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Author(s)	Jinno, Asumi; Maruyama, Yutaka; Ishizuka, Mayumi et al.
Citation	The Journal of Steroid Biochemistry and Molecular Biology, 98(1), 48-55 <a href="https://doi.org/10.1016/j.jsbmb.2005.07.003">https://doi.org/10.1016/j.jsbmb.2005.07.003</a>
Issue Date	2006-01
Doc URL	<a href="https://hdl.handle.net/2115/5818">https://hdl.handle.net/2115/5818</a>
Type	journal article
File Information	JSBMB98-1.pdf



## **Induction of cytochrome P450-1A by the equine estrogen equilenin, a new endogenous aryl hydrocarbon receptor ligand**

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## Abstract

Equilenin is one of ten kinds of estrogens that are found in pregnant mares' urine. It has been used extensively for estrogen replacement therapy in postmenopausal women. Typical inducers of the cytochrome P4501A1 (CYP1A1), such as TCDD, benzo(a)pyrene (B(a)P) and 3-methylcholanthrene, have a planar molecular structure in common and bind to the aryl hydrocarbon receptor (AhR). The structure of equilenin differs from classic estrogens by the presence of two additional double bonds in ring B of the steroid nucleus, and it is planar. This structural similarity of equilenin to the typical AhR agonist prompted us to investigate the capability of equilenin to induce CYP1A1 expression. Administration of equilenin to two mouse strains (C57BL and DBA) that exhibit different degrees of responsiveness to an Ah-receptor agonist and showed that equilenin was capable of dose-dependently increasing both the ethoxyresorufin-O-deethylase activity and CYP1a proteins in both strains of mice. Equilenin also induced CYP1A1 mRNA in treated HepG2 cell lines and transcriptional activity in an XRE-directed luciferase reporter gene. Competitive binding studies using C57BL AhR indicated equilenin weakly displaced <sup>3</sup>H-B(a)P from AhR. Together, these data show that equilenin, an equine steroid hormone, served as an AhR ligand in the present study.

**Key Words:** Steroid Hormone, CYP1A1, Aryl hydrocarbon receptor, Equilenin

## Introduction

Equilenin belongs to the equine estrogen family, which is composed of 10 different estrogens produced only during pregnancy, and is excreted in the urine of pregnant mares [1]. Equilenin binds to the estrogen receptor and exhibits estrogenic action, the potency of which is comparable to, if not exceeding, that of 17 $\beta$ -estradiol. Therefore, a preparation of the conjugated form of equilenin (Premarin), which is purified from the urine of pregnant mares, has been successfully used in the hormone-replacement therapy of postmenopausal symptoms.

Estrone and 17 $\beta$ -estradiol are estrogens that have a saturated B-ring, and are biosynthesized from cholesterol through a classic steroid synthetic pathway. Equilenin, which has a partially planar structure with aromatic A and B rings (Figure 1), is biosynthesized through a different pathway [2] from that of estrone and 17 $\beta$ -estradiol.

It was this planar structure that prompted us to think that equilenin may be a ligand to the arylhydrocarbon receptor (AhR) and might induce members of the CYP1 family. Induction of the CYP1 family is triggered by the activation of AhR by a group of organochlorides, such as co-planar polychlorinated biphenyls (PCBs) and polychlorinated dibenzo dioxins (PCDDs), and planar polycyclic aromatic hydrocarbons (PAHs), such as benzo(a)pyrene (B(a)P), 3-methylcholanthrene (3-MC),  $\beta$ -naphthoflavone and others. A group of lipophilic azo-naphthol compounds, such as 1-phenylazo 2-naphthol, with a structure capable of forming planar 3 fused 6-membered rings by the formation of intra-molecular hydrogen bonds can also be AhR ligands [3, 4]. As has been pointed out by this work and others [5, 6], planarity appears to be an important requirement for AhR ligands. Interestingly, a comparison of the chemical structures of these known AhR ligands with that of equilenin shows that the molecular structure of this planar estrogen can be almost perfectly superimposed on that of 3-MC (Figure 1). In this study, we found that this estrogen

indeed binds to AhR and induces CYP1A subfamily members. This is the first reported AhR ligand that is known to be biosynthesized endogenously.

## **Materials and Methods**

### ***Chemicals and reagents***

All the test substances and reagents used were of reagent grade including those described below. Equilenin, benzo(a)pyrene (B(a)P), resorufin, ethoxyresorufin, dimethyl sulfoxide (DMSO), dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), aprotinin, Triton X-100 and dextran-coated charcoal were purchased from Sigma, USA. HEPES, ethylenediaminetetraacetic acid (EDTA) was from Dojindo Laboratories, Japan; NADPH, glucose-6-phosphate (G-6-P) and glucose-6-phosphate dehydrogenase (G-6-PDH) was from Oriental Yeast, Japan; [<sup>3</sup>H]B(a)P (76.0 Ci/mmol) was from Amersham Pharmacia Biotech, UK. All other reagents were analytical grade or the highest quality available and purchased from Wako Pure Chemical Industries, Japan.

