Letter

Polycyclic aromatic hydrocarbons induce CYP3A5 gene expression via aryl hydrocarbon receptor in HepG2 cells

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ABSTRACT — The aryl hydrocarbon receptor (AhR) regulates expression of genes encoding drug/xenobiotic metabolizing enzymes. Cytochrome P450 (CYP) 3A5 is involved in drug metabolism. However, regulation of *CYP3A5* gene expression is not yet well understood. In this study, we aimed to investigate the effect of the ligands of AhR on *CYP3A5* gene expression. *CYP3A5* mRNA expression was induced by the polycyclic aromatic hydrocarbons (PAHs) such as 3-methylcholanthrene (3MC) and benzo[*a*]pyrene in HepG2 cells. To determine whether the PAHs induced *CYP3A5* gene expression via AhR, we generated AhR knockout (AhR KO) HepG2 cells. *CYP3A5* mRNA expression was not induced by 3MC treatment in AhR KO cells. In addition, we generated AhR rescue cells from AhR KO cells and evaluated *CYP3A5* mRNA expression. We found that *CYP3A5* mRNA expression was induced by 3MC treatment in AhR rescue cells. Taken together, these results demonstrated that *CYP3A5* mRNA expression was induced by PAHs via AhR in HepG2 cells. Our findings suggest that ligand-activated AhR affects CYP3A5-mediated drug metabolism.

Key words: Aryl hydrocarbon receptor, Polycyclic aromatic hydrocarbons, CYP3A5

INTRODUCTION

The aryl hydrocarbon receptor (AhR) belongs to the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family and functions as a ligand-activated transcription factor (Kawajiri and Fujii-Kuriyama, 2017). Following ligand binding, AhR translocates to the nucleus and heterodimerizes with aryl hydrocarbon receptor nuclear translocator (Arnt). The AhR/Arnt heterodimer binds to specific DNA sequences called xenobiotic response elements (XREs) and up-regulates the expression of AhR target genes such as *cytochrome P450 (CYP) 1A1*, *1A2*, and *1B1*. AhR has been mainly studied in the context of its ability to mediate the toxic effects resulting from exposure to dioxins and polycyclic aromatic hydrocarbons (PAHs) (Shimizu *et al.*, 2000; Mimura *et al.*, 1997). Recent studies have revealed that AhR plays pivotal roles such as

anti-cancer effect (Zhao *et al.*, 2012), energy metabolism (Wada *et al.*, 2016), immunity (Kimura *et al.*, 2008), and drug metabolism (Aleksunes and Klaassen, 2012).

The CYP3A subfamily is comprised of four genes, *CYP3A4*, *CYP3A5*, *CYP3A7*, and *CYP3A43*, and plays a major role in the metabolism of approximately 30% of clinically used drugs (Zanger and Schwab, 2013). Similarly, the other members of the CYP3A family, CYP3A5 is expressed in the human liver. *CYP3A5* gene has been found to have several single-nucleotide polymorphisms such as *CYP3A5**3, *CYP3A5**6 and *CYP3A5**7. The *CYP3A5**3 is the major allele in *CYP3A5*. This allele is caused due to the insertion of an early stop codon which produces a nonfunctional protein (Kuehl *et al.*, 2001). *CYP3A5**3 is observed in Europeans, Africans, East Asians, South Asians, and admixed Americans, with a frequency of 94%, 18%, 71%, 67%, and 80%, respec-

tively (Zhou *et al.*, 2017). The substrate specificities of CYP3A5 is overlapped with CYP3A4, but some differences in catalytic properties have been reported (Niwa *et al.*, 2008). It has been reported that various drugs, such as atazanavir, vincristine, midazolam, vardenafil, verapamil, and tacrolimus, are mainly metabolized by CYP3A5 (Tseng *et al.*, 2014).

