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Identification of amino acid residues in the ligand-binding domain of the aryl hydrocarbon receptor causing the species-specific response to omeprazole: possible determinants for binding putative endogenous ligands

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Running title: AhR Amino Acid Residues Required for Response to Omeprazole

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Abbreviations:

AhR, aryl hydrocarbon receptor

Arnt, AhR nuclear translocator

CYP, cytochrome P450

DMEM, Dulbecco's modified Eagle's medium

DMSO, dimethyl sulfoxide

FBS, fetal bovine serum

FICZ, 6-formylindolo[3,2-*b*]carbazole

ITE, 2-(1H-indol-3-ylcarbonyl)-4-thiazolecarboxylic acid methyl ester

LBD, ligand-binding domain

MEM, Eagle's minimum essential medium

OME, omeprazole

PBS, phosphate-buffered saline

PTK, protein tyrosine kinase

RT-PCR, reverse transcription polymerase chain reaction

SAhRM, selective AhR modulator

TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

XRE, xenobiotic response element

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Abstract

Omeprazole (OME) induces the expression of genes encoding drug-metabolizing enzymes such as cytochrome P450 1A1 (CYP1A1) *via* activation of the aryl hydrocarbon receptor (AhR) both *in vivo* and *in vitro*. However, the precise mechanism of OME-mediated AhR activation is still under investigation. While elucidating species-specific susceptibility to dioxin, we found that OME-mediated AhR activation was mammalian species specific. Moreover, we previously reported that OME has inhibitory activity toward CYP1A1 enzymes. From these observations, we speculated that OME-mediated AhR target gene transcription is due to AhR activation by increasing amounts of putative AhR ligands in serum by inhibition of CYP1A1 activity. We compared the amino acid sequences of OME-sensitive rabbit AhR and non-sensitive mouse AhR to identify the residues responsible for the species-specific response. Chimeric AhRs were constructed by exchanging domains between mouse and rabbit AhRs to define the region required for the response to OME. OME-mediated transactivation was observed only with the chimeric AhR that included the ligand-binding domain (LBD) of the rabbit AhR. Site-directed mutagenesis revealed three amino acids (M328, T353, and F367) in the rabbit AhR that were responsible for OME-mediated transactivation. Replacing these residues with those of the mouse AhR

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abolished the response of the rabbit AhR. In contrast, substitutions of these amino acids with those of the rabbit AhR altered non-sensitive mouse AhR to become sensitive to OME. These results suggest that OME-mediated AhR activation requires a specific structure within LBD that is probably essential for binding with enigmatic endogenous ligands.

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Introduction

Omeprazole (OME), a benzimidazole derivative, is a potent suppressor of gastric acid secretion and has been used to treat gastroesophageal reflux disease and duodenal ulcers (Lind et al., 1983). In human hepatoma cells and liver cells, OME is known to induce cytochrome P450 1A1 (CYP1A1) and CYP1A2 (Diaz et al., 1990; Curi-Pedrosa et al., 1994; Krusekopf et al., 1997; Yoshinari et al., 2008). The transactivation of these genes is known to be mediated by a ligand-dependent transcriptional factor, aryl hydrocarbon receptor (AhR) and AhR nuclear translocator (Arnt) (Burbach et al., 1992; Reyes et al., 1992). The AhR/Arnt heterodimer binds to the specific nucleic acid sequence known as the XRE, which is located in the 5′-flanking region of *Cyp1a1* and other AhR target genes (Denison et al., 1989). Although it is generally accepted that ligand binding is a key determinant for AhR-mediated transcriptional activity, even in the absence of exogenous ligands (Chang and Puga, 1998; Murray et al., 2005; Shiizaki et al., 2005), some reports have shown that OME induces CYP1A1 expression without binding to AhR using competitive binding assays (Daujat et al., 1992; Dzeletovic et al., 1997). Similar to typical AhR ligands, OME induces translocation of the AhR/Arnt complex to the nucleus and the complex binds to XRE of *Cyp1a1* enhancer region (Quattrochi and Tukey, 1993; Yoshinari et al., 2008). Therefore, OME is considered to

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activate AhR through a pathway other than ligand binding. Several reports have shown that the protein tyrosine kinase (PTK) pathway is involved in CYP1A1 induction by OME in rat and human hepatoma cells (Backlund et al., 1997; Kikuchi et al., 1998; Lemaire et al., 2004; Backlund et al., 2005). OME-mediated AhR signaling has been shown to be attenuated by *c-src* kinase inhibitors or by the expression of the dominant negative *c-src* protein. Furthermore, it has been suggested that the tyrosine residue at amino acid position 320 in the human AhR is a putative phosphorylation site required for OME-mediated AhR activation (Backlund et al., 2004). However, the molecular mechanisms of OME-mediated AhR activation have not been completely elucidated. In addition, OME-mediated AhR activation has been reported to be species-specific (Kikuchi et al., 1995, Dzeletovic et al., 1997). CYP1A1 induction by OME has been demonstrated in human, but not in mouse, primary hepatocytes, and hepatoma cell lines. However, the PTK pathway appears to be unrelated to this interspecies difference because the tyrosine 320 residue is conserved in mouse and human AhRs. By fusing mouse and human hepatoma cells, a gene locus was assigned as a human-specific gene responsible for CYP1A1 induction by OME (Kikuchi et al., 2002). However, specific genes exhibiting response to OME have not been identified. Thus, the mechanism underlying species-specific differences in OME-mediated AhR activation remains

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unclear. Elucidating such a mechanism could lead to a better understanding of OME-mediated AhR activation.

Recent studies have shown that AhR has physiological roles such as anti-inflammation and T cell differentiation (Kimura et al., 2008; Quintana et al., 2008). These discoveries lead to suggestions of using AhR ligands for chemotherapy. For example, selective AhR modulator (SAhRM) that exhibits anti-inflammatory properties without XRE-dependent xenobiotic gene expression has been proposed (Murray et al., 2010). Because some AhR ligands are already used in human therapy and because OME-mediated AhR activation is unique, OME and other imidazole compounds may be candidates for SAhRMs. Elucidation of the molecular mechanism of OME-mediated AhR activation will be important for such applications.

Recently, a report showed that AhR activation by CYP1A1-inhibiting chemicals is caused by an indirect mechanism involving disruption of the clearance of endogenous ligands (Wincent et al., 2012). We speculated that OME-mediated AhR activation is due to this indirect mechanism because OME is one of the CYP1A1-inhibiting chemicals (Shiizaki et al., 2008). Although controversial, species-specific response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is considered to be due to differences in the AhR molecular structure responsible for the ligand binding affinity (Bohonowych

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and Denison, 2007). If a putative ligand is related to OME-mediated AhR activation, a species-specific OME response would be generated because of the affinity of the putative ligand conferred by the AhR structure as well as response to TCDD. In addition, the structural differences in AhR will be due to particular amino acid residues.

In this study, we used AhRs derived from various laboratory animals and humans to demonstrate that species-specific activation by OME depends on specific amino acids within the AhR amino acid sequences.

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Materials and Methods

Chemicals. Omeprazole, 3-methylcholanthrene (3MC), ellipticine, kynurenic acid, and 2-(1H-indol-3-ylcarbonyl)-4-thiazolecarboxylic acid methyl ester (ITE) were purchased from Sigma-Aldrich (St. Louis, MO). TCDD (>99.5% purity) was obtained from Cambridge Isotope Laboratories (Andover, MA). β-Naphthoflavone was purchased from Wako Pure Chemical (Osaka, Japan). Indirubin was kindly provided by Dr. Tomonari Matsuda (Kyoto University, Kyoto, Japan). 6-Formylindolo[3,2-*b*]carbazole (FICZ) was purchased from Biomol International LP (Plymouth Meeting, PA). All chemicals were dissolved in dimethyl sulfoxide (DMSO) and added to media. The final concentration of DMSO was adjusted to 0.1% (*v/v*) in culture media.

CYP1A1 activity determined by P450-Glo Assays. CYP1A1 activity was determined using a P450-Glo CYP1A1 Assay (Promega, Madison, WI), according to the manufacturer's instructions. Sf9 cell microsomes containing recombinant human CYP1A1 were purchased from Becton Dickinson (Franklin Lakes, NJ). In brief, an aliquot (0.5 pmol) of microsomes was mixed with 100 mM KPO_4 buffer (25 μl)

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containing 120 μM of luciferin-CEE. Then, OME (final concentration 0.5-100 μM) or ellipticine (final concentration 0.1-10 μM) were added to reaction cocktails and incubated at 37°C for 10 min. After preincubation, 25 μl of 2× NADPH-regenerating system solution (2.6 mM NADP+, 6.6 mM glucose-6-phosphate, 0.4 unit/ml glucose-6-phosphate dehydrogenase, and 6.6 mM $MgCl_2$) were added and incubated at 37°C for 30 min. Luciferin obtained after conversion of luciferin-CEE by CYP1A1 was detected by adding the Luciferin Detection Reagent included in the assay kit, and luminescence was detected using the Wallac Arvo SX Multi-Label counter (PerkinElmer, Boston, MA).

