



Retinoids activate RXR/CAR-mediated pathway and induce CYP3A

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ABSTRACT

Retinoids and carotenoids are frequently used as antioxidants to prevent cancer. In this study, a panel of retinoids and carotenoids was examined to determine their effects on activation of RXR/CAR-mediated pathway and regulation of CYP3A gene expression. Transient transfection assays of HepG2 cells revealed that five out of thirteen studied retinoids significantly induced RXR α /CAR-mediated activation of luciferase activity that is driven by the thymidine kinase promoter linked with a PXR binding site in the CYP3A4 gene [tk-(3A4)₃-Luc reporter]. All-trans retinoic acid (RA) and 9-cis RA were more effective than CAR agonist TCBOPOP in induction of the tk-(3A4)₃-Luc reporter. Addition of retinoid and TCBOPOP further enhanced the inducibility and the induction was preferentially mediated by RXR α /CAR and RXR γ /CAR heterodimer. Chromatin immunoprecipitation assay showed that retinoids recruit RXR α and CAR to the proximal ER6 and distal XREM nuclear receptor response elements of the CYP3A4 gene promoter. The experimental data demonstrate that retinoids can effectively regulate CYP3A gene expression through the RXR/CAR-mediated pathway.

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1. Introduction

The cytochrome P450 enzymes (CYPs) 2B and 3A subfamilies are the important enzymes involved in the oxidative metabolism of endogenous and exogenous compounds. Their expression is highly inducible by drugs as well as by environmental pollutants. Pregnane X receptor (PXR) and constitutive androstane receptor (CAR), which heterodimerize with retinoid X receptor (RXR), are the principal regulators of hepatic CYP3A and CYP2B gene expression, respectively [1,2]. Studies have revealed that the cross-talk between PXR and CAR results in reciprocal activation of CYP2B and CYP3A genes [3–5]. Thus, dexamethasone, a PXR ligand, can induce Cyp2b10 in CAR-null mice, and phenobarbital (PB), a CAR activator, induces Cyp3a11 in PXR-null mice [4–6]. In addition to NR1 and NR2 sequences in the CYP2B promoter, CAR binds to the proximal response elements located in the CYP3A4 gene promoter region and can transcriptionally regulate CYP3A4 gene expression

[4,7,8]. Moreover, human PXR and human CAR can also bind and activate the NR3 site (DR4) in the CYP2B6 XREM (xenobiotic-responsive enhancer module located in the distal region) and the DR3 (direct repeats spaced by three nucleotides) and ER6 (an everted repeat with a 6-nucleotide spacer) sites in the CYP3A4 XREM [8,9].

Retinoids belong to the polyisoprenoid lipid family, which includes vitamin A (retinol) and its natural and synthetic analogs. In human, dietary animals and plants are the main sources of retinoids. A number of retinoids in this class possess anti-proliferative, differentiation, and pro-apoptotic effects [10]. Retinoids are used clinically to treat acute promyelocytic leukemia, skin cancer, Kaposi's sarcoma, and cutaneous T-cell lymphoma as well as acne and psoriasis [11]. The action of retinoids is mediated via activation of retinoid X receptors (RXRs). At least one-third of the nuclear hormone receptor superfamily members form dimmer with RXRs. RXR agonists activate certain RXR heterodimer complexes, which are termed permissive, in contrast, other non-permissive complexes do not respond to RXR agonists.

Retinoids exert complex effects on CYP gene expression. Several groups have reported the inductive effects of retinoids on CYP gene expression [12–14]. Conversely, it has been shown that 9-cis and all-trans RA repress phenobarbital-induced CYP2B1/2 in primary cultured rat hepatocytes and inhibit TCBOPOP-dependent CAR trans-activation of Cyp2b10 in mouse primary hepatocytes [15,16]. In both cases, the authors proposed that the inhibitory effect of RA was due to competition between CAR and RAR β for

Abbreviations: ALHD, aldehyde dehydrogenases; CYP, cytochrome P450; RA, retinoic acid; All-trans RP, all-trans retinol palmitate; PXR, pregnane X receptor; CAR, constitutive androstane receptor; RXR, retinoid X receptor; mCAR, mouse constitutive androstane receptor; mRXR, mouse retinoid X receptor; ER, everted repeat; DR, direct repeat; NR, nuclear receptor; NR1, NR2, nuclear receptor site1, 2; XREM, xenobiotic-responsive enhancer module; RT, reverse transcription; Gadph, glyceraldehyde-3-phosphate dehydrogenase; TCBOPOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene.

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binding to RXR. If this is the case, RA should also be able to inhibit other nuclear receptor-mediated pathways such as PXR/RXR. However, we recently reported that retinoids are capable of activating the RXR/hPXR-mediated pathway and RXR/VDR-mediated pathway leading to CYP3A4 induction in human hepatoma cells and mouse hepatocytes [17,18]. Thus, it seems RXRs are permissive partners for activation of RXR/PXR-mediated CYP3A gene expression. The CAR/RXR heterodimers are neither strictly permissive nor non-permissive for RXR signaling [19]. Instead, the effects of retinoids on activation of RXR/CAR are distinct in different contexts. These findings suggest that retinoids may have complex and variable effects on xenobiotic responses [19]. The current study examines the effects of retinoids on activation of CAR-mediated pathways in regulation of CYP3A and CYP2B gene expression.

