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Original Article

Sex-dependent difference in the hepatic and pulmonary toxicological effects in mice administrated 7-chlorinated benz[*a*]anthracene

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ABSTRACT — Chlorinated polycyclic aromatic hydrocarbons (Cl-PAHs) have recently been found in the environment at relatively high concentrations. However, their toxicological information has not been well documented. In this study, a 24 hr *in vivo* experiment was conducted to evaluate the sex-dependent difference of the acute toxicological effects of 7-chlorinated benz[*a*]anthracene (7-ClBaA) as a model Cl-PAH. 7-ClBaA or its parent chemical, BaA, was once orally administered to male or female ICR mice at concentrations of 1, 10, and 100 mg/kg body weight. The relative liver weights of the males were significantly increased at the highest dose of both chemicals compared to the vehicle controls, but the weights were comparable among all groups in the females. The plasma 7-ClBaA level was similar in both sexes, but significantly higher than that of BaA. 7-ClBaA dose-dependently induced expression of the genes *Cyp1a1*, *1a2*, and *1b1* in the liver and lung, and these stimulations were significantly higher in both organs and genders at a dose of 100 mg/kg 7-ClBaA compared with an equivalent amount of BaA, except in the case of hepatic *Cyp1a2* and *1b1* and pulmonary *Cyp1a2* in the female mice. The results suggest that acute toxicity of 7-ClBaA is gender- and organ-specific, and female mice might be less sensitive to acute toxicity of both 7-ClBaA and BaA than the males.

Key words: 7-Chlorinated benz[*a*]anthracene, Chlorinated polycyclic aromatic hydrocarbons, Cytochrome P450 1 family, Mice, Gender difference

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are by-products of the incomplete combustion of organic compounds, and are ubiquitous in water, soil, urban air, cigarette smoke, and foods (European Commission, 2002; IARC, 2010). Over the years, numerous studies have emphasized their toxicological effects, and suggested that some major PAHs, such as benz[*a*]anthracene (BaA) and benzo[*a*]pyrene (BaP), cause cancers in various organs and tissues (U.S. Environmental Protection Agency, 1999; European Commission, 2002). Importantly, intact PAHs themselves are chemically inert, but exert carcinogenic effects after metabolic activation by several drug-metabolizing enzymes, especially members of the cytochrome P450 (CYP) 1 family (Nebert *et al*., 2004). Notably, some PAHs show aryl hydrocarbon receptor (AhR)-dependent induction of several CYP 1 family members, mainly CYP1A1, 1A2 and 1B1, in organs such as the liver and lung (Shimada *et al.,* 2002). The carcinogenic metabolites of PAHs activated *via* multiple CYP enzymes then interact with DNA to initiate mutation and cell transformation (Guengerich and Shimada, 1991; Xue and Warshawsky, 2005). Therefore, a study on the effects of PAHs on AhR-mediated activities, particularly the induction of CYP1 family genes, is essential for understanding the toxicolog-

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ical effects of novel PAHs.

Meanwhile, their chlorinated compounds, Cl-PAHs, which are presumed to originate from the combustion of organic materials containing chlorine, have been widely detected in the environment in recent years (Ni and Zeng, 2012; Ohura *et al.*, 2005; Ohura, 2007; Sankoda *et al.*, 2012). However, toxicological information on these novel PAHs has been limited. Hence, it has become increasingly important to elucidate how the chlorination affects the toxicology of their parent PAHs. We recently synthesized 7-chlorinated BaA (7-ClBaA) (Fig. 1), one of the major environmental Cl-PAHs (Ohura, 2007), and reported on several of its biological characteristics. First, we found that 7-ClBaA is a potent AhR ligand in an *in vitro* assay system (Ohura *et al.*, 2007). Second, repeated oral exposure to 7-ClBaA for 14 days selectively induced hepatic *Cyp1a2* gene expression in male F344 rats, although treatment with the parent BaA induced *Cyp1a1*, *1a2* and *1b1* (Kido *et al.*, 2013). According to the Ames test, in the presence of rat recombinant *Cyp1a2,* 7-ClBaA induces frameshift mutations in *Salmonella typhimurium*, but BaA does not exert such mutagenicity (Kido *et al.*, 2013). Furthermore, after 14 days of oral administration of 7-ClBaA or BaA to male F344 rats, both chemicals were detected in their plasma, and also in their liver, muscle, kidney, spleen, heart, and lung (Sakakibara *et al.*, 2013). These results imply that chlorination might alter the toxicological effects of parent PAHs. On the other hand, gender is well recognized to be an important factor that influences the drug metabolism in experimental animals and also humans (Degawa *et al.*, 1985; Masubuchi *et al.*, 2011; Waxman and Holloway, 2009). However, to our knowledge, no information on sex-dependent differences in the toxicological effects of Cl-PAHs has been reported.