Plasmid Construction. The reporter plasmid pX4TK-Luc, which includes the firefly luciferase gene under the control of four copies of XRE and the thymidine kinase promoter, was a gift from K. Sogawa (Tohoku University, Sendai, Japan). The expression plasmids containing AhR cDNAs from six mammalian species were constructed as follows. cDNAs were prepared from mouse (*Mus musculus*, strain C57BL), human (*Homo sapiens*), rat (*Rattus norvegicus*, Holtzman), rabbit (*Oryctolagus cuniculus*, New Zealand White), hamster (*Mesocricetus auratus*, Syrian), and guinea pig (*Cavia porcellus*, Hartley). The open reading frames of each AhR were

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amplified by RT-PCR and inserted into the *Mlu*I and *Xho*I sites of the pCI-neo vector (Clontech, Palo Alto, CA). To construct the chimeric mouse/rabbit AhRs, AhR cDNA fragments based on the mouse and rabbit AhR ligand-binding domains (LBDs) (Fukunaga et al., 1995) were individually amplified, joined by overlapping PCR, and subcloned into the pCI-neo vector. Single point mutations were generated using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). All primers used in this study are shown in supplemental Table S1.

Cell Cultures and Transfection. The human epithelial carcinoma cell line HeLa and murine hepatoma cell lines Hepa1c1c and its derivative c12 were obtained from the American Type Culture Collection (Rockville, MD). HeLa cells were grown in MEM containing 10% charcoal-stripped FBS and 1× Antibiotic-Antimycotic (Invitrogen, Carlsbad, CA). Hepa1c1c and c12 cells were grown in DMEM containing 10% charcoal-stripped FBS and 1× Antibiotic-Antimycotic. All cultures were incubated at 37°C in 5% CO_2. Transfection was performed by a liposome method. In brief, 1 μg plasmid DNA and 4 μl Plus reagent (Invitrogen) were combined in 200 μl OPTI-MEM (Invitrogen). After incubation for 15 min, 2 μl Lipofectamine reagent (Invitrogen), diluted with 200 μl OPTI-MEM, was added. The cells were plated in 6-well tissue

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culture plates at 30–40% confluence a day before transfection. Liposomes were added in serum-free medium for 3 h and then replaced with MEM or DMEM containing 10% charcoal-stripped FBS without 1× Antibiotic-Antimycotic.

Luciferase Assay. The effects of OME and typical AhR agonists on XRE-dependent transcriptional activity were evaluated by cotransfecting the AhR expression plasmid, the reporter plasmid pX4TK-Luc (Mimura et al., 1999), and the Renilla luciferase (*R*luc) expression vector pRL-CMV (Promega) into cells. The transfected cells were washed with cold PBS and lysed in 25 μl 1× passive lysis buffer (Promega). Aliquots (10 μl) of the lysates were transferred to 96-well plates, and Luc^+ and *R*luc luminescence was measured using the Dual-Luciferase Reporter Assay System (Promega) in the Wallac Arvo SX Multi-Label counter. Transfection and translation efficiencies varied between independent experiments, and the results were normalized by calculating Luc^+/*R*luc ratios. All assays were performed in triplicate.

Immunoblotting. Immunoblotting was performed to evaluate expressed AhR protein levels after transient transfection experiments. Cells plated in 6-well plates were washed with PBS, lysed in 0.5 ml SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM

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Tris-HCl, pH 8) containing Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland), and sonicated to shear DNA. Protein concentration in cell lysates in the cell lysates was measured by the Bradford method using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany), and 20-μg aliquot of each sample was separated on an SDS-polyacrylamide gel. Immunoblotting for AhR was performed as described previously (Pollenz et al., 1994) with anti-human AhR antibody (SA-210; Biomol GmbH, Hamburg, Germany). Detection was performed with peroxidase-labeled anti-rabbit antibody using the ECL Plus detection system (GE Healthcare Bio-Sciences, Uppsala, Sweden). The bands were visualized and imaged using ChemiDoc XRS Plus (Bio-Rad Laboratories).

Generation of Stable Cell Lines Expressing Mutant AhRs. pCI-neo plasmids containing mouse and rabbit AhR cDNAs with point mutations were transfected into c12 cells. Stable transformants expressing AhRs were selected using G418 and screened for ethoxyresorufin-O-deethylase activity as described previously (Kennedy and Jones, 1994) after treatment with 10 nM TCDD. We chose and mixed more than six representative clones from transformants obtained with each plasmid and investigated CYP1A1 inducibility by TCDD and OME using real-time RT-PCR methods. In brief,

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the cells plated in 6-well plates at 80% confluence were exposed to 50 μM OME or 10 nM TCDD. After 12 h of exposure, total RNA was isolated using Isogen (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. An aliquot (2 μg) of total RNA was subjected to reverse transcription using MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA) and oligo-dT primers. An aliquot of cDNA (2 μl) or calibrator plasmid DNA (pCI-neo-mAhR) was amplified with master mixture (SYBR Premix Ex Taq, Takara Bio, Kyoto, Japan) containing gene-specific primers. The primer pair for mouse CYP1A1 cDNA amplification is shown in supplemental Table S1. The reaction mixture was amplified using the Smart Cycler System (Cepheid, Takara Bio) under the following conditions: an initial incubation at 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 10 s. To confirm amplification specificity, the PCR products were subjected to melting curve analyses. A calibration curve was generated by threshold cycles of calibrators of a known plasmid copy number. The initial quantity of target mRNA in the samples was determined by correlating their threshold cycles to the calibration curve.

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Results

OME inhibits CYP1A1 activity. To examine if OME directly inhibits CYP1A1 activity, recombinant human CYP1A1 proteins expressed by Sf9 cells were used in the inhibition study. In addition to ellipticine, a typical CYP1A1 inhibitor, OME also inhibited CYP1A1 activity The IC_{50} value was 6.77 μM in case of OME and 2.75 μM in ellipticine (Fig.1, data re-examined as reported in our previous study, Shiizaki et al., 2008). CYP1A1 activity was inhibited nearly 90% by more than 20 μM of OME, and this concentration correlated to the concentration of OME required for AhR activation (Diaz et al., 1990, Dzeletovic et al., 1997).

We confirmed species-specific induction of CYP1A1 by OME (Supplement Fig. S1A). Induction of CYP1A1 mRNA by OME occurred not only in human hepatoma HepG2 cells but also in mouse Hepa1c1c cells (Kikuchi et al., 1995). This induction was well correlated with XRE-driven reporter gene induction (Supplement Fig. S1B). We used this reporter assay system to evaluate OME-mediated AhR activation, which consequently led to CYP1A1 induction.

OME restores XRE-dependent transcription in CYP1A1-overexpressing SKII-1A1 cells and CYP1A1-deficient SK-Hep-1 cells. As reported in our previous

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study, in the presence of 10% FBS, the human hepatoma cell line SK-Hep-1, which is deficient in *CYP1A1* expression, showed higher basal transcription from the XRE-driven reporter gene without additional exogenous ligands than other human hepatoma cells (Shiizaki et al., 2005). We compared basal transcription levels of CYP1A1-overexpressing cell lines (SKII-1A1 cells) and found that basal transcription levels in these cells were significantly lower than those in wild-type SK-Hep-1 cells. Using this reporter system, OME or ellipticine were added to culture media of the wild-type SK-Hep-1 and SKII-1A1#2 cells (one of the CYP1A1-overexpressing clones). OME restored the transcription in the SKII-1A1#2 cells to a level similar to that in the wild-type cells (Fig. 2). Treatment of CYP1A1 inhibitor ellipticine indicated similar results. These observations suggest that OME does not transactivate XRE-driven reporter genes directly *via* AhR activation but instead inhibits transcription repression by CYP1A1 activity.

Charcoal-stripping of serum diminished the basal transcription level and OME-induced transcription of the XRE-driven reporter gene in HepG2 cells. Next, we tested the effect of FBS on the transcription induction of XRE-driven reporter genes by OME to provide indirect evidence of the presence of substances in FBS that might

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transactivate AhR. The human hepatoma cell line HepG2 and SK-Hep-1 cells are similar in terms of the expression levels of AhR and Arnt molecules (Shiizaki et al., 2005). The 4×XRE-Luc reporter gene and pCMV-Rluc were cotransfected into HepG2 cells. After transfection, the culture medium was changed to fresh DMEM containing normal FBS or charcoal-stripped FBS. Although OME-induced transcription was clearly observed in DMEM containing normal FBS, significantly less OME-induced transcription was observed in DMEM containing charcoal-stripped FBS (Fig. 3). Charcoal-stripping of FBS did not reduce 3MC (1 μM)-induced transcription. Together with this and above mentioned results (Fig. 1 and Fig.2), it is highly suspected that OME-mediated AhR activation of the XRE-dependent gene is due to the inhibition of the activity of CYP1A1 enzymes, which may metabolize putative AhR ligands contained in FBS.

Comparison of the responses of AhRs derived from various mammals to OME. While exploring the genes that cause species-specific susceptibility against dioxin, we prepared six AhR expression plasmids from six mammalian species using pCI-neo vectors. In these experiments, we used HeLa cells, which possess *CYP1A1* and express a similar level of ARNT molecules and a lower level of AhR molecules compared with

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HepG2 or SK-Hep-1 cells. We also used mouse hepatoma Hepa1c1c7 cells, which expressed a sufficient level of AhR. During this project, we used OME in addition to TCDD to examine the species-specific influence on AhR dependent transcription. XRE-driven reporter gene assays were performed by co-transfection of the abovementioned six AhR expression vectors and 4×XRE-Luc reporter genes.

The constitutive transcription of XRE-dependent luciferase differed among AhR species expressed in HeLa and Hepa1c1c7 cells (Fig. 4A and 4B). Although overexpressed AhR protein levels were almost similar in HeLa cell experiments, the highest basal transcription level was detected in the guinea pig AhR followed by the rabbit AhR (Fig. 4A). The pattern of basal transcription levels with each AhR in the Hepa1c1c7 cell experiments was similar to those in the HeLa cells; however, the levels did not correlate with the protein levels measured by immunoblotting (Fig. 4B). Of the six AhRs, the mouse AhR transfection caused the least increment in constitutive activity in both cells, as well as no significant induction of genes with the addition of OME (Fig. 4A and 4B). Considering the effects of exogenous AhRs transfection, reporter gene induction by OME was highest when the rabbit AhR was used, followed by the guinea pig AhR (Fig. 4A and 4B). However, in addition to the mouse AhR, rat and hamster AhRs were not activated by OME in mouse hepatoma cells. These results suggest that

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transcriptional activation by OME was dependent on the AhR species as well as the species of host cells.