2. Experimental

2.1. Materials

All-trans RA, 9-cis retinal, 13-cis retinol, 9-cis RA, all-trans retinol palmitate (all-trans RP), β -carotene, lycopene, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), 4-(E-2-[5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl]-1-propenyl) benzoic acid (TTNBP), and sterile dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO). Retinol acetate, 13-cis retinal, and fenretinide were purchased from Toronto Research Chemicals. Lutein was purchased from US Biological, and 13-cis RA was obtained from BIOMOL Research Laboratories.

2.2. Cell culture and transient transfection

CV-1 cells were maintained in Minimum Essential Medium (Mediatech, Herndon, VA, USA). HepG2 cells were cultured in Dulbecco's Modification of Eagle's Medium (Mediatech, Herndon, VA, USA). The media were supplemented with 10% charcoal-stripped fetal calf serum (Biomed, Foster City, CA, USA). Cells were cultured at 37 °C in 5% CO₂ atmosphere with a relative humidity of 95%. Cells were plated onto 24-well plates with a cell density of approximately 8×10^4 cells/well (CV-1 cells) and 2.5×10^5 cells/well (HepG2 cells). The plated cells were cultured overnight and then transfected using Lipofectamine (Invitrogen, Carlsbad, CA, USA) for CV-1 cells or Eugene 6 (Roche Diagnostics) for HepG2 cells with a mixture containing the tk-(3A4)₃-Luc reporter construct (300 ng), mRXR α and/or mCAR expression plasmid (50 ng each), and the internal control plasmid pRL-SV40 (10 ng). The pRL-SV40 renilla luciferase expression plasmid (Promega, Madison, WI, USA) was used for co-transfection as an internal control for normalization of transfection efficiency. The total amount of plasmid DNA was adjusted to 410 ng by addition of the control plasmid DNA lacking the cDNA. The tk-(3A4)₃-Luc reporter construct (provided by Dr. Wen Xie, University of Pittsburgh, Pittsburgh, PA, USA), containing three copies of an everted repeat with a everted repeat 6-nucleotide spacer (ER6) element from the CYP3A4 gene, was used as a reporter. Expression plasmids of mouse RXR α , β , or γ (gifts from Dr. Ronald Evans, Howard Hughes Medical Institute, CA, USA), mRXR α _{Y402A} (a gift from Dr. Hinrich Gronemeyer, Institut de Génétique et de Biologie Moléculaire et Cellulaire, France), and mouse CAR (a gift from Dr. Wen Xie) were used for co-transfection as indicated. After transfection, cells were treated with retinoids (10 μ M). Fresh medium and retinoids or TCPOBOP were provided every 24 h. After 48 h treatments, cells were harvested and luciferase assays were performed according to the manufacturer's protocol (Promega, Madison, WI, USA).

2.3. Isolation of mouse hepatocytes

The C57BL/6 mice were housed at 22 °C with a 12/12-h light/dark cycle and provided food and water *ad libitum*. All procedures were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Kansas University Medical Center Institutional Animal Care and Use Committee. Hepatocytes were isolated from 3-month-old mice, weighing 20–28 g using a modified *in situ* two-step collagenase perfusion method [20]. Briefly, livers were perfused *in situ* via the portal vein, first with calcium and magnesium free Hank's balanced salt solution (HBSS–; Invitrogen, Carlsbad, CA, USA) containing 0.5 mM EGTA and 10 mM HEPES (Invitrogen, Carlsbad, CA, USA) for 6–8 min, and then with HBSS with calcium and magnesium (HBSS+; Invitrogen) containing 10 mM HEPES, 0.5 mg/ml collagenase (Sigma–Aldrich Co., St. Louis, MO) and 0.05 mg/ml soybean type IIS trypsin inhibitor (Sigma–Aldrich Co., St. Louis, MO) for 6–7 min, at a flow rate of 10 ml/min. Perfused livers were gently isolated, decapsulated on ice, and dispersed in ice-cold HBSS–. Dispersed cells were filtered through 100 μ m nylon meshes, rinsed, suspended in ice-cold 35% (v/v) Percoll (Amersham Biosciences, Piscataway, NJ, USA) and centrifuged at $150 \times g$ for 10 min at 4 °C. Hepatocytes were rinsed and suspended in William's E culture medium (Sigma–Aldrich Co., St. Louis, MO) supplemented with 10 mM HEPES buffer (pH 7.4), 10% fetal calf serum (Biomed, Foster City, CA, USA), 2.5 μ g/ml insulin (Sigma–Aldrich Co., St. Louis, MO), 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were seeded in 24-well type I collagen-coated plates at 37 °C in 5% CO₂ with a relative humidity of 95%. Initial cell viability assessed by 0.4% Trypan blue stain (Sigma–Aldrich Co., St. Louis, MO) exclusion was greater than 80%. After 48-h culture, cells were treated with different compounds for 48 h. Fresh medium with retinoids or TCPOBOP were provided after the initial 24 h treatment.

