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Pyrene is a Novel Constitutive Androstane Receptor (CAR) Activator and Causes Hepatotoxicity by CAR

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are a class of ubiquitous persistent environmental pollutants which are primarily formed from the incomplete combustion of organic materials. Many potential sources of human exposure to PAHs exist, including daily exposures from the ambient environment or occupational settings. PAHs have been found to cause harmful effects on human health. Here, we evaluated the adverse effects of pyrene, a common PAH, on the liver. The present study demonstrates that pyrene is able to activate mouse constitutive androstane receptor (CAR). CAR protein, as measured by Western blot analysis, was observed to translocate into the nucleus from the cytoplasm in mouse liver after exposure to pyrene. Utilizing CAR null mice, we identified that CAR mediates pyrene-induced hepatotoxicity. Increased relative liver weight, hepatocellular hypertrophy, and elevated serum alanine aminotransferase levels were found in wild-type but not CAR null mice after orally administered pyrene. We further show that pyrene induced the expression of mouse liver metabolism enzymes including CYP2B10, CYP3A11, GSTm1, GSTm3, and SULT1A1, and caused hepatic glutathione depletion in wild-type but not CAR null mice. Moreover, by luciferase reporter assay and quantitative real-time PCR analysis, pyrene was found to be a potential inducer of CYP2B6 expression via activation of human CAR in HepG2 cells and human primary hepatocytes. Our observations suggest that pyrene is a novel CAR activator and that CAR is essential for mediating pyrene-induced liver injury.

Key words: hepatotoxicity; nuclear receptor CAR; pyrene

Polycyclic aromatic hydrocarbons (PAHs) contain 2 or more benzene rings which are primarily formed during the incomplete burning of coal, oil, or other organic substances such as tobacco and charbroiled meat (ATSDR, 1995). PAHs are persistent environmental pollutants, and can bind to the surface of particulate matter that is a major constituent of air pollution (Shi *et al.*, 2015). In 1976, the United States Environmental Protection Agency listed 16 PAHs as priority pollutants. In 2007, the global annual atmospheric emission of 16 PAHs was 504 Gg (Giga gram), one-fifth of which was contributed by China (Shen *et al.*, 2013). Several sources of PAHs exposure to humans include vehicle exhaust, cigarette smoke, industrial waste incineration, and contaminated food (ATSDR, 1995). PAHs exposure was associated with obesity in children aged 6–11 years in USA (Scinicariello and Buser, 2014). Commercial use of pyrene includes the manufacturing perinon pigments (WHO, 2003), and occupational exposures to PAHs additionally include inhalation of engine exhaust and involvement in industries such as oil refining, chemical production, and transportation that utilize products containing PAHs (ATSDR, 1995).

Of the 16 PAHs, pyrene (Fig. 1) occurs at relatively high concentrations in PAH mixtures. China is one of the countries with the highest emission rates of pyrene, and in 2007 emitted 4.9×10^3 Mg (Mega gram). The total global emission of pyrene in

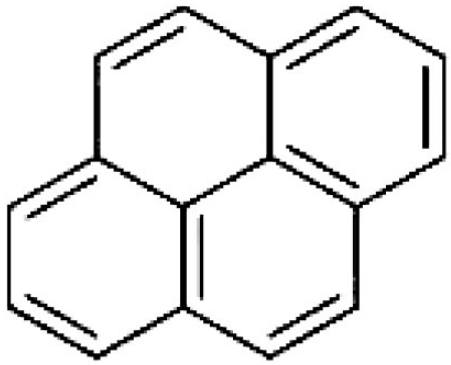

Fig. 1. Chemical structure of pyrene.

2007 was 1.9×10^4 Mg (Shen *et al.*, 2013). Pyrene is also one of the most highly concentrated PAHs detected in drinking water (WHO, 2003). In China, PAHs contamination has been reported in water, sediments, and soils of many rivers including the Yangtze River, Yellow River, and Songhua River, which are important drinking water and surface water sources (Floehr *et al.*, 2013; Feng *et al.*, 2014; Ma *et al.*, 2013). Additionally, a risk of indoor exposure to PAHs occurs cooking using coal briquettes or wood as the energy fuel or cooking oils that are heated to high temperatures. The concentration of 16 PAHs detected in a commercial Chinese kitchen was measured at 141.0 ng/m^3 including10.9 ng/m^3 of pyrene, and a higher concentration of PAHs (609.0 ng/m^3) was found in the Malay kitchen (See *et al.*, 2006).

Coke oven workers were reported to be exposed to coke oven emissions which primarily contain PAHs (Wu *et al.*, 1997). In these workers, serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were significantly elevated, as were exposure levels of 16 measured PAHs, of which pyrene contributed to approximately 25% of the exposure levels (Chen *et al.*, 2006). Pyrene also significantly increased rat serum AST levels after a single intraperitoneal injection (Yoshikawa *et al.*, 1985). Our previous study demonstrated that induction of mouse hepatic CYP1A2 and SULT1A1 mRNA levels by pyrene exposure was not mediated by the aryl hydrocarbon receptor and that further investigation was required (Lee *et al.*, 2007). However, studies on pyrene toxicity are still limited and the mechanism of pyrene-induced hepatotoxicity remains unknown.

The constitutive androstane receptor (CAR, NR1I3), a member of the nuclear receptor superfamily, plays an important role in the metabolism of xenobiotic and endogenous compounds by regulating its target genes (Baes *et al.*, 1994; Qatanani *et al.*, 2005; Wei *et al.*, 2000). CAR is highly expressed in the liver, and can recognize a broad spectrum of xenobiotics and endogenous substances. Various CAR activators including therapeutic drugs (eg, phenobarbital), environmental chemicals (eg, 6-(4-chlorophenyl)-imidazo[2,1-b]thiazole-5-carbaldehyde (CITCO)), and endogenous substances (eg, steroids) have been identified (Baes *et al.*, 1994; Maglich *et al.*, 2003; Tzameli *et al.*, 2000). CAR activation can upregulate expression of numerous xenobiotic metabolizing enzymes including phase I (eg, CYP2B subfamily) and phase II (eg, glutathione *S*-transferases) enzymes, as well as phase III transporters (eg, MRP4) (Assem *et al.*, 2004; Ueda *et al.*, 2002). CAR is also involved in regulation of energy metabolism and lipid homeostasis (Roth *et al.*, 2008), and plays a protective role such as promoting the excretion of bilirubin (Huang *et al.*, 2003). However, CAR was found to be a facilitator of chemical toxicity, mediating acetaminophen-induced hepatotoxicity (Zhang *et al.*, 2002), and promoting phenobarbital- and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP)-induced liver tumor formation (Huang *et al.*, 2005; Yamamoto *et al.*, 2004).