In the present study, therefore, we conducted *in vivo* experiments using male and female mice to evaluate sex-dependent differences in the toxicological effects of Cl-PAHs using 7-ClBaA as a model compound. Male and female ICR mice were given a single oral administration of one of several different doses of BaA or 7-ClBaA. Twenty-four hours later, their body and organ weights, plasma aspartate transaminase (AST) activity, and plasma BaA and 7-ClBaA concentrations were measured. In addition, the gene expression levels of *Cyp1a1, 1a2 and 1b1* in the liver and lung were analyzed using quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

MATERIALS AND METHODS

Chemicals

BaA was purchased from Sigma-Aldrich (St. Louis, MO, USA). Its chlorinated compound, 7-ClBaA, was synthesized *via* the chlorination of BaA according to our previous methods (Kido *et al.*, 2013; Ohura *et al.*, 2005). After synthesis, 7-ClBaA was purified chromatographically, and its purity was greater than 95%. All other reagents were of the highest grade available.

Animal experiments

Male and female ICR mice (n = 35 of each sex; age, 6 weeks; Japan SLC, Shizuoka, Japan) were randomly divided into 5 animals/group and 5 animals/cage. They were housed in a temperature (23 ± 1°C), humidity (50 ± 10%), and light (12-hr dark/12-hr light cycle, lights on at 7 am) controlled facility with free access to laboratory chow (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. After 7 days of acclimatization, each ani-

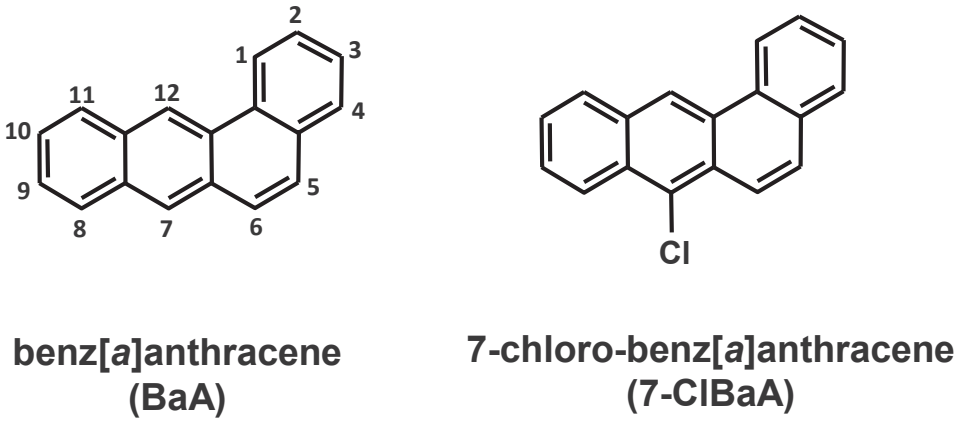

Fig. 1. Structures of BaA and 7-ClBaA.