### ***Animals***

C57BL mice (SLC, Japan) obtained at the age of 6 weeks were used in the experiments after acclimatization for 1 week. 1 mg/kg or 20 mg/kg solution of equilenin, or 20 mg/kg of B(a)P in corn oil was administered intraperitoneally to each group (three 7-week-old mice) repeatedly over a period of 3 days. Corn oil was administered to the control animals at the same dose volume (10 ml/kg). Twenty-four hr after the final administration, the mice were killed by decapitation under light anesthesia with CO<sub>2</sub>. The liver was excised immediately from each mouse, and perfused with an ice-cold 1.15% KCl solution to remove the blood. Microsomes were prepared by the method of Omura and Sato [7]. The protein concentration was determined by the method of Lowry *et al.* [8].

### ***Assay of ethoxyresorufin O-deethylation (EROD) activity***

The activity of ethoxyresorufin O-deethylation was determined by the method of Burke *et al.* [9] with slight modification. One ml of the reaction mixture containing an appropriate concentration of microsomal protein, 10 mM G-6-P, 10 mM MgCl<sub>2</sub> and 20 μM ethoxyresorufin in 20 mM Tris-HCl (pH 7.8) was preincubated for 5 min at 37°C. The reaction was started by adding 20 μl of a mixture of 50 mM NADPH and 200 U/ml of G-6-PDH. After incubation for 5 min, the reaction was terminated by adding 4 ml of cold methanol. The mixture was centrifuged at 3000 rpm for 5 min, and the supernatant methanol layer was collected for measurement of resorufin. Resorufin was measured using a fluorescence spectrophotometer (FP777, JASCO). The excitation wavelength was set at 528 nm and the emission wavelength at 590 nm.

### ***Western blot analysis***

Polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to the method of Laemmli [10], using 10% polyacrylamide and 15 μg microsomal protein. After electrophoresis, the proteins in the gel were transferred to a PVDF membrane by an electrophoretic transfer method. The antigen-antibody reactions were carried out after blocking of the

membrane with 5% skimmed milk solution using a goat anti-rat CYP1A1 serum (diluted 1:500, Daiichi Pure Chemicals, Japan) as the first antibody and a rabbit anti-goat IgG antibody conjugated with peroxidase (diluted 1:200, Chemicon, USA) as the second antibody. Each antigen-antibody reaction was conducted for 1 hr at 37°C. After the antigen-antibody reaction, a color development reaction was performed using a mixture of diaminobenzidine and H<sub>2</sub>O<sub>2</sub> (0.025% 3,3-diaminobenzidine, 0.0075% H<sub>2</sub>O<sub>2</sub>-Tris-HCl buffer, pH 7.4), and the staining intensities of the protein bands on the membrane were analyzed by NIH Image [11].

#### ***Cell culture maintenance and passage***

Human hepatocarcinoma cells, HepG2, and human fetal kidney cells, 293T, were cultured at 37°C in an atmosphere of 5% CO<sub>2</sub> and 100% humidity. For maintenance of the cells, DMEM (GIBCO BRL, USA) supplemented with 10% bovine serum albumin (Moregate, Australia), penicillin, streptomycin and amphotericin B (antibiotic antimycotic; GIBCO BRL) was used. When the cells reached a confluent condition, the maintenance medium was removed, and the cells were washed with Dulbecco's phosphate-buffered saline (D-PBS). After washing, a solution containing 0.25% trypsin and 0.25% EDTA to HepG2 cells or 0.05% trypsin and 0.25% EDTA to 293T cells was added, and the cells were allowed to stand at 37°C until the cell layer was removed from the surface of the culture flask. The cells were collected and centrifuged at 110×g for 3 min to remove the supernatant solution. The cell pellet was appropriately suspended in the buffer, diluted and inoculated to a new culture flask.

#### ***Assay of CYP1A1 mRNA induced by equilenin***

HepG2 cells were plated on a 6-well plate at a cell density of  $1.25 \times 10^7$  cell/well and cultured for 24 hr. The medium was replaced with medium containing 0, 1, 3, 10 or 30 μM of equilenin dissolved in DMSO. The final concentration of DMSO was 0.1%. After exposure of the cells to the test substance for 24 hr, the medium was removed, and the cells were washed twice with D-PBS. Total RNA was extracted using an Rneasy Mini Kit (QIAGEN, Germany) according to the manufacturer's instruction manual. RNA (0.1 μg) was reverse-transcribed to cDNA using the RT enzyme. Using this cDNA as a template, CYP1A1 mRNA was assayed using an ABI PRISM7700 (PE Applied Biosystems, USA). The specific primers and TaqMan probe used in this assay are as follows: Human CYP1A1 primers: (Forward) 5'-ttcgtacctaccaaccctt-3', (Reverse) 5'-tgtctgtgatgtcccggatgt-3', TaqMan probe: 5'-catgcagaagatggcaaggagcactacaa-3'

A 50 μl aliquot of the Taqman Universal PCR Master Mix containing an appropriate amount of the template cDNA, 3 μM of primers and 2 μM of Taqman probe was prepared, and real-time PCR was performed by 50 cycles of incubation at 95°C for 15 sec, followed by incubation at 60°C for 1 min. The PCR product was subcloned into a pCR2.1 vector (Invitrogen, USA) to prepare the standard curve.