The present study investigates the mechanism of pyrene-induced hepatotoxicity. We demonstrate that pyrene is a novel CAR activator and induces hepatotoxicity with elevated serum ALT levels, hepatocellular hypertrophy, lower glutathione levels, and induces expression of some CAR-related hepatic enzymes in wild-type but not CAR null mice. We further demonstrated that pyrene is a potential human CAR activator that induces *CYP2B6* gene expression in human primary hepatocytes.

MATERIALS AND METHODS

Animals and treatment. Male CAR null mice on the C57BL/6 genetic background (provided by Dr. Masahiko Negishi, National Institute of Environmental Health Sciences/National Institutes of Health, Research Triangle Park, North Carolina) and their littermate male wild-type mice were used for this study. Mice were housed in an environmentally temperature-controlled room with a 12-h light/dark cycle with access to rodent chow and water *ad libitum*. All of the animal handling was performed in accordance with ethical guidelines with regard to human care and treatment of Wenzhou Medical University. At 8 weeks of age, mice (6–7 mice per group) were orally administered 2 doses of pyrene (150 or 300 mg/kg body weight) or corn oil as the vehicle once daily for 4 consecutive days. On day 5, all animals were sacrificed. To assess the effect of CAR activation by pyrene, a single dose of TCPOBOP (Sigma-Aldrich) at 3 mg/kg was injected intraperitoneally (i.p.) into mice once as a positive control. Blood was collected and the serum was separated. Livers were removed. Samples were stored at −80°C until use.

Chemicals. Pyrene (CAS no. 129-00-0, 99%) was purchased from Sigma-Aldrich (St. Louis, Missouri). All other chemicals were obtained from commercial sources at the highest grade of purity available.

Cell culture. HepG2 cells were maintained in Eagle's minimal essential medium (MEM) (Invitrogen, Carlsbad, California) supplemented with 10% fetal bovine serum (Gibco, Grand Island, New York), 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate (Gibco), and penicillin/stretomycin (Gibco) at 37°C under 5% CO_2. Human primary hepatocytes were obtained from Invitrogen and cultured as previously described (Shi *et al.*, 2014; Zhang *et al.*, 2012). Briefly, hepatocytes were maintained in William's E medium without Phenol Red (Sigma-Aldrich) supplemented with ITS+1 liquid media supplement (Sigma-Aldrich), HEPES buffer (Sigma-Aldrich), L-glutamine (Sigma-Aldrich), and dexamethasone (Sigma-Aldrich). No cytotoxicity in HepG2 cells or human primary hepatocytes was observed in current pyrene treatments.

Luciferase reporter assay. HepG2 cells were seeded in 24-well plates and transiently transfected with hCAR/pCR3 and CYP2B6-PBREM/pGL3 basic luciferase plasmids (kind gifts of Dr. Masahiko Negishi's lab) (Sueyoshi *et al.*, 1999) along with the internal control pRL-TK plasmid (Promega, Madison, Wisconsin) using Lipofectamine 2000 (Invitrogen). After 24 h, the cells were treated with solvent (0.1% DMSO), CITCO (2μM), pyrene (25, 50 μM). Twenty-four hours later, the cells were harvested. Dual-Glo luciferase assay system (Promega) was performed to

Table 1. Primer Sequence for Quantitative Real-Time PCR

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
CYP2B10	TGCTGTCGTTGAGCCAACC	CCACTAAACATTGGGCTTCCT
CYP1A1	GCCACATCCGGGACATCACAG	GCTGGACATTGGCATTCTCGT
CYP1A2	AGTACATCTCCTTAGCCCCAG	GGGTCCGGGTGGATTCTTC
CYP3A11	AGAACTTCTCCTTCCAGCCTTGTA	GAGGGAGACTCATGCTCCAGTTA
UGT1A1	CACCTGAAGCCTCAATACACAT	CAGTCCGTCCAAGTTCCACC
UGT1A6	GTTTCTCTTCCTAGTGCTTTGGG	CCTCGTTCACTGAGATGTTCTAC
SULT1A1	CAACATGGAGCCCTTGCGTAA	ATGAGCACATCATCAGGCCAG
GSTm1	GTGACGCTCCCGACTTTGACAG	CGACAAGTAAGGCAGATTGG
GSTm3	GGACTGACTCACTCCATCCG	CTGGCTTCGGTCAAAGTTGGG
Mouse GAPDH	AGAACATCATCCCTGCATCCA	CCGTTCAGCTCTGGGATGAC
CYP2B6	AGACGCCTTCAATCCTGACC	CCTTCACCAAGACAAATCCGC
Human GAPDH	CCCATCACCATCTTCCAGGAG	GTTGTCATGGATGACCTTGGC

measure luciferase activities and firefly luciferase activities were normalized with *Renilla* luciferase activities.

Histological study. A section of liver tissue was fixed in 10% neutral buffered formalin and embedded in paraffin for histological examination. Liver tissue sections were stained with hematoxylin and eosin.

Measurement of ALT activity. For evaluation of liver injury, serum ALT levels were measured using a transaminase CII-Test kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instructions.