mal was given a single oral administration of BaA or 7-ClBaA. Briefly, the test solutions described above were prepared at concentrations of 1, 10 or 100 mg/10 mL of peanut oil, and then each solution was intragastrically administered at 10 mL/kg body weight to achieve a dose of 1, 10 or 100 mg/kg body weight. The vehicle control group was administered the same volume of peanut oil. Twenty-four hours after the administration, including overnight fasting, mice were anesthetized abdominally with sodium pentobarbital, and blood samples were collected *via* cardiac puncture using heparinized syringes. The liver and lung were removed and weighed immediately after the collection of the blood samples. Then several specimens of each organ were immersed in RNALater® (Ambion Incorporated, Austin, TX, USA) at 4°C overnight, and stored at -20°C until RNA extraction. The plasma fraction was collected after centrifugation at 3,000 rpm for 10 min, and kept under -80°C.

All procedures were conducted according to the guidelines for the care and use of laboratory animals of the National Institute of Animal Health (Tsukuba, Japan). BaA and 7-ClBaA are toxicants and carcinogens; hence, laboratory coats, gloves and masks were worn at all times, and contaminated materials were incinerated.

Plasma activity of AST

The activity of the plasma biomarker for hepatic injury, aspartate transaminase (AST), was determined using a dry-chemistry blood analyzer (Spotchem EZ SP-4430) for clinical chemistry (Arkray Inc., Kyoto, Japan). Tests were carried out in accordance with the manufacturer's instructions by using Spotchem II AST strips.

Concentrations of 7-ClBaA and BaA residues in plasma

The analysis of BaA and 7-ClBaA in plasma samples was carried out using the method described in our previous report, with modifications (Sakakibara *et al*., 2013). For the preparation of plasma samples, 1 mL of ammonium sulfate (3 M) and 3 mL of *n*-hexane including 40 ng perylene-d_{12} as an internal standard were added to each blood sample (150~400 mg) in 10 mL glass centrifuge tubes and vortexed for ~1 min. After centrifuging at 3,000 rpm for 10 min, the organic fraction was collected into another 10 mL glass centrifuge tube and dried under N_2 at 40°C. The residue was weighed and then redissolved in 1 mL of *n*-hexane. The solution was loaded into a Sep-Pak Plus Silica column (Waters, MA, USA) and further purification was achieved by elution of 10 mL *n*-hexane. Finally, the eluted solution was concentrated to a volume of ca. 500 μL in *n*-hexane for gas chromatography–mass spectrometry (GC/MS) analysis. The target compounds were quantified on a GC/MS (Jms Q-1000GC; JEOL, Tokyo, Japan) equipped with an InertCap-5MS/NP (30 m × 0.25 mm i.d. × 0.25 μm film thickness; GL Science, Tokyo, Japan) fused silica capillary column operated in EI+ mode (70 eV). For quantitative analysis, the chosen fragmentations were monitored in the selective ion monitoring mode: m/z 228 for BaA, m/z 262 for Cl-BaA, and m/z 264 for perylene-d_{12} as the internal standard. The limits of quantification for BaA and 7-ClBaA were 20 ng/mL and 5 ng/mL, respectively.

Gene expression analysis

Total RNA was extracted from each liver (20 mg each) using a QuickGene RNA Tissue Kit SII (RT-S2) and QuickGene-810 Nuclear Acid Isolation System (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's instructions. The ratio of the optical densities from the RNA samples measured at 260 nm to the optical densities measured at 280 nm was used to evaluate the purity of nucleic acid, and total RNA concentrations were determined based on the absorbance at 260 nm. The quality of total RNA was further estimated by the integrity of 28S and 18S rRNAs. Two hundred ng of extracted RNA was reverse-transcribed in a final volume of 10 μL using a PrimeScript® RT reagent kit (RR037A; Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions.