It has been reported that *CYP3A5* gene expression is regulated by nuclear receptors such as the glucocorticoid receptor (GR), constitutively activated receptor (CAR), and pregnane X receptor (PXR) (Hukkanen *et al.*, 2003; Burk *et al.*, 2004). CYP3A5 expressors have a high ability to metabolize tacrolimus and require 1.5- to 2-fold higher dosage of tacrolimus compared to the usual dosage (Birdwell *et al.*, 2015). Clarification of the CYP3A5 expression mechanism is pivotal for personalized medicine. Since AhR plays an important role in drug metabolism, we predicted that AhR regulates *CYP3A5* gene expression. We searched for XRE sequence upstream of *CYP3A5* gene using a web-based tool, The University of California, Santa Cruz Genome Browser (<http://www.genome.ucsc.edu/>). The XRE sequence was present approximately -2.1 kb upstream from the transcription start site of the *CYP3A5* gene in the human genome. In this study, we investigated the effect of AhR ligands on *CYP3A5* gene expression in HepG2 cells.

MATERIALS AND METHODS

Chemicals

3-methylcholanthrene (3MC) and benzo[*a*]pyrene (B[*a*]P) were purchased from FUJIFILM Wako (Osaka, Japan).

Cell culture, generation of stable AhR knockout (KO) cells, and AhR rescue cells

HepG2 hepatocellular carcinoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle medium (Nacalai Tesque, Kyoto, Japan), supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 100 units/mL penicillin, and 100 µg/mL streptomycin (Nacalai Tesque). The cells were cultured at 37°C with 5% CO₂ with saturated humidity. AhR knockout (AhR KO) HepG2 cells were established according to our previously described method (Yamashita *et al.*, 2018, 2019) with modifications. The gRNAs sequences for the human AhR gene were 5'-ccggGCGACTGGCGTAGGTGATGT-3'/5'-aaacACATCACCTACGCCAGTCGC-3'. The gRNAs were annealed and cloned into a pGuide-it vector

(Clontech Laboratories, Inc., Mountain View, CA, USA). AhR rescue cells, which stably expressed myc-tagged fusion AhR, were generated by transfecting AhR KO cells with pEBMulti-Hyg/myc-AhR plasmid and subjecting the cells to serial hygromycin selections. The pEBMulti-Hyg/myc-AhR plasmid was constructed from pcDNA5/TO myc AhR plasmid, using the Infusion cloning kit (TaKaRa, Shiga, Japan), by inserting the myc tag and AhR gene into *Hind* III, *Bam*HI, and *Eco*R V, *Xho* I sites, respectively.

Real-time RT-PCR

Total RNA was isolated from the cells using ISOGEN II (Nippon Gene Co. Ltd, Tokyo, Japan) according to the manufacturer's protocol. cDNA was synthesized using the ExScript RT reagent kit (TaKaRa). Quantitative real-time RT-PCR was carried out using SYBR premix ExTaq (TaKaRa) on a Smart Cycler II system (Cepheid Inc., Sunnyvale, CA, USA). The following primer pairs were used: *CYP3A5*: forward 5'-GCTCGCAGCCCAGTCAATA-3', reverse 5'-AGGTGGTGCCTTATTGGGC-3', *CYP1A1*: forward 5'-GTCATCTGTGCCATTGCTTTG-3', reverse 5'-CAACCACCTCCCCGAAATTATT-3' and ribosomal protein lateral stalk subunit P0 (*RPLP0*): forward 5'-GGCGACCTGGAAGTCCAAC-3', reverse 5'-CCATCAGCACCACAGCCTTC-3'. *RPLP0* was used as a control.

Western blot analysis

The cells were harvested and lysed in sodium dodecyl sulfate (SDS) sample buffer containing 125 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% dithiothreitol, and 0.01% bromophenol blue. Whole-cell lysates were resolved by SDS-polyacrylamide gel electrophoresis and subsequently transferred onto a polyvinylidene fluoride membrane (Immobilon; Millipore, Burlington, MA, USA). Immunoblotting was performed using anti-AhR (D5S6H) (1:2000 dilution; Cell Signaling Technology, Danvers, MA, USA) and anti-β actin (1:500 dilution; Santa Cruz Biotechnology, Dallas, TX, USA) as the primary antibodies. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG (1:4000 dilution; Cell Signaling Technology) were used as secondary antibodies. Band intensities were detected using a LumiVision PRO 400EX (Aisin Seiki, Aichi, Japan) and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Plasmid construction