Responses of chimeric AhRs to OME. According to our observations stated above, we speculated that the rabbit AhR must contain a domain responsible for OME-induced XRE-dependent transcription. Six chimeric AhRs were constructed using mouse and rabbit AhR cDNAs (Fig. 5A). Among the chimeric constructs, MR1, RM2, and MRM1, which included the rabbit LBD (amino acids 261–393), showed OME-dependent reporter gene induction. MR2, RM1, and RMR1, which included the mouse LBD (amino acids 257–389), did not respond to OME, despite the other regions of these chimeras were of the rabbit AhR (Fig. 5A). To determine the minimum requirement for the response to OME, chimeric AhRs were constructed with half LBDs. However, no constructs with partial rabbit AhR LBDs responded to OME (Fig. 5B). These data suggest that the response to OME required the complete rabbit AhR LBD, and that multiple amino acid combinations specific to the rabbit AhR should be indispensable for OME-mediated AhR activation.

Determination of the amino acid residues responsible for OME-mediated AhR

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activation. Next, we conducted a study of a single amino acid substitution in the rabbit AhR that would eliminate OME-mediated AhR activation. There are 16 amino acid differences between the rabbit and mouse AhR LBDs (Fig. 6A). As shown in Fig. 6B, the basal transcriptional activity of rabbit AhR mutants was variously changed by a single amino acid substitution. Most of the mutants except M328I, T353A, and F367L did not influence so much to the OME-mediated transcriptional level. These three mutants reduced induction to less than one-half of Wild-type rabbit AhR which showed an approximately 3-fold change by OME. The OME-mediated inductive ratio of M328I, T353A, and F367L was 1.7-, 1.2-, and 1.0-fold, respectively. Difference in properties between mouse AhR and rabbit AhR is the basal transcriptional activity and OME-induced transcriptional activity. By plotting these properties to emphasize differences of AhR mutants, we found the three mutant AhRs, M328I, T353A, and F367L indicated intermediate properties of the mouse AhR and rabbit AhR (Fig. 6C).

In addition, we constructed double and triple substitutions of these three amino acid residues in the rabbit AhR. As shown in Fig. 7A, the combinations of M328I and T353A or T353A and F367L substitutions impaired OME-mediated transcriptional induction. Moreover, the triple substitutions of M328I, T353A, and F367L exhibited properties that were remarkably similar to those of the mouse AhR, i.e., low constitutive activity

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and OME unresponsiveness. In contrast, mouse AhRs with single amino acid substitutions of I324M, A349T, or L363F, which are residues equivalent to M328, T353, and F367 in the rabbit AhR, slightly introduced OME responsiveness with increased levels of constitutive activities (Fig. 7B). However, triple substitutions of these three residues in the mouse AhR resulted in an approximately 5-fold induction by OME, which was not observed in wild-type mice.

Among the three amino acids, we focused on rabbit F367 because this residue is rabbit-specific (Fig. 8A). In addition, the guinea pig AhR was as responsive to OME as the rabbit AhR, and the amino acid corresponding to rabbit F367 is V368 in the guinea pig AhR, which is different in the other four species (Leu). Therefore, we constructed several mutated mouse and guinea pig AhRs to confirm that Val in this position was effective for the response to OME. As shown in Fig. 8B, the mouse AhR with triple substitutions of L363V, I324M, and A349T responded more to OME compared with double substitutions of I324M and A349T. In contrast, the response of the guinea pig AhR to OME was abolished by the V368L substitution. These results suggest that the combination of three amino acid residues of the AhR, i.e., Met, Thr, and Phe, are required for the response to OME, and also that Phe can be replaced with Val. The importance of these three amino acid residues in the OME response was confirmed by

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human AhR mutants as well as guinea pig AhR (Supplemental Fig.S2). When two amino acid residues (330M and 355T) in human AhR were changed to mouse-type amino acid (330I and 355A), OME response was attenuated. Contrary to this, 369F, which is particularly in rabbit AhR, represent higher OME-response than wild type human AhR.

CYP1A1 gene induction by OME restored the three amino acids substitutions in the mouse AhR and abolished them in the rabbit AhR. To confirm the results that M328, T353, and F367 in the rabbit AhR are responsible for the OME-mediated AhR activation, we established cell lines that stably expressed AhRs, including the triple point mutations. The c12 cell line, which is a Hepa1c1c-derived sub-line lacking endogenous AhR expression, was used for this purpose, and examined for CYP1A1 induction by TCDD or OME exposure. As shown in Fig. 9, CYP1A1 mRNA induction by OME was similar to that observed in the reporter assays (Fig. 7), i.e., the induction was limited to the cells expressing the wild-type rabbit AhR or the mouse AhR with the three amino acid substitutions. TCDD-induced CYP1A1 mRNA induction was observed at similar levels in all cell lines expressing both wild-type and mutated AhRs, suggesting that TCDD-induced AhR activation was not influenced by these amino acid

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substitutions.

Responses of mutated AhRs to typical AhR ligands. Next, we characterized the responses of the mutated AhRs to typical AhR ligands, including AhRs with substitutions of the three amino acids involved in the response to OME. As shown in Fig. 10 and Table 1, EC_{50} values calculated from the dose–response curves of three typical chemical ligands were slightly changed (within 2-fold) by these three amino acid substitutions that drastically reversed OME responsiveness. We also tested candidates for endogenous ligands, namely ITE (Song et al., 2002), FICZ (Wei et al., 1998), indirubin (Adachi et al., 2001), and kynurenic acid (DiNatale et al., 2010), which have induction potencies that differ between mouse and rabbit AhRs. FICZ induced transcriptional activity at a lower concentration with the rabbit AhR than with the mouse AhR, whereas indirubin had a reverse effect. However, as with the chemical ligands, the dose-response curves and EC50 values for the putative endogenous ligands were not much changed by the three amino acid substitutions. (Fig. 11 and Table 1). These results show that these three amino acid residues define OME sensitivity but do not influence the responsiveness of the other AhR ligands tested in this study.

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Discussion

This study aimed to identify and experimentally validate the factor(s) generating species-specific responsiveness of AhR to OME. First, we speculated that OME-mediated AhR activation is due to increasing amounts of putative AhR ligands resulting from the inhibition of CYP1A1 activity by OME. This hypothesis is based on the following observations: (1) OME-mediated AhR activation required CYP1A1 activity and was influenced by endogenous ligands included in serum. (2) The concentration of OME activating AhR was higher than with that of the typical AhR ligand and was similar to the concentration of OME required for CYP1A1 inhibition. (3) The comparison of AhRs from six mammalian species revealed that transcriptional activation of the reporter gene by OME seemed to correlate with the basal transcription level, reflecting the response of the putative endogenous ligand. If a type of ligand mediates OME-dependent AhR activation, OME responsiveness would be attributable to the AhR itself, independent of host cells. In the present study, we constructed chimeric AhRs from mouse and rabbit AhRs, which indicated that OME responsiveness required multiple amino acid residues present in the LBD of the rabbit AhR. These amino acid residues were not present in the mouse AhR. In conclusion, we demonstrated that M328, T353, and F367 of the rabbit AhR were required for OME

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responsiveness.

These results are fundamentally different from those of previous reports on cell-specific factors that define OME responsiveness (Kikuchi et al., 1995; Dzeletovic et al., 1997; Kikuchi et al., 2002). Some of the differences may be due to the species used because most of the abovementioned studies used the human AhR, whereas we primarily used the rabbit AhR, which is highly sensitive to OME. In a previous report (Dzeletovic et al., 1997), the human AhR transiently expressed in mouse hepatoma c12 cells was not activated by OME. We performed a similar experiment with c12 cells stably expressing the rabbit AhR, and these cells exhibited inducible expression of CYP1A1 by OME. (Fig. 9). We thought that the stably expressed rabbit AhR altered intracellular conditions, e.g., the basal level of CYP1A1 expression, to respond to the AhR activator. In fact, the c12-derived cells expressing the rabbit AhR exhibited a raised level of constitutive CYP1A1 expression and remarkable CYP1A1 induction (>15-fold) upon TCDD exposure.

Several investigations have identified specific amino acid residues that are important for AhR activation. We previously reported that L318 in the mouse AhR is required for the response to β-naphthoflavone (βNF) (Goryo et al., 2007). An AhR polymorphism (A375V) in C57BL/6 and DBA/2 mice results in a difference in TCDD

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binding affinity and consequent toxicity (Ema et al., 1994). Two polymorphisms, V325I and A381S, in the common tern and chicken AhRs are responsible for sensitivity to TCDD (Karchner et al., 2006). Interestingly, the V325I polymorphism in the bird species corresponds to the mouse AhR I324, one of the critical amino acids for OME responsiveness. In the tern AhR, Ile is required in this position for high sensitivity to TCDD. The single mutation I324M in the mouse AhR was insufficient for producing a response to OME, but instead increased basal transcriptional activity. The amino acid residue at this position could be close to TCDD and may be one of the key amino acids for ligand binding and constitutive activity. As mentioned in the Results section, F367 is specific to the rabbit AhR. In the guinea pig AhR, the corresponding amino acid is V363, and the L363V substitution was an effective alternative to the phenylalanine required for reconstituting the response to OME in the mouse AhR (Fig. 6). It is interesting that there are differences between Leu and Val despite their structural similarity. Ala at position 349 in the mouse AhR is also conserved in rat and hamster AhRs, which exhibit only a slight response to OME. In human and guinea pig AhRs, which are OME sensitive, the corresponding amino acid at this position is threonine, identical to the rabbit AhR. Overall, these three amino acid residues correspond strongly to the species differences in the response to OME. The differences in intensity of the response to OME

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among the animal species can be explained well by this comparative approach.