A total of 1 μL of cDNA solution was added to 19 μL of the PCR mixture containing VeriQuest Probe qPCR Master Mix (10 μL; Affymetrix Inc., Santa Clara, CA, USA), DNase/RNase-free water (7 μL), house-keeping gene solution (glyceraldehyde-3-phosphate dehydrogenase: *Gapdh*; 1 μL), and the individual target primer (1 μL). All primers used in this study were obtained from Applied Biosystems as follows: *Cyp1a1*, Assay ID, Mm00487218_m1; *Cyp1a2*, Mm00487224_m1; and *Cyp1b1*, Mm00487229_m1. The qRT-PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems). The relative expression level of the target gene was calculated by the comparative automatic threshold cycles method using *Gapdh* as a calibrator. The relative differences in expression levels between groups were expressed using cycle time (Ct) values and the relative differences between groups were expressed as relative increases, setting the vehicle control group to 1.0. Each experiment was carried out in duplicate.

Statistical analyses

Statistical analyses were undertaken using the software program Stat View for Windows (Version 5.0; SAS

Institute, Cary, NC, USA). The statistical significance of differences between the control group and treated groups was determined using two-way ANOVA followed by Tukey's analysis. The results were considered to be significant if the probability of error was < 5%.

RESULTS

Body and liver weights

The body weights in both sexes were not significantly changed over the 24 hr after administration of either compound (Table 1). On the other hand, the relative liver weights (% of body weight) of male mice showed a dose-dependent increase in both treatment groups, and the difference in the group treated with 100 mg/kg body weight of either compound was significantly higher compared to the vehicle control. However, the liver weights in females were similar to those in the controls.

Plasma AST activity

The male mice treated with BaA showed significantly increased plasma AST activity in the 100 mg/kg group (Fig. 2). However, the male mice treated with 7-ClBaA showed a similar level of plasma AST activity compared with the controls, and a significantly lower level of this activity in the 100 mg/kg group compared to the 100 mg/kg BaA group. On the other hand, no notable alterations in AST activity were observed in either treatment group in female mice.

Plasma levels of BaA and 7-ClBaA

The residue levels of BaA and 7-ClBaA in the plasma are shown in Fig. 3. Both chemicals were detected in the plasma, which was collected 24 hr after the single oral administration. The plasma amounts of both compounds were dose-dependently increased, and were not significantly different between the two chemicals when doses of 1 or 10 mg/kg doses were used. However, at a dose of 100 mg/kg, the 7-ClBaA levels were significantly higher than those of BaA in both male and female mice. The gender difference in BaA or 7-ClBaA accumulation in the plasma was negligible. The mean concentrations of BaA and 7-ClBaA in the plasma in the 100 mg/kg group were 620 and 2,890 ng/g lipid weight in male mice, and 769 and 2,775 ng/g lipid weight in female mice, respectively.

Induction of the *Cyp1a1*, *1a2*, and *1b1* genes in the mouse liver and lung

In male mice orally administered BaA or 7-ClBaA, qRT-PCR analysis showed dose-dependent inductions of *Cyp1a1* and *1a2* in the liver and lung, and both gene expressions were significantly greater in the group treated with 100 mg/kg 7-ClBaA than in the group administered 100 mg/kg BaA (Fig. 4). Moreover, *Cyp1a1* expression in the lung of male mice treated with 10 mg/kg 7-ClBaA was also significantly greater than that in the lung of male mice treated with 10 mg/kg BaA. A similar dose-dependent induction of *Cyp1a1* and *1a2* in the liver and lung of female mice was also observed. However, the intensity of *Cyp1a2* expression in the female mouse lung was much weaker compared to that in the male mice. The alteration of *Cyp1b1* expression was not notable in the livers and lungs collected from both male and female mice administered BaA in this study. On the other hand, treatment with 7-ClBaA at 100 mg/kg significantly increased *Cyp1b1* expression in both organs from male mice compared to that in controls, and the difference between the two chemicals was also significant at a dose of 100 mg/kg body weight. In addition, *Cyp1b1* expression in female mice treated with 7-ClBaA showed the same pattern, but there was no significant difference between the two chemicals in the liver.

