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Activation of Retinoic Acid Receptors by Dihydroretinoids[S]

Alexander R. Moise, Susana Alvarez, Marta Domínguez, Rosana Alvarez, Marcin Golczak, Glenn P. Lobo, Johannes von Lintig, Angel R. de Lera, and Krzysztof Palczewski

Department of Pharmacology, Case School of Medicine, Case Western Reserve University, Cleveland, Ohio (A.R.M., M.G., G.P.L., J.V.L., K.P.); and Departamento de Química Orgánica, Facultad de Química, Universidade de Vigo, Vigo, Spain (S.A., M.D., R.A., A.R.d.L.)

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ABSTRACT

Vitamin A-derived metabolites act as ligands for nuclear receptors controlling the expression of a number of genes. Stereospecific saturation of the C_{13}-C_{14} double bond of all-*trans*-retinol by the enzyme, retinol saturase (RetSat), leads to the production of (*R*)-all-*trans*-13,14-dihydroretinol. In liver and adipose tissue, expression of RetSat is controlled by peroxisome proliferator-activated receptors (PPAR) α and γ, respectively. Expression of RetSat in adipose tissue is also required for PPARγ activation and adipocyte differentiation, but the involved mechanism is poorly understood. In this study, we examined the potential of (*R*)-all-*trans*-13,14-dihydroretinol and its metabolites to control gene transcription via nuclear receptors. Using a cell-based transactivation assay to screen 25 human nuclear receptors for activation, we found that dihydroretinoids have a narrow transcriptional profile limited primarily to activation of retinoic acid receptors (RARs). Although (*R*)-all-*trans*-13,14-dihydroretinoic acid exhibited comparable potency to retinoic acid in promoting the interaction of RARs with a coactivator peptide in vitro, its potency in activating RAR-controlled genes in cell-based assays was much lower than that of retinoic acid. As an explanation for the weak RAR agonist activity of dihydroretinoids in cell-based assays, we propose that both delivery of ligand to the nucleus and RAR activation favor retinoic acid over dihydroretinoids. Discrimination between the cognate ligand, retinoic acid, and close analogs such as dihydroretinoids, occurs at multiple levels and may represent a mechanism to modulate retinoid-dependent physiological processes.

Vitamin A and its derivatives, commonly referred to as retinoids, play a critical role in vision and the regulation of embryonic development, cell differentiation, proliferation, and apoptosis. Visual function is conferred by photoreceptor molecules composed of rod and cone opsin proteins coupled to 11-*cis*-retinaldehyde (Wald, 1965; Palczewski, 2006). The all-*trans*-retinoic acid (atRA) metabolite is a potent signaling molecule during embryogenesis, regulating tissue development and homeostasis. atRA exerts its actions by regulating the expression of specific subsets of genes within target tissues via ligand-activated transcription factors known as retinoic acid receptors (RARs), which work as heterodimers with retinoid X receptors (RXRs) (Giguere et al., 1987; Petkovich et al., 1987; Altucci and Gronemeyer, 2001).

Major efforts have been made to establish whether retinol is converted to other bioactive metabolites besides atRA or 11-*cis*-retinaldehyde. These studies led to the discovery of several retinoids with potential biological activity, but only a few enzymes have been identified that could produce them in vivo (Buck et al., 1991; Heyman et al., 1992; Schuchardt et al., 2009). We originally identified and characterized the enzyme known as retinol saturase (RetSat) that catalyzes the saturation of all-*trans*-retinol (atROL) through a stereospecific reaction that generates (*R*)-all-*trans*-13,14-dihydroretinol [(*R*)-DROL] (Moise et al., 2004, 2007, 2008). The homolog of RetSat in zebrafish, zRetSatA, has an additional specificity, generating all-*trans*-7,8-dihydroretinol in addition to (*R*)-DROL (Moise et al., 2007, 2008). (*R*)-DROL is converted in vivo to DRA through the same metabolic pathway involved in

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ABBREVIATIONS: atRA, all-*trans*-retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; RetSat, retinol saturase; atROL, all-*trans*-retinol; DROL, all-*trans*-13,14-dihydroretinol; DRA, all-*trans*-13,14-dihydroretinoic acid; PPAR, peroxisome proliferator-activated receptor; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; ERR, estrogen related receptor; FRET, fluorescence resonance energy transfer; CMV, cytomegalovirus; TTNPB, 4-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid; HEK, human embryonic kidney; PBS, phosphate-buffered saline; CRABP, cellular retinoic acid binding protein; GFP, green fluorescent protein; RBP4, retinol-binding protein 4; SRC-1, steroid receptor coactivator; DMF, dimethylformamide.

