Vitamin D Receptor Activation Enhances Benzo[a]pyrene Metabolism via CYP1A1

Expression in Macrophages

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Abbreviations: BaP, benzo[a]pyrene; AHR, aryl hydrocarbon receptor; CYP, cytochrome P450; 1,25(OH)2D3, 1α,25-dihydroxyvitamin D3; VDR, vitamin D receptor; RXR, retinoid X receptor; ER, everted repeat; PXR, pregnane X receptor; CAR, constitutive androstane receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; ADTT, (25R)-25-adamantyl-1α,25-dihydroxy-2-methylene-22,23-didehydro-19,26,27-trinor-20-epi-vitamin D3; ADMI3,
(25S)-26-adamantyl-1α,25-dihydroxy-2-methylene-22,23-didehydro-19,27-dinor-20-epi-vitamin D3; PCR, polymerase chain reaction; siRNA, small interfering RNA; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; LCA, lithocholic acid; XRE, xenobiotic responsive element
Abstract

Benzo[a]pyrene (BaP) activates the aryl hydrocarbon (AHR) and induces the expression of genes involved in xenobiotic metabolism, including cytochrome P450 (CYP) 1A1. CYP1A1 is involved not only in BaP detoxification but also in metabolic activation, which results in DNA adduct formation. Vitamin D receptor (VDR) belongs to the NR1I subfamily of the nuclear receptor superfamily, which also regulates expression of xenobiotic metabolism genes. We investigated the cross-talk between AHR and VDR signaling pathways and found that 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], a potent physiological VDR agonist, enhanced BaP-induced transcription of CYP1A1 in human monocytic U937 cells and THP-1 cells, breast cancer cells and kidney epithelium-derived cells. 1,25(OH)₂D₃ alone did not induce CYP1A1 and 1,25(OH)₂D₃ plus BaP did not increase CYP1A2 or CYP1B1 mRNA expression in U937 cells. Combination of 1,25(OH)₂D₃ and BaP increased CYP1A1 protein levels, BaP hydroxylation activity and BaP-DNA adduct formation in U937 cells and THP-1 cells more effectively than BaP alone. The combined effect of 1,25(OH)₂D₃ and BaP on CYP1A1 mRNA expression in U937 cells and/or THP-1 cells was inhibited by VDR knockdown, VDR antagonists, and α-naphthoflavone, an AHR antagonist. Electrophoretic mobility shift assays and chromatin immunoprecipitation assays showed that VDR directly bound to an everted repeat 8 motif in the human CYP1A1 promoter. Thus, CYP1A1 is a novel VDR target gene involved in xenobiotic metabolism. Induction of CYP1A1 by the activation of VDR and
AHR may contribute to BaP-mediated toxicity and the physiological function of this enzyme.
Introduction

Polycyclic aromatic hydrocarbons such as benzo[a]pyrene (BaP) are environmental pollutants produced by the combustion of cigarettes, creosote railroad ties, and coke ovens (Miller and Ramos, 2001; Uno and Makishima, 2009). BaP is implicated as a causative agent in malignancies, such as lung and head-and-neck cancers, and atherosclerosis as a consequence of cigarette smoking (Alexandrov et al., 2010; Shimada and Fujii-Kuriyama, 2004). BaP inhalation activates the aryl hydrocarbon receptor (AHR), which forms an active transcription factor heterodimer with the AHR nuclear translocator, and induces expression of a group of genes called the \(Ah\) gene battery, which includes the phase I enzymes [cytochrome P450 (CYP) 1A1 (gene symbol, \textit{CYP1A1}), \textit{CYP1A2}, \textit{CYP1B1}, and NAD(P)H:quinone oxidoreductase 1] and the phase II enzymes (glutathione S-transferase A1 and UDP glucuronosyltransferase 1A6) (Nebert et al., 2000).

BaP mediates carcinogenic, mutagenic and cytotoxic effects after conversion to toxic metabolites through an AHR-dependent mechanism of metabolic activation (Miller and Ramos, 2001; Shimada and Fujii-Kuriyama, 2004). BaP is first oxidized by CYP1A1 and CYP1B1 to phenols, such as 3-hydroxy-BaP and 9-hydroxy-BaP, and epoxides, such as BaP-7,8-epoxide (Shimada, 2006; Uno and Makishima, 2009). BaP-7,8-epoxide is then metabolized by epoxide hydrolase to BaP-7,8-diol, which serves as substrate for a second CYP-dependent oxidation, generating the toxic compound
BaP-7,8-diol-9,10-epoxide. Among the metabolites in BaP-treated cells, (+)-BaP-7,8-diol-9,10-epoxide-2 is the most reactive carcinogen (Alexandrov et al., 2010; Shimada, 2006).

Original studies on mutant Hepa-1 cells that are resistant to BaP-induced growth suppression have shown that BaP resistance is associated with mutations in the Cyp1a1 gene and dysfunction of the AHR transcription factor (Hankinson et al., 1991). Expression of exogenous CYP1A1 in CYP1A1-deficient cells restores the formation of BaP-induced DNA adducts (Maier et al., 2002). These findings indicate that metabolic activation of BaP requires the AHR-CYP1A1 cascade. In contrast, BaP-induced DNA adducts are increased in the liver and BaP clearance from the blood is slower in CYP1A1-null mice (Uno et al., 2001). Overexpression of CYP1A1 in hepatocytes suppresses BaP-induced DNA adduct formation and AHR transactivation (Endo et al., 2008). CYP1A1 may be involved in both metabolic activation and detoxification of BaP depending on conditions (Uno et al., 2006).