pGL3-XRE, an XRE-driven firefly luciferase-express-

ing plasmid, was previously constructed by inserting three tandem repeats of the consensus XRE-containing sequence (5'-TCTTCTCCACGCAACTCCGA-3') into the upstream region of the firefly luciferase gene in the pGL3-promoter vector (Promega, Madison, WI, USA) (Sanada *et al.*, 2009). The underlined nucleotides are the consensus XRE.

Luciferase reporter analysis

Using PEI Max reagent (Polysciences, Warrington, PA, USA), cells were co-transfected with both pGL3-XRE luciferase reporter plasmid and pGL4.74 (hRluc/TK; Promega, San Luis Obispo, CA, USA) carrying the *Renilla* luciferase gene as an internal standard. Following overnight culture in growth medium, the cells were treated with 3MC for 24 hr. The cells were then lysed with passive lysis buffer and luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. The activities of firefly luciferase were normalized to those of *Renilla* luciferase.

Statistical analysis

Statistical comparisons were performed using one-way analysis of variance followed by Dunnett's multiple comparison test as the post-hoc test. Unpaired Student's t-test was performed to compare the differences between two groups. Results with $p < 0.05$ were considered as statistically significant. Statistical analyses performed using KaleidaGraph software, version 4.1.1 (Synergy Software, Eden Prairie, MN, USA).

RESULTS AND DISCUSSION

In silico analysis revealed the XRE sequence was present approximately -2.1 kb upstream from the transcription start site of the *CYP3A5* gene in the human genome. To determine the effects of AhR ligands, 3MC and B[a]P, on *CYP3A5* gene expression, we measured the mRNA expression levels of *CYP3A5* and the typical AhR target gene, *CYP1A1*, in HepG2 cells. Both *CYP3A5* and *CYP1A1* mRNA expression levels were increased by 3MC (1 μ M) or B[a]P (2 μ M) treatment (Fig. 1A), and the effect of 3MC was seen in a dose-dependent manner (Fig. 1B). In general, AhR binds to XRE in the promoter region of the gene thereby enhancing its transcription. Therefore, we constructed a reporter luciferase plasmid with the *CYP3A5* gene promoter region (-2463/-1 bp) containing XRE and evaluated the effect of 3MC on luciferase activity. The luciferase activity was not elevated by 3MC treatment (data not shown). This

Fig. 1. *CYP1A1* and *CYP3A5* mRNA expression was induced by PAHs. (A) HepG2 cells were treated with 3MC (1 μ M), B[a]P (2 μ M) or solvent (0.1% DMSO). (B) HepG2 cells were treated with increasing concentrations of 3MC. After 24 hr, cells were harvested and the mRNA expression of *CYP1A1* and *CYP3A5* was measured by real time RT-PCR. The results are normalized against *RPLP0* mRNA expression levels and are presented as the average of fold induction of HepG2 cells over the solvent control (mean $\pm$ S.D., $n = 3$ or 4). ** $p < 0.01$. Images show the representative results of two independent experiments.

result suggests that AhR regulates *CYP3A5* gene expression via other regions containing XRE, such as intron of *CYP3A5* gene or distant from *CYP3A5* gene. AhR may also regulate the expression and/or activating transcription factors involved in the induction of *CYP3A5* gene expression. Further investigation is needed to clarify the mechanism of induction of *CYP3A5* gene expression by AhR.

