−/−) mice

	Ahr (+/+)				Ahr (−/−)			
	BP-tetrol I	BP-tetrol II	BP-tetrol III	Sum	BP-tetrol I	BP-tetrol II	BP-tetrol III	Sum
Kidney	25.6 ± 4.9	7.7 ± 2.3	5.9 ± 1.9	39.2 ± 8.4	127.6 ± 59.2	42.4 ± 11.5	19.4 ± 6.3	189.4 ± 73.3*
Plasma	40.4 ± 12.9	48.0 ± 14.2	42.2 ± 13.2	130.6 ± 39.5	113.7 ± 55.2	116.4 ± 60.0	63.9 ± 32.0	294.0 ± 145.4
Heart	89.9 ± 19.3	63.7 ± 16.5	40.3 ± 9.3	194.0 ± 43.6	178.7 ± 29.0	97.2 ± 19.9	42.0 ± 6.3	317.9 ± 54.1*
Lung	107.9 ± 33.4	48.7 ± 18.4	25.1 ± 9.1	181.7 ± 59.7	258.4 ± 70.0	134.7 ± 40.4	41.8 ± 10.4	434.9 ± 118.8*
Liver	84.2 ± 24.6	47.1 ± 19.3	27.9 ± 11.0	159.3 ± 50.4	261.8 ± 41.0	471.1 ± 79.5	87.1 ± 19.1	819.9 ± 112.7*
Spleen	194.0 ± 26.2	64.3 ± 9.4	30.6 ± 4.8	289.0 ± 34.9	737.2 ± 122.3	287.4 ± 22.7	131.4 ± 15.2	1156.0 ± 134.8*

BP-tetrol levels (fmol/mg proteins) from the protein adduct hydrolysis expressed as the means ± S.D. of data from six individual animals in Ahr (+/+) vs. Ahr (−/−) mice.
* Statistically significant difference between Ahr (+/+) and Ahr (−/−) mice at $p < 0.05$ (independent samples t-test with Welch correction).

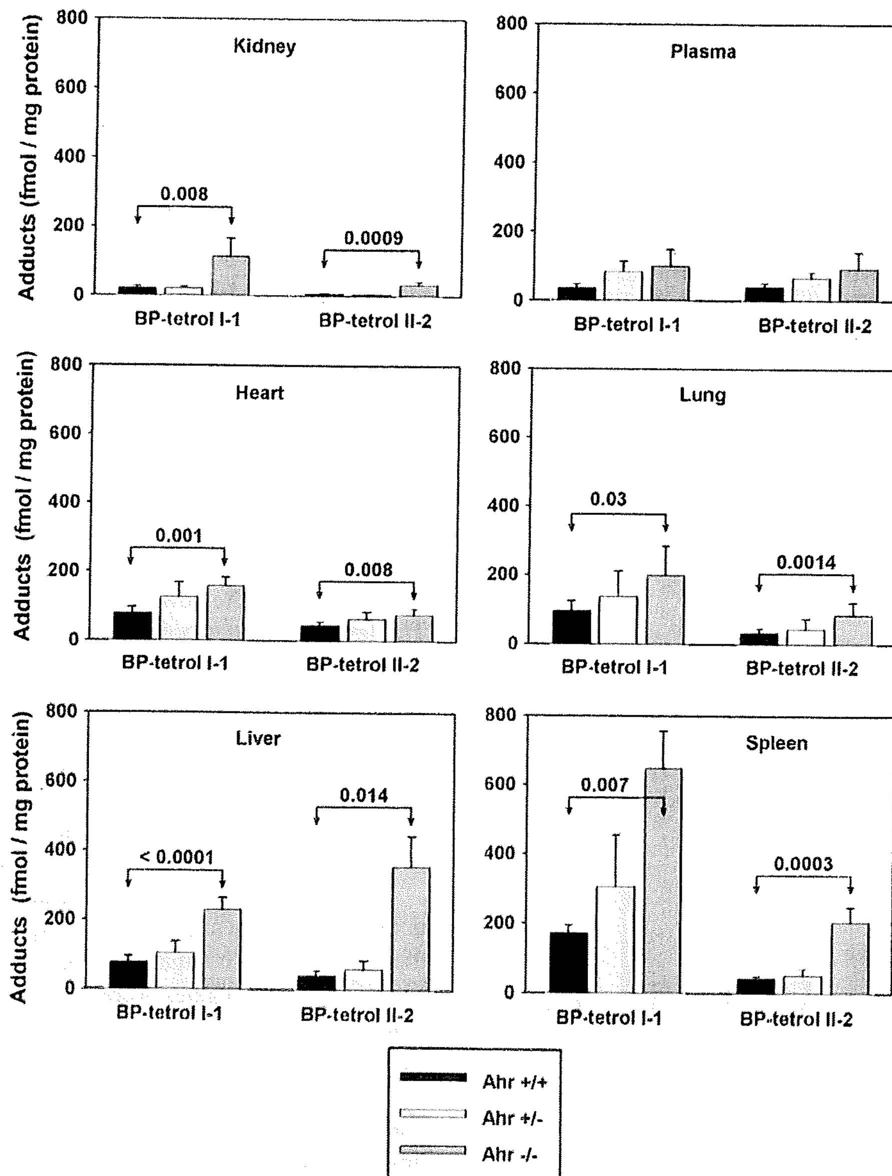

Fig. 5. The BP-tetrol I-1 and BP-tetrol II-2 in kidney, plasma, heart, lung, liver and spleen. In all cases, the tetrol levels are significantly higher in the Ahr (−/−) as compared to the Ahr (+/+). Means were compared by the independent samples t-test with Welch correction.