The active form of vitamin D3, 1,25-dihydroxyvitamin D3 [1,25(OH)2D3], binds to the vitamin D receptor (VDR; NR1I1) and regulates numerous physiological and pharmacological processes, including bone and calcium metabolism, cellular growth and differentiation, immunity, and cardiovascular function (Choi and Makishima, 2009; Nagpal et al., 2005). Natural and synthetic VDR ligands inhibit the proliferation and/or induce the differentiation of various types of malignant cells, including myeloid leukemia.
On ligand binding, VDR undergoes conformational changes that result in dynamic interaction with the heterodimer partner retinoid X receptor (RXR; NR2B) and exchange of cofactor complexes (Makishima and Yamada, 2005). Corepressors bind to the VDR-RXR heterodimer in the absence of ligand and ligand binding reduces the affinity of corepressors and increases the affinity for coactivators, a structural transition that induces transcription of specific genes. The VDR-RXR heterodimer binds preferentially to a vitamin D response element that consists of a two hexanucleotide (AGGTCA or a related sequence) direct repeat motif separated by three nucleotides. An inverted palindrome of the hexanucleotide motif, also called everted repeat (ER) element (Mangelsdorf and Evans, 1995), separated by six, seven, eight, or nine nucleotides has been also identified as vitamin D response elements in genes including CYP3A4 (Choi and Makishima, 2009; Thummel et al., 2001). VDR has been found to act as a receptor for secondary bile acids, including lithocholic acid (LCA) and 3-ketocholanic acid, and to induce the expression of CYP3A enzymes (Makishima et al., 2002). CYP3A enzymes catalyze the metabolic conversion of a wide variety of xenobiotics and endogenous substrates, including bile acids, to more polar derivatives (Xie and Evans, 2001).

VDR belongs to the NR1I nuclear receptor subfamily along with pregnane X receptor (PXR; NR1I2) and constitutive androstane receptor (CAR; NR1I3), both of which play a role in the regulation of xenobiotic metabolism. These findings suggest that
VDR may be involved in the regulation of xenobiotic-metabolizing enzymes other than CYP3A. Recently, CAR has been found to induce \textit{CYP1A1} and \textit{CYP1A2} expression by binding to a common regulatory element in the human \textit{CYP1A1} and \textit{CYP1A2} genes in hepatocytes (Yoshinari et al., 2010). In this study, we report that VDR activation enhances AHR-induced \textit{CYP1A1} expression in human macrophage-derived cells.
Materials and Methods

Chemical Compounds. 1,25(OH)\textsubscript{2}D\textsubscript{3}, BaP and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) were purchased from Wako Pure Chemical Industries (Osaka, Japan), and \(\alpha\)-naphthoflavone was from Sigma-Aldrich (St. Louis, MO). LCA acetate, ADTT \([(25\text{R})-25\text{-adamantyl-1}\alpha,25\text{-dihydroxy-2-methylene-22,23-didehydro-19,26,27-trinor-20-epi-vitamin D}_3]\) and ADMI3 \([(25\text{S})-26\text{-adamantyl-1}\alpha,25\text{-dihydroxy-2-methylene-22,23-didehydro-19,27-dinor-20-epi-vitamin D}_3]\) were synthesized in the Sachiko Yamada laboratory (Igarashi et al., 2007; Ishizawa et al., 2008; Nakabayashi et al., 2008).

Cell Culture. Human monocyte/macrophage-derived cells (U937 and THP-1; RIKEN Cell Bank, Tsukuba, Japan) and human breast cancer MCF-7 cells (American Type Culture Collection, Rockville, MD) were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin at 37\(^\circ\)C in a humidified atmosphere containing 5% CO\textsubscript{2}. Human kidney HEK293 cells (RIKEN Cell Bank) were cultured in Dulbecco's modified Eagle medium containing 5% fetal bovine serum. Cell viability after all of the treatments was more than 90%, as determined by exclusion of trypan blue.

Reverse Transcription and Real-time Quantitative Polymerase Chain Reaction. Total RNAs from samples were prepared by the acid guanidine thiocyanate-phenol/chloroform method (Matsunawa et al., 2009; Tavangar et al., 1990).
cDNAs were synthesized using the ImProm-II Reverse Transcription system (Promega Corporation, Madison, WI). Real-time polymerase chain reaction (PCR) was performed on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using Power SYBR Green PCR Master Mix (Applied Biosystems). Intron-spanning primers were as follows: CYP1A2 (GenBank accession no. NM_000761), 5'-AGT GGG AGA TCT TCC TCT TC-3' and 5'-GGT GTC TTC TTC AGT TGA TG-3'; VDR (GenBank accession no. NM_000376), 5'-CAC GTC ACT GAC GCG GTA CTT-3' and 5'-GCT GAC CTG GTC AGT TAC AGC A-3'; AHR (GenBank accession no. NM_001621), 5'-GTA AGT CTC CCT TCA TAC C-3' and 5'-AGG CAC GAA TTG GTT AGA G-3'. Other primers were reported previously (Matsunawa et al., 2009). The RNA values were normalized to the amount of β-actin mRNA, or mRNA copy numbers were determined using expression plasmids for a standard curve in real-time PCR reactions (Uno et al., 2006).