#### ***Luciferase assay***

The promoter sequence of human CYP1A1, which was integrated into a pRNH241c vector provided by Dr. Piechocki and Dr. Hines (Department of Pharmacology, Wayne State University School of Medicine, USA) [12], was inserted to an enhancer region of a PGL3 Reporter Vector (Promega, USA) coding a luciferase gene of the firefly. For correction of transfection efficiency,

the HepG2 cells were transfected simultaneously with pPL Control Vector (Promega) coding *Renilla* luciferase. The transfection was performed using Lipofect AMINE PLUS (GIBCO BRL) according to the manufacturer's instruction manual. A serum-free medium was used for transfection, and was replaced with medium containing serum 3 hr after the transfection. The cells were treated 12 hr after the medium change with DMSO (0.1%) as a control and equilenin or B(a)P at final concentrations of 1, 10 and 30  $\mu$ M and 0.1, 0.5 and 1  $\mu$ M, respectively, for 24 hr. The activity of luciferase was measured 24 hr after addition of the test substances. The assay of luciferase was performed using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instruction manual. The chemiluminescence was measured using a Luminescencer JNR (ATTO, Japan). The measurement was conducted continuously for 2 min after addition of luminescent substrate, and the integrated value for 2 min was used.

#### ***Expression of fusion protein of recombinant AhR with histidine tag***

cDNA corresponding to the open reading frame of the C57BL mouse AhR was obtained using C57BL mouse liver total RNA by an RT-PCR method. The primers used were C57BLAhR: (Forward) 5'-ccatcgatagagcagcggcgcca-3', (Reverse) 5'-gctagctcaatggtgatggtgatgactctgcacctgcttaggaa-3'. The sequence of the restriction site for Cla I was attached to the 5' end of the forward primer. The sequence of the restriction enzyme site for Nhe I was attached to the reverse primer. The sequence coding 6 molecules of histidine was also attached to the reverse primer. AhR cDNA and the expression vector pCAGGS [13] were treated with restriction enzymes and ligated using a TAKARA ligation kit version 2 (Takara, Japan) according to the manufacturer's instruction manual. The recombinant expression vector thus obtained was transfected into *E. coli*, JM109 according to the conventional procedure, amplified by culture and collected by a Plasmid Purification Midi Kit (QIAGEN).

The AhR expression vector (10  $\mu$ g/150 mm dish) was transfected into 293T cells using Lipofect AMINE PLUS (GIBCO BRL) according to the manufacturer's instruction manual. A serum-free medium was used in transfection and was replaced with a serum-containing medium at 3 hr after the transfection. Seventy-two hr after starting the transfection, the cells were washed with D-PBS twice, detached from the flask with a cell scraper and collected by centrifugation at 3000 rpm for 5 min at 4°C. The collected cells were re-suspended in HBS buffer (10 mM HEPES, 0.15 M NaCl, 1 mM DTT, 0.01% TritonX-100, 1 mM PMSF and 1  $\mu$ g/ml aprotinin, pH 7.5) and homogenized by sonication for 30 sec. The cell homogenate was centrifuged at 10,000 $\times$ g for 30 min at 4°C, and the supernatant fraction was collected.

The recombinant fusion protein of AhR with a His-tag in the cell extract was purified using a HisTrap Kit (Amersham Pharmacia Biotech). According to the manufacturer's instruction manual, a solution of Ni<sup>2+</sup> was applied to a 1 ml HiTrap Chelating HP column, which was then washed with ultra-pure water. After washing, the column was equilibrated with 10 ml of a starting buffer (phosphate buffer, 10 mM imidazole). The cell extract was filtered through a membrane filter (0.45  $\mu$ M), combined with imidazole at a final concentration of 10 mM and applied to the equilibrated column. Proteins were eluted with 5 ml of an elution buffer (phosphate buffer, 500 mM imidazole). The eluate was collected in fractions of 0.5 ml each. The flow rate of the buffer was set at 1 ml/min and all the procedures were performed at 4°C. The protein concentrations in the fractions were determined,

and three fractions containing a high concentration of protein were identified (1.5 ml). Western blot analysis of the fractions was also conducted using anti mouse AhR (Novus Biologicals, Inc., USA) to detect the expressed protein with a size of about 93 kDa. The fractions containing high protein concentrations (1.5 ml) was applied to a HiTrap Desalting column (Amersham Pharmacia Biotech) to remove the salt and exchange the buffer. The column was equilibrated with an elution buffer (25 mM HEPES, 1.5 mM EDTA, 1 mM DTT, pH 7.4) at a volume not less than 15 ml. After application of the sample, the elution buffer was passed through the column by a pump at a flow rate of 2.5 ml/min, and the eluate was collected in 5 fractions of 1 ml each. All the procedures were performed at 4°C. The protein concentration in each fractions was determined to identify the fractions containing the protein. The fractions were frozen in liquid nitrogen and stored at -80°C until use.