It has been reported that the induction of *CYP3A4* gene expression by PAHs occurs via PXR, but not via AhR, in HepG2 cells (Kumagai *et al.*, 2012). Therefore, we established AhR knockout (AhR KO) HepG2 cells, using the CRISPR/Cas9 system, to examine whether activated AhR was involved in the up-regulation of *CYP3A5* gene expression. AhR protein was not detected in AhR

Fig. 2. Expression of AhR protein and induction of luciferase activity by 3MC were not observed in AhR KO cells. (A) Whole-cell lysates of HepG2 cells and AhR KO cells were resolved by SDS-PAGE and proteins were detected by immunoblotting using antibodies against AhR and β-actin. (B) HepG2 cells and AhR KO cells were transfected with the pGL3-XRE luciferase reporter plasmid and pGL4.74. The following day, cells were treated with 3MC (1 μM) or solvent (0.1% DMSO). After 24 hr, luciferase activity was measured using the Dual-Luciferase Reporter Assay System. The results are shown as the fold induction of HepG2 cells over the solvent control after normalization with *Renilla* luciferase (mean ± S.D., n = 4). **p < 0.01. Images show the representative results of two independent experiments.

Fig. 3. *CYP1A1* and *CYP3A5* mRNA expression was not induced by PAHs in AhR KO cells. AhR KO cells were treated with 3MC (1 μM), B[a]P (2 μM) or solvent (0.1% DMSO). After 24 hr, cells were harvested and the mRNA expression levels of *CYP1A1* and *CYP3A5* were measured by real time RT-PCR. The results are normalized against *RPLP0* mRNA expression levels and are presented as the average of fold induction of the solvent control (mean ± S.D., n = 3 or 4). **p < 0.01. Images show the representative results of two independent experiments.

PAHs are ubiquitous environmental pollutants produced during the incomplete combustion of organic materials. It is also known that cigarette smoke contains various PAHs and activates AhR. Based on our results, we

KO cells as shown in Fig. 2A. Furthermore, XRE-driven luciferase activity was increased by 3MC in wild type (WT) HepG2 cells, but not in AhR KO cells (Fig. 2B). These results confirmed that AhR KO cells were established. Next, we evaluated the mRNA expression of *CYP1A1* and *CYP3A5* in AhR KO cells. *CYP1A1* and *CYP3A5* mRNA expression levels were not increased by 3MC treatment (Fig. 3).

The CRISPR/Cas9 system has an off-target effect in which other genes can be possibly edited (Zhang *et al.*, 2015). Therefore, we established two kinds of cells from AhR KO cells: AhR rescue cells and control cells. The former is derived by transfecting the AhR KO cells with an episomal vector containing the *AhR* gene (AhR rescue), and the latter by transfecting the cells with an empty vector (control cells). As shown in Fig. 4A, AhR protein expression was observed in AhR rescue cells, but not in control cells. In addition, the mRNA expression levels of *CYP1A1* and *CYP3A5* were significantly increased by 3MC in AhR rescue cells (Fig. 4B). These results indicated that the results shown in Fig. 3 were not due to the off-target effects of the CRISPR/Cas9 system, and that induction of *CYP3A5* mRNA expression requires AhR expression.

Fig. 4. *CYP1A1* and *CYP3A5* mRNA expression was induced by 3MC in AhR rescue cells. (A) Whole-cell lysates of control cells and AhR rescue cells were resolved by SDS-PAGE and proteins were detected by immunoblotting using antibodies against AhR and β-actin. (B) AhR rescue cells were treated with 3MC (1 μM) or solvent (0.1% DMSO). 24 hr later, the cells were harvested and the mRNA expression of *CYP1A1* and *CYP3A5* was measured by real time RT-PCR. The results are normalized against *RPLP0* mRNA expression levels and are presented as the average of the fold induction of the solvent control (mean ± S.D., n = 3 or 4). **p < 0.01. Images show the representative results of two independent experiments.

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predicted that smokers might have elevated *CYP3A5* gene expression compared to non-smoker. However, one study suggested that cigarette smoking decreased the mRNA levels of *CYP3A5* in alveolar macrophages (Hukkanen *et al.*, 2003). Therefore, further studies are needed to determine the mechanism of regulation of *CYP3A5* gene expression by AhR in various cell types or tissues.

Taken together, the present study demonstrated that PAHs induced *CYP3A5* mRNA expression in HepG2 cells via AhR. Further studies on the molecular mechanism of *CYP3A5* up-regulation by AhR may provide useful information for personalized medicine.

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

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