Quantitative real-time PCR analysis. Human primary hepatocytes were treated with solvent (0.1% DMSO), CITCO (2 μM), or pyrene (25, 50 μM). Twenty-four hours later, the cells were harvested. Total RNA was extracted from mouse livers or primary hepatocytes using TRIzol reagent (Invitrogen). cDNA was synthesized from total RNA by iScript select cDNA synthesis kit (Bio-Rad, Hercules, California). Quantitative real-time PCR was performed with iTaq Universal SYBR Green Supermix (Bio-Rad) by CFX96 Touch real-time PCR detection system (Bio-Rad). Primer sequences are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The relative quantity was normalized to endogenous control values and calculated by using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis. Liver nuclear extracts were used for determination of CAR activation by pyrene in mouse liver, and prepared by NE-PER nuclear and cytoplasmic extraction reagents from Thermo Fisher Scientific (Rockford, Illinois) according to the manufacturer's instructions. Nuclear fractions (for CAR) and samples of liver homogenate (for CYP2B10, CYP1A1/2) were subjected to 10% polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane (Invitrogen). The membrane was blocked with 5% nonfat milk blocking buffer and blotted with CAR monoclonal antibody developed by Perseus Proteomics (Tokyo, Japan) and purchased from R&D Systems (Minneapolis, Minnesota), or CYP2B10 (Millipore, Darmstadt, Germany), or CYP1A1/2 (group antibody, reacting with CYP1A1 and CYP1A2, Bioworld Technology, Louis Park, Minnesota), or GAPDH (Cell Signaling Technology, Danvers, Minnesota) or lamin β (Santa Cruz Biotechnology, Santa Cruz, California) primary antibody. Subsequently, they were incubated with HRP-conjugated antimouse IgG (Cell Signaling Technology), or anti-rabbit IgG (Cell Signaling Technology). The protein bands on the membranes were developed using SuperSignal Western Femto kit (Pierce Biotechnology, Rockford, Illinois) followed by exposure on Kodak BioMax MR film (Kodak, Rochester, New York).

Quantification of hepatic total glutathione. In brief, a section of liver tissue was homogenized with cold 50 mM MES buffer (pH 6.0) containing 1 mM EDTA. An equal volume of the MPA reagent (Sigma-Aldrich) was added and mixed by vortexing, then samples were incubated for 5 min, centrifuged, and the supernatant was collected. We add TEAM reagent (Sigma-Aldrich) to the supernatant, vortexed immediately, and measurement was performed with the freshly prepared assay cocktail. Total glutathione (GSH) levels were measured by a GSH assay kit (Cayman Chemical Company, Ann Arbor, Michigan) according to manufacturer's instructions.

Statistical analysis. Data are expressed as the mean ± SD. Multiple comparisons were determined using Dunnett's test following ANOVA by using JMP 8.0 software (SAS Institute, Cary, North Carolina). Values of $p < .05$ were considered statistically significant.

RESULTS

Pyrene Activates CAR in Wild-Type Mouse Liver

It is well known that CAR translocates into the nucleus from the cytoplasm upon its activation. TCPOBOP, a potent and specific mouse CAR agonist, can effectively activate mouse CAR. We used TCPOBOP-treated mice as positive control for investigation of CAR activation. To determine whether pyrene can activate CAR, we analyzed CAR protein levels in mouse liver nuclear extracts of control, TCPOBOP-, or pyrene-treated groups. As shown in Figure 2A, CAR protein levels increased in wild-type mice by pyrene or TCPOBOP treatment compared with the control, whereas it was detected in neither control nor the pyrene or TCPOBOP administration group of CAR null mice as expected. These results indicate that CAR can be activated by pyrene in wild-type mouse liver. Expression of CAR target gene CYP2B10 is known to be upregulated after CAR activation. We verified that CYP2B10 mRNA expression was significantly induced in wild-type mice treated with pyrene or TCPOBOP compared with the control group. However, as expected, pyrene or TCPOBOP treatment had no such effect on CYP2B10 mRNA expression in CAR null mice (Fig. 2B). These data confirm that pyrene is able to active mouse CAR in the liver.

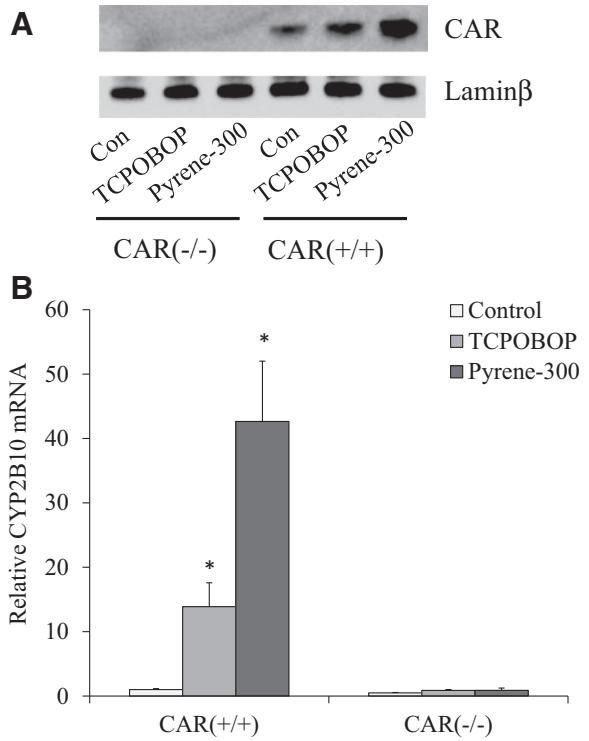

Fig. 2. Constitutive androstane receptor (CAR) activation by pyrene. Liver nuclear extracts were isolated from wild-type (CAR+/+) or CAR null mice (CAR−/−) which were orally administered corn oil (control) or pyrene (300 mg/kg) once daily for 4 days or TCPOBOP (i.p. 3 mg/kg) once as a positive control. A, CAR protein levels were detected by Western blot with anti-CAR antibody. Lamin β was used as a loading control. B, CYP2B10 mRNA expression levels were measured by quantitative real-time PCR. GAPDH was used as an internal control. Data are expressed as the mean ± SD. N = 6–7 mice per group. *indicates a significant difference at $p < .05$ in comparison with control group of the same genotype.