2.4. Real-time PCR

Hepatocytes were harvested after 48 h treatments and total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from total RNA (1 μ g) using random primer and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cyp3a11 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) TaqMan PCR primers and fluorescent probes (Sigma–Aldrich Co., St. Louis, MO) were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). The sequences are shown as follows, in the order of forward primer, reverse primer, and probe (FAM, 5-carboxyfluorescein; BHQ1, Black Hole Quencher 1): Cyp3a11, 5'-TCACAGACCAGAGACGATTAAGA-3', 5'-CCCGCCGTTTGTGAAG-3', 6FAM-TGTGCTAGTGAAGGAATGTTTCT-BHQ1; and GAPDH, 5'-TGTGTCCGTCGTGGATCTGA-3', 5'-CTGCTTCACCACCTTCTGA-3', 6FAM-CCGCCTGGAGAACTGCCA-BHQ1. To avoid potential genomic DNA contamination, 5' and 3' primers were designed to span exon–exon junctions. Moreover, the primers and probe were confirmed to be specific using BLAST. TaqMan PCR assays were performed in 96-well optical plates on an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Cycling parameters for each of the PCR reactions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Cyp3a11 mRNA level was normalized against that of mouse GAPDH. Fold induction values were calculated using $\Delta\Delta C_t$ method according to manufacturer's instructions.

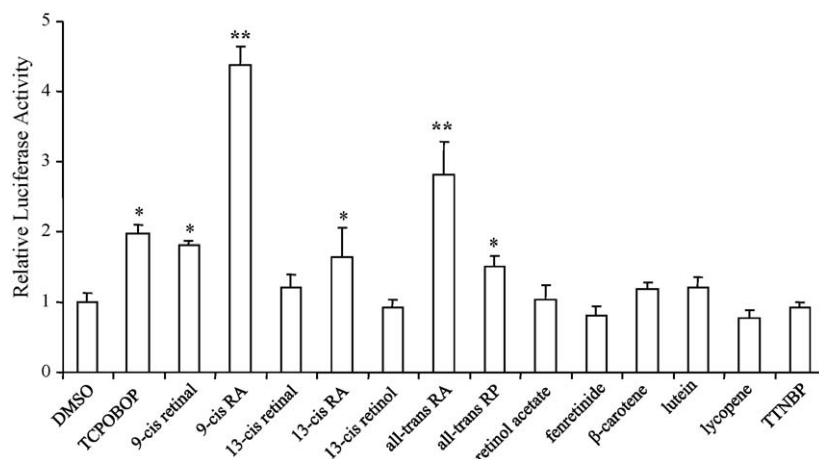


Fig. 1. Activation of the RXR α /CAR-mediated pathway by retinoids. HepG2 cells were transiently transfected with mRXR α and mCAR expression plasmids (50 ng each), the tk-(3A4)₃-Luc reporter construct (300 ng), and the renilla luciferase expression vector (10 ng). The transfected cells were treated with TCPOBOP (10 μ M), the indicated retinoids (10 μ M), or DMSO (0.1%) for 48 h and then assayed for luciferase activity. The results are expressed as relative fold changes of luciferase activity to DMSO control. Each value represents the mean \pm SD of three independent experiments. * P < 0.05, ** P < 0.01, compared to control (DMSO).

2.5. Chromatin immunoprecipitation (ChIP) assay

HepG2 cells were grown to 80% confluence in 6-well plates then transfected with mCAR and mRXR α expression plasmids (0.2 μ g). Following the treatment of retinoids or TCPOBOP for 48 h, the cells were harvested for ChIP assay using a ChIP Assay kit (Upstate Biotechnology, Lake Placid, NY, USA) according to the manufacturer's protocol. Briefly, cells were washed twice with PBS and cross-linked with 1% formaldehyde at room temperature for 10 min, then sonicated on ice with 10-s ultrasound bursts generated by a Model 500 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA, USA) at 10% power for a total of 1 min. One-tenth of the chromatin solution was reserved for subsequent amplification of total input. Soluble chromatin was immunoprecipitated with antibodies against RXR α , CAR or nonimmune rabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and then incubated with protein A-agarose beads. The beads were washed and the samples were eluted. Cross-links were reversed by heating samples at 65 $^{\circ}$ C overnight and DNA was purified using a Qiaquick Spin Column (QIAGEN, Valencia, CA, USA). To identify the immunoprecipitated DNA fragments, PCR was performed using specific primers flanking either the proximal ER6 region (–281 to –80; sense: 5'-GGCGATTTAATAGATTTTATGC-3'; antisense: 5'-TGCTCTGCCTGCAGTTGGAA-3') or distal XREM region (dDR3/ER6; –7771 to –7562; sense: 5'-CCCAATTAAAGGTCATAAA-3'; antisense: 5'-CAGAAGTTCAGCTGTGATTC-3') of CYP3A4 promoter. The number of amplification cycles used for each target gene was empirically determined. Amplified fragments were analyzed on a 2% agarose gel. A gel-imager with Quantity One software version 4.5.0 (Bio-Rad Laboratories, Hercules, CA, USA) was used to visualize and quantitate the intensity of the bands.