**Western Blotting Analysis.** For VDR and AHR expression, nuclear extracts were prepared as previously described (Inaba et al., 2007; Schreiber et al., 1989). For CYP1A1 expression, microsomes (S9 fraction) from cells were prepared as previously described (Endo et al., 2008; Uno et al., 2001). Western blot analysis was performed using a monoclonal anti-VDR antibody (Santa Cruz Biotechnology, Santa Cruz, CA), a polyclonal anti-AHR antibody (R&D Systems Inc., Minneapolis, MN), a polyclonal anti-CYP1A1 antibody (Daiichi Pure Chemicals, Tokyo, Japan) and a monoclonal
anti-β-actin antibody (Sigma-Aldrich), visualized with an enhanced chemiluminescence detection system or an alkaline phosphatase conjugate substrate system (Endo et al., 2008).

**Enzyme Activity Assays.** Extracts from microsomal fractions were subjected for enzyme activity assays. BaP hydroxylation was assayed as previously reported (Endo et al., 2008; Nebert and Gelboin, 1968). Briefly, 200 μg of microsomal protein was incubated in 100 mM potassium phosphate buffer (pH 7.4) containing 1 mg/ml bovine serum albumin, 80 μM BaP and 0.5 mM NADPH at 37°C for 20 minutes. The reaction was stopped by the addition of acetone/n-hexane (1:3). After the organic phase was extracted with NaOH, the concentration of 3-hydroxy-BaP was measured spectrofluorometrically with activation at 396 nm and fluorescence at 522 nm.

**Measurement of DNA Adducts.** BaP-induced DNA adducts were determined by a 32P-postlabeling method (Endo et al., 2008; Talaska et al., 1996; Uno et al., 2001). After DNA extraction from cells, hydrolysis to 3'-phosphodeoxynucleotides with micrococcal endonuclease and spleen phosphodiesterase, and removal of non-adducted 3'-phosphodeoxynucleotides with n-butanol extraction, the 3'-phosphodeoxynucleosides were labeled at the 5' positions with [32P]ATP and T4 polynucleotide kinase. Two-dimensional thin-layer chromatography on polyethylenimine cellulose sheets was used to resolve the 32P-labeled DNA adducts (Randerath and Randerath, 1964), which were then visualized and quantified by scintillation counting (Packard 1900 CA,
Specific activity of isotope-labeled ATP was expressed as relative adduct labeling values normalized with amounts of DNA in each sample.

**RNA Interference.** Small interfering RNAs (siRNAs) directed against VDR (Dharmacon M-003448) and control siRNA (Dharmacon D-001206) were purchased from Thermo Fisher Scientific (Waltham, MA). siRNA oligonucleotides were transfected into cells using DharmaFECT1 Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions.

**Electrophoretic Mobility Shift Assays.** Electrophoretic mobility shift assays (EMSAs) were performed as reported previously (Endo-Umeda et al., 2012; Yoshikawa et al., 2001). Briefly, receptor proteins were in vitro translated with a TNT Quick Coupled Transcription/Translation System (Promega Corporation). Sequences for double stranded oligonucleotides are shown in Fig. 5A. Binding reactions were performed in a buffer containing 10 mM Tris-HCl (pH 7.6), 50 mM KCl, 0.05 mM EDTA, 2.5 mM MgCl₂, 8.5% glycerol, 1 mM dithiothreitol, 0.5 μg/ml poly(dI-dC), 0.1% Triton-X100, and nonfat milk. Unlabeled probes and anti-VDR antibody (Santa Cruz Biotechnology) were used for competition experiments and supershift experiments, respectively. Samples were separated on 5% polyacrylamide gels and were visualized with autoradiography.

**Chromatin Immunoprecipitation.** Chromatin immunoprecipitation (ChIP) was performed as reported previously (Matsunawa et al., 2009; Shang et al., 2000). After nuclear proteins were cross-linked to DNA in 1% formaldehyde for 15 min, cells were
washed and lysed in lysis buffer (50 mM Tris-HCl, pH 8.1, 1% SDS, 10 mM EDTA). After sonication and removal of cellular debris, the lysates were diluted in ChIP dilution buffer (16.7 mM Tris-HCl, pH 8.1, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl). ChIP was performed with control IgG antibody, anti-AHR antibody, or anti-VDR antibody (Santa Cruz Biotechnology). DNA was purified with MonoFas DNA Purification Kit (GL Sciences, Torrance, CA). PCR was performed using GoTaq Master Mix (Promega) with the following primers: 5'-GAA CGC TGG GCG TGC AGA TGC CTC-3' and 5'-CAC TAA GGC GAT CCT AGA GGC TG-3', detecting the region -375 to -693 in CYP1A1 promoter as shown in Fig. 5A. The PCR products were separated by electrophoresis in 2% agarose gel.

