Microbial Metabolites of Omeprazole Activate Murine Aryl Hydrocarbon Receptor In Vitro and In Vivo

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Received May 8, 2014; accepted July 24, 2014

ABSTRACT

Omeprazole (OME), a proton pump inhibitor used to treat gastritis, is also an aryl hydrocarbon receptor (AhR) activator. OME activates AhR in human hepatocytes and hepatoma cells, but not in mice in vivo or in vitro. We recently discovered that this species-specific difference results from a difference in a few amino acids in the ligand-binding domain of AhR. However, OME activates both mouse and human AhRs in the yeast reporter assay system. Nevertheless, the cause of this discrepancy in OME responses remains unknown. Here, we report that CYP1A1 mRNA expression in mouse cecum was elevated after OME administration, although the mouse is regarded as an OME-unresponsive animal. Using the yeast reporter assay system with human and murine AhRs, we found AhR agonist-like activity in the cecal extracts of OME-treated mice. We speculated that OME metabolites produced by cecal bacteria might activate murine AhRs in vivo. In high-performance liquid chromatography (HPLC) analysis, AhR agonist-like activity of cecal bacterial culture and cecal extracts were detected at the same retention time. AhR agonist-like activity was also detected in the HPLC fractions of yeast culture media containing OME. This unknown substance could induce reporter gene expression via mouse and human AhRs. The agonist-like activity of the OME metabolite was reduced by concomitant α-naphthoflavone exposure. These results indicate that a yeast-generated OME metabolite elicited the response of mouse AhR to OME in the yeast system, and that bacterial OME metabolites may act as AhR ligands in human and mouse intestines.

Introduction

Omeprazole (OME), a potent proton pump inhibitor, has been used to treat gastroesophageal reflux disease and duodenal ulcers (Lind et al., 1983). OME can induce CYP1A1 and CYP1A2 in human hepatocytes and hepatoma cells (Diaz et al., 1990; Curi-Pedrosa et al., 1994; Krusekopf et al., 1997; Yoshinari et al., 2008). These drug-metabolizing enzymes are induced by polycyclic aromatic hydrocarbons via the aryl hydrocarbon receptor (AhR) (Burbach et al., 1992). AhR is a ligand-dependent transcriptional factor that is present in the cytoplasm in the absence of ligands. Typical AhR ligands include 2,3,7,8-tetrachlorodibenzo-p-dioxin, 3-methylcholanthrene (3-MC), and β-naphthoflavone (BNF). After AhR binds to these ligands, it is translocated into the nucleus where it forms a heterodimer with the AhR nuclear translocator (Arnt) (Reyes et al., 1992). The AhR/Arnt heterodimer then binds to a xenobiotic response element (XRE), a specific DNA sequence that is located in the 5' flanking region of AhR target genes such as CYP1A1 (Denison et al., 1989).

Interestingly, a competitive ligand-binding assay revealed that OME induces CYP1A1 without binding to AhR (Daujat et al., 1992). Nevertheless, similar to the typical AhR ligands, OME induces AhR/Arnt translocation into the nucleus, which then binds to the XRE sequence required for CYP1A1 induction (Quattrochi and Tukey, 1993; Yoshinari et al., 2008). Several studies reported that OME-mediated AhR activation occurred via a protein tyrosine kinase pathway in both rat and human hepatoma cells (Backlund et al., 1997; Kikuchi et al., 1998; Lemaire et al., 2004; Backlund and Ingelman-Sundberg, 2005). Thus, the molecular mechanisms of OME-mediated AhR activation are not completely understood.

OME-mediated AhR activation is species-specific. OME-mediated CYP1A1 induction was identified in human primary hepatocytes and hepatoma cells, but not in mouse cells (Kikuchi et al., 1995). Meanwhile, in the yeast reconstituted AhR assay system, OME activated both mouse and human AhRs (Dzeletovic et al., 1997). Therefore, the response to OME seems to be independent of the animal species from which AhR originates. In addition, a putative species-specific factor that influences the OME response has been postulated.
In contrast to these observations, we recently found that OME-mediated transcriptional activation of the XRE-driven reporter gene was dependent on the species from which AhR originated but independent of the host cell species where AhR was expressed (Shizaki et al., 2014). Specifically, human AhR introduced into murine hepatoma cells was activated by OME, whereas mouse AhR showed no response to OME, although it was expressed in human cells. Thus, with regard to the response of OME to mouse AhR, the experimental results obtained in yeast and mammalian cells appear inconsistent.