Table 2. Relative Liver Weights of Both Genotypes Mice After Pyrene Exposure

		Pyrene (mg/kg)		
	Mice	Control	150	300
Liver/body weight (%)	CAR (+/+)	4.447 ± 0.416	5.679 ± 0.428*	5.420 ± 0.221*
	CAR (−/−)	4.679 ± 0.342	4.776 ± 0.483	4.834 ± 0.137

Data are the mean ± SD. N = 6–7 mice/group, *$p < .05$, significantly different from control group in the same genotype.

CAR Mediates Pyrene-Induced Hepatotoxicity

To investigate the role of CAR in mediating the effect of pyrene exposure in liver, wild-type and CAR null mice were orally administered 2 doses of pyrene (150, 300 mg/kg/day) or corn oil as control for 4 days. We found that the relative liver weights significantly increased in pyrene-exposed wild-type mice compared with the control group, but not in CAR null mice (Table 2). Hepatocellular hypertrophy was observed in the group exposed to the higher dose of pyrene (Fig. 3A), and serum ALT levels were significantly elevated in both pyrene exposure groups in wild-type mice compared with the control group (Fig. 3B). However, CAR null mice showed no such pyrene-induced hepatotoxicity. These results indicate that CAR plays a critical role in liver injury by pyrene exposure.

Effects of CAR on Hepatic Metabolism Enzymes Expression

CAR is well known to regulate the expression of various hepatic metabolism enzymes. To investigate the effects of CAR activation after pyrene exposure, we performed quantitative real-time PCR and Western blotting analyses to measure hepatic phase I and phase II enzyme expression levels.

As shown in Figure 4A, CYP2B10 mRNA expression levels were significantly induced in a dose-dependent manner in wild-type mice after pyrene exposure. In contrast, there was no effect of pyrene on CYP2B10 mRNA expression in CAR null mice. These results were strongly consistent with its protein expression (Fig. 4J). This indicated that pyrene induced CYP2B10 expression in a CAR-dependent manner. We found that CYP1A1 (Fig. 4B) and CYP1A2 (Fig. 4C) mRNA expression levels were significantly increased in both pyrene-exposed wild-type and CAR null mice. In line with their mRNA expression patterns, CYP1A1 and CYP1A2 protein expression levels also increased in both wild-type and CAR null mice after pyrene exposure by CYP1A1/2 antibody detection (Fig. 4J). These results suggest that CAR does not mediate the induction of CYP1A1 and CYP1A2 expression by pyrene. Additionally, CYP3A11 mRNA expression levels were significantly increased in wild-type mice treated with the higher dose of pyrene, whereas no changes in CYP3A11 mRNA expression levels were observed in CAR null mice (Fig. 4D). We further investigated whether CAR regulated hepatic phase II enzyme expression by pyrene treatment. As shown in Fig. 4E–G, phase II enzymes GSTm1, GSTm3, and SULT1A1were significantly increased in wild-type but not CAR null mice, indicating that CAR is involved in regulating the expression of these genes after pyrene treatment. We also measured UGT1A1 and UGT1A6 gene expression. However, pyrene treatment did not significantly affect UGT1A1 mRNA levels either in wild-type or in CAR null mice (Fig. 4H). UGT1A6 mRNA levels were significantly decreased in wild-type mice treated with the higher dose of pyrene, but not in CAR null mice, suggesting that the higher exposure dose of pyrene may have an inhibitory effect on UGT1A6 mRNA expression that is mediated by CAR (Fig. 4I). Taken together, CAR plays a vital role in regulating the expression of some liver metabolism enzymes by pyrene exposure.

CAR is Involved in Pyrene-Induced Hepatic GSH Depletion

We further investigated the effect of pyrene exposure on mouse liver GSH content. We found that the higher exposure dose of pyrene caused GSH levels to significantly decrease in wild-type mice, but not in CAR null mice, suggesting GSH depletion contributed to pyrene-induced hepatotoxicity and that depletion was CAR-dependent (Fig. 5).

Pyrene Induces CYP2B6 Expression in Human Primary Hepatocytes

CYP2B6 is a typical target gene of human CAR. Activated CAR transcriptionally regulates *CYP2B6* gene expression through a response element (eg, PBREM) located in the *CYP2B6* promoter region (Honkakoski *et al.*, 1998). To investigate whether pyrene activates human CAR to induce *CYP2B6* gene expression, we performed a luciferase reporter gene assay and measured CYP2B6 mRNA expression. The CYP2B6-PBREM luciferase reporter construct was cotransfected into HepG2 cells with a human CAR expression construct. After pyrene treatment, the luciferase activity of CYP2B6-PBREM significantly increased in a dose-dependent manner (Fig. 6A). Furthermore, consistent with the above luciferase assay results, CYP2B6 mRNA expression levels were significantly induced after pyrene treatment in