The results of our study suggest some possibilities for elucidating the underlying mechanism of OME-mediated AhR activation. In our experiments, the response required an OME-sensitive AhR that depended on three amino acid residues located in LBD. Some reports have suggested that PTK is associated with OME-mediated AhR activation (Kikuchi et al., 1998; Backlund et al., 2005). In one report, the Y320 residue of the human AhR was determined to be a putative phosphorylation site required for OME responsiveness (Lemaire et al., 2004). However, this tyrosine residue and the proximal nine amino acids are conserved in all of the AhRs tested in the present study. Furthermore, the three amino acid residues that we have found to be essential are neither putative phosphorylation sites nor are they included in the PTK consensus. Hence, AhR phosphorylation by PTK may be influenced by a conformational change resulting from these three amino acids. The mutant AhRs generated in the present study will be useful for further investigations of the relationship between OME and PTK. Other than the involvement of PTK, a possible mechanism for OME-mediated AhR activation is the presence of some type of ligand. A possible type of ligand is an endogenous (intrinsic) ligand in the culture medium or one that is generated intracellularly. In general, an endogenous ligand is considered to be inactivated rapidly

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by CYP enzymes (Chang and Puga, 1998; Adachi et al., 2001; Spink et al., 2003). In the presence of a CYP inhibitor, the endogenous ligand remains and may activate the AhR. In fact, the inhibitory effects of OME on CYP1A1, 2C19, and 3A4 have been reported previously (XQ et al., 2004; Shiizaki et al., 2008). By disrupting an autoregulatory loop consisting of CYP1A1 and FICZ, one of the endogenous ligands, a recent report showed that CYP1A1 upregulation by CYP1A1-inhibiting chemicals was due to an indirect mechanism (Wincent et al., 2012). On the basis of this theory, we confirmed CYP1A1 inhibition by OME (Fig. 1) and demonstrated that CYP1A1 activity was required for OME-mediated AhR activation (Fig. 2). These results were quite similar to the results obtained from CYP1A1 inhibitor ellipticine. Furthermore, OME-mediated AhR activation was partially reduced due to charcoal-stripping of serum, which is considered to be a supplier of endogenous ligands (Fig. 3). Thus, we tested four putative endogenous ligands, including FICZ; however, AhR activation by these ligands was not significantly influenced by the three amino acid substitutions. We concluded that these four putative endogenous ligands are not involved in the indirect mechanism of OME-mediated AhR activation. Therefore, an enigmatic ligand may be involved. The possible mechanism related to the indirect activation of AhR by OME was illustrated in supplemental Fig.S3. Another possible ligand is an unstable and short-lived metabolite

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of OME, which would be difficult to detect by general experimental procedures. Hu et al. developed a sensitive assay that indicated that OME itself could bind to AhR, although with a very low affinity (Hu et al., 2007). The results of Fig.2 involve the possibility that CYP1A1 produces the putative active metabolite, and we do not have data that deny this hypothesis at present. If some ligand is related to OME-mediated AhR activation, it must differ in its affinity for rabbit and mouse AhRs, and the difference would be attributable to the three amino acid residues identified in this study.

In conclusion, three amino acids in the LBD of AhR define species-specific differences in OME-mediated AhR activation. These findings will be useful for elucidating the molecular mechanism underlying OME-mediated AhR activation.

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Authorship Contributions

Participated in research design: Shiizaki and Ohsako.

Conducted experiments: Shiizaki and Ohsako.

Contributed new reagents or analytic tools: Shiizaki, Ohsako, Kawanishi, and Yagi.

Performed data analysis: Shiizaki.

Wrote or contributed to the writing of the manuscript: Shiizaki, Ohsako, and Yagi.

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Footnotes

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Figure Legends

Fig. 1. Inhibition of human CYP1A1 activity by omeprazole. CYP1A1 activity was determined using P450-Glo Assays and Sf9 cell microsomes containing recombinant human CYP1A1. Luciferin-CEE (a specific substrate for CYP1A1) and microsomes were incubated at 37°C for 30 min with various concentrations of omeprazole or ellipticine. Each data point represents the mean of duplicate determinations.

Fig. 2. OME restores XRE-dependent transcription in CYP1A1-overexpressing SKII-1A1 cells and CYP1A1-deficient SK-Hep-1 cells. CYP1A1-deficient SK-Hep-1 (Wt) and its derivative cell lines overexpressing CYP1A1 (SKII-1A1#2) cultured in medium containing 10% FBS were transfected with the 4×XRE-Luc reporter gene and pCMV-Rluc. After transfection, fresh medium containing 100 μM omeprazole (closed column), 5 μM ellipticine (gray column) or DMSO (CT, open column) was added. Data are represented as the ratio of firefly luciferase activity to Renilla luciferase activity observed 16 h after OME was added. (Fluc/Rluc, mean ± SD, n = 3) (* $P < 0.05$, ** $P < 0.01$, n.s.: no significant difference to control).

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Fig. 3. Effects of FBS on XRE-dependent transcriptional activation. HepG2 cells were transfected with the 4×XRE-Luc reporter gene and pCMV-Rluc. After transfection, the culture medium was changed to fresh DMEM containing normal FBS (nFBS, open column) or charcoal-stripped FBS (cFBS, closed column). Twenty-four hours after medium exchange, 50 or 100 μM of OME was added. Significant reductions in OME-induced transcription was noted with charcoal stripping; however, 3MC (1 μM)-induced transcription was not affected. Data are represented as mean ± SD (n = 3) 16 h after OME addition. (* $P < 0.05$, ** $P < 0.01$, n.s.: no significant difference).

Fig. 4. Comparison of the induction of XRE-dependent transcription by OME through six mammalian AhR species. HeLa (**A**) and Hepa1c1c (**B**) cells were transfected with an empty vector (pCI-neo, CT) or six mammalian AhR expression plasmids (generated from the mouse, rat, human, rabbit, guinea pig, and hamster) together with the XRE-driven pX4TK-Luc reporter gene and pCMV-Rluc. After 24 h, the cells were exposed to 100 μM OME or 0.1% DMSO. After 16 h of incubation, the cells were lysed, and firefly luciferase and Renilla luciferase activities were measured. Data represent the mean ± SD of normalized firefly luciferase/Renilla luciferase activities of three independent experiments. OME-treated groups (closed columns) were

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compared with DMSO controls (open columns) using one- or two-way analysis of variance, and statistically significant differences are denoted by asterisks (* $P < 0.05$, ** $P < 0.01$). Total cell lysates were subjected to immunoblotting to confirm the expression level of each AhR (lower panels).

Fig. 5. Construction of chimeric AhRs and their responses to OME and TCDD. (A) Schematic diagrams of the six chimeric constructs (MR1, MR2, RM1, RM2, MRM1, and RMR1) generated from parts of cDNAs of the rabbit (white) and mouse AhRs (gray) for the first screening. HeLa cells were transfected with expression plasmids containing chimeric AhRs or the empty vector together with pX4TK-Luc and exposed to 100 μM OME, 10 nM TCDD, or 0.1% DMSO. Induction of the reporter gene by these chimeric AhRs after exposure to OME (left) and TCDD (right). Data represent means of the fold induction above the control from two independent experiments. Immunoblotting was performed to confirm the expression level of each AhR (lower panel). (**B**) The second screening to narrow the responsible domain. Four more chimeric constructs (MRM2, MRM3, RMR2, and RMR3) were generated and subjected to the same assay. Immunoblotting was performed to confirm the expression level of each AhR (lower panel).

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Fig. 6. Effects of point mutations on the response of the rabbit AhR to OME. (A) Amino acid alignment of mouse and rabbit AhR LBDs. Identical amino acid residues are indicated by asterisks. Differing amino acids are indicated in bold font. Underlined sequences indicate the overlapping sequence for connecting with the mouse and rabbit LBDs shown in Fig. 5B. **(B)** Amino acids within the rabbit AhR LBD were individually replaced with the corresponding amino acids of the mouse AhR. RMR1 is the construct as shown in Fig. 5A. Asterisks indicate the mutated AhR in which two adjacent amino acids were replaced simultaneously. HeLa cells were transfected with expression plasmids containing mutated AhRs, pX4TK-Luc, and pCMV-Rluc. After 24 h, the cells were exposed to 100 μM OME or 0.01% DMSO for 16 h. Data represent the means of normalized firefly luciferase/Renilla luciferase activities of two independent experiments. Total cell lysates were prepared 24 h after transfection and analyzed using immunoblotting to confirm the expression level of each AhR (lower panels). **(C)** The relationship between basal transcriptional activity and the OME-mediated transcriptional activity were plotted. Open circle, Wild type rabbit AhR; Closed circle, rabbit AhR mutants; Open triangle, wild type mouse AhR; solid triangle, RMR1 chimera AhR.