atRA formation (Moise et al., 2005). The dihydroretinoid pathway is depicted in Fig. 1. DRA, as well as all-*trans*-7,8-dihydroretinoic acid, produced in zebrafish, are selective agonists for RAR but not RXR (LeMotte et al., 1996; Moise et al., 2005, 2008). Similar to atRA, levels of DRA are controlled in vivo in both a temporal and spatial manner through enzymes and other factors involved in their synthesis and breakdown (Moise et al., 2005). Thus, high levels of DRA, atRA, or all-*trans*-7,8-dihydroretinoic acid have been shown to induce the expression of CYP26A1-C1 enzymes that catalyze ring oxidation reactions of these compounds (Moise et al., 2005, 2007).

Several observations suggest that RetSat, and enzymatic products of RetSat, play an important role in adipocyte differentiation and/or responses mediated by the peroxisome proliferator-activated receptors (PPAR) α and γ. Expression of RetSat in the liver and kidney is dramatically up-regulated during fasting (Sun et al., 2008), whereas in adipose tissue, it is highly up-regulated during adipocyte differentiation (Schupp et al., 2009). Both observations can be explained by the fact that RetSat expression is controlled by PPARα in liver and kidney and by PPARγ in fat through a peroxisome proliferator-response element found in intron 1 of the *RETSAT* gene (Sun et al., 2008; Schupp et al., 2009). Receptors PPARα and PPARγ are key participants in the regulation of lipid metabolism and adipocyte differentiation, respectively, and are pharmacological targets for hypolipidemic (fibrate) and antidiabetic (thiazolidinedione) drug classes. That *RETSAT* is a fibrate/thiazolidinedione-sensitive gene suggests that its products could be involved in insulin sensitivity. Indeed, RetSat expression is modulated by a high-fat diet (López et al., 2004; Lopez et al., 2005; Schupp et al., 2009) and suppressed in insulin-resistant states, as noted in obese patients and genetically obese (*ob/ob*) mice (Schupp et al., 2009). In addition, ablation of RetSat expression in the NIH 3T3-L1 cell culture model of adipogenesis blocks adipocyte differentiation, whereas ectopic expression of enzymatically active RetSat promotes it (Schupp et al., 2009). However, exogenous supplementation with the product of RetSat, DROL, does not promote adipocyte differentiation so it does not compensate for the effect of ablating RetSat expression (Schupp et al., 2009). In fact, treatment of 3T3-L1 cells with (*R*)-DROL leads to inhibition of adipogenesis through activation of RARs (Moise et al., 2008). These results suggest that in addition to DROL there could be other dihydroretinoids responsible for the proadipogenic effects of RetSat. Indeed, dihydroretinoid species besides DROL can be detected in various tissues of animals maintained on a normal diet (Schmidt et al., 2003; Moise et al., 2004), but it is not clear whether these compounds exert proadipogenic effects. Therefore, to gain a better understanding of the physiological role of dihydroretinoids and vitamin A in general, we used a large nonbiased screen to determine the capability of dihydroretinoids to activate nuclear receptors.

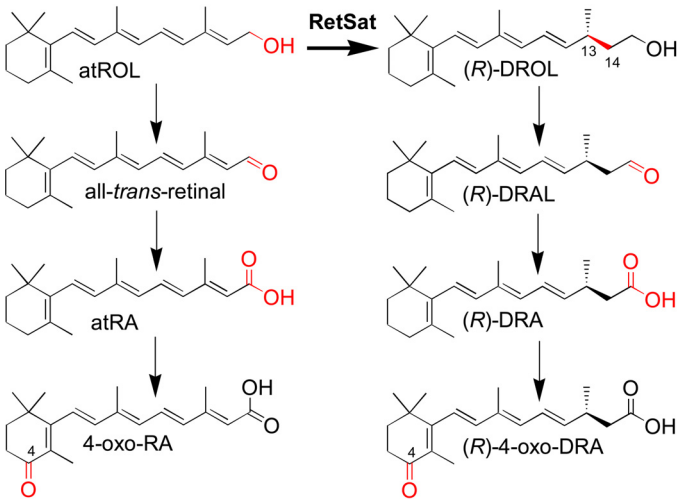

Fig. 1. Dihydroretinoid pathway of vitamin A metabolism. RetSat catalyzes the key irreversible reaction converting atROL into DROL to initiate the dihydroretinoid pathway shown.