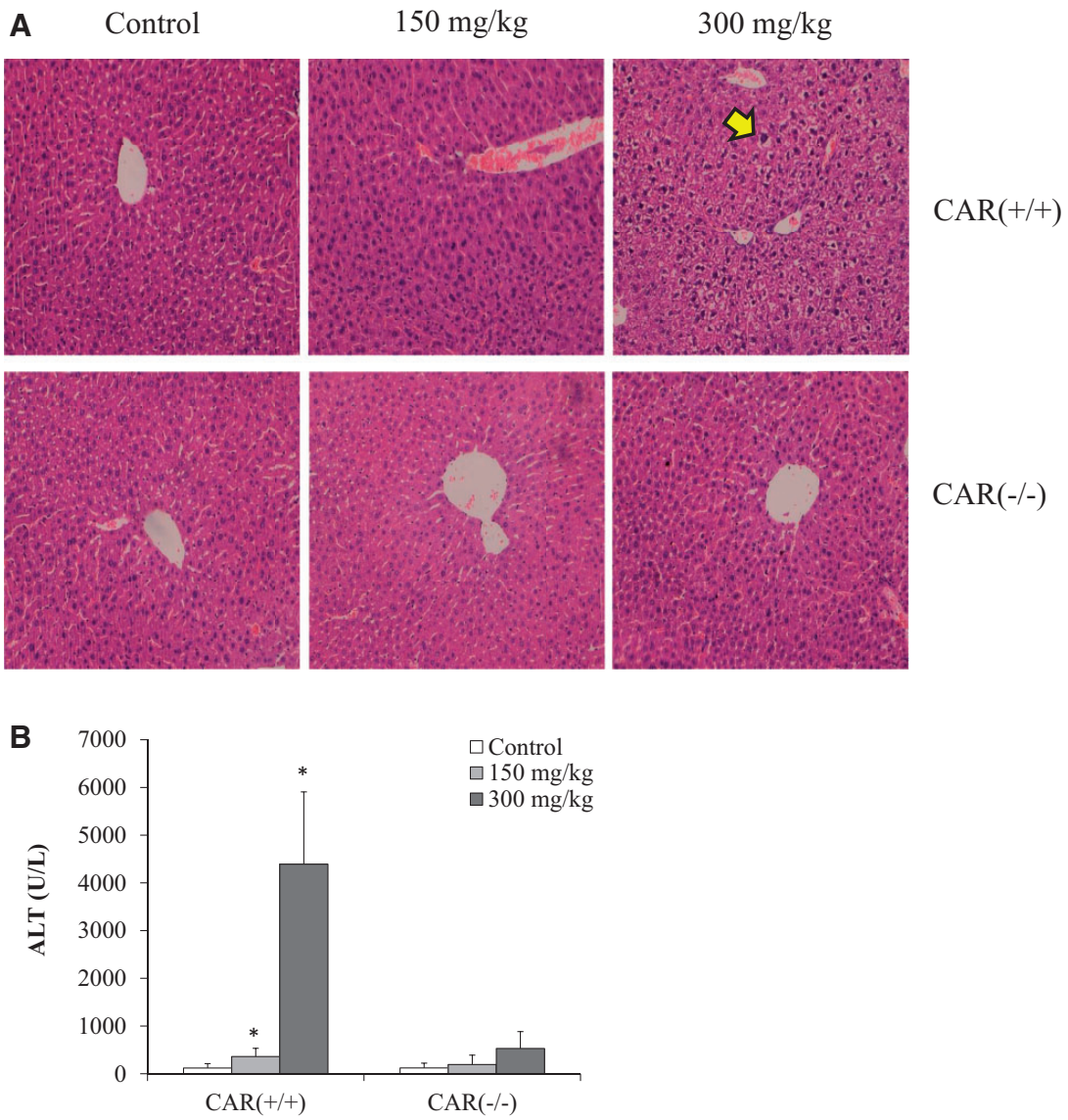

Fig. 3. Effects of pyrene exposure on mouse liver. Mice were orally administered 150 or 300 mg/kg body weight of pyrene or corn oil as the control once daily for 4 consecutive days. A, Liver histopathological findings in wild-type (CAR+/+) and CAR null mice (CAR−/−) were determined by hematoxylin and eosin staining (×200 magnification). Arrow indicates abnormal histological changes (hepatocellular hypertrophy). B, Serum ALT levels in wild-type (CAR+/+) and CAR null mice (CAR−/−). Data are expressed as the mean ± SD. $N = 6–7$ mice per group. $^*p < .05$, significantly different from control group of the same genotype.

human primary hepatocytes (Fig. 6B and C). These findings suggest that pyrene is able to activate human CAR to induce CYP2B6 mRNA expression. Thus, in addition to causing mouse liver injury, pyrene is a potential human CAR activator that affects the expression of human liver metabolism enzymes such as CYP2B6.

DISCUSSION

Environmental pollution has become a great worldwide concern, particularly in China. With industrial development in China, many kinds of pollutants are subsequently released into the environment and adversely affect human health. As persistent organic pollutants, environmental contamination by PAHs has become ubiquitous worldwide. To our knowledge, the present study is the first to demonstrate the molecular mechanism of pyrene-induced hepatotoxicity. We identified that pyrene, an important component of PAH mixtures, is a novel CAR activator, causes hepatic GSH depletion, induces mouse hepatotoxicity in a CAR-dependent pathway, and affects the expression of various CAR-related hepatic metabolism enzymes. Importantly, we found that pyrene is a potential human CAR activator that upregulates *CYP2B6* gene expression in human primary hepatocytes.

In the current study, wild-type and CAR null mice were orally administered pyrene for 4 days. It is well known that nuclear translocation of CAR from the cytoplasm is the initial and essential step for its activation. Nuclear CAR protein levels were increased in pyrene-exposed wild-type mice but in not CAR null mice, indicating CAR is able to be activated by pyrene. By hepatic histological staining, hepatocellular hypertrophy was observed in wild-type mice treated with the higher exposure dose of pyrene. Compared with the control, the relative liver weight of the higher exposure dose of pyrene group significantly increased in wild-type mice. However, CAR null mice showed no such adverse effects after exposure to pyrene.