#### ***Assay of equilenin binding to C57BL AhR***

A 200 µl aliquot of the desalted fraction of AhR (100 µg protein/ml) was incubated with 10 nM [<sup>3</sup>H]B(a)P for 1 hr at 4°C in the presence or absence of equilenin (10 – 300 µM) or 3-MC (0.1 – 10 nM). [<sup>3</sup>H]B(a)P, equilenin and 3-MC were dissolved in DMSO, and added to the incubation mixture at a final concentration of 1%. Non-specific binding was obtained by incubating 10 nM [<sup>3</sup>H]B(a)P with an excessive amount (1000-fold) of non-radioactive B(a)P. After incubation, dextran-coated charcoal was added to the incubation mixture at a final concentration of 1 mg/ml, and the mixture was further incubated for 30 min at 4°C in order to remove the free fraction of the radioactive ligand. After centrifugation at 100×g for 10 min, a 150 µl-aliquot of the supernatant fraction was mixed with 10 ml of emulsifying liquid scintillator, Scintisol EX-H (Dojindo Laboratories), and the radioactivity was measured after 3 hr using a liquid scintillation counter, TRI-CARB 2100TR (Packard Instrument Co., USA).

#### ***Statistical analysis***

Statistical analyses were performed by ANOVA and post hoc tests (Fisher's PLSD and Scheffe's test). The values were expressed as the mean value ± standard error, and the difference at a level of significance less than 0.05 (P < 0.05) was regarded as statistically significant.

## **Results**

#### ***Induction of CYP1A by equilenin***

B(a)P at the dose of 20 mg/kg/day and equilenin at doses of 1 and 20 mg/kg/day were administered to 3 groups of C57BL mice for three days. The vehicle control group received corn oil. Although the dose of 20 mg/kg for equilenin seems quite high to measure estrogenic effects, it is common to treat animals with CYP1A1 inducers such as B(a)P at doses of 20-80 mg/kg/day. Therefore, we used this dose of equilenin for comparison. No significant health effect on mice was observed during the treatment period with either treatment. Administration of equilenin and B(a)P at the doses of 20 mg/kg/day for three days significantly increased the activity of the CYP1A-dependent drug-metabolizing enzyme, ethoxyresorufin O-deethylation (EROD), in C57BL mice (Figure 2A). Western blot analysis using rat CYP1A1 antibody revealed increased expression of CYP1A

protein in liver microsomes of mice (Figure 2B, 2C). The potency of the induction by equilenin was slightly more than a half of that by B(a)P at the same dose.

#### ***Induction of CYP1A by equilenin in HepG2 cells***

The effect of treatments with 1, 3, 10 and 30  $\mu\text{M}$  equilenin on the HepG2 cells was investigated. HepG2 cells have endogenous AhR. Assay for CYP1A1 mRNA in equilenin-treated HepG2 cells was conducted using a Taqman probe and primer set that is specific to human CYP1A1 mRNA. The induction of mRNA by equilenin was concentration-dependent, and at a concentration of 30  $\mu\text{M}$  of equilenin, about 15-fold induction was observed (Figure 3).

#### ***Effect of equilenin on activation of transcription via XRE***

In order to investigate the mechanism of CYP1A induction observed in C57BL mice and in HepG2 cells, a luciferase reporter assay system was constructed by transiently transfecting HepG2 cells with the CYP1A1 5' flanking reporter plasmids, pGL3-XRE, together with the pGL-TK control plasmid (Materials and Methods), and transcriptional activation by XRE after addition of equilenin was investigated using this assay system. Equilenin at concentrations of 1, 10 and 30  $\mu\text{M}$  showed the activation of transcription equivalent to the levels of activation by B(a)P at concentrations of 0.1, 0.5 and 1  $\mu\text{M}$  (Figure 4), indicating that the potency of equilenin is about one tenth to one thirtieth of that of B(a)P.

#### ***Affinity of equilenin to AhR***

AhR protein of C57BL mice was prepared as described in Materials and Methods. Affinity of equilenin to C57BL AhR was evaluated by a competition assay using radiolabeled B(a)P, an archetypal ligand of AhR. The results obtained were compared with those obtained with 3-MC. 3-MC at concentrations of 0.1 – 10 nM showed concentration-dependent inhibition of the binding of 10 nM [ $^3\text{H}$ ]B(a)P with AhR, and the inhibition was almost 100% at a concentration of 10 nM. Equilenin at a concentration of 10  $\mu\text{M}$  caused 25% inhibition and at 300  $\mu\text{M}$ , about 50% inhibition (Figure 5). Although equilenin was found to be a far weaker ligand to AhR than 3-MC, the results nevertheless indicate that it binds to AhR.

## **Discussion**

The arylhydrocarbon receptor is a transcriptional factor that has a bHLH (basic-helix-loop-helix)-PAS (Per/ARNT/Sim) structure and forms a complex with two molecules of 90 kDa-heat shock protein (HSP90) in the cytosol. AhR binds exogenous ligands such as PAHs, PCBs and PCDDs in the cytosol, then migrates into the nucleus and forms a heterodimer with the arylhydrocarbon receptor nuclear translocator (ARNT) after dissociation from HSP90. This heterodimer was demonstrated to activate gene transcription by binding to a xenobiotic responsive element (XRE) that has a 5'-GCGTG-3' DNA sequence as a core motif, and which is present in the regulatory region of a number of target genes such as CYP1A1 [14], CYP1A2, CYP1B1, NAD(P)H-quinone oxidoreductase (NQO)-1, the Ya subunit of glutathione-S-transferase (GST), UDP-glucuronosyl transferase (UGT1) and aldehyde dehydrogenase (ALDH)-3.

