Biochemical Pharmacology 79 (2010) 270–276

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Retinoids activate RXR/CAR-mediated pathway and induce CYP3A

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ARTICLE INFO

Article history:
Received 19 June 2009
Accepted 11 August 2009

Keywords:
RXR
CAR
Retinoid
CYP3A
Nuclear receptor

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 RXRα/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 RXRα/CAR and RXRγ/CAR heterodimer. Chromatin immunoprecipitation assay showed that retinoids recruit RXRα 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). Atleast 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 TCPOBOP-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 RARβ 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; TCPOBOP, 1,4-bis[2-(3,5-dichloro-pyridyloxy)]benzene.

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0006-2952/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.bcp.2009.08.012

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-tet-rahydro-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 (Biomeda, Foster City, CA, USA). Cells were cultured at 37 °C in 5% CO_2 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 Fugene 6 (Roche Diagnostics) for HepG2 cells with a mixture containing the tk-$(3A4)_3$-Luc reporter construct (300 ng), mRXRα 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 RXRα, β, or γ (gifts from Dr. Ronald Evans, Howard Hughes Medical Institute, CA, USA), mRXRα_{Y402A} (a gift from Dr. Hinirch 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 μM). 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 μm 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 (Biomeda, Foster City, CA, USA), 2.5 μg/ml insulin (Sigma–Aldrich Co., St. Louis, MO), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were seeded in 24-well type I collagen-coated plates at 37 °C in 5% CO_2 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 μg) 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′-TCACAGACCCAGAGACGATTAAGA-3′, 5′-CCCGCCGGTTTGTGAAG-3′, 6FAM-TGTGCTAGTGAAGGAATGTTTTTCT-BHQ1; and GAPDH, 5′-TGTGTCCGTCGTGGATCTGA-3′, 5′-CCTGCTTCACCACCTTCTTGA-3′, 6FAM-CCGCCTGGAGAAACCTGCCA-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 ΔΔCt method according to manufacturer's instructions.