Materials and Methods

Materials and Chemical Syntheses. atRA was purchased from Sigma (St. Louis, MO), and all other ligands were obtained from Phenex Pharmaceuticals AG (Ludwigshafen, Germany). Chemical syntheses of (*R*)-DROL, (*S*)-DROL, (*R*)-DRA, and (*S*)-DRA were performed, and the enantiomeric purity of dihydroretinoids was verified by chiral HPLC as described previously (Moise et al., 2008). Compounds were delivered to the assays in dimethyl sulfoxide (DMSO).

Cellular-Based Assays of Nuclear Receptor Activation. The receptor screen was accomplished with reagents and assay services provided by Phenex Pharmaceuticals AG as reported previously (Albers et al., 2006). The reporter plasmid pFR-Luc contained a synthetic promoter with five tandem repeats of yeast GAL4-binding sites that control the expression of the *Photinus pyralis* (American firefly) luciferase gene. Individual nuclear receptors, expressed as fusions of the ligand-binding domain of the nuclear receptor and the DNA-binding domain of the yeast GAL4 protein, were cloned into a pCMV-BD vector (Stratagene, La Jolla, CA). In the case of estrogen-related receptor (ERR) α and γ and the androgen receptor, the nuclear receptor fusion protein and reporter constructs were coexpressed with the PPARγ coactivator 1 as a pTRex construct (Invitrogen Carlsbad, CA). This was done to enhance the constitutive nuclear receptor activity of these receptors while still allowing the nuclear receptor to be modulated by interacting compounds. In the case of constitutive androstane receptor, a FRET-based assay was performed to monitor the interaction between the activated nuclear receptor and nuclear receptor coactivator 3, which was fused to the pCMV-BD vector and the nuclear receptor ligand-binding domain to the pCMV-AD vector and assayed as described previously (Albers et al., 2006). Experiments were performed with 11 dilutions of (*R*)-DROL compound or known agonist plus a solvent control, all in triplicate. *Renilla reniformis* luciferase, driven by a constitutive promoter, was included as an internal control to improve experimental accuracy. To find the most appropriate initial range of concentrations for dose-response experiments, a preliminary experiment was performed to detect nonspecific reduction of *Renilla* luciferase activity (e.g., by cytotoxicity, inhibition of *R. reniformis* luciferase enzyme activity, or an inhibitory effect on cellular enzyme production). A clear reduction of *R. reniformis* luciferase reporter activity was observed at 25 μM for (*R*)-DROL and at 1.5 μM for (*R*)-DRA. Because test compound concentrations that reduced *R. reniformis* luciferase reporter activity by more than 30% might be cytotoxic, we chose 25 μM as the highest test concentration for (*R*)-DROL in our screen of 25 nuclear receptors. Likewise, we chose 1.5 μM as the highest concentration for (*R*)-DROL, (*S*)-DROL, (*R*)-DRA, (*S*)-DRA, atRA, and TTNPB in screens involving human and rat RARs. All transient transfections were done in HEK 293 cells at >90% confluence by a polyethylenimine-based procedure. HEK 293 cells were cultured in Dulbecco's modified Eagle's medium and 10% fetal calf serum, and maintained at 37°C, 5% CO_2, and 100% humidity. Stock compounds, originally dissolved in DMSO, were prediluted in medium and added

4 h after addition of the transfection mixture (final vehicle concentration, <0.1%). Cells were incubated for another 16 h before firefly luciferase and *R. reniformis* luciferase activities were measured sequentially in the same cell extract as described previously (Dyer et al., 2000). Data points at the extreme left of all plotted curves represent values generated by the control vehicle in the assay.