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Fig. 7. Effect of single, double, and triple mutations on the response of rabbit and mouse AhRs to OME. (**A**) Rabbit AhRs with combinations of the three mutations of M328I, T353A, and F367L were transfected into HeLa cells along with the reporter genes. The combinations of the three mutations are indicated in the bottom table of the graph. CT, empty expression vector; Wt, wild-type rabbit AhR. The cells were exposed to 100 μM OME or 0.01% DMSO for 16 h. Data represent the means of normalized firefly luciferase/Renilla luciferase activities of two independent experiments. (**B)** Mouse AhRs with combinations of the three mutations of I324M, A349T, and L363F were prepared, and luciferase assays were performed as described above. Wt, wild-type mouse AhR. Total cell lysates were prepared 24 h after transfection and analyzed using immunoblotting to confirm the expression level of each AhR (lower panels).

Fig. 8. Effect of single, double, and triple mutations on the response of mouse and guinea pig AhRs to OME. (A) Alignment of the LBD amino acid sequences for six mammalian AhRs. The numbers indicate the amino acid positions in the mouse AhR (top line) and guinea pig AhR (bottom line). The three candidate amino acids that are crucial for the response to OME are in bold font. **(B)** Mouse AhRs with combinations of the three mutations and the guinea pig AhR with the V368L substitution were

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transfected into HeLa cells along with the reporter genes. Wt indicates wild-type mouse AhR or wild-type guinea pig AhR. The cells were exposed to 0.01% DMSO or 100 μM OME for 16 h. Data represent the means of normalized firefly luciferase/Renilla luciferase activities of two independent experiments. Total cell lysates were prepared 24 h after transfection and analyzed using immunoblotting to confirm the expression level of each AhR (lower panel).

Fig. 9. CYP1A1 mRNA induction by OME in stable transformants of mutant AhRs. AhR-deficient mouse hepatoma c12 cells were transfected with the expression vector of the mouse AhR, rabbit AhR, or their derivative mutated AhRs. Mouse 3mut: mouse AhR with I324M, A349T, and L363F substitutions. Rabbit 3mut: rabbit AhR with M328I, T353A, and F367L substitutions. After transfection, a heterogeneous population of G418-resistant cells was maintained and exposed to 0.1% DMSO (solvent control), 10 nM TCDD or 100 μM OME. After 16 h of incubation, total RNA was isolated, and mouse CYP1A1 mRNA was measured using real-time RT-PCR as described in the Materials and Methods section. Data represent mean ± SD (n = 3). OME-treated groups were compared with DMSO controls using one-way analysis of variance, and statistically significant differences are denoted by asterisks (* $P < 0.05$, ** $P < 0.01$,

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n.s.: no significant difference).

Fig. 10. Response of mouse and rabbit AhR mutants to OME, TCDD, 3MC, and βNF. HeLa cells were transfected with reporter genes and (**A**) the mouse wild-type AhR (Mouse AhR Wt); (**B**) the mouse AhR with the triple substitutions I324M, A349T, and L363F (Mouse 3mut); (**C**) the rabbit wild-type AhR (Rabbit AhR Wt); and (**D**) the rabbit AhR with the triple substitutions M328I, T353A, and F367L (Rabbit 3mut). Open circles represent 1, 10, 25, 50, and 100 μM OME; solid diamonds represent 0.01, 0.1, 0.3, 1, 3, 10, and 20 nM TCDD; open triangles represent 1, 10, 30, 100, 200, and 1000 nM 3MC; and solid squares with dotted lines represent 1, 10, 100, 300, 1000, and 2000 nM βNF. Data represent the means of normalized firefly luciferase/Renilla luciferase activities from two independent experiments. EC_{50} values were calculated and are summarized in Table 1.

Fig. 11. Response of mouse and rabbit AhR mutants to known endogenous ligands: indirubin, FICZ, ITE, and kynurenic acid. HeLa cells were transfected with reporter genes and various AhRs: (**A**) the mouse wild-typeAhR (Mouse AhR Wt); (**B**) the mouse AhR with the triple substitutions I324M, A349T, and L363F (Mouse 3mut); (**C**) the

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wild-type rabbit AhR (Rabbit AhR Wt); (**D**) and rabbit AhR with the triple substitutions M328I, T353A and F367L (Rabbit 3mut). Open circles represent 0.1, 1, 10, 30, 100, and 1000 nM indirubin; open diamonds represent 0.1, 1, 10, 30, 100, and 1000 nM FICZ; solid triangles represent 0.1, 1, 10, 30, 100, and 1000 nM ITE; and solid squares represent 0.1, 1, 3, 10, and 30 μM kynurenic acid. Data represent the means of normalized firefly luciferase/Renilla luciferase activities from two independent experiments. EC_{50} values were calculated and are summarized in Table 1.

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Table 1. Summary of EC_{50} values of endogenous and synthetic chemical ligands

	mouse (wt)	mouse (3mt)	rabbit (wt)	rabbit (3mt)
OME	NR	17.4 μM	19.2 μM	NR
TCDD	2.05 nM	1.97 nM	1.98 nM	1.25 nM
3MC	18.2 nM	12.5 nM	47.5 nM	76.2 nM
β-NF	167 nM	188 nM	218 nM	272 nM
FICZ	3.34 nM	5.26 nM	18.8 nM	24.0 nM
Kynurenic acid	8.74 μM	9.73 μM	13.1 μM	12.8 μM
ITE	30.5 nM	38.4 nM	19.9nM	36.7 nM
Indirubin	358 nM	187 nM	131 nM	138 nM

[a]mouse (3mt): mouse AhR containing I324M, A349T, and L363F substitutions.

[b]rabbit (3mt): rabbit AhR containing M328I, T353A, and F367L substitutions.

[c]NR: no response observed.

[d]OME: Omeprazole.

[e]3-Methylcholanthrene.

[f]β-Napthoflavone.

Fig.1

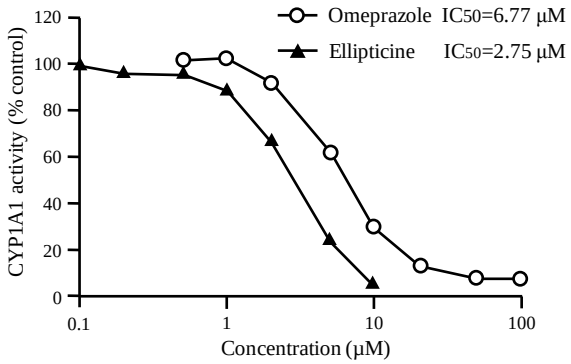

Omeprazole IC50=6.77 μM
Ellipticine IC50=2.75 μM
CYP1A1 activity (% control)
120
100
80
60
40
20
0
0.1
1
10
100
Concentration (μM)

Fig.2

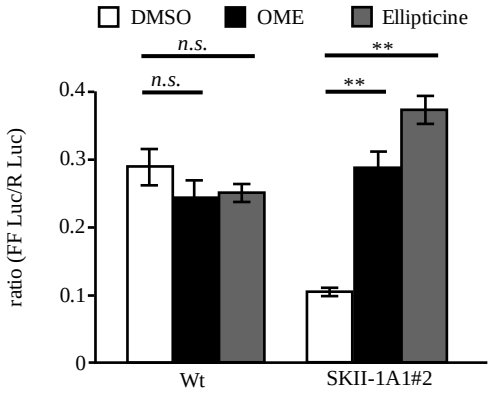

DMSO
OME
Ellipticine
n.s.
**
n.s.
**
0.4
0.3
0.2
0.1
0
ratio (FF Luc/R Luc)
Wt
SKII-1A1#2

Fig.3

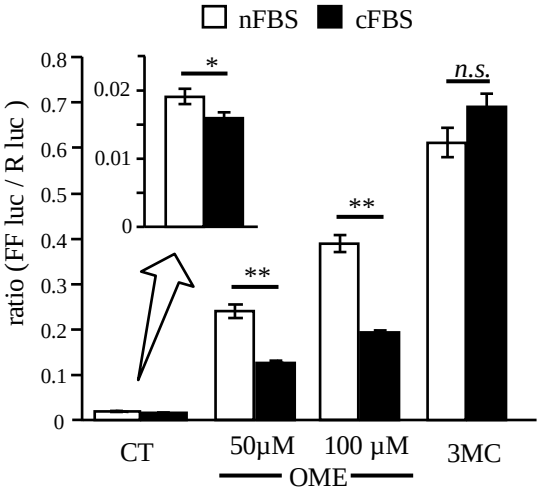

nFBS
cFBS
ratio (FF luc / R luc)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.02
0.01
0
*
**
**
n.s.
CT
50μM
100 μM
3MC
OME

Fig.4

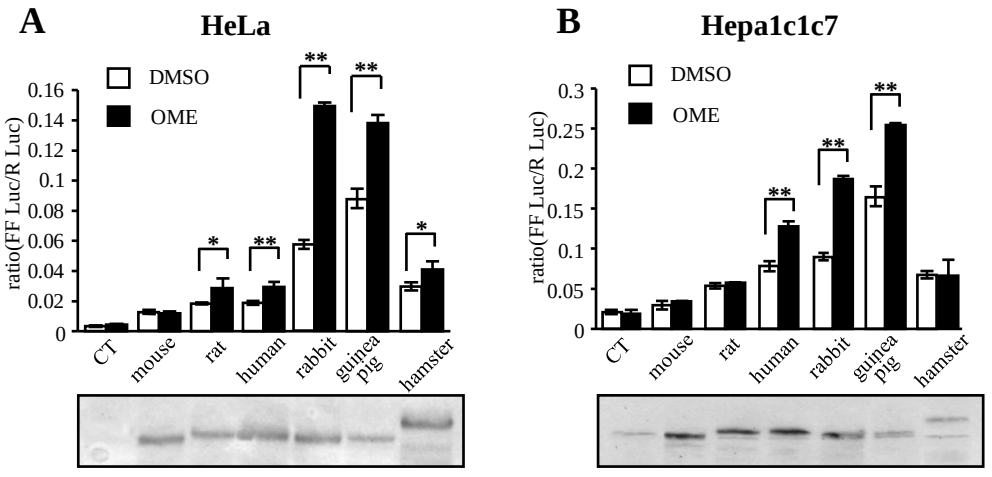

A
HeLa
DMSO
OME
ratio(FF Luc/R Luc)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
CT
mouse
rat
human
rabbit
guinea pig
hamster
B
Hepa1c1c7
DMSO
OME
ratio(FF Luc/R Luc)
0.3
0.25
0.2
0.15
0.1
0.05
0
CT
mouse
rat
human
rabbit
guinea pig
hamster