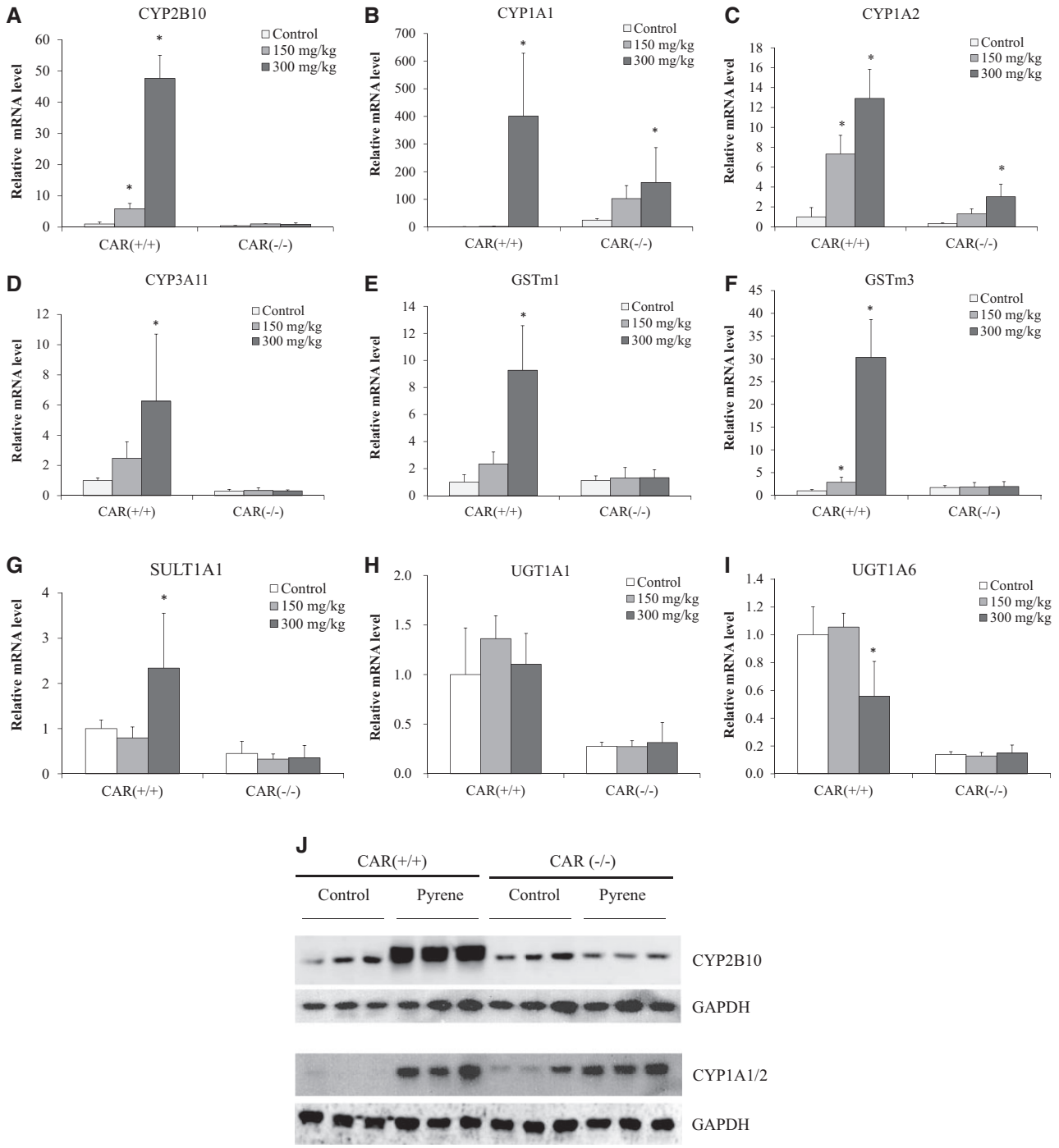

Fig. 4. Effects of pyrene exposure on liver metabolism enzyme expression in wild-type (CAR+/+) and CAR null mice (CAR−/−). A, CYP2B10; B, CYP1A1; C, CYP1A2; D, CYP3A11; E, GSTm1; F, GSTm3; G, SULT1A1; H, UGT1A1; I, UGT1A6. Data are expressed as the mean ± SD. $N = 6–7$ mice per group. mRNA expression levels in whole liver lysates were measured by quantitative real-time PCR. GAPDH was used as an internal control. *indicates a significant difference at $p < .05$ in comparison with control group of the same genotype. J, CYP2B10, CYP1A1, and CYP1A2 protein expression levels. Among control or 300 mg/kg pyrene exposure groups, 3 mice per group were selected for Western blot analysis. GAPDH was used as a loading control.

Elevated serum ALT levels are a well-known indicator of liver injury. We found that serum ALT levels significantly and dose--dependently increased in wild-type mice but not in CAR null mice when exposed to pyrene. These results suggest that pyrene induces liver injury by activation of CAR. Several studies have identified that CAR plays a crucial role in chemical-induced liver hepatotoxicity. For example, CAR has been found to be a key regulator of acetaminophen-induced injury with elevated serum ALT levels and hepatocyte damage in wild-type mice but not CAR null mice (Zhang *et al.*, 2002). Yamazaki *et al* demonstrated that 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) is able to activate CAR and that CAR is essential for DDC-induced liver injury in wild-type mice but not CAR null mice (Yamazaki *et al.*, 2011). These findings also suggest that wild-type mice may be much more susceptible to certain chemical-induced hepatotoxic effects than those that are deficient for