3. Results

3.1. Retinoids trans-activate the RXR α /CAR-mediated pathway

To determine whether retinoids can activate RXR α /CAR-mediated pathway and regulate CYP3A gene expression, transient transfection assays were performed using HepG2 cells. We used HepG2 cells because this cell line expresses low level of CAR, and endogenous CYP2B6 mRNA cannot be induced by TCPOBOP [21]. HepG2 cells were transfected with CAR and RXR α expression plasmids and the reporter construct tk-(3A4)₃-Luc, which contains three copies of the ER6 motif located in the regulatory region of the

CYP3A4 gene. Fig. 1 shows that CAR agonist TCPOBOP, which typically induces CYP2B gene expression, induced tk-(3A4)₃-Luc reporter activity by twofold in the presence of CAR and RXR α expression plasmids. This result is consistent with published findings [7,19] and indicates cross-talk between CAR and PXR in activating CYP3A. Among the thirteen retinoids and carotenoids studied, five increased tk-(3A4)₃-Luc activity in HepG2 cells when CAR and RXR α were expressed. There was no induction when CAR and RXR α were not included in the transfection (data not shown). 9-Cis RA (4.4-fold) and all-trans RA (2.8-fold) were more effective than TCPOBOP and demonstrated the highest inducibility. All-trans retinol palmitate (all-trans RP), 9-cis retinal, and 13-cis RA moderately induced reporter activity with a similar induction fold as TCPOBOP.

Combination treatments were performed to test the interaction between retinoids and TCPOBOP (Fig. 2). The fold induction was further increased when both retinoids and TCPOBOP were used to treat the cells in comparison with when single chemical was used. For example, the fold induction was 2.0, 4.4, and 9.4 when TCPOBOP, 9-cis RA, and combination of both were used to treat the cells, respectively.

3.2. Retinoids induce Cyp3a11 mRNA

Retinoid-mediated Cyp3a11 induction was confirmed using mouse primary hepatocytes by real-time PCR. The expression of Cyp3a11 mRNA was monitored in primary hepatocytes treated with TCPOBOP, retinoids, and combination of TCPOBOP plus retinoids. As anticipated, basal Cyp3a11 mRNA was detectable in mouse primary hepatocytes, and Cyp3a11 mRNA was induced 10.5-fold by TCPOBOP (Fig. 3). The five retinoids which induced CAR/RXR-mediated activation of tk-(3A4)₃-Luc also increased Cyp3a11 mRNA levels in primary mouse hepatocytes. However, the relative strength of these retinoids in the activation of tk-(3A4)₃-Luc in HepG2 cells was different from the induction of Cyp3a11 mRNA in primary mouse hepatocytes. This is probably due to the presence of different levels of cofactors or enzymes in these two types of cells. 9-Cis retinal, which only moderately trans-activated tk-(3A4)₃-Luc reporter in HepG2 cells, exhibited the highest Cyp3a11 mRNA induction in mouse primary hepatocytes. Consistent with transient transfection assay results, the levels of Cyp3a11 mRNA were further increased when both retinoids and TCPOBOP were utilized than when a single agent was used (Fig. 3). We cannot rule out the possibility that retinoids might activate