Table 1. Effects of BaA and 7-ClBaA administration on body and liver weights in mice

	Control	BaA (mg/kg BW)			7-ClBaA (mg/kg BW)		
		1	10	100	1	10	100
Male							
Body weights (g)	29.4 ± 0.7	30.2 ± 0.2	30.3 ± 0.5	29.3 ± 0.9	29.3 ± 0.7	29.5 ± 0.6	31.3 ± 1.0
Liver weight (% BW)	3.60 ± 0.30	3.65 ± 0.07	3.90 ± 0.08	4.09 ± 0.27*	3.68 ± 0.15	3.85 ± 0.18	3.97 ± 0.21*
Female							
Body weights (g)	25.6 ± 0.4	25.1 ± 0.4	25.0 ± 0.2	26.1 ± 0.3	26.5 ± 0.5	25.9 ± 1.1	23.8 ± 0.9
Liver weight (% BW)	3.68 ± 0.09	3.51 ± 0.24	3.52 ± 0.14	3.82 ± 0.42	3.86 ± 0.42	3.54 ± 0.10	3.72 ± 0.38

Data indicate mean ± S.E. (n = 5). BW: body weight. *Significantly different at $P < 0.05$ vs the vehicle control.

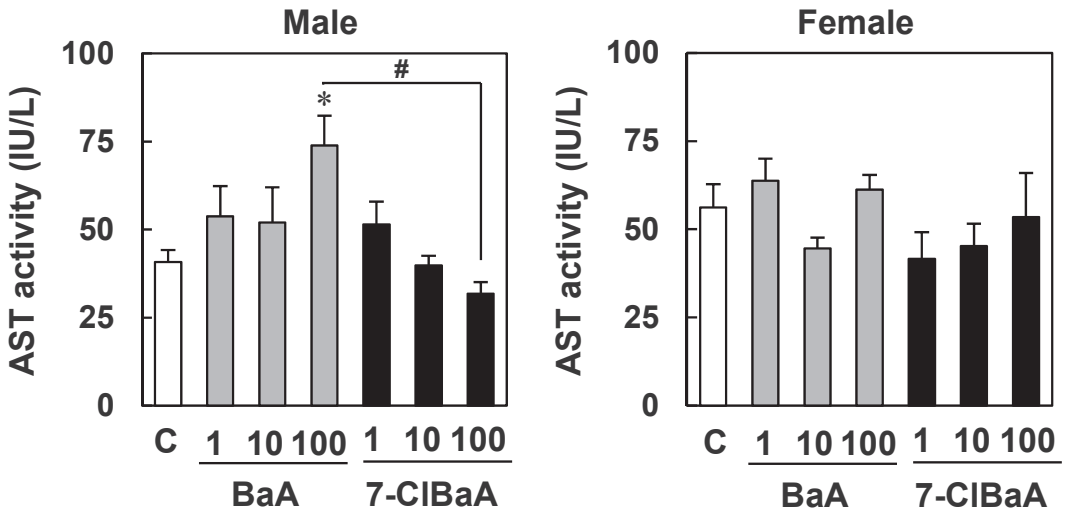

Fig. 2. Effects of BaA and 7-ClBaA on plasma AST activity. Male and female ICR mice were administered a single oral dose of benz[*a*]anthracene (BaA) or 7-chlorinated benz[*a*]anthracene (7-ClBaA) at 1, 10, and 100 mg/kg body weight. Values are the means ± S.D. (n = 5). *Significantly different at $P < 0.05$ vs the vehicle control, #at $P < 0.05$ vs the 100 mg/kg treatment groups by Tukey's analysis.