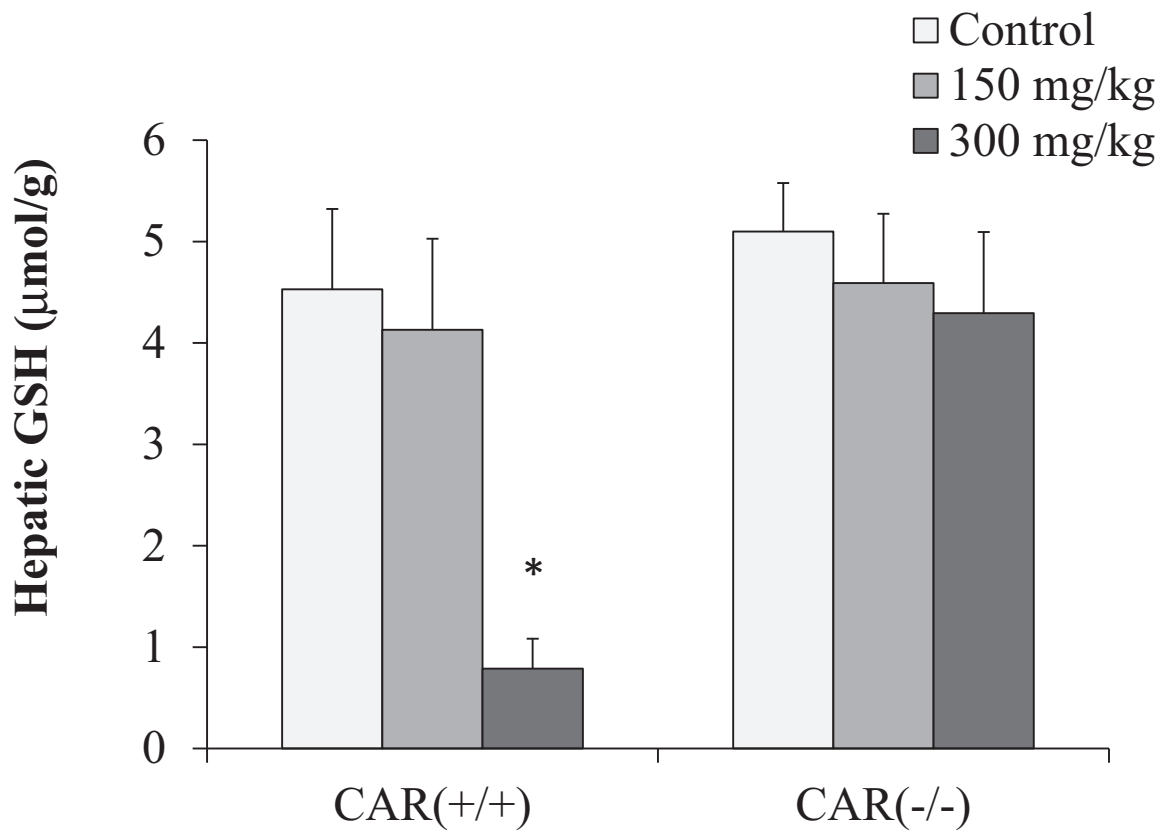

Fig. 5. Effects of pyrene exposure on hepatic glutathione (GSH) levels in wild-type (CAR+/+) and CAR null mice (CAR−/−). Hepatic GSH levels were significantly reduced in wild-type mice but not CAR null mice by higher exposure dose of pyrene. Data are expressed as the mean ± SD. $N = 6–7$ mice per group. $^{*}p < .05$, significantly different from control group of the same genotype.

CAR expression. Notably, we showed that wild-type mice displayed lower GSH levels compared with CAR null mice at the higher exposure dose of pyrene. This suggests that GSH depletion may contribute to pyrene-induced hepatotoxicity. Similarly, GSH depletion was also associated with acetaminophen-induced liver toxicity (Zhang *et al.*, 2002).

Many previous studies have identified that CAR plays an important role in the regulation of gene expression in response to stimulation by exogenous chemicals as well as endogenous compounds (Aleksunes and Klaassen, 2012; Qatanani *et al.*, 2005; Ueda *et al.*, 2002; Yamazaki *et al.*, 2011). In this study, we demonstrate that pyrene is a CAR activator which results in adverse effects on the liver. These findings led us to investigate whether pyrene affects hepatic gene expression via the canonical CAR activation pathway. As a prototypical CAR target gene, both the mRNA and protein expression levels of CYP2B10 were notably induced in pyrene-exposed wild-type mice, whereas no such effects were observed in CAR null mice. These data strongly suggest that CAR can be activated by pyrene and subsequently up-regulate CYP2B10 expression. The induction of CYP2B10 expression by CAR activation has also been identified in other studies. Activation of CAR by well-known CAR agonist phenobarbital significantly induced hepatic CYP2B10 expression (Luisier *et al.*, 2014; Ueda *et al.*, 2002; Yamamoto *et al.*, 2004). Recently, Sueyoshi *et al.* (2014) reported that the flame retardant BDE-47 effectively activated CAR and induced CYP2B10 expression levels in wild-type mice but not CAR null mice. Dong *et al.* (2009) demonstrated that streptozotocin increased expression of CYP2B10 and CYP3A11 in wild-type mice, whereas the induction was completely absent in CAR null mice. In the present study, we further demonstrated that CYP3A11, GSTm1, GSTm3, and SULT1A1 mRNA expression levels were significantly increased by pyrene exposure in a CAR-dependent manner, suggesting CAR is involved in the regulation of these genes in response to pyrene treatment. Upregulation of GSTm3 expression by activated CAR has also been found in mice treated with phenobarbital (Luisier *et al.*, 2014). Utilizing activators of CAR, Klaassen and colleagues found that expression levels of some GST enzymes including GSTm1 and GSTm3 mRNA were significantly induced in wild-type but not CAR null mice (Aleksunes and Klaassen, 2012; Knight *et al.*, 2008). Additionally, Zhang *et al.* (2002) identified that expression of a GST enzyme, GSTpi, strongly induced by phenobarbital or TCPOBOP treatment in wild-type but not CAR null mice. In our study, increased GSTm1 and GSTm3 expression in wild-type mice after pyrene treatment may enhance hepatic GSH depletion, contributing to pyrene-induced liver toxicity that is mediated by CAR activation. We observed a significant decrease of UGT1A6 mRNA expression in the higher pyrene exposure group in the wild-type mice but not in CAR null mice, suggesting that the higher dose of pyrene may inhibit UGT1A6 expression by CAR. Additionally, we observed that pyrene induced the mRNA and protein expression levels of CYP1A1 and CYP1A2 in both wild-type and CAR null mice, suggesting induction of CYP1A1 and CYP1A2 expression by pyrene exposure is not mediated specifically by CAR. This result was concordant with a previous study which showed that phenobarbital or TCPOBOP induced CYP1A2 expression in both wild-type and CAR null mice (Zhang *et al.*, 2002).