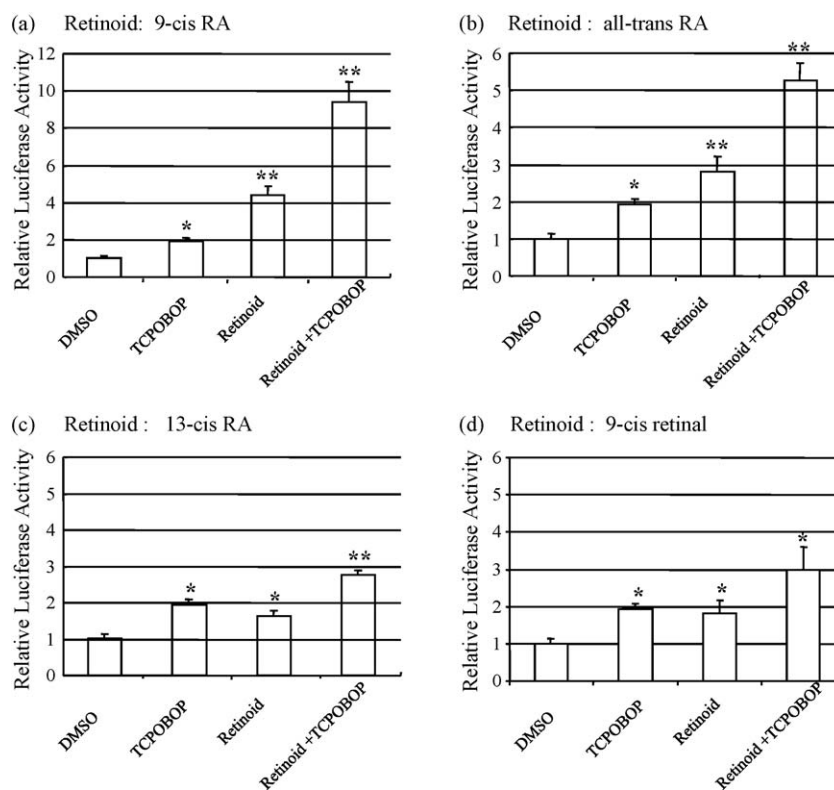


Fig. 2. The combination effects of retinoids plus TCPOBOP on activation of RXR α /CAR activation. HepG2 cells were transiently transfected with the mRXR α and mCAR expression plasmids (50 ng each), the tk-(3A4)₃-Luc reporter construct (300 ng), and the renilla luciferase expression vector (10 ng). Cells were treated with DMSO (0.1%), retinoid (Re, 10 μ M), TCPOBOP (10 μ M), or their combination for 48 h. Then cells were harvested for luciferase assays. Each value represents the mean \pm SD of three independent experiments. * P < 0.05, ** P < 0.01, compared to control (DMSO).

nuclear receptors other than RXR/CAR to induce Cyp3a11 expression. However, the fold induction was much greater using a combination of CAR agonist and retinoids than when a single chemical was used, suggesting that retinoids could further activate TCPOBOP-activated RXR/CAR signaling pathway. The experimental data, therefore, strongly suggest that retinoids play a role to activate the RXR/CAR signaling pathway and induce Cyp3a11 gene expression in mouse primary hepatocytes. On the other hand, the

retinoids employed were ineffective in inducing the levels of Cyp2b10 mRNA (data not shown).

3.3. Differential activation of RXR isoforms and CAR by retinoids

To understand the differential role of RXR α , β , and γ in retinoid-mediated CYP3A induction through the CAR signaling pathway, expression plasmids of RXR α , β , γ , and CAR as well as tk-(3A4)₃-Luc reporter were transfected into HepG2 cells. The cells were treated with DMSO, TCPOBOP, and retinoids for 48 h after transfection. The results revealed that expression of RXR α , β , and γ in HepG2 cells leads to differential induction of luciferase activity in response to retinoids and TCPOBOP (Fig. 4). 9-Cis retinal, 9-cis RA, 13-cis RA, all-trans RA, and all-trans RP preferentially activated the RXR α /CAR- and RXR γ /CAR-, but not RXR β /CAR-mediated pathways. Only 9-cis RA and all-trans RA could modestly induce the luciferase activity mediated by RXR β /CAR. Fenretinide, a synthetic retinoid, did not induce luciferase activity through any of the RXR isoforms.

3.4. Retinoids differentially activate the RXR α homodimers and RXR α /CAR heterodimers

To investigate the role of RXR α homodimer and RXR α /CAR heterodimer in activation of tk-(3A4)₃-Luc reporter, CV-1 cells, which have low levels of endogenous nuclear receptors, were transfected with the RXR α expression plasmid and the tk-(3A4)₃-Luc reporter construct in the absence or presence of a CAR expression plasmid. Transfected cells were treated with DMSO, TCPOBOP, or retinoids for 48 h. In the absence of CAR, TCPOBOP did not induce the reporter activity, suggesting that the level of endogenous CAR in CV-1 cells were too low to activate tk-(3A4)₃-

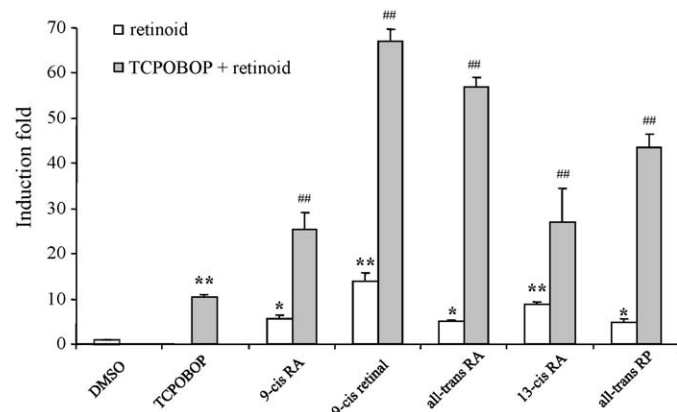


Fig. 3. The effect of retinoids and TCPOBOP on Cyp3a11 gene expression in primary mouse hepatocytes. Forty-eight hours after plating, mouse hepatocytes were treated with DMSO (0.1%), retinoids (10 μ M), TCPOBOP (10 μ M), or their combination for 48 h. Total RNA was extracted; GAPDH and Cyp3a11 mRNA levels were quantified by TaqMan real-time PCR. The results are expressed as relative fold changes of compound-treated to DMSO-treated control. Each value represents the mean \pm SD of each treatment in triplicate. * P < 0.05, ** P < 0.01, compared to DMSO, ### P < 0.01, compared to TCPOBOP.