**Statistical Analyses.** All values are shown as means ± S.E.M. The two-tailed, unpaired Student’s t test was performed to assess significant differences.
Results

1,25(OH)2D3 Enhances the Expression of CYP1A1 induced by BaP. We previously reported that AHR activation by BaP and TCDD enhances the expression of CYP24A1 in human monocyte/macrophage-derived THP-1 cells treated with 1,25(OH)2D3 (Matsunawa et al., 2009). To further investigate the reciprocal effects of VDR and AHR signaling, we treated monocyte-derived U937 cells and THP-1 cells with BaP and/or 1,25(OH)2D3 and examined the expression of CYP1A1, an AHR target gene that has been shown to be involved in metabolic activation and detoxification of BaP (Uno and Makishima, 2009). Treatment with BaP (0.3 μM) induced the expression of CYP1A1 in U937 cells and THP-1 cells, while 1,25(OH)2D3 (30 nM) did not show significant induction (Fig. 1A). Interestingly, CYP1A1 mRNA expression induced by BaP was further increased by co-administration of 1,25(OH)2D3 in these cells (Fig. 1A). Combined BaP and 1,25(OH)2D3 treatment also increased CYP1A1 mRNA expression to a higher degree than by BaP alone in breast cancer MCF-7 cells and in kidney epithelium-derived HEK293 cells (Fig. 1A). Previous reports have demonstrated expression of functional VDR and AHR proteins in U937 cells, THP-1 cells, MCF-7 cells and HEK293 cells (Amano et al., 2009; Campbell et al., 2000; Hayashi et al., 1995; Inaba et al., 2007; Ishizawa et al., 2008; Zhang et al., 2008). We compared mRNA and protein levels of VDR and AHR in these cell lines. VDR mRNA expression levels in U937 cells, THP-1 cells, MCF-7 cells and HEK293 cells were 1,008 ± 126 copies, 51 ± 9 copies, 690
± 88 copies and 526 ± 28 copies/μg total RNA, respectively, while those for AHR were 856 ± 244 copies, 107 ± 19 copies, 7,232 ± 922 copies and 1,900 ± 217 copies/μg total RNA, respectively (Fig. 1B). We next examined expression of nuclear VDR and AHR protein levels. As reported previously (Amano et al., 2009), VDR protein expression was observed in U937 cells and, to a lesser extent, in THP-1 cells in the absence of ligand (Fig. 1C). BaP plus 1,25(OH)2D3 increased VDR protein levels in these cells as well as in MCF-7 cells and HEK293 cells (Fig. 1C). AHR protein expression was observed in all cell lines both with and without combined BaP and 1,25(OH)2D3 treatment (Fig. 1C). There were some discrepancies between mRNA and protein levels of VDR and AHR (Fig. 1, B and C). It may be due to translational or post-translational regulation of these proteins. As reported previously (Ishizawa et al., 2008; Matsunawa et al., 2009), 1,25(OH)2D3 treatment effectively induced expression of the VDR target CYP24A1 in U937 cells and THP-1 cells (Fig. 1D), indicating that VDR functions in these cells. BaP (1 μM) did not induce CYP1A2 or CYP1B1 expression in U937 cells, and the combination of BaP and 1,25(OH)2D3 had no effect on expression of these genes (Fig. 1E). These findings indicate that combined BaP and 1,25(OH)2D3 treatment effectively induces CYP1A1 mRNA expression.

Next, we examined the effects of several concentrations of BaP in combination with 1,25(OH)2D3 on CYP1A1 mRNA expression in U937 cells. In the absence of 1,25(OH)2D3, BaP at 0.1 μM increased CYP1A1 mRNA expression, an effect not seen at
0.01 μM or 0.03 μM (Fig. 2A). Interestingly, in combination with 10 nM 1,25(OH)2D3, BaP at 0.01 μM effectively induced CYP1A1 expression. While BaP alone exhibited maximal CYP1A1 induction at 0.1 μM, the combination of 1,25(OH)2D3 further enhanced CYP1A1 expression in U937 cells treated with 0.1 μM, 0.3 μM and 1 μM BaP (Fig. 2A). 1,25(OH)2D3 alone up to 100 nM did not increase CYP1A1 expression. 1,25(OH)2D3 at 3 nM to 30 nM increased CYP1A1 expression induced by 0.3 μM BaP (Fig. 2B). Thus, enhanced BaP-induced CYP1A1 expression by 1,25(OH)2D3 is concentration-dependent.