Fig.5

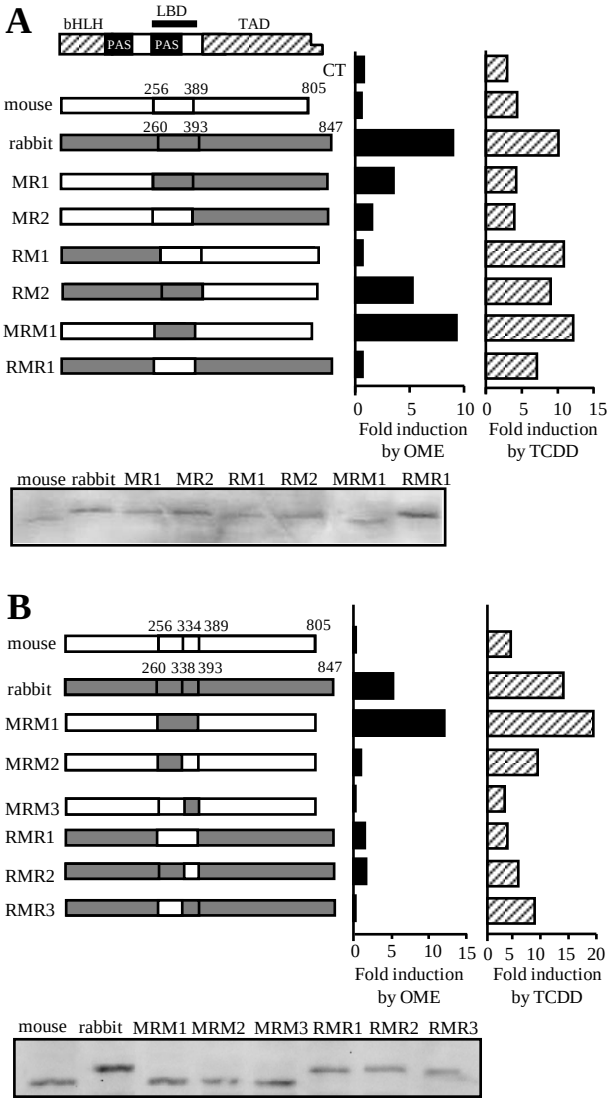

A
bHLH
LBD
TAD
PAS
PAS
CT
mouse
rabbit
MR1
MR2
RM1
RM2
MRM1
RMR1
256 389 805
260 393 847
0 5 10
Fold induction by OME
0 5 10 15
Fold induction by TCDD
mouse rabbit MR1 MR2 RM1 RM2 MRM1 RMR1
B
mouse
rabbit
MRM1
MRM2
MRM3
RMR1
RMR2
RMR3
256 334 389 805
260 338 393 847
0 5 10 15
Fold induction by OME
0 5 10 15 20
Fold induction by TCDD
mouse rabbit MRM1 MRM2 MRM3 RMR1 RMR2 RMR3

Fig.6

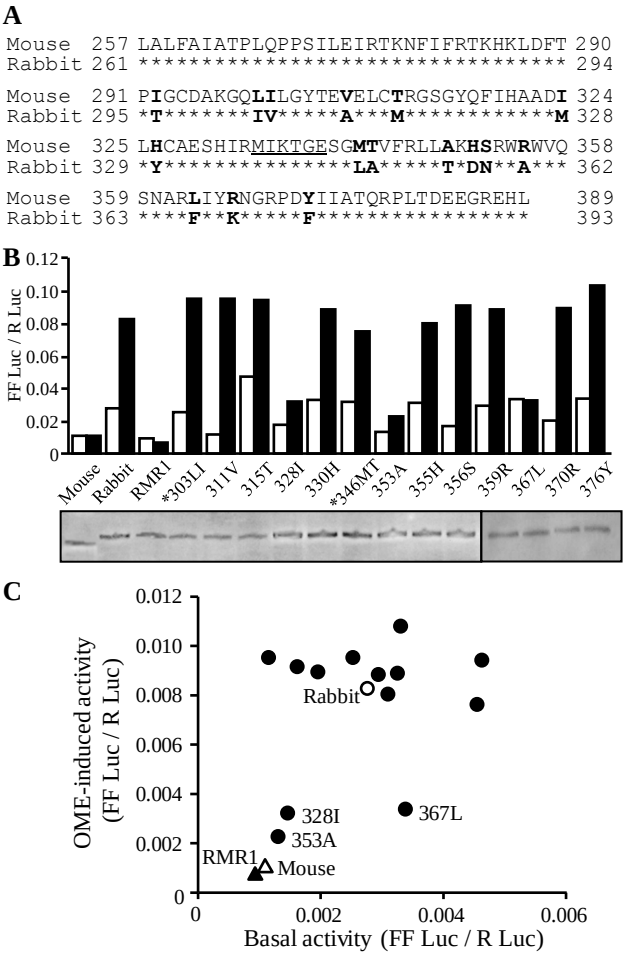

A
Mouse 257 LALFAIATPLQPPSILEIRTKNFIFRTKHKLDFT 290
Rabbit 261 ********************************** 294
Mouse 291 PIGCDAKGQLILGYTEVELCTRGSGYQFIHAADI 324
Rabbit 295 *T*******IV*****A***M**********M 328
Mouse 325 LHCAESHIRMIKTGESGMTVFRLLAKHSRWRWVQ 358
Rabbit 329 *Y**************LA*****T*DN**A*** 362
Mouse 359 SNARLIYRNGRPDYIIATQRPLTDEEGREHL 389
Rabbit 363 ****F**K*****F**************** 393
B
FF Luc / R Luc
0.12
0.10
0.08
0.06
0.04
0.02
0
Mouse
Rabbit
RMR1
*303LI
311V
315T
328I
330H
*346MT
353A
355H
356S
359R
367L
370R
376Y
C
OME-induced activity (FF Luc / R Luc)
0.012
0.010
0.008
0.006
0.004
0.002
0
Rabbit
328I
367L
353A
RMR1
Mouse
0
0.002
0.004
0.006
Basal activity (FF Luc / R Luc)

Fig.7

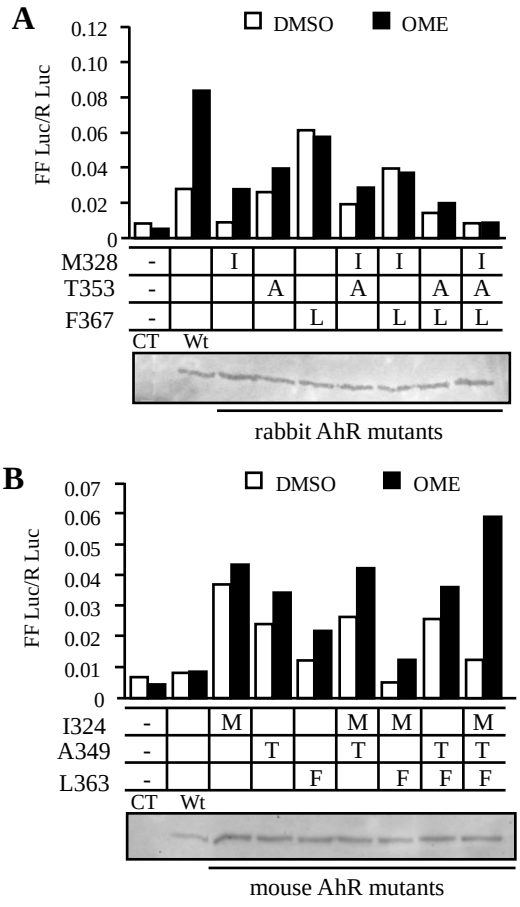

A
DMSO
OME
FF Luc/R Luc
0.12
0.10
0.08
0.06
0.04
0.02
0
M328
T353
F367
CT
Wt
rabbit AhR mutants
B
DMSO
OME
FF Luc/R Luc
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
I324
A349
L363
CT
Wt
mouse AhR mutants

Fig.8

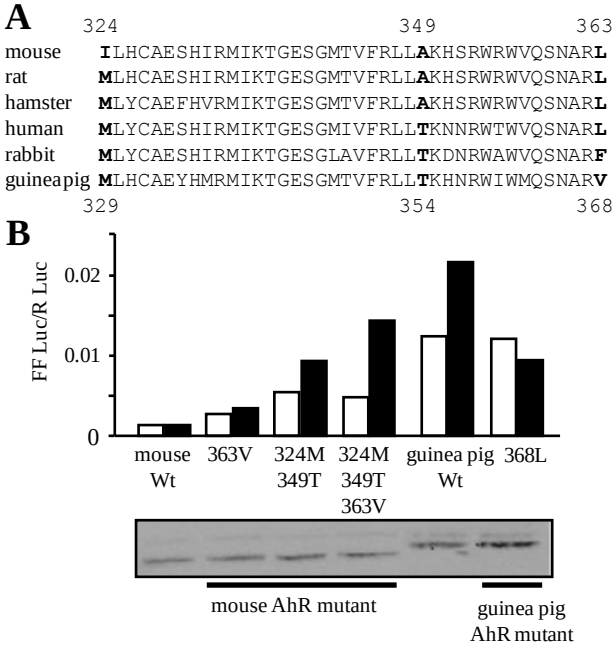

A
324
349
363
mouse ILHCAESHIRMIKTGESGMTVFRLLAKHSRWRWVQSNARL
rat MLHCAESHIRMIKTGESGMTVFRLLAKHSRWRWVQSNARL
hamster MLYCAEFHVRMIKTGESGMTVFRLLAKHSRWRWVQSNARL
human MLYCAESHIRMIKTGESGMIVFRLLTKNNRWTWVQSNARL
rabbit MLYCAESHIRMIKTGESGLAVFRLLTKDNRWAWVQSNARF
guineapig MLHCAEYHMRMIKTGESGMTVFRLLTKHNRWIWMQSNARV
329
354
368
B
FF Luc/R Luc
0.02
0.01
0
mouse Wt
363V
324M 349T
324M 349T 363V
guinea pig Wt
368L
mouse AhR mutant
guinea pig AhR mutant