A recent report indicated that AhR-deficient (AhR−/−) mice develop spontaneous cecal tumors, and that a natural AhR ligand suppresses tumorigenesis in ApoE−/− mice, a mouse model of familial adenomatous polyposis (Kawajiri et al., 2009). To identify the AhR-activating chemical that prevents colorectal tumorigenesis, we administered various AhR ligands to C57BL/6 mice. We also administered OME because it has been reported to have inhibitory effects on colorectal carcinogenesis (Penman ID et al., 1993). During the course of the experiment, we found that several animals in the OME-treated group expressed high CYP1A1 mRNA levels in their ceca. This was an unexpected finding because the C57BL/6 mouse has been regarded as an OME-unresponsive animal. Moreover, CYP1A1 induction varies considerably among animals. We speculated that CYP1A1 induction was due to differences in the animals' intestinal environments, such as the presence of enterobacterial flora. In the present study, we examine whether OME metabolites produced by enterobacteria and yeast can activate mouse AhR.

**Materials and Methods**

**Chemicals.** Omeprazole (5-methoxy-2-[(4-methoxy-3,5-dimethyl-2-pyridyl) methyl]sulfanyl]-1H-benzimidazole) and 3-MC were purchased from Sigma-Aldrich (St. Louis, MO). BNF and α-naphthoflavone (ANF) were purchased from Wako Pure Chemical (Osaka, Japan). All substances, including high-performance liquid chromatography (HPLC) fraction samples, were dissolved in dimethylsulfoxide (DMSO) and added to the culture media. The DMSO concentration was adjusted to 0.1% (v/v) in the media.

**Plasmids.** Plasmids used in this study were constructed and described in our previous report (Shizaki et al., 2014). We used the reporter plasmid pX4TK-Luc that carries the firefly luciferase gene under the control of four copies of XRE. Transfection was performed using the liposome method.

**Cell Culture and Transfection.** The human epithelial carcinoma cell line HeLa (HeLa S3) and mouse hepatoma cell line Hepa-1c1c were obtained from Dr. S. Ohsako (The University of Tokyo, Tokyo, Japan). The human epithelial carcinoma cell line HeLa (HeLa S3) and mouse hepatoma cell line Hepa-1c1c were obtained from the American Type Culture Collection (Rockville, MD). HeLa cells were grown in Eagle's minimum essential medium (MEM) containing 10% charcoal-stripped fetal bovine serum and 1× Antibiotic-Antimycotic (Invitrogen, Carlsbad, CA), and were incubated at 37°C in 5% CO₂ atmosphere. Hepa-1c1c cells were grown in similar culture conditions except Dulbecco’s minimum essential medium was used instead of MEM. Transfection was performed by the liposome method. In brief, 1 μg of plasmid DNA and 4 μl of PLUS-Reagent (Invitrogen) were combined in 200 μl of OPTI-MEM (Invitrogen). After incubation for 15 minutes, 2 μl Lipofectamine reagent (Invitrogen) diluted with 200 μl of OPTI-MEM was added. The cells were plated in well-tissue culture plates at 30–40% confluence 1 day before transfection. Liposomes were added to the cells in serum-free medium for 3 hours, and the medium was then replaced with MEM containing 10% charcoal-stripped fetal bovine serum without 1× Antibiotic-Antimycotic.

**Luciferase Assay for AhR Ligand Activity in Human and Mouse Cells.** The effects of OME and typical AhR agonists on AhR-mediated transcriptional activity were quantified by cotransfecting the AhR expression plasmid (pCI-neo-AhR or pCI-neo-mAhR), XRE-driven reporter plasmid pX4TK-Luc, and plasmid pRL-CMV (Promega, Madison WI, USA) encodes a Renilla luciferase gene (Rluc) driven by a CMV promoter into cells. The transfected cells were washed with cold phosphate-buffered saline (PBS) and lysed in 25 μl of 1× passive lysis buffer (Promega). Aliquots (10 μl) of the lysates were transferred to 96-well plates, and Luc+ and Rluc luminescence was measured using the Dual-Luciferase Reporter Assay System (Promega) with the Wallac ARVO SX multilabel counter (Perkin Elmer, Norwalk, CT). Because transfection efficiencies varied among the experiments, the transcriptional activity was normalized by Luc/Rluc intensity ratios.