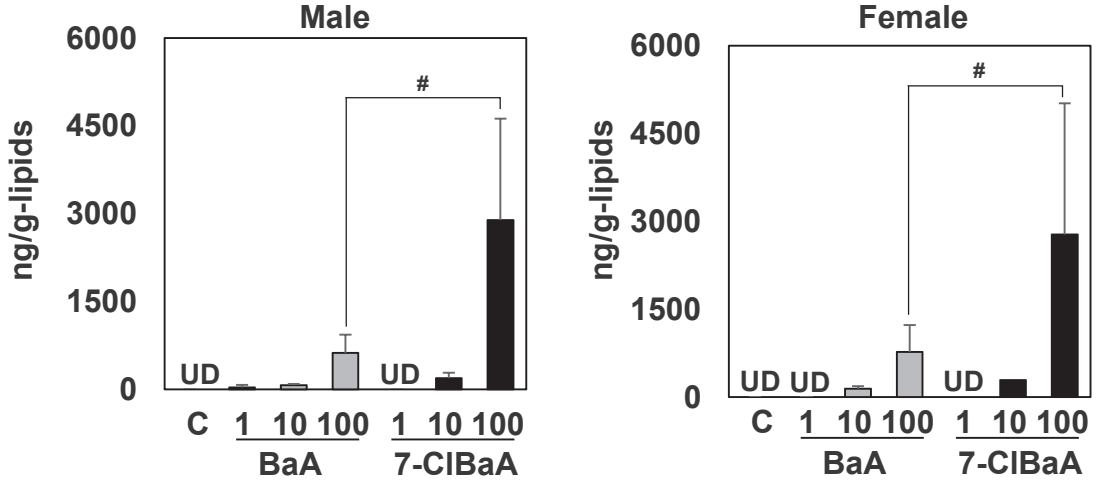

Fig. 3. Plasma concentrations of BaA and 7-ClBaA. Male and female ICR mice were administered a single oral dose of benz[*a*]anthracene (BaA) or 7-chlorinated benz[*a*]anthracene (7-ClBaA) at 1, 10, and 100 mg/kg body weight. Values are the means ± S.D. (n = 5). #at $P < 0.05$ vs the 100 mg/kg treatment groups by Tukey's analysis. UD, undetected (< 20 ng/mL for BaA and < 5 ng/mL for 7-ClBaA).

DISCUSSION

In this study, for the first time, we evaluated the sex-dependent differences in the toxicological effects of 7-ClBaA using male and female ICR mice. When mice received a single oral administration of 7-ClBaA or BaA up to 100 mg/kg body weight, their body weights were not changed over the 24 hr after administration of either compound. In contrast, the relative hepatic weights of male mice were significantly increased at the high doses