By investigating the structure-activity relationship of the ability of 40 azo-compounds to induce CYP1A1, Fujita et al.[3, 4] concluded that lipophilic azo-naphthol compounds, such as 1-phenylazo-2-naphthol, capable of forming coplanar 3 fused 6-membered rings with a molecular encumbrance area between 90 and 140 square angstroms through intra-molecular hydrogen bonding have the ability to induce CYP1A1 and UGT activities. They pointed out that lipophilicity, a planar structure with a minimum of three fused rings arranged in an angular form like the bay-region of carcinogenic PAH and size (molecular encumbrance area less than 150 square angstrom) are the important determinants for non-chlorinated organics to become an AhR ligand. Lewis *et al.* investigated the structure-activity relationship among the CYP1A1 inducers, and demonstrated also that the chemical structure is closely related to the reactivity [15] with the receptor. Typical CYP1A1 inducers such as TCDD, 3-MC, B(a)P and  $\beta$ -naphthoflavone and the AhR ligands possessed a common chemical structure, planarity and size within a rectangle of  $6.8 \times 13.7 \text{ \AA}$ .

The compounds with a benzimidazole structure, typically omeprazole, have been known to induce CYP1A1 in cultured human cells [16]. Kikuchi *et al.* showed that the induction of CYP1A1 by omeprazole was not inhibited by  $\alpha$ -naphthoflavone, an AhR antagonist, but the induction was inhibited by tyrosine kinase inhibitors, demonstrating that the signal transduction system mediated by a ligand-independent tyrosine kinase is involved in the induction [17]. On the contrary, Dzeletovic *et al.* demonstrated that the ligand binding site of AhR is related to the reactivity to omeprazole [18], suggesting a possibility of sulfenamide generated by acid decomposition of omeprazole with a very short half-life being produced and bound to AhR. Omeprazole has a planar benzimidazole structure. The possible addition of the third ring fused to the two-ring structure by intramolecular hydrogen bonding between NH of imidazole and sulfoxide oxygen may also fulfill the structural requirement for an AhR ligand as in the case of 1-phenylazo-2-naphthol.

Substances such as omeprazole, primaquine [19] and carbaryl [20] are classified as atypical CYP1A1 inducers; several mechanisms of induction have been proposed for them [21-24]. All of these inducers, however, have planar structures of two fused rings, and if those rings are joined by another ring formed by the intramolecular hydrogen bonding either before, if possible, or after hydroxylation of the ring by metabolism, they might be able to bind to AhR. It is then hypothesized that these compounds or metabolites, which are capable of forming additional rings, fuse to two coplanar ring structures by intramolecular hydrogen bonding to induce CYP1A1 by acquiring the ability to bind to AhR. The hydroxylation of these compounds would be possible *in vivo*, but in an AhR binding assay without a drug-metabolizing mechanism, these compounds would not be hydroxylated and unable to form intramolecular hydrogen bonding and therefore, may not successfully compete for the binding or be a very weak ligand. In fact, Denison et al. showed that carbaryl was a very weak AhR ligand by using a low nonsaturating concentration of radiolabeled TCDD in the ligand-binding assay [5, 22].

A recent study [25] indicated that 1 mM primaquine successfully displaced 5 nM tritium-labeled TCDD from AhR, while 3 mM omeprazole failed to displace it. These authors suggested that high- and low-affinity ligands for the AhR interact with different residues of the AhR ligand-binding pocket.

Induction of Cyp1a was observed after administration of equilenin to C57BL mice (Figure 2). Focusing attention on the fact that equilenin has a planar structure similar to 3-MC, a known AhR ligand, it is highly likely that equilenin binds to AhR and, therefore, induces CYP1A in mice via the AhR-mediated mechanism. In order to confirm this possibility, we first investigated the effect of equilenin on HepG2 cells, a cell line that has been frequently used as the hepatic model, to confirm that the observed increase in CYP protein level is indeed an induction involving a transcriptional process. In the assay of mRNA using a probe and primer set specific to human CYP1A1 mRNA, a significant increase in mRNA level was observed after treatment of HepG2 cells with equilenin at a concentration of 30  $\mu$ M.

To investigate the involvement of AhR in the induction of CYP1A by equilenin, a binding assay using expressed AhR was conducted. We transfected C57BL-AhR expression vector (pCAGGS) with His-tag sequence into 293T cell, and AhR in cytosolic fraction was purified using anti-His-tag antibody. AhR possessed affinities to the AhR ligands, B(a)P and 3-MC, and we did not re-add any co-factor proteins, such as HSP90, after the purification step. In the binding assay using [<sup>3</sup>H]B(a)P, 25-50% inhibition of the binding of 10 nM radiolabeled B(a)P with AhR was achieved by equilenin in a concentration range of 10  $\mu$ M–300  $\mu$ M. If one assumes that 50% inhibition is an equipotent concentration of the challenger (equilenin) to the challenged (B(a)P), then the binding affinity of equilenin is some 1/30,000 of that of B(a)P. About 50% inhibition was observed with 1 nM 3-MC, and with 10 nM 3-MC, 100% inhibition. The affinity of equilenin to AhR was much lower than that of 3-MC, which appears to have higher affinity to AhR than B(a)P, but nevertheless, it was shown to bind. The structure of equilenin partially overlaps with that of 3-MC. The large difference in AhR binding affinity despite the structural similarity may be attributed to the OH bond of equilenin, which reduces lipophilicity, and to its saturated C ring, which reduces planarity.