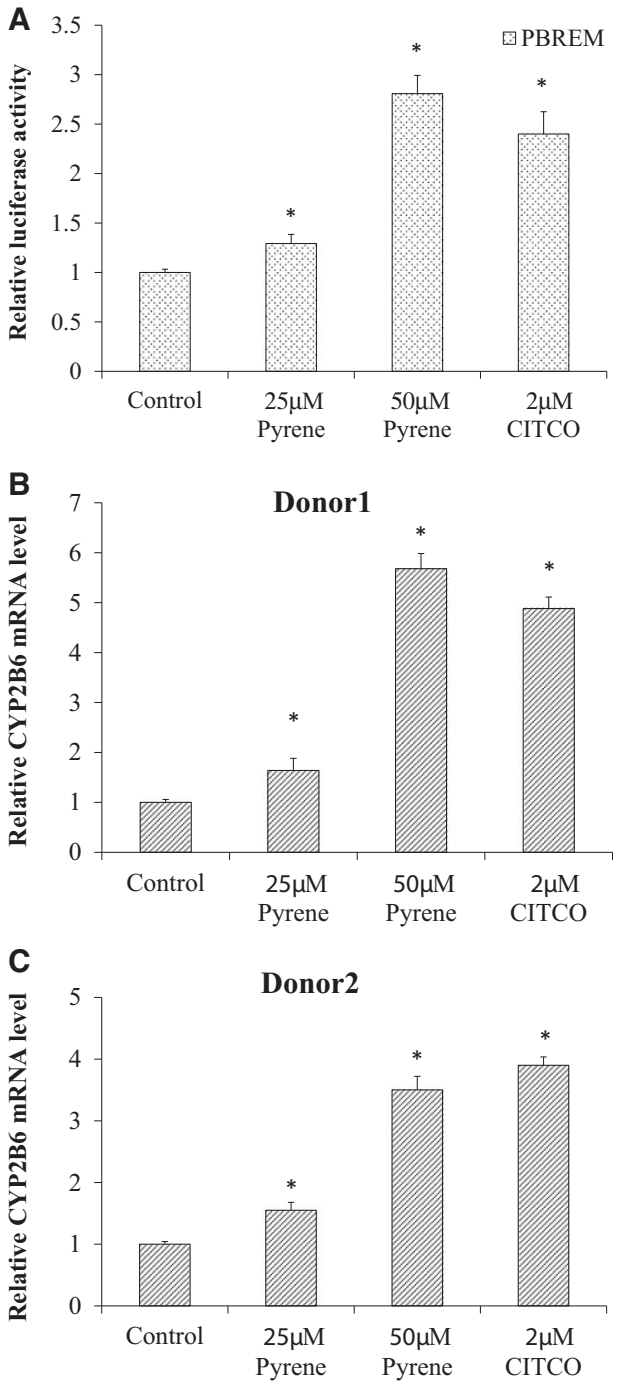

Fig. 6. Effects of pyrene treatment on *CYP2B6* expression. A, Transactivation of *CYP2B6* reporter gene by human CAR in HepG2 cells. *CYP2B6* reporter construct containing PBREM was transiently transfected into HepG2 cells together with human CAR expression vector. Transfected cells were treated with pyrene (25, 50 μM), CITCO (2 μM), or DMSO (0.1%) as the vehicle for 24 h. Luciferase activities were determined as described in the methods. B, C, Induction of CYP2B6 mRNA levels in human primary hepatocytes by pyrene. Human hepatocytes from 2 donors were treated with pyrene (25, 50 μM), CITCO (2 μM), or DMSO (0.1%) for 24 h. Total RNA was extracted from hepatocytes and subjected to quantitative real-time PCR analysis of CYP2B6 mRNA expression levels. CITCO treatment was used as a positive control demonstrating human CAR activation. Data represent the mean ± S.D. of 3 independent experiments or triplicate determinations. $^{*}p < .05$, significantly different from control (DMSO).

Our data indicate that mouse CAR can be activated by pyrene exposure. We further investigated the effects of pyrene on the activation of human CAR. In humans, a PBREM has been identified in the *CYP2B6* promoter region and that is functionally characterized as a CAR binding site in response to chemical stimulation. Thus, CAR is considered to be a key regulator of the *CYP2B6* gene through binding of the PBREM (Honkakoski *et al.*, 1998; Sueyoshi *et al.*, 1999). Reporter gene assays showed that the luciferase activity of CYP2B6-PBREM was significantly induced by pyrene in HepG2 cells, indicating that pyrene is a likely human CAR activator. Furthermore, we confirmed that CYP2B6 mRNA expression levels were significantly induced in human primary hepatocytes by pyrene. These findings suggest that pyrene has the ability to activate human CAR, which results in expression of CAR target genes such as *CYP2B6*. Moreover, by activating of CAR, pyrene may potentially affect the hepatic response to xenbiotic exposure including environmental contaminants, therapeutic drugs, and endogenous compounds by altering xenobiotic metabolism gene expression. Induction of CYP2B6 expression was also found in human primary hepatocytes in response to other CAR activators such as phenobarbital, CITCO, and BDE47 (Sueyoshi *et al.*, 2014).

Leung *et al* detected 16 PAHs in air samples from 5 cities (Hong Kong, Guangzhou, Xiamen, Xi'an, and Beijing) in China. Pyrene concentrations in the air samples from these 5 cities were 0.28, 0.38, 0.24, 8.00, and 11.35 ng/m^3, respectively (Leung *et al.*, 2014). Roggi *et al.* (1997) reported that the dietary intake of pyrene in a general adult population ranged between 1.8 and 5.8 μg/day. Although the daily exposure levels for humans are lower than the exposure doses used in the present study, pyrene may have a cumulative effect on human health, resulting in chronic toxicity. Hence, the adverse effects of pyrene on human health should not be ignored because PAH mixtures containing pyrene have been found to be harmful to occupational workers' health (Chen *et al.*, 2006; Wu *et al.*, 1997) and are associated with childhood obesity (Scinicariello and Buser, 2014). To protect human health and reduce the exposure risk of PAHs, it is important to control sources of pollution such as decreasing industrial emissions that contain PAHs, as well as regulating food safety and reducing workplace chemical exposure level. Our present work contributes a better understanding of the mechanism of PAHs-induced hepatotoxicity and describes a novel pathway of liver injury by pyrene exposure.

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