The effects of combined BaP and 1,25(OH)2D3 on CYP1A1 protein levels and enzyme activity were examined. BaP treatment induced CYP1A1 protein expression in U937 cells and the addition of 1,25(OH)2D3 further increased CYP1A1 protein level, while 1,25(OH)2D3 alone caused modest induction (Fig. 3A). Whereas CYP1A1 proteins were detected only at low levels in THP-1 cells treated with BaP or 1,25(OH)2D3 alone, combined BaP and 1,25(OH)2D3 treatment effectively induced CYP1A1 protein expression in THP-1 cells (Fig. 3A). We examined BaP hydroxylation activity by detecting conversion of BaP to 3-hydroxy-BaP. CYP1A1, but not CYP1A2 or CYP1B1, mediate this cellular reaction (Endo et al., 2008). Cells were treated with BaP and/or 1,25(OH)2D3 for 24 hours, and microsomes were isolated for enzyme activity assays. BaP treatment effectively increased BaP hydroxylation activity in U937 cells (Fig. 3B). Interestingly, 1,25(OH)2D3 slightly increased BaP hydroxylation activity in U937 cells (Fig. 3B), consistent with CYP1A1 protein expression (Fig. 3A) but not with mRNA
expression (Fig. 1A and Fig. 2). 1,25(OH)2D3 may increase CYP1A1 protein levels and enzymatic activity through an unknown post-translational mechanism. Combined BaP and 1,25(OH)2D3 treatment further increased BaP hydroxylation activity in U937 cells (Fig. 3A). BaP treatment also increased BaP hydroxylation activity in THP-1 cells, but the enzyme activity levels were very weak when compared to U937 cells (Fig. 3B). This difference is likely due to lower CYP1A1 protein levels in THP-1 cells (Fig. 3A). Although 1,25(OH)2D3 alone was not effective, it enhanced BaP hydroxylation activity in THP-1 cells treated with BaP (Fig. 3B). Exogenous CYP1A1 expression increases and decreases BaP-DNA adduct formation in Hepa-1 cells and HepG2 cells, respectively (Endo et al., 2008; Maier et al., 2002). We next examined the effect of 1,25(OH)2D3 on BaP-DNA adduct formation. BaP-DNA adduct formation was detected in BaP-treated U937 cells and THP-1 cells, and combined 1,25(OH)2D3 treatment further increased BaP-DNA adducts in these cells (Fig. 3C). Therefore, BaP and 1,25(OH)2D3 co-treatment increases CYPIA1 protein levels, BaP hydroxylation activity and BaP-DNA adduct formation in U937 cells and THP-1 cells.

Both VDR Activation and AHR Activation are Involved in CYPIA1 Transcription. VDR is activated by LCA and its derivatives, such as LCA acetate, as well as 1,25(OH)2D3 (Ishizawa et al., 2008). LCA acetate alone did not induce CYPIA1 mRNA expression in U937 cells (Fig. 4A). Similarly to 1,25(OH)2D3, LCA acetate
further increased CYP1A1 mRNA levels induced by BaP (Fig. 4A), suggesting that CYP1A1 transcription is regulated by VDR activation. To examine the VDR dependency of CYP1A1 induction, we knocked down VDR using siRNA. VDR knockdown attenuated CYP1A1 induction by combination of BaP and 1,25(OH)2D3 in U937 cells and THP-1 cells (Fig. 4B). In addition, we examined the effect of VDR antagonists, ADTT and ADMI3 (Igarashi et al., 2007; Nakabayashi et al., 2008). Both ADTT and ADMI3 effectively reduced CYP1A1 expression induced by BaP plus 1,25(OH)2D3 in THP-1 cells (Fig. 4C). Thus, VDR activation is involved in effective CYP1A1 induction.

The effect of 1,25(OH)2D3 on CYP1A1 expression was examined in U937 cells treated with TCDD. TCDD is a high-affinity AHR ligand that is virtually not metabolized in cells (Bock and Kohle, 2006). 1,25(OH)2D3 increased CYP1A1 expression induced by TCDD (Fig. 4D). Next, we examined the effect of α-naphthoflavone, an AHR antagonist (Gasiewicz and Rucci, 1991), on CYP1A1 expression induced by BaP plus 1,25(OH)2D3 in U937 cells. The addition of α-naphthoflavone inhibited CYP1A1 induction by BaP alone and combined treatment of BaP and 1,25(OH)2D3 (Fig. 4E). These findings indicate that both AHR and VDR are necessary for CYP1A1 induction by BaP plus 1,25(OH)2D3.

**VDR-RXR Binds to the CYP1A1 Promoter.** VDR, PXR and CAR belong to the NR1I subfamily of the nuclear receptor superfamily and regulate common target genes, such as CYP3A4 (Makishima et al., 2002; Thummel et al., 2001; Xie et al., 2000). The
CAR-RXR heterodimer binds to an ER8 element in the human CYPIA1 promoter (Yoshinari et al., 2010). We performed EMSAs using oligonucleotide probes containing the ER8 element and a known xenobiotic-responsive element (XRE) (WT in Fig. 5A). The VDR-RXR heterodimer bound to isotope-labeled XRE-ER8 (Fig. 5B). Complex formation was inhibited by addition of unlabeled XRE-ER8 (Fig. 5B). Mutation of the XRE and a proximal half-site of ER8 (MT2 in Fig. 5A) inhibited binding of VDR-RXR to isotope-labeled wild-type XRE-ER8 but mutation of both hexanucleotides of ER8 (MT1 in Fig. 5A) failed to exhibit competition (Fig. 5B). Addition of anti-VDR antibody induced a supershift of the VDR-RXR complex with XRE-ER8 (Fig. 5B). The results indicate that VDR-RXR directly binds to ER8 in the CYPIA1 promoter.