To confirm and to measure transcriptional activation of the XRE sequence, a reporter gene assay was performed to find that equilenin at a concentration of 1, 10 and 30  $\mu$ M increased the transcriptional activation to an extent similar to that induced by 0.1, 0.5 and 1  $\mu$ M B(a)P, respectively. Therefore, the potency of XRE activation of equilenin was 1/10 to 1/30 of that of B(a)P. It is interesting that the potency of XRE activation by equilenin and B(a)P did not reflect the large difference in affinity of these compounds to AhR. Equilenin may be converted in the cells to more potent ligand by metabolism, or alternatively, may activate XRE in some ligand-independent manner. Further studies are needed to elucidate these possibilities.

A preparation purified from estrogens of pregnant mares (Premarin) is used clinically in the treatment of postmenopausal symptoms as a hormone-replacement therapy. Premarin contains equilenin as the sulfo-conjugate. After administration at the therapeutic dose, Premarin undergoes a deconjugation reaction, and the blood concentration of equilenin reaches a maximum of 2.3 nM. This concentration is nowhere near the concentration of equilenin that induced CYP1A1 in HepG2 cells, but we do not know the local concentration of orally administered Premarin after first-pass uptake by the liver, as well as the extent of accumulation of this hormone with repeated use.

Previous studies on estrogens with an unsaturated B-ring have been focused on its risk of carcinogenesis. It has been reported that 17 $\beta$ -dihydroequilenin is hydroxylated at the 4-position by CYP1A1 and CYP1B1, and autoxidized to a quinone form, which

causes the DNA injury [26]. Therefore, induction of CYP1A1 may increase this risk. This DNA-damaging action by 17 $\beta$ -dihydroequilenin is known to be higher than that of 4-hydroxy estrone, the endogenous hormone, in the cells having estrogen receptors [27]. 17 $\beta$ -Estradiol is known to undergo hydroxylation at the 2-position by CYP1A1/2 [28, 29] and at the 4-position by CYP1B1 [30]. The difference in the CYP isoforms responsible for hydroxylation is probably due to the B-ring of these estrogens being aromatic. According to the results of previous studies [31, 32] in Syrian hamsters, a mixture of Premarin hydroxylates, equilenin and equilin, caused tumorigenesis at a rate of 100% while the rate of tumorigenesis was 75% and 0% after treatment with either equilin or equilenin, respectively. The metabolite of CYP, hydroxylated equilenin, has been considered to be involved in the tumorigenesis. Because the estrogens with the unsaturated B-ring are hydroxylated at the 4-position, it has been reported that hormone replacement therapy over a long period using Premarin is accompanied by an increased risk of carcinogenesis.

Induction of drug-metabolizing enzymes via AhR in response to xenobiotic compounds is regarded as a rational response to achieve the purpose of metabolizing and excreting the ligand xenobiotics. There were physiological studies using AhR-knockout mice [33-36], however, the role of AhR has not been clarified in detail yet. Although several compounds have been proposed as candidates for endogenous AhR ligands [37, 38], none of them has been identified as a biosynthesized endogenous ligand to date, and AhR is still classified as the orphan receptor. We have shown that equilenin, an endogenous steroid hormone, served as the AhR ligand in the present study. We do not think that equilenin can be generalized as the physiological AhR ligand because it is not found in animals other than pregnant mares. However, we believe that the results obtained here are very important in understanding the role of the AhR and give incites into the further study.

### **Acknowledgement**

This work was supported by Grants-in-Aid for Scientific Researches (Projects 15201009 and Priority Areas [A] Projects 13027201) from the Japanese Ministry of Education, Science, Sports, Culture and Technology.

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## Figure legends

**Figure 1:** Chemical structures of equilenin and CYP1A1 inducers.

**Figure 2:** Effects of equilenin on EROD activities and CYP1A expression levels in C57BL and DBA mice. C57BL and DBA2 mice (6 weeks old) were administered either a daily ip injection of corn oil (control), B[a]P (20 mg / kg / day), or equilenin (1 and 20 mg / kg / day) for three days. The data are the means  $\pm$  SD (n=3). Black bars represent C57BL mice, and white bars DBA mice. \*Significantly different from controls (p<0.05). (A): Effects of equilenin on EROD activities. (B): Effects of equilenin on CYP1A expression levels. (C): CYP1A expression levels estimated using NIH image.

**Figure 3:** Effect of equilenin on the CYP1A1 mRNA expression level in HepG2 cells. Cells were treated with DMSO (0.1%) and various concentrations of equilenin (0–30  $\mu$ M) for 24 hr. The relative amount of CYP1A1 mRNA was determined by real-time quantitative PCR. The data represent the relative amounts of CYP1A1 compared to the control. The data are the means  $\pm$  SD of three experiments. \* Significantly different (p<0.05) from DMSO control.