Cell-free Cofactor Binding Assays. Cell-free cofactor binding assays were carried out with reagents and assay services provided by Phenex Pharmaceuticals AG. These consisted of a GST fusion of an individual human RARβ or RARγ ligand-binding domain with an N-terminally biotinylated peptide from the cofactor SRC-1 (amino acid residues between 676 and 700), designed around the nuclear receptor-binding LXXLL-motif. The RAR ligand-binding domain was expressed as a GST fusion by using a recombinant baculovirus in SF9 cells. Cells were lysed by sonication, and the fusion proteins purified over glutathione-Sepharose (GE Healthcare) according to manufacturer's instructions. Assays were performed in a 384-well plate, each well containing a final volume of 25 μl consisting of 10 mM Tris/HCl, pH 6.8, 5 mM $MgCl_2$, 400 mM KCl, and 0.9 μg/μl bovine serum albumin. Detection was achieved with an europium-labeled anti-GST antibody AD0064 (PerkinElmer Life and Analytical Sciences, Waltham, MA) and streptavidin fused to allophycocyanin (ProZyme, Inc., Hayward, CA) as described previously (Albers et al., 2006). Assay components were mixed and then equilibrated for 1 h at room temperature. Measurements were obtained by using an EnVision (PerkinElmer Life and Analytical Sciences) multiplate reader set at 320 nm for excitation and at 615 nm (acceptor signal) and 665 nm (donor signal) for emission readout wavelengths as described previously (Otte et al., 2003; Albers et al., 2006). For analysis of dose-response curves, ratios were plotted against logarithms of concentrations and 50% effective concentrations (EC_{50}) were calculated by Prism software (GraphPad Software Inc., San Diego, CA).

Data Evaluation and Threshold Definition. Primary readouts of the assay results were loaded into assay evaluation software (Genedata Screener; Genedata AG, Basel, Switzerland). Quality control was done in the module 'Assay Analyser', and outliers were masked and excluded from further analysis. FRET data were calculated by using the following equation: $Y = 1000 \times$ (measurement value at 655 nm)/(measurement value at 615 nm), where Y represents the interaction of coactivator and receptor as measured by FRET. We also used a linear transformation of the Y values, after which the vertical axis for all assays ranged from 0 to 100%, where 0% represents the vehicle control and 100% represents the maximal stimulation control at saturating concentrations of the reference compound. The linearly transformed FRET assay results were transferred to the Prism program to generate graphs and dose-response curves. For cell-based assays, data obtained from measuring GAL4-driven firefly luciferase activity were normalized to the absorbance of the transfection control, constitutively expressed *R. reniformis* luciferase. Data derived from cell-based assays were first expressed as relative luciferase units and then transferred to Prism to generate graphs and dose-response curves. Curves were fitted onto the data with a four parameter logistics model, according to the following formula: Y = (baseline response) + [(maximum response) − (baseline response)]/$(1 + 10^{(\log EC_{50} - X)n_H})$, where X is the logarithm of the concentration, Y is the activation response, and n_H is the Hill slope. The threshold for an agonist activity as well as for an antagonist activity at ERRα and ERRγ was defined as 15% of the efficacy of the appropriate reference compound.

Retinoid Analyses. All experimental procedures related to extraction, derivatization, and separation of retinoids and dihydroretinoids were carried out under dim red light. HEK cells were incubated with retinoids, washed twice with ice-cold phosphate-buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, and 10 mM sodium phosphate, pH 7.4, and then harvested by scraping. The cell pellet was homogenized in 3 volumes (v/w) of PBS and 6 volumes of ethanol for 30 s in a Dounce homogenizer. Alternatively, nuclear and cytoplasmic extracts were prepared by using the mammalian nuclear protein extraction reagent (Pierce, Rockford, IL) and following the manufacturer's instructions. The organic extraction protocol for polar retinoids from cell pellets or cell extracts was described previously (Moise et al., 2005). Polar retinoids were deprotonated by adding NaOH to 1 ml of each ethanolic extract (NaOH, 75 mM final concentration), and nonpolar retinoids were extracted with 5 ml of hexane. The extraction was repeated once, and the combined organic phases were dried under vacuum, resuspended in hexane, and examined by normal-phase HPLC with an Ultrasphere Si (5 μm, 4.6 × 250 mm column; Beckman Coulter, Fullerton, CA). Elution was carried out with an isocratic solvent system consisting of 10% ethyl acetate in hexane (v/v) for 25 min at a flow rate of 1.4 ml/min at 20°C with detection at 325 and 290 nm for nonpolar retinoids and dihydroretinoids, respectively. For polar retinoids, the aqueous phase was acidified with 12 N HCl, and extracted with 5 ml of hexane. The organic phases of the polar retinoid extractions were combined, dried, resuspended in a solvent composed of 80% CH_3CN, 10 mM ammonium acetate, 1% acetic acid, and examined by reversed-phase HPLC. Analysis of polar retinoids from tissues was performed by reversed-phase HPLC with a narrow bore, 120-Å, 5-μm, 2.1 × 250 mm, Denali C18 column (Grace-Vydac, Hesperia, CA). The solvent system was composed of buffer A (80% CH_3CN, 10 mM ammonium acetate, and 1% acetic acid) and buffer B (60% methanol and 40% *tert*-butyl methyl ether). HPLC elution conditions were 0.3 ml/min at 20°C, using 100% buffer A for 20 min, 100% buffer B for the next 10 min, followed by equilibration in buffer A for 10 min. Elution of atRA and DRA was monitored by an online diode array detector of a liquid chromatography unit (1200 Series; Agilent Technologies, Santa Clara, CA) with detection set at 350 and 290 nm for atRA and DRA, respectively. Nonpolar retinoids were identified by comparing their spectra and elution times with those of authentic standards.