**Animal Exposure to OME.** C57BL/6J mice were obtained from CLEA Japan (Tokyo, Japan) and maintained under specific pathogen-free conditions. Animal experiments were approved by the Saitama Cancer Center Animal Care and Use Committee. Two-month-old male C57BL/6J mice were used in the present experiments. The mice were given OME (75 mg/kg per 10 ml) or 3-MC (5 mg/kg per 10 ml) in an oil-water emulsion by gavage. The doses of these chemicals were determined from previous reports (Watson and Smith, 2001; Kawajiri et al., 2009). To prepare the emulsion, the chemicals were suspended in EtOH by sonication, with the addition of olive oil and saline containing Tween 80, and mixed using a double syringe and a three-way stopcock.

The final composition of the oil-water emulsion included 2% EtOH, 23% olive oil, and 0.5% Tween 80 in saline. The control-group mice received an emulsion without chemicals. Twenty-four hours after a single oral administration of the chemicals, the cecum was removed and its contents were collected. The cecum was washed with ice-cold PBS and subjected to RNA isolation.

**Measurement of CYP1A1 mRNA in Mouse Cecum by Real-Time Reverse-Transcription Polymerase Chain Reaction.** Total RNA was isolated from cecal tissue using Isogen (Nippon Gene, Tokyo, Japan). 24 hours after administration of the chemicals. An aliquot (2 μg) of total RNA was subjected to reverse transcription using Superscript III (Invitrogen) and oligo-dT primers. Real-time reverse-transcription polymerase chain reaction (PCR) was carried out according to the methods described in our previous report (Shizaki et al., 2008). In brief, an aliquot of cDNA (1 μl of a 2.5 μl total reaction volume) or calibrator plasmid DNA (pCl-neo-mouse CYP1A1) was amplified with the master mixture (SYBR Premix Ex Taq; Takara Bio, Kyoto, Japan) containing gene-specific primers. The primer pair for mouse CYP1A1 cDNA amplification was as follows: forward primer, 5′-TTAAAAACAGCCCGGCTGTGAA-3′; reverse primer, 5′-AAGTAGG-GACGGCCACAAGTC-3′. To verify cDNA synthesis and amplification, glyceraldehyde-3-phosphate dehydrogenase mRNA was amplified using the following primers: forward primer, 5′-AATGTTGAAAGGTCGGTGTTG-3′; reverse primer, 5′-GAGATGTTGATGGCCCTCC-3′. To confirm amplification, the PCR products were subjected to agarose gel electrophoresis. A calibration curve was generated using the 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.

**Yeast Reporter Assay for AhR Ligand Activity, OPUH (Osaka Prefecture University human AhR-assay yeast) and OPUH (Osaka Prefecture University mouse AhR-assay yeast) are the reporter assay yeast strains that express human and mouse AhR/Arnt, respectively, controlled by XRE, which were established in our previous study (Kawanishi et al., 2003). The assay in the present study was performed according to the procedures described in the previous report. In brief, the yeast strains were grown overnight at 30°C in the synthetic defined (SD) medium lacking tryptophan, uracil, and leucine. Five microliters of the overnight culture yeast was added to 100 μl of synthetic medium containing 1% galactose and 1% glucose in each well of the 96-well microtiter plates. One microliter of the sample dissolved in DMSO was added to each well, and the microliter plates were incubated for 16 hours at 30°C. To calculate the agonist potencies of AhR ligands, various concentrations of BNF (3 × 10⁻⁷ to 10⁻⁶ M) were measured simultaneously as a positive control. After incubation, each yeast suspension (10 μl) was added to 100 μl of Z-buffer (60 mM NaHPO₄, 40 mM Na₂HPO₄, 1 mM MgCl₂, 10 mM KCl, 2 mM dithiothreitol, and 0.2% sarcosyl, adjusted to pH 7) that contained 1 mg/ml o-nitrophenol-β-D-galactopyranoside, and was incubated for 60 minutes at 37°C. The absorbance at 405 and 595 nm was measured to determine the amount of o-nitrophenol generated and yeast cell density, respectively. Increases in induction were calculated using the following formula: (Absorbance [Abs]₄₀₅/Abs₅₉₅ in compound exposure − (Abs₄₀₅/Abs₅₉₅ in compound-free control)) × 100%. A calibration curve was plotted using the values obtained from BNF exposure (10 nM to 1 μM), and the agonist-like activity of the samples against AhR was calculated as BNF equivalent quantity (BNF-Eq.).