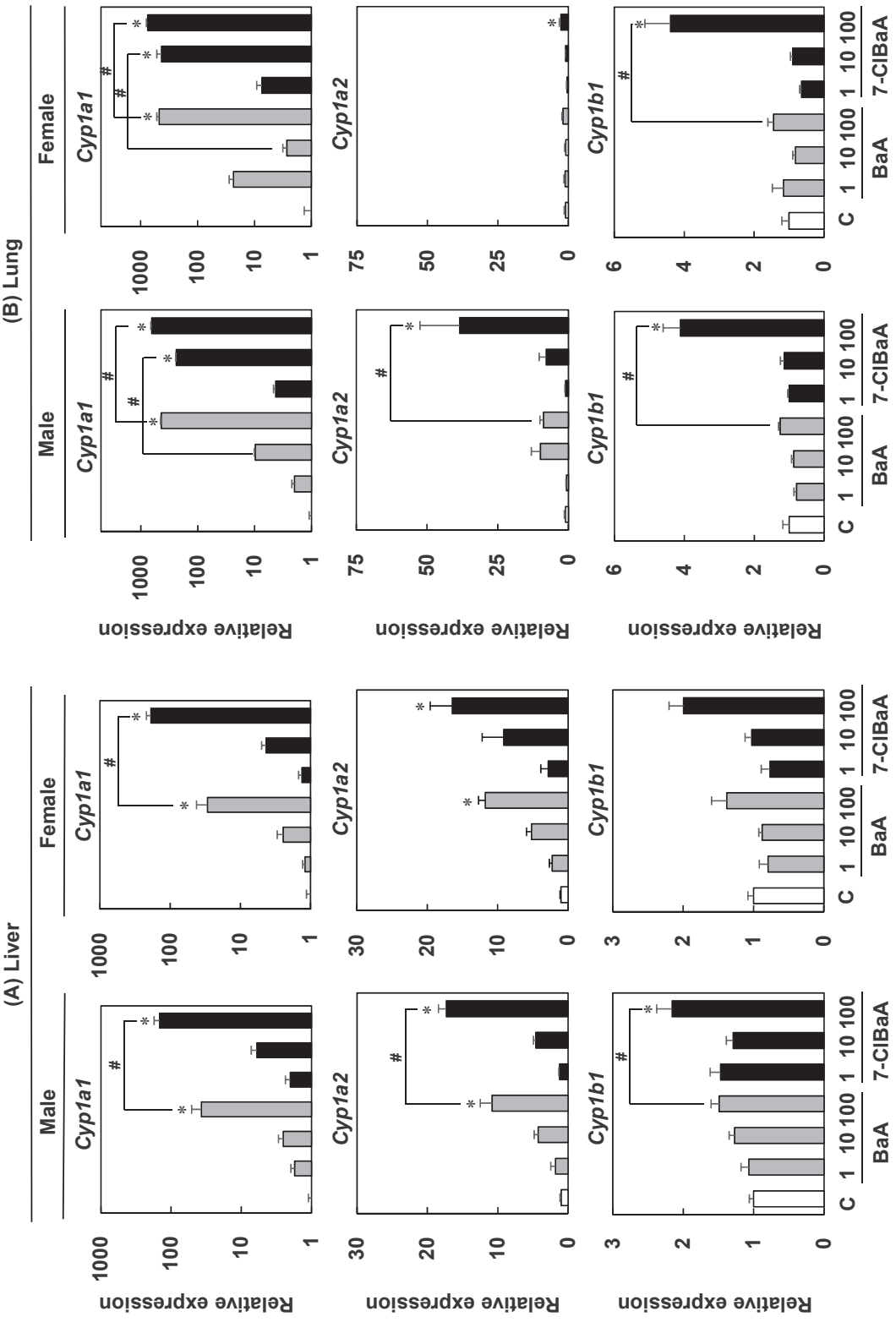

Fig. 4. Liver (A) and lung (B) expression of the *Cyp1a1*, *1a2*, and *1b1* genes related to drug metabolism after oral administration of BaA or 7-ClBaA to male and female ICR mice. Values are the means ± S.D. (n = 5). *Significantly different at $P < 0.05$ vs the vehicle control, #at $P < 0.05$ vs the 10 and 100 mg/kg treatment groups by Tukey's analysis.

(100 mg/kg) of BaA and 7-ClBaA compared to the vehicle controls, but these weights in female mice were similar among all of the groups. Likewise, the results for plasma AST activity were discordant between the 7-ClBaA and BaA administration groups in male mice; this activity was significantly increased in the group treated with 100 mg/kg BaA, but remained at a similar level in the group treated with 100 mg/kg 7-ClBaA compared to the control, although their relative liver weights were similarly increased at 24 hr after treatment. For the female mice, there was no marked alteration in AST activity in any of the treatment groups. Increased relative liver weights are commonly seen in both sexes in various animal models after administration of a wide range of PAHs; however, such effects may precede the onset of more serious hepatic effects (ATSDR, 1995). Mice acutely administered BaA twice by oral gavage showed increased incidences of hepatomas and pulmonary adenomas as compared to the incidence rates in the controls after nearly 19 months (ATSDR, 1995).