Finally, we performed ChIP assays to examine direct VDR binding to the CYPIA1 promoter in cells using anti-AHR or anti-VDR antibodies and PCR for the -379 to -693 CYPIA1 promoter region, which contains the XRE (-489 to -495) and the ER8 (-506 to -525) (Fig. 5A). Six hours after ligand addition, BaP and 1,25(OH)2D3 recruited AHR and VDR, respectively, to the CYPIA1 promoter in U937 cells, and combination of these compounds did not further increase the recruitment of AHR or VDR (Fig. 5C). At 24 hours, VDR recruitment was slightly higher with BaP plus 1,25(OH)2D3 compared to treatment of 1,25(OH)2D3 alone (Fig. 5C). BaP and 1,25(OH)2D3 also induced recruitment of AHR and VDR, respectively, to the CYPIA1 promoter in THP-1 cells (Fig. 5D). Therefore, VDR regulates CYPIA1 transcription by direct binding to the promoter in
monocyte/macrophage-derived cells.
Discussion

We show here that VDR activation enhanced the CYP1A1 expression and activity induced by AHR ligands in monocyte/macrophage-derived U937 cells and THP-1 cells. EMSA and ChIP assays demonstrate that VDR binds to the ER8 motif located in the proximal promoter of the human CYP1A1 gene. CAR, another NR1I subfamily nuclear receptor, also binds to the same motif and its activation induces expression of both CYP1A1 and CYP1A2 in hepatocytes (Yoshinari et al., 2010). In contrast to CYP1A1 induction, the combined treatment of BaP and 1,25(OH)2D3 did not induce CYP1A2 expression in U937 cells (Fig. 1). While administration of BaP to mice induces Cyp1a1 mRNA levels in liver, small intestine, spleen and bone marrow, it induces Cyp1a2 mRNA expression in liver, small intestine and to a lesser degree in spleen, but not in bone marrow (Uno et al., 2006). Although the human CYP1A1 and CYP1A2 genes are located in a head-to-head orientation on chromosome 15 and share a common regulatory region (Ueda et al., 2006), transcription of these genes may be regulated by additional tissue- or cell-type-specific mechanisms. Bone marrow-derived cells may be less responsible for CYP1A2 induction.

While CAR activation induces expression of CYP1A1 and CYP1A2 independent of AHR (Yoshinari et al., 2010), use of an AHR antagonist, α-naphthoflavone, shows that the effect of 1,25(OH)2D3 on CYP1A1 induction requires AHR activation (Fig. 4). These findings indicate that VDR signaling is only effective in augmenting CYP1A1
transcription activated by AHR in monocyte/macrophage-derived cells. VDR siRNA and VDR antagonists decreased \textit{CYP1A1} expression induced by BaP plus 1,25(OH)\textsubscript{2}D\textsubscript{3} (Fig. 4), suggesting that VDR bound to the \textit{CYP1A1} promoter is functionally active. The combination of BaP and 1,25(OH)\textsubscript{2}D\textsubscript{3} did not further increase the recruitment of AHR or VDR to the \textit{CYP1A1} promoter (Fig. 5). Although AHR activation modifies the transcriptional activity of estrogen receptor through direct association, a direct interaction between AHR and VDR has not been detected (Ohtake et al., 2003). These findings suggest that AHR and VDR bind independently to the \textit{CYP1A1} promoter. The combination of ligands may effectively induce \textit{CYP1A1} mRNA expression through formation of a multimeric complex where AHR interacts indirectly with VDR via coregulatory proteins. Further studies are needed to elucidate the molecular mechanisms of CYP1A1-selective action of VDR on a regulatory region of the \textit{CYP1A1-CYP1A2} gene locus.

Increased expression of CYP1A1 by BaP plus 1,25(OH)\textsubscript{2}D\textsubscript{3} resulted in enhanced BaP-DNA adduct formation (Fig. 3). BaP is first oxidized by CYP1A1 and CYP1B1 to phenols, such as 3-hydroxy-BaP and 9-hydroxy-BaP, and epoxides, such as BaP-7,8-epoxide (Shimada and Fujii-Kuriyama, 2004). We observed enhanced BaP hydroxylation to 3-hydroxy-BaP in cells treated with BaP and 1,25(OH)\textsubscript{2}D\textsubscript{3} (Fig. 3), a finding consistent with increased CYP1A1 expression. BaP-7,8-epoxide is then metabolized by epoxide hydrolase to BaP-7,8-diol, which serves as a substrate for a
subsequent CYP-dependent oxidation, generating the toxic compound BaP-7,8-diol-9,10-epoxide (Shimada and Fujii-Kuriyama, 2004). BaP-7,8-diol is also metabolized to BaP-7,8-dione by aldoketoreductase, and BaP-1,3-, 1,6-, and 3,6-diones are thought to be formed by metabolism of BaP to phenols by CYP enzymes (Shimada, 2006). These BaP quinones are also involved in DNA adduct formation. Glutathione S-transferase suppresses BaP-induced DNA adduct formation by conjugation of reactive BaP metabolites (Uno and Makishima, 2009). The formation of BaP-induced DNA adducts is decreased in Hepa-1 c37 cells, a CYP1A1-deficient mutant clone of Hepa-1 cells (Maier et al., 2002). Stable transfection of a Cyp1a1 expression plasmid restores adduct formation to the level of parent Hepa-1 cells (Maier et al., 2002). Exogenous expression of the oncogenic AML1-ETO fusion protein in U937 cells up-regulates CYP1A1 expression and increases BaP-DNA adduct formation (Xu et al., 2007). These findings are similar to our result showing that CYP1A1 induction by BaP plus 1,25(OH)2D3 was associated with BaP-DNA adduct formation (Fig. 3C). By contrast, DNA adduct formation is increased in CYP1A1-deficient mice (Uno et al., 2004), and overexpression of CYP1A1 in hepatocytes suppresses BaP-induced DNA adduct formation (Endo et al., 2008). CYP1A2 is also involved in the suppression of BaP-DNA adduct formation but it does not cause 3-hydroxy-BaP formation (Endo et al., 2008). CYP1A2 expression was not increased in U937 cells treated with BaP plus 1,25(OH)2D3 (Fig. 1E). Insufficient expression of CYP1A2 or other detoxifying enzyme(s) may
contribute to the accumulation of BaP metabolites and to DNA adduct formation. Monocytes and monocyte-derived leukemia cells exhibit antimicrobial activity by producing reactive oxygen species in response to 1,25(OH)₂D₃ (Levy and Malech, 1991; Sly et al., 2001). BaP enhances differentiation of monocytic THP-1 cells induced by 1,25(OH)₂D₃ (Matsunawa et al., 2009). Increased reactive oxygen species associated with monocyte/macrophage differentiation may modify BaP metabolism and promote DNA adduct formation. In addition to monocytic differentiation, AHR activation enhances VDR-dependent expression of CYP24A1, which stimulates 1,25(OH)₂D₃ inactivation (Matsunawa et al., 2009). Thus, combined administration of BaP and 1,25(OH)₂D₃ enhances BaP-DNA adduct formation and 1,25(OH)₂D₃ catabolism through CYP1A1 and CYP24A1, respectively. These signaling pathways may be related to BaP-induced toxicities.