**Aneurobic Culture of Cecal Bacteria and Aerobic Liquid Yeast Culture for the Extraction of OME Metabolites.** The cecal contents from three C57BL/6J mice were suspended in 10 ml of PBS and filtered through a 40-μm cell strainer. An aliquot of filtrate was inoculated in 60 ml of Gifu anaerobic Microbial Metabolites of Omeprazole Activate Murine AhR 1691
medium broth (GAM; Nissui Seiyaku Co., Ltd., Tokyo, Japan) containing 10 μM OME, and was dispensed into five six-well culture plates. The plates were incubated anaerobically overnight in Anaero Pack (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) at 37°C. To obtain OME metabolites by yeast, Saccharomyces cerevisiae strain W303a was cultured at 30°C for 8 hours in aerobic conditions in 100 ml of Yeast Extract-Peptone-Dextrose (YPD) medium or SD medium containing 10 μM OME.

**Extraction of OME Metabolites from Cecal Contents and Bacteria and Yeast Culture Media.** The cecal contents were suspended in 10 ml of PBS and extracted by sonication for 10 minutes at 4°C. The suspension was centrifuged at 8000g for 10 minutes at 4°C, and the supernatant was filtered with a 0.45-μm filter (Nihon Millipore Ltd., Yonezawa, Japan). The culture media for the cecal bacterial mixture and yeast were treated using the same procedures described for cecal extraction. The filtered fluids were passed through Waters Sep-Pak Plus C18 Environmental cartridges (Waters, Milford, MA) at a flow rate of 10 ml/min (Kawanishi et al., 2004). Each cartridge was washed with 10 ml of pure water three times, and the bound substances were eluted from the cartridges with 2 ml of acetonitrile. The eluent was then dried in a vacuum concentrator, and the resultant residue was dissolved in 25 μl of DMSO. These extract samples were applied to yeast reporter assay and HPLC analysis.

**HPLC Analysis of OME Metabolites.** Analyses of OME metabolites in cecal contents and yeast and cecal culture media were performed using HPLC (Agilent 1100 series HPLC system equipped with an Agilent G1315B photodiode array detector; Agilent Technologies, Waldbronn, Germany). The extract containing OME metabolites was eluted at a flow rate of 1 ml/min for 60 minutes using the TSKgel ODS-100V 3-μm column (TSK, Tokyo, Japan) with a linear gradient from 50 to 90% acetonitrile. The eluent was detected with UV-visible detection at 280 nm. Fractions were collected every minute (1 ml) for 60 minutes and were dried and dissolved in 10 μl of DMSO. AHR ligand activity in each fraction was determined by yeast reporter assays using OPUM and OPUH yeasts. Data were calculated by a standard curve obtained from the BNF-exposed yeasts and were indicated as BNF-Eq (final concentration in assay medium: 1/100 dilution of DMSO dissolved in each fraction).