Our findings clearly indicate that a sex-dependent difference existed in the acute hepatic toxicity of BaA and 7-ClBaA orally administered to ICR mice, with the female mice appearing to be less sensitive to both chemicals than males. The factors responsible for these variations remain unclear. Masubuchi and co-workers reported that female CD-1 mice were less sensitive than males to hepatotoxicity induced by acetaminophen as assessed by increases in serum alanine aminotransferase (ALT), and suggested one possibility was that a higher exposure dose of acetaminophen may have masked the difference in the severity of liver injury (Masubuchi *et al*., 2011). In contrast, female mice were also reported to show higher susceptibilities to the heterocyclic amines which are potent mutagens that act via induction of the AhR pathway, than male mice (Matsukura *et al*., 1981). We previously reported that 7-ClBaA is a potent AhR ligand using an *in vitro* assay system (Ohura *et al*., 2007). Furthermore, we also indicated using an Ames test that 7-ClBaA exerted mutagenic activity without metabolic activation, although BaA was inert (Kido *et al*., 2013). Therefore, 7-ClBaA appears to have an alternative toxicological pathway that is not linked to the AhR and metabolic activation in addition to its stimulation of the AhR pathway. PAHs can affect the expression of multiple other genes by way of both AhR-dependent and -independent mechanisms (Mimura and Fujii-Kuriyama, 2003). Hence, further studies are required to clarify the gender-specific mechanism of Cl-PAHs toxicity.

We found that intact BaA and 7-ClBaA were dose-dependently accumulated in the plasma collected from the mice 24 hr after the administrations. Although a sex-dependent difference was not observed in the plasma levels, the 7-ClBaA levels in both male and female mice were significantly higher than those of BaA in the group given the highest dose (100 mg/kg). This suggests that chlorination of BaA increases the persistence of 7-ClBaA in the mice.

In our previous study using male Fischer 344 rats, oral administration of 7-ClBaA (10 mg/kg body weight) for 14 consecutive days selectively induced the hepatic *Cyp1a2* gene, although BaA treatment induced *Cyp1a1*, *1a2* and *1b1* (Kido *et al*., 2013). Contrary to our expectations, single oral administration of 7-ClBaA to mice induced the expression of all three of these CYP genes in the liver in a dose-dependent manner. Furthermore, similar inductions were observed in the female liver, and in the lungs of both male and female mice, indicating that 7-ClBaA induces all three CYP genes more effectively in mice than rats. When comparing the plasma levels of both chemicals between our previous rat study and this mouse study, the level of 7-ClBaA was significantly lower than the level of BaA in the Fischer 344 rats treated repeatedly over 14 days, but it was significantly higher than the BaA level in the mice exposed orally once. Hence, it was indicated that the higher persistence of 7-ClBaA in the plasma circulation might stimulate hepatic and pulmonary gene expression of all CYP1 family members, including *Cyp1a1*, *1b1* and *1a2*, but relatively low levels of 7-ClBaA might selectively and potently induce *Cyp1a2*. The differences in body accumulations of the target compounds and the expression of CYP1 genes may also be related to the different periods of exposure and/or the different species studied (Souma *et al*., 2006).

In conclusion, this was the first study to evaluate the acute toxicological effects of 7-ClBaA according to the sex-dependent difference based on *in vivo* experiments. For the male mice, 7-ClBaA at 100 mg/kg body weight increased relative liver weight after 24 hr, although its parent chemical, BaA increased both liver weight and AST activity. On the other hand, these alterations were not observed in the female mice, indicating that female mice might be less sensitive to acute toxicity of both chemicals than the males. In addition, the gene expressions of *Cyp1a1*, *1a2*, and *1b1* showed significantly greater induction in the liver and lung of mice of both sexes in the 7-ClBaA-treatment group compared to the BaA-treatment group. Thus, chlorination of BaA appeared to exhibit gender-specific toxicity, and increased the accumulation of 7-ClBaA in the body while also enhancing CYP1A gene expression in mice. These findings provide novel information; however, additional long-term exposure experi-

ments with more analytical parameters will be needed to understand the adverse biological effects and mechanisms of toxic action on animals and humans and to establish guidelines for the risk assessment of this class of compounds.

Conflict of interest---- The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENTS

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