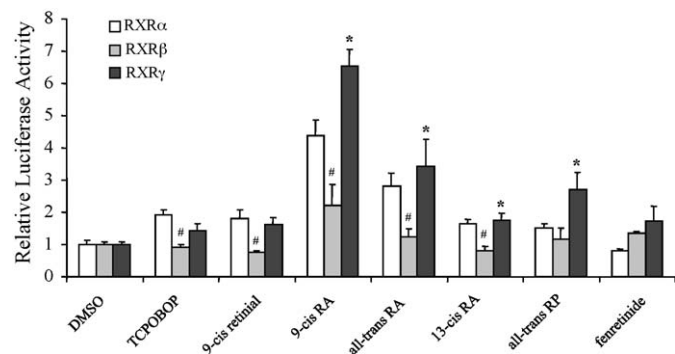


Fig. 4. Differential activation of RXR isoforms and CAR by retinoids in HepG2 cells. HepG2 cells were transiently transfected with the mCAR expression plasmids (50 ng), one of the mRXR isoforms (α , β , and γ) (50 ng), the tk-(3A4)₃-Luc reporter construct (300 ng), and the renilla luciferase expression vector (10 ng). Cells were treated with DMSO (0.1%), retinoid (10 μ M), TCPOBOP (10 μ M) for 48 h and then assayed for luciferase activity. Each value represents the mean \pm SD of three independent experiments. * P < 0.05, compared to RXR α within each treatment group. # P < 0.05 compared to RXR β within each treatment group.

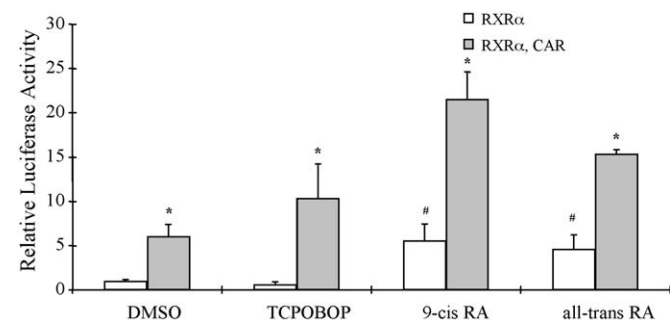


Fig. 5. Retinoids preferentially activate RXR α /CAR heterodimer rather than RXR α homodimer. CV-1 cells were transiently transfected with the tk-(3A4)₃-Luc reporter construct (300 ng), the renilla luciferase expression vector (10 ng), and the mRXR α and mCAR expression plasmids as indicated (50 ng each). Cells were treated with DMSO (0.1%), TCPOBOP (10 μ M), or retinoids (10 μ M) for 48 h. Cells were then harvested for luciferase assays. Each value represents the mean \pm SD of three independent experiments. * P < 0.05, compared to RXR α within each treatment group. # P < 0.05, compared to control (DMSO) treatment group without CAR expression plasmid.

Luc (Fig. 5). Expression of RXR α alone in the cells treated with 9-cis RA or all-trans RA induced the reporter activity about fivefold in CV-1 cells. When both RXR α and CAR were expressed, luciferase activity was induced by DMSO treatment (6.0-fold) indicating the constitutive activation nature of CAR. In addition, all-trans RA

(15.3-fold) and 9-cis RA (21.6-fold) were able to further induce tk-(3A4)₃-Luc reporter activity when both RXR α and CAR were expressed in the CV-1 cells (Fig. 5).

A mutant RXR α plasmid mRXR α _{Y402A}, which preferentially forms homodimers rather than heterodimers [22], was used to confirm that retinoids preferentially activate the RXR α /CAR heterodimer-mediated pathways. Transfection of wild type RXR α or mRXR α _{Y402A} produced over twofold induction of the reporter activity upon 9-cis RA treatment of HepG2 cells. However, all-trans RA, which could induce tk-(3A4)₃-Luc activity in CV-1 cells, neither activated RXR α homodimer nor mRXR α _{Y402A} homodimer in HepG2 cells (Fig. 6a). In comparison with transfection of RXR α and CAR expression plasmids into cells in which retinoids significantly induced luciferase activity, expression of mRXR α _{Y402A} and CAR significantly reduced retinoid-mediated induction of luciferase activity (Fig. 6b). These findings indicated that it is the heterodimer of RXR α /CAR that mediates retinoid-induced tk-(3A4)₃-Luc activity.