**Electrophoretic Mobility Shift Assay.** Electrophoretic mobility shift assay (EMSA) of the AhR-Arnt complex was carried out based on the method of a previous report (Bank et al., 1992). Hepa-1c1c cells were harvested and homogenized in HEDG buffer (HEPES/EDTA/DTT/glycerol buffer) containing 1 mM dithiothreitol, 10% glycerol, 96 mM KCl, and 50 mM KCl, and protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany), and were centrifuged at 20,000 × g for 20 minutes at 4°C. Twenty-microgram aliquots of cytosol protein were incubated with DMSO, 300 nM 3-MC, 25 μM OME, or 1% of HPLC fraction 32 for 2 hours at room temperature. Then the reaction was mixed with 2.5 pmol of biotin-labeled XRE-oligonucleotide in the binding buffer and incubated for 15 minutes at room temperature. Final compositions in the binding buffer were 25 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany), and were centrifuged at 20,000 × g for 20 minutes at 4°C. Twenty-microgram aliquots of cytosol protein were incubated with DMSO, 300 nM 3-MC, 25 μM OME, or 1% of HPLC fraction 32 for 2 hours at room temperature. Temperature. Then the reaction was mixed with 2.5 pmol of biotin-labeled XRE-oligonucleotide in the binding buffer and incubated for 15 minutes at room temperature. Final compositions in the binding buffer were 25 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 96 mM KCl, and 50 μg/ml poly[dI-dC] (poly[dI-dC] (poly[dI-dC] (poly[dI-dC] (poly[dI-dC] deoxynucleotidylic acid); Sigma-Aldrich). The oligonucleotide sequences containing XRE were as follows: 5′-biotin-ACATCTCTCATCGAGTCCTTCTTCTGCTGTCGTAACACTGATGCTGAGTCCTTATCCGAGTGG-3′ and 5′-biotin-CCACCTCGGAAATGATGCTGAGTCCTTATCCGAGTGG-3′. The oligonucleotide sequences containing mutated XRE were as follows: 5′-biotin-ACATCTCTCATCGAGTCCTTCTTCTGCTGTAACACTGATGCTGAGTCCTTATCCGAGTGG-3′ and 5′-biotin-CCACCTCGGAAATGATGCTGAGTCCTTATCCGAGTGG-3′. The AhR/Arnt/DNA complexes were separated on 6% PAGE. After electrophoresis, gels were denatured in alkaline solution (0.5 M NaOH, 1.5 M NaCl), neutralized in Tris-NAc buffer (Tris-HCl (pH 7.5), 1.5 M NaCl), and electrobotted to positively charged nylon membrane (Bio-Rad, Richmond, CA). Signals were detected using High Sensitive Streptavidin–horseradish peroxidase (Thermo Fisher Scientific, Waltham, MA) and ECL Plus Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK).

**Statistical Analysis.** The F test was used to compare variations, and Student’s t test was used to evaluate significant differences between two groups.

**Results**

**Species-Specific Response of AhR to OME in Mammalian and Yeast Cells.** To measure ligand-induced AhR activation, XRE-driven reporter gene assays were performed in mammalian and yeast cells that expressed human or mouse AhR. OME and 3-MC (positive control) showed ligand activity for human AhR in both human and yeast cell reporter assays (Fig. 1, A and C). Although OME showed ligand activity for mouse AhR expressed in yeast (Fig. 1D), OME did not show any activity when mouse AhR was expressed in HeLa cells (Fig. 1B). These data agree with the results of previous studies (Dzeletovic et al., 1997; Shii zaki et al., 2014) and suggest that OME-induced mouse AhR activation depends on the cells in which AhR is expressed.

**CYP1A1 Induction by OME Administration in Mouse Cecum.** As shown in Fig. 2, CYP1A1 mRNA, one of the most responsive AhR target genes, was significantly induced in the ceca of 3-MC–treated mice. CYP1A1 mRNA in OME-treated mice also increased, but it was highly diverse (F = 12.1 > F0.05 = 5.05), and the difference in the level was not statistically significant (P = 0.146, Student’s t test, unequal variances). These results indicate that OME administration induced CYP1A1 expression in the cecum of the mouse, which is regarded as an OME-unresponsive animal. However, unlike 3-MC administration, these effects seemed highly diverse among OME-treated animals.