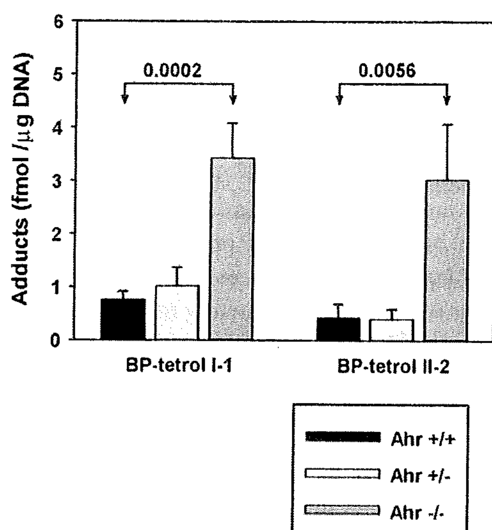

Fig. 6. BP-tetrol I-1 and BP-tetrol II-2 measured after hydrolysis of DNA-adducts in liver of BP exposed mice. Levels of both tetrols were significantly higher in Ahr (−/−) compared to Ahr (+/+). Means were compared by the independent samples t-test with Welch correction ($p<0.05$).

4. Discussion

In the present study, the metabolism of BP given by gavage in Ahr knockout, heterozygotes and wild type mice has been compared. A significant accumulation of unmetabolized BP and increased levels of adducts and metabolites were found in Ahr (−/−) as compared to Ahr (+/−) and Ahr (+/+) mice. The BP-tetrol levels showed an inverse relationship compared to the Ahr gene–dose. In the liver of the Ahr (−/−) mice, the levels of BP-tetrol II-2 were higher than BP-tetrol I-1. These results indicate that the Ahr (−/−) mice have a higher formation of the less carcinogenic BPDE II than of the ultimate carcinogenic BPDE I in the liver. However, this pattern was not observed in the other tissues. The use of tetrols as surrogate markers for protein adducts is a highly specific and valuable biomarker method (Jansen et al., 1994; Rahn et al., 1982). Although less sensitive, the HPLC-fluorescence method gives valuable structural information regarding the formation of diolepoxides in the BP metabolism compared to ^{32}P-postlabeling (Eriksson et al., 2004).

The gastrointestinal tract and the liver are important in detoxicating orally administrated BP (Nebert, 1989). In the present study, organs distal to the stomach (the site of administration) like lung and spleen from Ahr (−/−) mice showed the highest levels of unmetabolized BP as compared to the other organs in Ahr (−/−) mice, and to the lung and spleen in the Ahr (+/+) and Ahr (+/−) mice.

Table 2
BP metabolite levels in internal organs and plasma from Ahr (+/+) and Ahr (−/−) mice

	Ahr (+/+)					Ahr (−/−)				
	BP	Protein adducts	Free tetrols	Diols/phenols	Conjuagtes	BP	Protein adducts	Free tetrols	Diols/phenols	Conjugates
Lung	175 ± 115	182 ± 60	26 ± 10	ND	150 ± 49	56306 ± 14724*	435 ± 119*	1219 ± 235*	ND	1754 ± 1522*
Spleen	219 ± 130	289 ± 35	15 ± 2	ND	59 ± 28	41085 ± 27678*	1156 ± 135*	1055 ± 132*	ND	1230 ± 657*
Plasma	580 ± 161	131 ± 39	–	–	–	20620 ± 10667*	294 ± 145	–	–	–
Heart	162 ± 144	194 ± 44	6 ± 3	ND	53 ± 12	17272 ± 9325*	318 ± 54*	573 ± 109*	ND	507 ± 294*
Kidney	833 ± 542	39 ± 8	25 ± 12	158±92	15222 ± 5319	14278 ± 2364*	189 ± 73*	857 ± 280*	2650 ± 627	20606 ± 4167
Liver	65 ± 46	159 ± 50	20 ± 9.1	93±70	1341 ± 355	8070 ± 5558*	820 ± 113*	695 ± 295*	1912 ± 591	6479 ± 3392*

Metabolites (fmol/mg proteins) expressed as the means ± S.D. of data from six individual animals in Ahr (+/+) vs. Ahr (−/−) mice.
* Statistically significant difference between Ahr (+/+) and Ahr (−/−) mice at $p<0.05$ (independent samples t-test with Welch correction); ND, not detected; –, not measured.