Fig.9

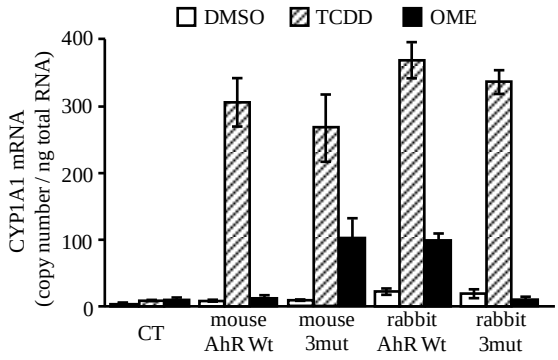

DMSO
TCDD
OME
CYP1A1 mRNA
(copy number / ng total RNA)
400
300
200
100
0
CT
mouse
AhR Wt
mouse
3mut
rabbit
AhR Wt
rabbit
3mut

Fig.10

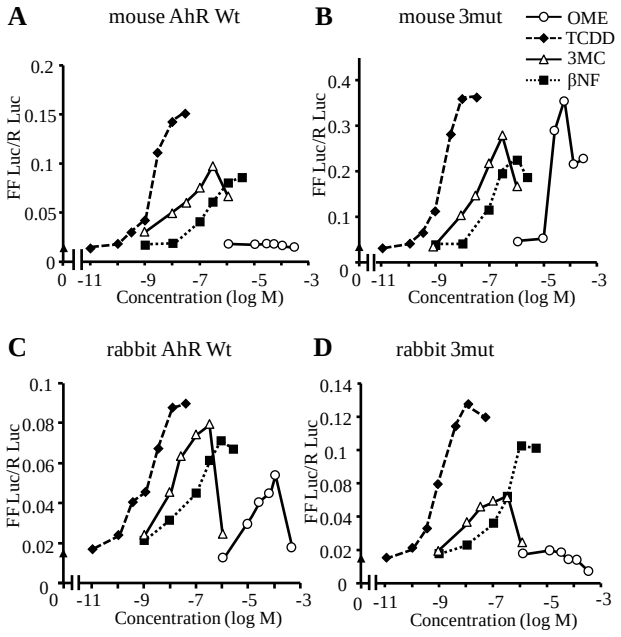
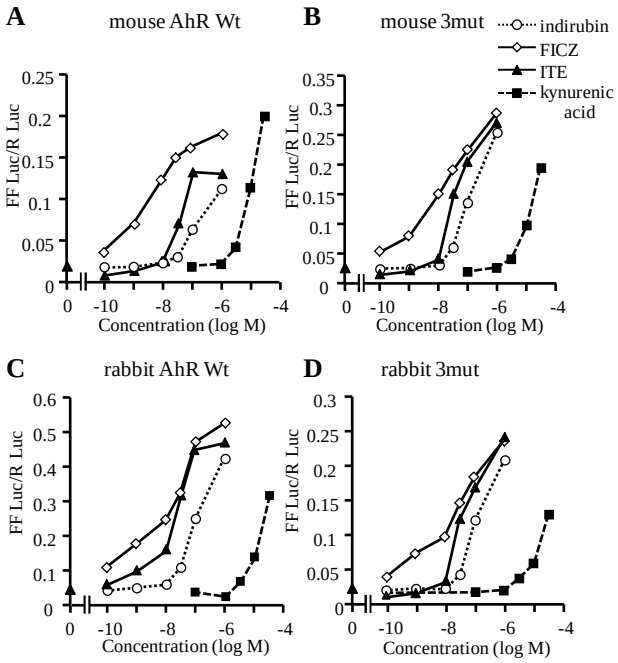

A
mouse AhR Wt
B
mouse 3mut
OME
TCDD
3MC
βNF
FF Luc/R Luc
Concentration (log M)
C
rabbit AhR Wt
D
rabbit 3mut

Fig.11


A
mouse AhR Wt
B
mouse 3mut
indirubin
FICZ
ITE
kynurenic acid
FF Luc/R Luc
Concentration (log M)
C
rabbit AhR Wt
D
rabbit 3mut

Supplementary Information

Molecular Pharmacology

Identification of amino acid residues in the ligand-binding domain of the aryl hydrocarbon receptor causing the species-specific response to omeprazole: possible determinants for binding putative endogenous ligands

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Division of Environmental Health Sciences, Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan (S. O.)

Department of Life Science, Dongguk University, Seoul, Korea (T. Y.)

Department of Biology, Graduate School of Science, Osaka Prefecture University, Osaka, Japan (M. K., T. Y.)

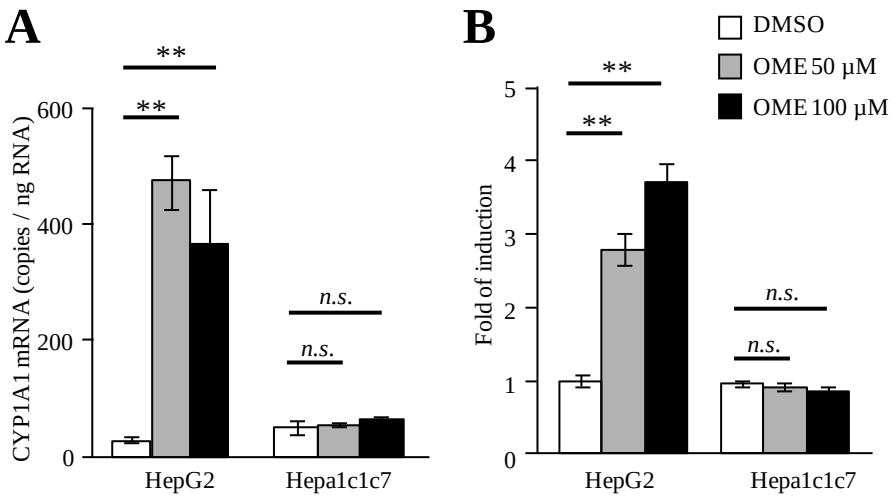

Figure S1. OME induced CYP1A1 and XRE-driven reporter genes in hepatoma cells of humans but not of mice. Differences in the response to omeprazole (OME) between human and mouse cell lines. A) Effects of OME on CYP1A1 mRNA expression in HepG2 cells and Hepa1c1c7 cells. The cells were plated on 6-well tissue culture plates and exposed to DMSO (open column: solvent control) or OME (gray column: 50 µM, black column: 100 µM). After 8 h of exposure, total RNA was isolated and human and mouse CYP1A1 mRNA and detected using real-time RT-PCR. Data represent CYP1A1 mRNA copy number in 1 ng total RNA (mean ± SD, n = 3). B) Effects of omeprazole on XRE-dependent transcriptional activation in HepG2 and Hepa1c1c7 cells. The cells were transfected with pCI-neo-hAhR, 4×XRE-Luc reporter gene, and pCMV-Rluc (internal control). Twenty-four hours after transfection, 50 or 100 µM of OME was added to the medium. Data represent mean ± SD (n = 3) 16 h after OME addition. (* $P < 0.05$, ** $P < 0.01$, n.s.: no significant difference).

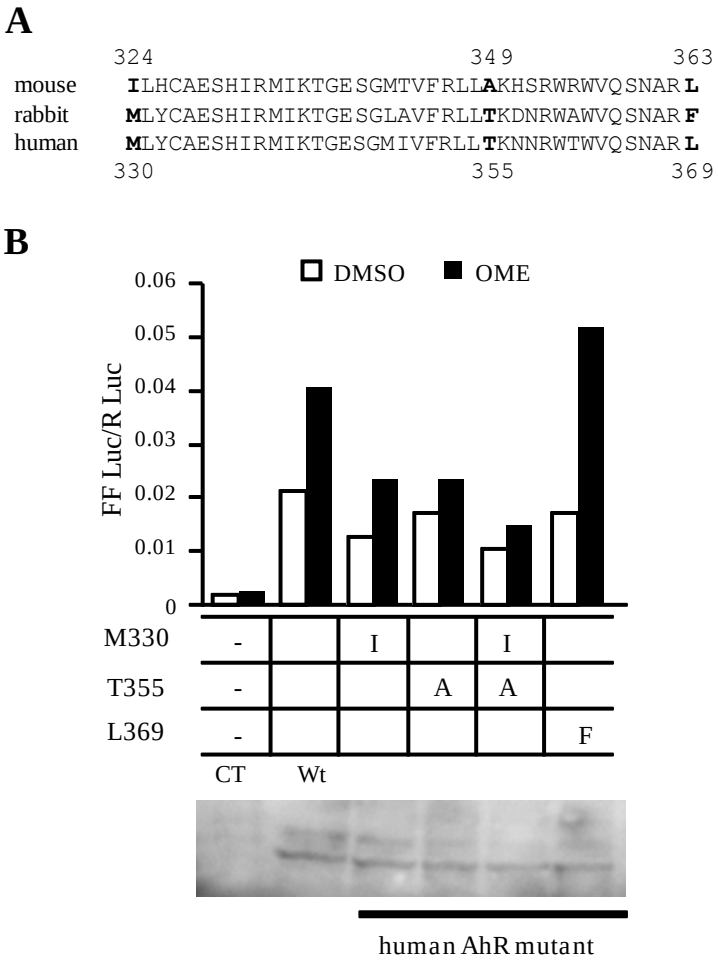

Figure S2. Effects of mutations on the response of human AhR to OME. A) Alignment of the LBD amino acid sequences for mouse, rabbit and human AhRs. The numbers show the amino acid positions in the mouse AhR (top line) and human AhR (bottom line). The three candidate amino acids that are crucial for the response to OME are in bold font. **B**) Human AhRs with combinations of the three mutations were transfected into HeLa cells along with the reporter genes. Wt indicates wild-type human AhR. The cells were exposed to 0.01% DMSO or 100 μM OME for 16 h. Data represent the means of normalized firefly luciferase/Renilla luciferase activities of two independent experiments. Total cell lysates were prepared 24 h after transfection and analyzed by immunoblotting to confirm the expression level of each AhR (lower panel).