**AhR Agonist–Like Activity in Mouse Cecal Contents after OME Administration.** We speculated that one possible cause for the differences in the induction levels of cecum CYP1A1 among the OME-treated mice is the commitment of enterobacteria. Differences in the enterobacterial flora would generate different metabolites in ceca,
some of which might have AhR ligand potency. We therefore examined AhR ligand activity of the cecal content extract and found that the extracts had varying levels of activity in both yeast and mammalian reporter assays (Fig. 3). In the yeast reporter assay, the mean value of AhR ligand activity in the OME-treated group was higher than that in the control group ($F = 14.8; F_{0.01} = 5.35$). Bars and values in the graph indicate the mean for each group. One sample each from the control and OME-treated groups indicated no CYP1A1 cDNA amplification. Note the scale is logarithmic (n.s., not significant). (B) After real-time PCR, amplified products were visualized by agarose gel electrophoresis; representative results from three animals are shown. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

AhR Ligand Activity in Cecal Bacterial and Yeast Cultures. We attempted to isolate AhR ligand compounds from cecal contents after OME treatment. However, cecal contents were believed to be insufficient to isolate enigmatic active compounds because their amounts were limited and heterogeneous in each mouse. We therefore attempted to culture mouse cecal bacteria in anaerobic conditions. Table 1 shows the AhR ligand activity of the anaerobic culture medium of cecal bacteria as determined by the reporter assay yeasts expressing mouse AhR (OPUM). The GAM broth, a general culture medium for cultivating anaerobic bacteria, contained AhR ligand activity from the outset; this activity was reduced by the addition of OME and/or cecal bacteria. We then conducted a similar experiment using yeast instead of enterobacteria (Table 2). The yeast SD medium showed less activity compared with the YPD medium. In the absence of yeast, the extract from the SD medium not incubated after adding OME indicated AhR ligand activity that disappeared after 8 hours of incubation. In the yeast medium, AhR ligand...
activity was observed in the SD medium extract after 8 hours of incubation. These results led us to two different interpretations: the yeast generated AhR ligand compounds from OME during incubation, or the yeast maintained OME intact without inactivation. Therefore, we further investigated AhR ligand activity in the cecal contents and bacterial culture using HPLC.

**HPLC Analysis of Cecal Contents and Cecal Bacterial Culture.**

Figure 4 shows the HPLC chromatogram for the cecal content extract and cecal bacterial culture. OME and its metabolites were detected by measuring the absorbance at 280 nm because they are expected to contain an imidazole group. The cecal content extracts of OME-treated animals that showed activity higher than that in control animals were mixed and then analyzed. AhR ligand activity was observed in multiple HPLC fractions in the reporter assay yeasts expressing mouse AhR (Fig. 4, A and B). Fractions 18, 27, and 32 contained active compounds specific to OME treatment. Fraction 27 corresponded to the peak for pure OME in HPLC analysis (Fig. 4B). We observed the ligand activity of the cecal bacterial culture extracts in flow-through fractions and fraction 32 (Fig. 4C). Extracts from the GAM broth or medium after the culture of cecal bacteria also indicated ligand activity, but only in the flow-through fractions (data not shown). Fractions 39–42 were dark brown and toxic to yeast. These results suggest that fraction 32 contained ligand compounds that were produced specifically by OME treatment.

**HPLC Analysis of Yeast Metabolite in Yeast Culture Medium.**

We expected that AhR ligand–like compounds in cecal bacterial culture would be one of the OME metabolites generated by enterobacteria. We speculated that the estimated AhR-activating OME metabolite in cecal culture would also be generated in yeast culture and contribute to the activation of mouse AhR in the yeast reporter assay. Figure 5 shows the HPLC chromatogram of the extract from the OME-containing SD medium incubated with or without yeast. Similar to the cecal bacterial culture and cecum content extracts, fractions 32 and 33 showed ligand activity in the reporter assay yeasts expressing mouse AhR (OPUM), and the activity was generated specifically by the yeast culture (Fig. 5B). For the extract of the medium with and without yeast (Fig. 5A), fraction 35 also indicated ligand activity in the reporter assay yeasts.
expressing human AhR (OPUH), but not mouse AhR (OPUM). These results indicate that compounds generated by yeast from OME have ligand activity for mouse AhR and were included in fractions 32 and 33. These findings agree with the results for the cecal content extracts and cecal bacterial culture. Therefore, we used only fraction 32 in subsequent experiments.