3.5. Retinoids recruit RXR α and CAR to the promoter region of the CYP3A4 gene

ChIP assay showed that 9-cis retinal, 9-cis RA, and TCPOBOP alone could recruit CAR and RXR α to the dXREM and ER6 in the regulatory region of the CYP3A4 gene. The recruitment was further enriched when both retinoid and TCPOBOP were used (Fig. 7). Nonspecific IgG control did not immunoprecipitate appreciable amounts of promoter DNA.

These results demonstrate that 9-cis retinal and 9-cis RA are effective in recruiting RXR α /CAR to the nuclear receptor response elements in the CYP3A4 gene.

4. Discussion

The nuclear receptor CAR has been shown to be responsible for many important xenobiotic responses [23]. It functions as a heterodimeric partner of RXR and can recruit coactivators in the presence and absence of ligands [24,25]. Besides CYP2B, CAR has been reported to directly regulate the transcriptional activity of the CYP3A4 gene both *in vitro* and *in vivo* [8]. Recently, Faucette et al. [26] reported that CAR exhibits a weak induction of CYP3A4 relative to CYP2B6 because of its weak binding and functional activation of the CYP3A4 ER6. The current paper demonstrates that several retinoids can activate the RXR/CAR-mediated pathway and induce CYP3A expression. Moreover, a strong induction is achieved with combination treatment of TCPOBOP and retinoids, which implicates the importance of CAR in regulating CYP3A gene expression.

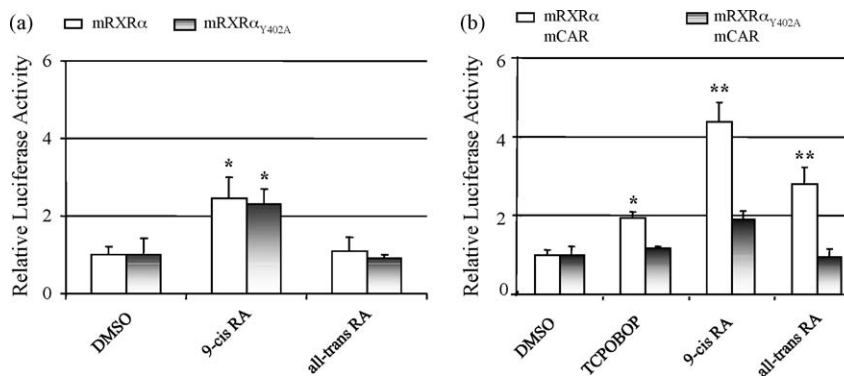


Fig. 6. The differential effects of RXR α homodimer and RXR α /CAR heterodimer on retinoid-induced tk-(3A4)₃-Luc activity in HepG2 cells. HepG2 cells were transiently transfected with the tk-(3A4)₃-Luc reporter construct (300 ng), the renilla luciferase expression vector (10 ng), and (a) mRXR α or mRXR α _{Y402A} (50 ng each), or (b) mCAR plus mRXR α or mRXR α _{Y402A} (50 ng each) expression plasmid. Cells were treated with indicated RA (10 μ M) for 48 h, and then harvested for luciferase assays. Each value represents the mean \pm SD of three independent experiments. * P < 0.05, ** P < 0.01, compared to control (DMSO).

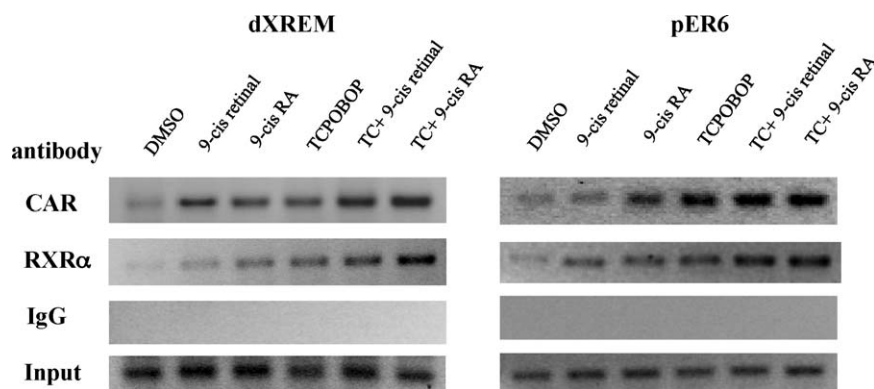


Fig. 7. Recruitment of CAR and RXR α to the CYP3A4 promoter by retinoids. Cells were treated with vehicle (0.1% DMSO), 9-cis retinal, 9-cis RA, TCPOBOP (TC), or their combinations for 48 h and then subjected to ChIP analysis using antibodies against CAR, RXR α , or IgG. DNA precipitates were isolated and PCR was used to analyze the DNA fragments containing distal XREM (left panel) or proximal ER6 (right panel) response element. Ten percent of the total cell lysate was used as input.