CYP1A1 is involved in the phase I metabolism of xenobiotics, such as BaP, and endogenous compounds, including estradiol and eicosanoids (Nebert and Karp, 2008; Uno and Makishima, 2005). Induced differentiation of myeloid leukemia cells by 1,25(OH)₂D₃ is associated with increased expression of enzymes involved in eicosanoid metabolism, such as arachidonate 5-lipoxygenase and prostaglandin-endoperoxide synthase 1 (Amano et al., 2009; Matsunawa et al., 2009). Prostaglandin production plays a role in inducing differentiation of myeloid leukemia cells (Rocca et al., 2004). CYP1A1 induced in monocyte-derived cells may be involved in monocyte/macrophage function.
by altering eicosanoid metabolism. Recently, AHR has been implicated in the regulation of immunity and inflammation (Kerkvliet, 2009). TCDD pretreatment enhances cholestasis-induced liver damage and proinflammatory cytokine production, phenotypes that are further exaggerated in CYP1A1/CYP1A2-null mice (Ozeki et al., 2011). The AHR-CYP1A cascade may regulate inflammatory responses in immune cells, including monocytes and macrophages. The enhancing effect of 1,25(OH)2D3 on CYP1A1 mRNA expression was also observed in breast cancer- and kidney epithelium-derived cells. Further studies are needed to elucidate the physiological role of CYP1A1 in monocytes/macrophages and other cell types.
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Authorship Contributions

*Participated in research design*: Matsunawa, and Makishima

*Conducted experiments*: Matsunawa, Akagi, Uno, and Endo-Umeda

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Footnote

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Legends for Figures

Fig. 1. Effect of BaP and 1,25(OH)_2D_3 treatment on mRNA expression of CYP1 enzymes.

(A) Combined effect of BaP and 1,25(OH)_2D_3 on CYP1A1 mRNA expression in monocytic leukemia-derived U937 cells, THP-1 cells, breast cancer MCF-7 cells, and kidney epithelium-derived HEK293 cells. Cells were cultured with 0.3 μM BaP and/or 30 nM 1,25(OH)_2D_3 for 24 hours. (B) Real-time quantitative reverse transcription-PCR analysis of VDR and AHR in U937 cells, THP-1 cells, MCF-7 cells and HEK293 cells.

(C) Western blotting analysis of VDR and AHR in U937 cells, THP-1 cells, MCF-7 cells and HEK293 cells. Cells were cultured with vehicle control or 1 μM BaP plus 30 nM 1,25(OH)_2D_3 for 24 hours. Each lane was loaded with 50 μg of nuclear proteins. (D) Effect of 1,25(OH)_2D_3 on expression of the VDR target gene CYP24A1 in U937 cells and THP-1 cells. Cells were cultured in the absence or presence of 30 nM 1,25(OH)_2D_3 for 24 hours. (E) BaP and 1,25(OH)_2D_3 are not effective in inducing CYP1A2 and CYP1B1 expression in U937 cells. Cells were cultured with 1 μM BaP and/or 30 nM 1,25(OH)_2D_3 for 24 hours. The values represent means ± S.E.M. of triplicate assays. *p < 0.05; **p < 0.01.