AhR Agonist–Like Activity of the OME Bacterial Metabolite Detected by the Mammalian Cell Reporter Assay. HPLC analysis and yeast reporter assays revealed that the OME metabolite generated by yeast and enterobacteria may be an AhR ligand. The next question was whether this metabolite of the microbial culture was also an AhR ligand in mammalian cells. Figure 6A shows the agonist-like activity of the OME bacterial metabolite against mouse AhR detected by the XRE-driven reporter gene. In this assay, the OME metabolite in each fraction was determined using OPUH (middle graph) or OPUM (lower graph). Open triangles indicate the elution position of the pure OME. Note the different y-axis scales in the graphs. mAU, milli-absorbance units; n.d., not detected.

Fig. 5. HPLC analysis of the yeast culture medium and AhR ligand activity of HPLC fractions. (A) Upper graph: The solid-phase extract of the yeast culture medium was separated by HPLC using a C18 reverse-phase column. Elution was performed using the methods described in the legend for Fig. 4. AhR ligand activity in each fraction was determined by the yeast reporter assay using OPUH (middle graph: human AhR) or OPUM (lower graph: mouse AhR) and was represented as BNF-Eq. (B) The extract of yeast culture medium containing OME was analyzed. AhR ligand activity in each fraction was determined using OPUH (middle graph) or OPUM (lower graph). Open triangles indicate the elution position of the pure OME. Note the different y-axis scales in the graphs. mAU, milli-absorbance units; n.d., not detected.

Fig. 6. Ligand activity of HPLC-fractionated OME metabolites against mouse (A) or human (B) AhR expressed in HeLa cells. HeLa cells were transfected with reporter genes (pX4TK-Luc and pRL-CMV) and mouse AhR expression plasmid (pCI-neo-mAhR) or human AhR expression plasmid (pCI-neo-hAhR). Cells were exposed to DMSO (CT, solvent control), 50 μM OME (OME), 0.3 μM 3-MC (3-MC), or the DMSO-dissolved HPLC fraction (from left to right, 0.25%, 0.5%, 1%) 24 hours after the transfection. CBM-32, fraction 32 separated from the cecal bacterial culture medium including 100 μM OME; YM-32 and YM-35, fraction 32 or 35 separated from the yeast culture medium including 100 μM OME. Final concentrations of DMSO were adjusted to 1% in all assays. After incubating for 16 hours, the cells were lysed, and firefly luciferase (Fluc) and Renilla luciferase activity were measured. Data represent the average ± standard deviation of normalized firefly luciferase/Renilla luciferase activity (×100) from triplicated experiments. Asterisks indicate significant differences between treatment versus control groups (*P < 0.05; **P < 0.01; ***P < 0.001). CT, control.
oligonucleotides, which indicated the presence of a XRE-specifically-bound complex including AhR/Arnt in YM-32. These results demonstrated that the cytosolic mouse AhR was transformed to the form able to bind to XRE after YM-32 treatment.

**Discussion**

In this study, we elucidated two unsolved mysteries. The first was the reason for mouse AhR being responsive to OME only in the yeast reporter assay. The second was the reason for CYP1A1 being occasionally induced in mouse cecum by OME administration. An answer to these questions is that yeast and enterobacteria metabolically generate AhR ligands from OME. This explanation is based on the evidence that AhR ligand activity separated from the extracts of enterobacteria, and yeast culture was found in the same HPLC fraction that was different from the pure OME fraction. The fraction containing the estimated OME metabolite indicated agonist-like activity for both mouse and human AhR. The agonist-like activity of the fraction increased in a dose-dependent manner, but was reduced by the AhR antagonist. We therefore concluded that the AhR ligand, generated metabolically by enterobacteria from OME, induced CYP1A1 in mouse cecum and the reporter gene in the yeast.

The species-specific differences in AhR activation by OME are thought to result from intracellular environment but not AhR itself (Kikuchi et al., 2002). The insensitivity to OME in mouse hepatocyte and OME reactivity of mouse AhR expressed in yeast agreed with this theory (Dzeletovic et al., 1997), and were reconfirmed in this study. In our recent study, however, OME-mediated AhR activation was dependent on the AhR species. In particular, mouse AhR did not respond to OME even when it was expressed in human cells (Shiizaki et al., 2014). Although there seems to be a contradiction between these two insights, the present findings that an AhR ligand metabolically generated by yeast activates mouse AhR expressed in yeast might explain such conflicts without discrepancy.