Our transfection data show retinoids induce higher or similar reporter activity compared with the typical CAR agonist TCPOBOP. These findings are in agreement with the literature. Previous studies show a synthetic RXR agonist, LG1069, activates a CYP3A reporter containing an ER6 element through RXR/CAR-mediated pathway [19]. In addition, our data also show that retinoids as well as TCPOBOP induce Cyp3a11 gene expression in mouse primary hepatocytes suggesting the role retinoids play in the induction of Cyp3a11 in normal hepatocytes. It is known that PXR [4,8], CAR [27], and VDR [28,29] can directly regulate CYP3A expression *in vivo*. Thus, we cannot exclude the possibility that these nuclear receptors might also participate in retinoid-mediated Cyp3a11 induction in mouse primary hepatocytes. However, the role of CAR in mediating retinoid-induced Cyp3a11 expression is evidenced by enhanced Cyp3a11 expression when a combination of retinoids and TCBOPOP were used as well as in the transient transfection experiment using CV-1 cells, which have low endogenous nuclear receptors. It has been shown that the addition of 9-cis RA and TCPOBOP simultaneously can increase the affinity for coactivators by improving the entropic component of binding [30]. Thus, the enhanced induction of Cyp3a expression by combination treatment can be due to an increase in binding affinity of a cofactor or recruitment of additional and/or different cofactors. Moreover, the ChIP assay data also showed combinational treatment of retinoids and TCPOBOP enhanced the recruitment of CAR and RXR α to the regulatory region of the CYP3A4 gene. However, it should be noted that the expression of exogenously expressed receptors may be much greater than what is found in normal cells, thus the results of modulated CYP3A4 expression will need to be confirmed in normal cells (i.e., primary hepatocytes) or *in vivo*.

In primary hepatocytes, the induction effects of certain retinoids are different from those observed in the HepG2 cells. All-trans RP, 9-cis retinal, and 13-cis RA, which only moderately activate luciferase reporter activity in HepG2 cells, highly induce Cyp3a11 mRNA level in mouse primary hepatocytes. This discrepancy may be due to differential retinoid metabolism in primary mouse hepatocytes and established human cell line. Retinyl palmitates, retinol, and retinal can be converted into retinoic acid by aldehyde dehydrogenases (ALHDs), CYP2C19, CYP3A4, and CYP1A1/2 in hepatocytes. Primary hepatocytes maintain most phase I and phase II enzymes necessary for retinoid metabolism, while cell lines may express a limited range of CYP450 and conjugation enzymes [31]. The difference in expression of transcription cofactors in these two cell types might also contribute to differential inducibility.

Though the activation of CAR may aid in the clearance of the toxic xenobiotics and endogenous molecules, its activity can also be deleterious, as CAR-mediated induction of cytochrome P450

contributes to cocaine-induced hepatotoxicity [32]. CAR has also been proven to be responsible for the induction of hepatomegaly and liver tumors by xenobiotic stresses [23,33,34]. Furthermore, the interindividual variations in CAR activity have a significant impact on the metabolism of a wide range of pharmacological agents and other foreign compounds, and could be the basis for the relatively rare but clinically significant hepatotoxicity [35]. In this sense, further investigation of the activation of downstream target genes of CAR by retinoids may facilitate better understanding of the diverse effects of retinoid therapy and may help avoid adverse drug–drug interactions.

Retinoids are used as cancer therapeutic and chemopreventive agents. They exert multiple biological effects due to their diverse structures, nuclear receptor binding affinities, and toxicity profiles [10]. The concentration of retinoids used in our experiments might not be physiologically relevant. However, it is likely to have a pharmacological or potential toxicological effect when high dose of retinoids are used. The rapid resistance of therapeutic doses of retinoids has not been studied [36]. Some of the CYP enzymes under retinoid-mediated transcriptional control [12,13] are themselves responsible for retinoid metabolism [37,38]. Thus, induction of CYP3A by retinoids may enhance the metabolism of retinoids themselves, which in turn may partially account for the development of retinoid resistance.

CYP3A4, the predominant CYP gene expressed in liver and small intestine, is involved in the metabolism of approximately 60% of all therapeutic drugs and is reported to be involved in many clinically important drug interactions [39,40]. CYP3A4 expression is affected by factors such as hormonal status, nuclear receptor activity, xenobiotic exposure, and possibly by genetic polymorphisms. We show enhanced induction of CYP3A by retinoids and CAR agonists. Increased expression of CYP3A4 may produce adverse drug–drug interactions [40]. While this effect could improve the clearance of potentially toxic compounds, it could also lead to reduction in treatment efficacy by rapidly decreasing drug concentrations below therapeutic effect ranges. Additionally, CYP3A up-regulation may cause acute toxicity through production of detrimental metabolites. The xenobiotic induction of drug-metabolizing enzymes underlies many reported drug interactions and is vital for patients subjected to combination drug therapy [39]. Our findings provide insights into the appropriate therapeutic use of retinoids during clinical treatment.

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