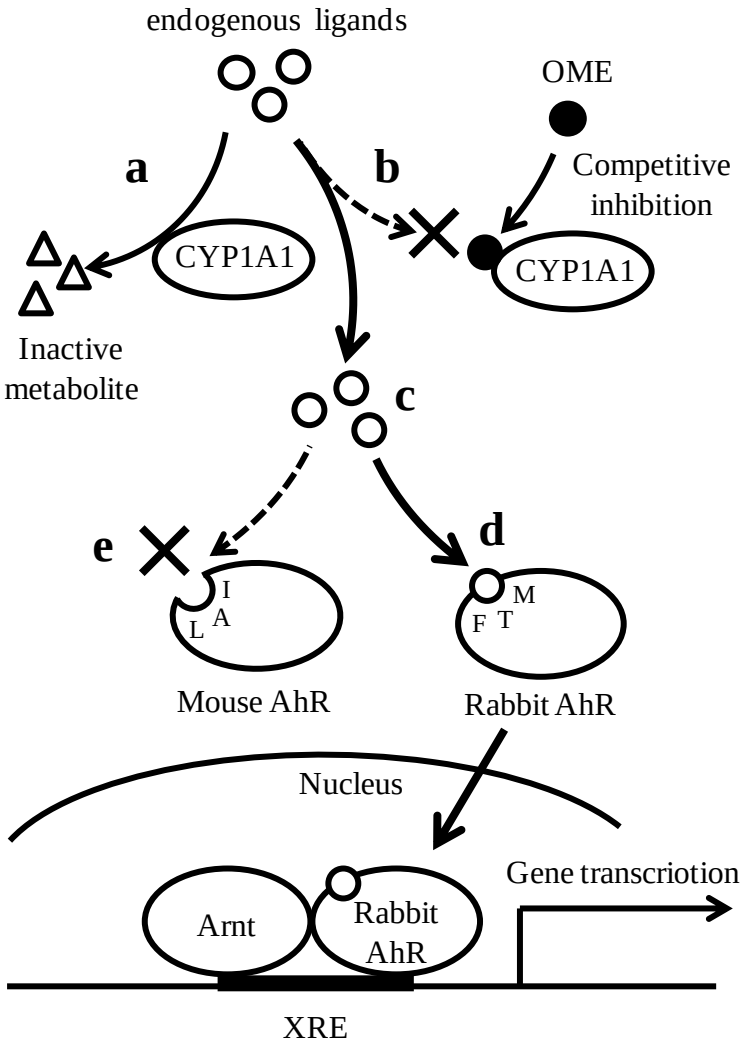

Figure S3. Schema of indirect activation of AhR by OME. a) In the normal state, CYP1A1 transforms endogenous ligands (indicated as open circle) to inactivated metabolites (indicated as triangle). **b)** When OME exists, it inhibits CYP1A1 which inactivates endogenous ligand competitively. **c)** Consequently, the endogenous ligand remains in culture medium. **d)** The accumulated endogenous ligand in cells binds to AhR and activates it. **e)** Binding of endogenous ligand is weak against mouse AhR but strong against rabbit AhR, and three amino acid residues in the LBD of AhR determine the endogenous ligand-binding affinity.

Supplemental Table S1. Primers used in this experiment

	Forward primer	Reverse primer
Primers for cloning of AhR		
Human AhR	TTCTCGAGATGAACAGCAGCAGCGCCAACATCAC	GGACGCGTTACAGGAATCCACTGGATGTCAAAT
Mouse AhR	AACTCGAGATGAGCAGCGGCGCCAACATCACCTA	GGACGCGTTCAACTCTGCACCTTGCTTAGGAATG
Rat AhR	AACTCGAGATGAGCAGCGGCGCCAACATCACCTA	CCACGCGTTACAGGAATCCGCTGGGTGTGATATC
Rabbit AhR	AACTCGAGATGAACGGCGGCGGCGCCAA	AAACGCGTTCACAGCAGTCGGCCGGACG
Guinea pig AhR	AACTCGAGATGAACAGCGGCGGCGCCAACATCAC	CGACGCGTTTACAGGAATCCACTGGGTGTCAAGT
Hamster AhR	AACTCGAGATGAGCAGCGGCGCCAACATCACCTA	AAACGCGTCTACAGGAATCCGCTGGGTGTGATAT
Primers for chimeric AhR construction		
mrAhR780–812	CTGGCTTTGTTTGCAATAGCTACTCC	GGAGTAGCTATTGCAAACAAAGCCAG
mrAhR1000–1024	ATGATTAAGACTGGAGAAAGTGGC	GCCACTTTCTCCAGTCTTAATCAT
mrAhR1156–1180	GATGAAGAAGGAAGAGAGCATTTAC	GTAAATGCTCTCTTCCTTCTTCATC
Primers for site-directed mutagenesis		
rAhR295I	CTAGACTTCACACCTATTGGCTGTGATGC	GCATCACAGCCAATAGGTGTGAAGTCTAG
rAhR303LI	GATGCTAAAGGACAGCTCATCTTGGGCTAC	GTAGCCCAAGATGAGCTGTCCTTTAGCATC
rAhR311V	GGGCTACACAGAGGTAGAGCTGTGCATGAG	CTCATGCACAGCTCTACCTCTGTGTAGCCC
rAhR315T	GCAGAGCTGTGCACGAGAGGGTCGGG	CCCGACCCTCTCGTGCACAGCTCTGC
rAhR328I	CATGCTGCTGATATCCTGTACTGTGCCGAG	CTCGGCACAGTACAGGATATCAGCAGCATG
rAhR330H	CTGCTGATATGCTGCACTGTGCCGAGTCC	GGACTCGGCACAGTGCAGCATATCAGCAG
rAhR346MT	GAGAAAGTGGCATGACAGTTTTCAGGCTTC	GAAGCCTGAAAACTGTCATGCCACTTTCTC
rAhR353A	CAGTTTTCAGGCTTCTTGCAAAAGATAATC	GATTATCTTTTGCAAGAAGCCTGAAAACTG
rAhR355H	GCTTCTTACAAAACATAATCGATGGGCTTG	CAAGCCCATCGATTATGTTTTGTAAGAAGC
rAhR356S	CTTCTTACAAAAGATAGTCGATGGGCTTGG	CCAAGCCCATCGACTATCTTTTGTAAGAAG
rAhR359R	AAAAGATAATCGATGGCGTTGGGTCCAGTC	ATTAGACTGGACCCAACGCCATCGATTATC
rAhR367L	GTCTAATGCACGCTTGATTTATAAGAATGG	CCATTCTTATAAATCAAGCGTGCATTAGAC
rAhR370R	TGCACGCTTTATTTATAGGAATGGAAGACC	CTGGTCTTCCATTCCTATAAATAAAGCGTG
rAhR376Y	GGAAGACCAGATTATATCATTGCAACTC	GAGTTGCAATGATATAATCTGGTCTTCC
mAhR324M	GCTGCAGACATGCTTCACTGTGCAGAATCC	GGATTCTGCACAGTGAAGCATGTCTGCAGC
mAhR349T	GTTTTCCGGCTTCTTACAAAACACAGTC	GACTGTGTTTTGTAAGAAGCCGGAAAAC
mAhR363F	CAATGCACGCTTTATTTACAGAAATG	CATTTCTGTAAATAAAGCGTGCATTG
mAhR363V	GTCCAATGCACGCGTGATTTACAGAAATGG	CCATTTCTGTAAATCACGCGTGCATTGGAC
gpAhR368L	GTCTAATGCACGTTTAGTTTATAAAAATGG	CCATTTTTATAAACTAAACGTGCATTAGAC
hAhR330I	CATGCAGCTGATATCCTTTATTGTGCCGAG	CTCGGCACAATAAAGGATATCAGCTGCATG
hAhR355A	GTTTTCCGGCTTCTTGCAAAAAACAACCG	CGGTTGTTTTTTGCAAGAAGCCGGAAAAC
hAhR369F	GTCTAATGCACGCTTTCTTTATAAAAATGG	CCATTTTTATAAAGAAAGCGTGCATTAGAC
Primer for real-time RT-PCR of mouse CYP1A1		
mCYP1A1RTm	TAAAACACGCCCGCTGTGAA	AAGTAGGAGGCAGGCACAATGTC