The higher levels of BP in the Ahr (−/−) mice may be explained by reduced metabolism in the liver and therefore less presystemic elimination through the gut. This has also been observed with Ah non-responsive mice that were orally exposed to BP. The systemic exposure to BP increased in organs distal to the site of administration, as spleen and bone marrow, resulting in higher toxicity of BP and increased cell turnover (Galvan et al., 2003; Nebert, 1989). The high levels of adducts, metabolites and BP found in our Ahr (−/−) mice should then induce a higher genotoxic effect in the knockout as compared to the wild type.

Recently, it was found that deletion of the Cyp1a1 and Cyp1a1/1b1 genes resulted in a slow clearance of BP and increased DNA adducts in liver, spleen and bone marrow as measured by ^{32}P-postlabeling (Uno et al., 2004, 2006). In these studies, the mice received an oral dose of 125 mg/kg/day of BP continuously. The higher adduct levels were explained by a reduced phase II conjugation due to a loser coupling between the metabolizing enzymes in the knockout mice and the phase II conjugating enzymes, indicating that the induction of Cyp1a1 was protective and more important in the detoxification than the bioactivation of BP. Meanwhile, Cyp1b1 was suggested to be more important in the bioactivation of BP leading to adduct formation in spleen and bone marrow, and damage to the immune system (Uno et al., 2004, 2006).

In the present study, the basal expression of Cyp1a1 in lung and liver were almost not detectable, but highly induced in the wild type mouse. In contrast, basal expression of Cyp1b1 was present in lung and liver of all genotypes. In the Ahr (+/+) mice, the Cyp1b1 was induced in the lung, but not in the liver. We have not found any sign of induction of Cyp1a1/1b1 in the Ahr (−/−) mice. Nevertheless, the BP metabolism in our study could partly be explained by constitutive expression of Cyp1b1. The BP metabolism observed in the Ahr (−/−) may also be evidence of an Ahr independent bioactivation and biotransformation of BP (Kondraganti et al., 2003). There are other Cyp isoforms (Cyp1a2, Cyp2–Cyp4) that may be involved (Shou et al., 1994), together with other oxidative enzymes. Kondraganti et al. (2003) found that an i.p. dose of BP induced formation of equal amounts of hepatic BP-DNA adducts in both Ahr (+/+) and Ahr (−/−) mice. Although BP did not induce Cyp1a1/1a2 in Ahr (−/−), they measured basal expression of Cyp1a1/1b1 in both Ahr (+/+) and Ahr (−/−) liver. Based on these results they proposed the existence of Ahr independent bioactivation of BP in the knockout mice. There are also reports of Ahr independent induction of Cyp1a1/1a2 and Cyp1b1 (Delescluse et al., 2000; Galvan et al., 2005; Nakatsuru et al., 2004), that may depend on the type of PAH and the administration route. Nakatsuru et al. (2004) found that dibenzo[*a,l*]pyrene (DB[*a,l*]P), after skin application, induced low levels of Cyp1a1 in the Ahr (−/−) mouse skin, while BP and 7,12-dimethylbenz[*a*]anthracene (DMBA) was not found to induce any Cyp1a1. Galvan et al. observed Ahr independent bioactivation of BP and DMBA in bone marrow in Ahr non-responsive mice. The mice were given an i.p. dose of BP, which apparently increased the systemic exposure and toxicity of BP (Galvan et al., 2005). Finally, data from Cyp knockout studies could also support the idea of an Ahr independent bioactivation of BP (Uno et al., 2004, 2006).

There appears to be an active phase II conjugation in the Ahr (−/−) due to the formation of conjugates in all the tissues examined. This could be the result of a basal expression of phase II enzymes or an Ahr independent phase II induction. At the same time, the higher levels of adducts and free tetrols found in the Ahr (−/−) points towards a reduced phase II conjugation (Nebert et al., 2004). The higher levels of conjugates in the Ahr (−/−) might also be the effect of a reduced transport of water soluble compounds out of the cell, rather than an increased formation of conjugates (Ebert et al., 2005). Thus, in the absence of a functional Ahr receptor, and a proper induction of the phases I and II, and probably phase III enzymes (Klaassen, 2002; Xu et al., 2005), there appears to be a lower metabolic clearance of BP resulting in increased levels of DNA and protein adducts, metabolites and conjugates in the Ahr (−/−) mice.

The Ahr (−/−) mice have been found to be protected against BP induced carcinogenicity in the skin. On the other hand, there are reports indicating that the Ahr (−/−) mice may not be less susceptible to BP induced adduct formation (Kondraganti et al., 2003). In addition, toxicity of BP increases in Ahr non-responsive mice (Galvan et al., 2003; Galvan et al., 2005; Nebert, 1989) and Cyp knockout mice (Uno et al., 2004, 2006).

In conclusion, increased levels of protein and DNA adducts, metabolites and unmetabolized BP in mice lacking the Ahr were observed after oral exposure to BP. This may partly be explained by an Ahr independent and/or delayed bioactivation of BP in the Ahr knockout mice. The DNA-adduct level resulting from exposure to PAH has been suggested to represent not only a molecular exposure marker, but also a marker of cancer risk. Further studies are needed to clarify the persistence of BP adducts in Ahr (−/−) after oral exposure and their relation to cancer.

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