**Figure 4:** XRE–Luciferase Reporter Assay. Induction of Luciferase activity by B[a]P (A) and equilenin (B) in HepG2 cells. HepG2 cells were transiently transfected with the CYP1A1 5' flanking reporter plasmids, pGL3-XRE, together with the pRL-TK control plasmid. Twelve hr after transfection, the medium was changed and the cells were treated for 24 hr with DMSO, various concentrations of B[a]P (0–1  $\mu$ M) or equilenin (0–30  $\mu$ M). The data are the means  $\pm$  SD of three experiments. \* Significantly different (p<0.05) from DMSO control.

**Figure 5:** Competitive binding assay of 3-MC and equilenin for specific binding of 10 nM [<sup>3</sup>H]B(a)P. C57BL-AhR expression vector (pCAGGS) with His-tag sequence was transfected into 293T cell, and AhR in cytosolic fraction was purified using anti-His-tag antibody. AhR incubated with 10 nM [<sup>3</sup>H]B(a)P at 4 °C for 1 hr in the absence or presence of 3-MC (0–10 nM) or equilenin (0–300  $\mu$ M). The data are the means  $\pm$  SD of four experiments. \* Significantly different (p<0.05) from control.

Figure 1

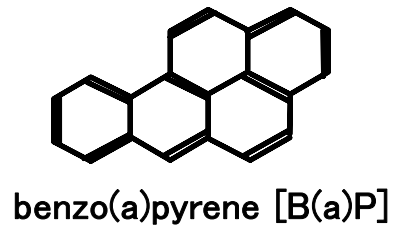
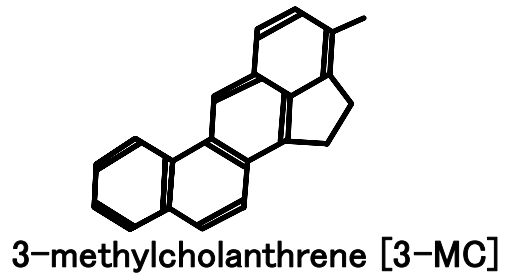
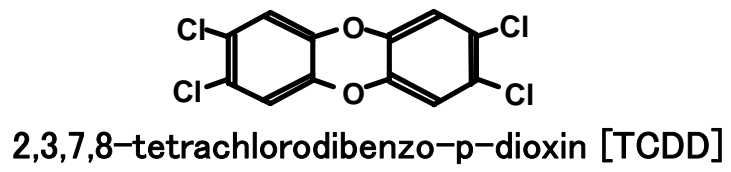
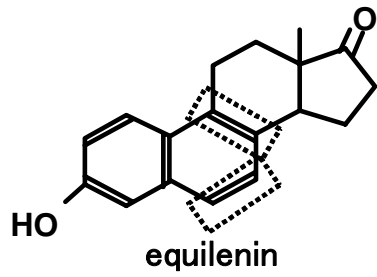