We identified candidates for OME-derived AhR ligand compounds in yeast culture medium from HPLC fractions 32 and 35 (Fig. 5B). The compound in HPLC fraction 32 was considered identical to that contained in cecal contents and cecal bacterial culture (Fig. 4). Fraction 35 separated from the medium without yeast also indicated AhR ligand activity, but it was shown only in the human AhR–expressing yeast reporter assay (Fig. 5A). The compound contained in this fraction was thought to be a derivative of OME, independent of yeast metabolic activity, and not a valid AhR ligand because it showed no activity in mouse AhR–expressing reporter yeast or HeLa cells expressing human AhR (Fig. 6). Furthermore, the transcriptional activation of reporter gene was decreased by concomitant exposure of AhR antagonist ANF (Fig. 7), and EMSA analysis showed that the compound included in HPLC fraction 32 transformed mouse cytosolic AhR to the form able to bind to XRE. We conclude that CYP1A1 induction in the cecum of OME-treated mice was due to a ligand compound contained in fraction 32 generated by enterobacteria. As shown in Fig. 4D, we detected AhR ligand–like activity in OME metabolite from cecal bacterial culture. However, in the repeated experiment using bacteria provided by other mouse ceca, the intensities of activity in fraction 32 were highly diverse. Thus, it was conceivable that the variety of induction of CYP1A1 in mouse ceca shown in Fig. 2 was due to individual differences of enterobacterial flora in mouse ceca. Actually, influences of drug metabolite caused by the change of enterobacterial flora have been reported (Matsumoto et al., 2012).

In our previous report, we constructed several OME-responsive or -unresponsive AhRs by a few amino acid substitutions of mouse and rabbit AhR. These mutant AhRs responded to fraction 32 without remarkable differences from wild-type AhR (data not shown). Thus, AhR agonist–like activity included in fraction 32 did not seem to be related to species-specific responsiveness to OME.

OME-mediated AhR activation has not been thought to require ligand binding, although several studies concluded that ligand binding is the key determinant of AhR-mediated transcription (Gasiewicz and Rucci, 1991; Murray et al., 2005). The results of in vitro binding assays appear to provide credible evidence that OME can activate
AhR without ligand binding (Daujat et al., 1992; Dzeletovic et al., 1997; Backlund and Ingelman-Sundberg, 2004); however, these data were observed in the assays in which OME was added after cytosol isolation. When OME was treated before cytosol isolation, a reduction in 2,3,7,8-tetrachlorodibenzo-p-dioxin binding to AhR was observed in rabbit hepatocytes (Lesca et al., 1995). In the electrophoresis mobility shift assay, OME failed to shift the AhR/Arnt complex signal when it was added to the cytosol isolated from human tumor cells (Daujat et al., 1992; Backlund et al., 1997). In contrast, cytosol from cells pretreated with OME showed a retardation band specific to the AhR/Arnt complex (Quattrochi and Tukey, 1993). Taken together, these previous reports do not contradict our present findings that the OME metabolite is an active compound with AhR ligand activity and is generated by yeast and enterobacteria. However, further studies will be needed to isolate and determine the active metabolite of OME.

Our study showed that the response of mouse AhR to OME in the yeast system is due to the yeast-generated OME metabolite, which indicates that OME bacterial metabolites are AhR ligands in the intestines of mice and humans. These results provide significant information regarding the safety of OME, which is an uncommon CYP1A1-inducible therapeutic drug.

Acknowledgments
The authors thank Seiichiro Ohsako (University of Tokyo) and Kazuhiro Sogawa (Tohoku University) for providing the plasmids. The authors thank Togo Ikuta (Saitama Cancer Center) for helpful suggestions pertaining to the study on the safety of OME, which is an uncommon CYP1A1-inducible enzyme.

Authorship Contributions
Participated in research design: Shiizaki, Yagi.
Conducted experiments: Shiizaki, Yagi.
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Performed data analysis: Shiizaki.
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References

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