Assays of CRABPII Nuclear Translocation. A construct encoding human cellular retinoic acid binding protein II (CRABPII) with a C-terminal green fluorescent protein (GFP) tag expressed under the control of a CMV promoter was obtained from Origene Technologies, Inc. (Rockville, MD). HEK 293 cells were cultured in Dulbecco's modified Eagle's medium and 10% fetal calf serum, and maintained at 37°C, 5% CO_2, and 100% humidity. HEK 293 cells were transfected with CRABPII-GFP by using Lipofectamine 2000 (Invitrogen), and 24 h later they were induced with either DRA or atRA at 10^{-9} M, 10^{-8} M, and 10^{-7} M, or with DMSO vehicle alone. Two hours after induction, cells were rinsed in PBS, fixed in 4% paraformaldehyde, counterstained with nuclear stain 4′,6-diamidino-2-phenylindole and visualized by fluorescence microscopy.

Competition Assays. RAR transactivation assays were performed as described previously (Moise et al., 2005, 2008). The atRA-responsive F9 cell line was transfected with a reporter construct composed of an atRA response element derived from the human RARβ promoter placed upstream of the *Escherichia coli lacZ* gene. This reporter cell line, designated SIL15-RA (Wagner et al., 1992), was a gift from Dr. Michael Wagner (State University of New York Downstate Medical Center, Brooklyn, NY) and Dr. Peter McCaffery (University of Massachusetts Medical School, Worcester, MA). Cells were grown in L15-CO2 media and stimulated for 24 h in the dark at 37°C and 100% humidity with indicated concentrations of atRA alone or combined with either 10^{-7} M (*R*)-DRA or 10^{-7} M (*S*)-DRA. Cells then were lysed and assayed for expression of β-galactosidase with the β-galactosidase enzyme assay system (Promega, Madison WI).

Expression and Purification of Human Serum Retinol-Binding Protein 4. Human RBP4 cDNA cloned into a pET3a expression vector was a kind gift from Dr. J. W. Kelly (Scripps Research Institute, La Jolla, CA). RBP4 expression in *E. coli* was carried out as described previously (Golczak et al., 2008). In brief, RBP4 was expressed in BL-21 DE3 cells by using a standard protocol. Bacterial cells were harvested and lysed by osmotic shock. Insoluble material was pelleted, washed three times with 20 mM

Tris/HCl buffer, pH 8.0, and solubilized in 7 M guanidine hydrochloride and 10 mM dithiothreitol. Buffer (25 mM Tris/HCl, pH 8.8) then was added to dilute the guanidine hydrochloride concentration to 5.0 M. After overnight incubation at 4°C, insoluble material was removed by ultracentrifugation (120,000*g*, 1 h, 4°C), and the collected supernatant was used to implement RBP4 refolding. RBP4 was refolded by dropwise addition of solubilized material to a mixture containing 25 mM Tris/HCl, pH 8.8, 0.3 mM cystine, 3.0 mM cysteine, 1 mM EDTA, followed by 1 mM atROL delivered in ethanol at 4°C. The reaction was carried out for 5 h at 4°C with vigorous mixing. The precipitate was removed by ultracentrifugation (120,000*g*, 1 h at 4°C), and the supernatant was dialyzed against 10 mM Tris/HCl buffer, pH 8.0, at 4°C overnight, filtered, and loaded on a DE53 cellulose chromatography column. Refolded holo-RBP4 was eluted by a linear gradient of NaCl (0–300 mM) in 10 mM Tris/HCl buffer, pH 8.0. Collected fractions were examined by SDS-PAGE and UV-visible spectroscopy. Fractions containing RBP4 with an absorbance ratio at 280 nm/330 nm of 0.9 or higher were pooled, concentrated, and stored at −80°C until further use. Apo-RBP was prepared according to a procedure described previously (Cogan et al., 1976) by extracting refolded holo-RBP with 3 volumes of diethyl ether for 2 h at room temperature. The organic phase containing atROL was separated by centrifugation for 5 min, at 4000*g*. The efficiency of atROL extraction was monitored by recording the absorption spectra of the aqueous phase. Apo-RBP was repurified on Sephadex G-200 gel filtration column equilibrated with 20 mM Tris/HCl buffer, pH 7.4, and 150 mM NaCl.