Figure 2

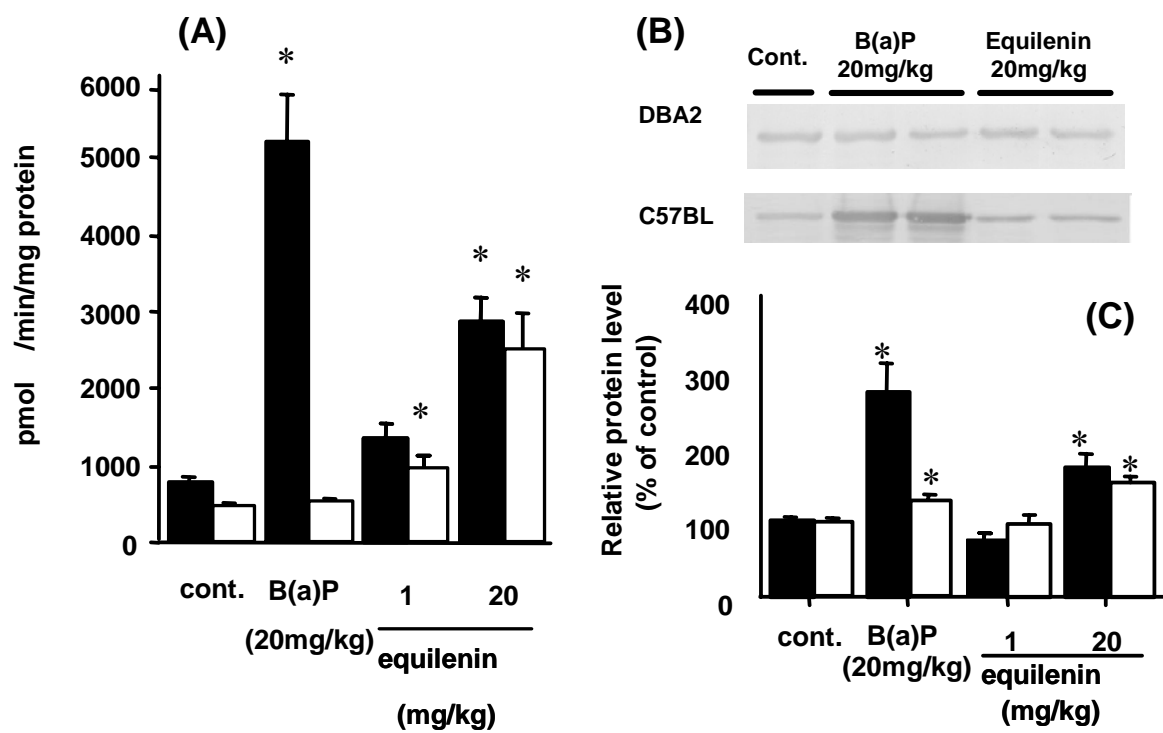


Figure 3

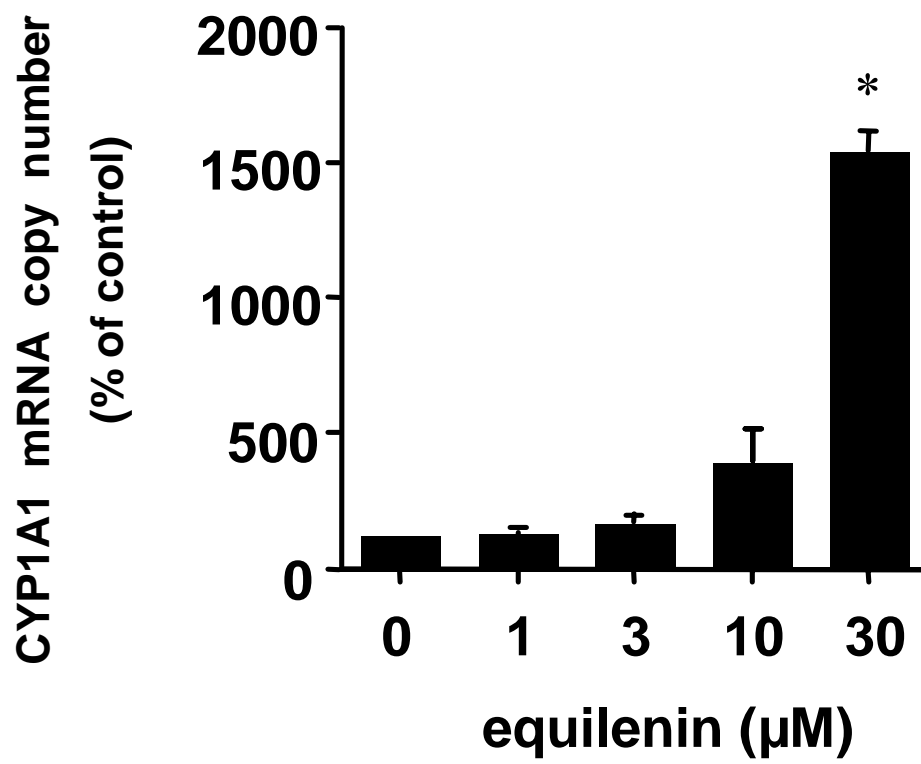


Figure 4

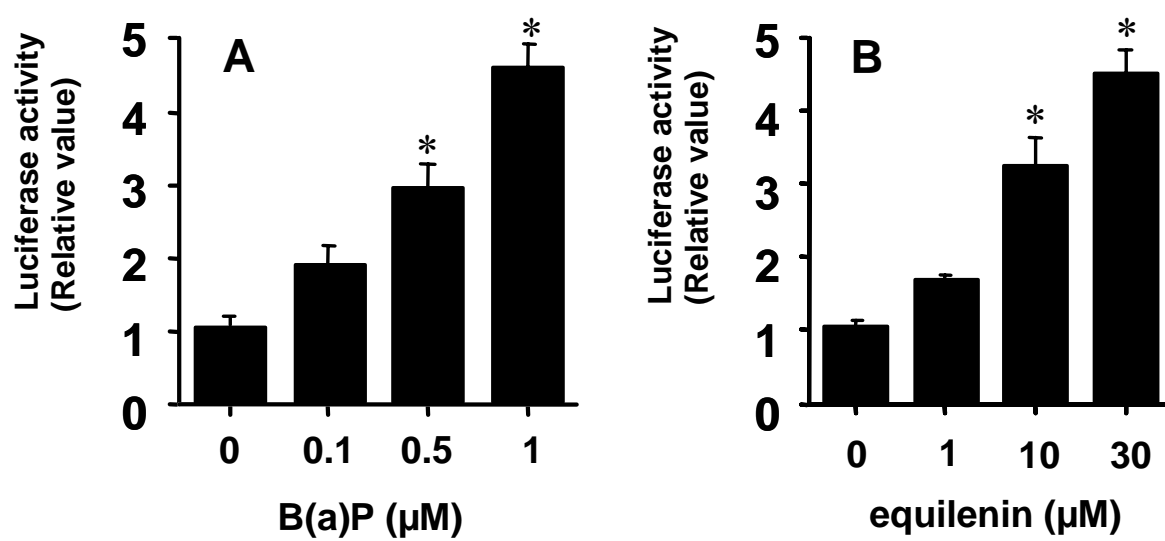


Figure 5