Fig. 2. Concentration-dependent effects of BaP (A) and 1,25(OH)_2D_3 (B) on CYP1A1 mRNA expression in U937 cells. (A) Cells were treated with the indicated concentrations of BaP in the absence or presence of 10 nM 1,25(OH)_2D_3 for 24 hours. *p < 0.05; **p <
0.01 compared with vehicle control. \( #p < 0.05; ###p < 0.001 \) compared with 1,25(OH)\(_2\)D\(_3\) alone. \( \dagger p < 0.05; \dagger \dagger p < 0.01 \). (B) Cells were treated with the indicated concentrations of 1,25(OH)\(_2\)D\(_3\) in the absence or presence of 0.3 \( \mu \)M BaP for 24 hours. \(*p < 0.05; **p < 0.01. \) The values represent means \( \pm \) S.E.M. of triplicate assays.

Fig. 3. Effect of BaP and 1,25(OH)\(_2\)D\(_3\) treatment on CYP1A1 protein expression (A), BaP hydroxylation activity (B) and DNA adduct formation (C). For Western blotting and BaP hydroxylation assays, cells were cultured with 0.3 \( \mu \)M BaP and/or 30 nM 1,25(OH)\(_2\)D\(_3\) for 24 hours. Western blotting was repeated with similar results. For DNA adduct detection, U937 cells and THP-1 cells were cultured with 0.3 \( \mu \)M BaP and/or 30 nM 1,25(OH)\(_2\)D\(_3\) for 24 hours and 48 hours, respectively. \(*p < 0.05; **p < 0.01; ***p < 0.001. \) ND, not detected. The values represent means \( \pm \) S.E.M. of triplicate assays.

Fig. 4. CYP1A1 mRNA induction requires activation of VDR and AHR. (A) CYP1A1 expression is induced by BaP plus LCA acetate. U937 cells were cultured with 0.3 \( \mu \)M BaP and/or 30 \( \mu \)M LCA acetate for 24 hours. (B, C) CYP1A1 induction by BaP plus 1,25(OH)\(_2\)D\(_3\) is decreased by VDR siRNA (B) and VDR antagonists (C). (B) U937 cells or THP-1 cells were transfected with control siRNA (Cont-si) or VDR siRNA (VDR-si) and treated with vehicle control or 1 \( \mu \)M BaP plus 30 nM 1,25(OH)\(_2\)D\(_3\). (C) THP-1 cells were cultured with vehicle control or 1 \( \mu \)M BaP plus 30 nM 1,25(OH)\(_2\)D\(_3\) in combination
with vehicle control (Cont), 1 μM ADTT or 1 μM ADMI3. (D) CYPIA1 expression is induced by TCDD plus 1,25(OH)2D3. U937 cells were cultured with 0.1 nM TCDD and/or 10 nM 1,25(OH)2D3 for 24 hours. (E) CYPIA1 induction by BaP plus 1,25(OH)2D3 is inhibited by α-naphthoflavone. U937 cells were cultured with 1 μM BaP and/or 10 nM 1,25(OH)2D3 in the absence or presence of 10 μM α-naphthoflavone (aNF) for 24 hours. The values represent means ± S.E.M. of triplicate assays. *p < 0.05; **p < 0.01; ***p < 0.001.

Fig. 5. An ER8-type vitamin D response element in the human CYPIA1 promoter. (A) Schematic illustration of the human CYPIA1 promoter. An ER8 motif (-506 to -525) is located near a XRE (-489 to -495). Mutations in oligonucleotides used for EMSAs are shown as MT1 and MT2, compared with the wild-type sequence (WT). PCR primers for ChIP assays amplify fragments (-379 to -693) containing the XRE and ER8 motifs. (B) EMSAs show direct binding of VDR-RXR to the human CYPIA1 ER8 motif. WT oligonucleotides (WT) shown in (A) were labeled and incubated with VDR and/or RXRα proteins. Preincubation with 100-fold molar excess of unlabeled WT, MT1 or MT2 oligonucleotides, shown in (A), and anti-VDR antibody was performed for competition and supershift, respectively. (C) U937 cells were cultured with 0.3 μM BaP and/or 30 nM 1,25(OH)2D3 for 6 hours and 24 hours and were subjected to ChIP analysis. PCR results using control IgG, anti-VDR antibody and anti-RXRα antibody were shown in agarose
gel electrophoresis. (D) THP-1 cells were cultured with 0.3 μM BaP and/or 30 nM 1,25(OH)₂D₃ for 6 hours and were subjected to ChIP analysis. EMSAs and ChIP assays were repeated with similar results.
Figure 1

A. CYP1A1 mRNA

B. VDR mRNA

C. HEK293 cells

D. CYP24A1 mRNA

E. U937 cells

F. CYP1A2 mRNA

G. CYP1B1 mRNA
Figure 5, A and B

A

CYP1A1

-379

-489

-495

-506

-525

-693

XRE

ER8

CYP1A2

+1

WT

CCGGC TCGCGTG AGAACGCCTG CGACC CAGCCCTG AGGTCA CGGGG

MT1

CCGGC TCGCGTG AGAACGCCTG CTACAC CAGCCCTG ATGTAA CGGGG

MT2

CCGGC TCTATT AGAACGCCTG CTACAC CAGCCCTG AGGTCA CGGGG

B

VDR

- - + + + + + +

RXR

- + - + + + + +

unlabeled WT

- - - - - + - -

unlabeled MT2

- - - - - + - -

unlabeled MT1

- - - - - - + -

anti-VDR antibody

- - - - - - - +

supershift

VDR-RXR

free probe
Figure 5, C and D