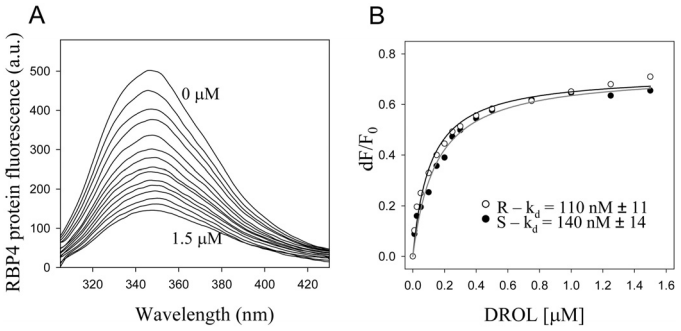

Fig. 2. Binding of (*R*)-DROL and (*S*)-DROL to RBP4 determined by quenching of protein fluorescence. Purified apo-RBP4 was diluted in 20 mM Tris/HCl buffer, pH 7.6, containing 50 mM NaCl and 5% glycerol (v/v) to a final concentration of 0.05 μM. Increasing quantities of (*R*)-DROL or (*S*)-DROL were added in 1-μl aliquots of DMF to 2 ml of total sample. After each addition, the titrated solution was mixed and incubated for 1 min before excitation at 285 nm and recording of emission spectra. A, fluorescence spectra of RBP4 recorded after addition of increasing concentrations of (*R*)-DROL. B, changes in maximum emission in response to increasing (*R*)-DROL or (*S*)-DROL concentrations were monitored at 345 nm, corrected for background, dilution, and inner filter effects, and used to estimate the apparent K_d values shown. a.u., arbitrary units; F, protein fluorescence.

Fluorescence Binding Assays. A spectrofluorometric technique was used to study the binding of (*R*)-DROL or (*S*)-DROL to RBP4. All measurements were performed with a fluorometer (model LS55; PerkinElmer Life and Analytical Sciences). Binding of (*R*)-DROL or (*S*)-DROL was evaluated by monitoring the quenching of protein fluorescence by increasing concentrations of ligand. With the excitation wavelength set at 285 nm, emission spectra were recorded from 300 to 520 nm with bandwidths for excitation and emission fixed at 10 nm. Titrations were carried out at 20°C in 20 mM Tris/HCl buffer, pH 7.6, containing 50 mM NaCl and 5% glycerol (v/v). (*R*)-DROL or

TABLE 1

Pharmacological screen of nuclear receptor activation by (*R*)-DROL

The compound (*R*)-DROL was used to screen a panel of human nuclear receptors. Activation was assayed by monitoring the expression of a luciferase reporter in transfected cells. The EC_{50} and efficacy of (*R*)-DROL were compared with those of known agonists. Assays were carried out as described under *Materials and Methods*.

Receptor	Systematic Name	Control Ligand	EC_{50}	
			Control Ligand	(*R*)-DROL
			nM	*μM*
TRα	NR1A1	Triiodothyronine	2.7	N.A.
TRβ	NR1A2	Triiodothyronine	6.8	N.A.
RARα	NR1B1	TTNPB	0.90	6.6
RARβ	NR1B2	TTNPB	0.59	1.9
RARγ	NR1B3	TTNPB	0.55	1.8
PPARα	NR1C1	GW7647	3.0.2	3.0[a]
PPARβ	NR1C2	GW501516	0.94	N.A.
PPARγ	NR1C3	Rosiglitazone	56	N.A.
LXRβ	NR1H2	T0901317	257	N.A.
LXRα	NR1H3	T0901317	105	N.A.
FXRα	NR1H4	GW4064	143	N.A.
VDR	NR1I1	1,25-Dihydroxy-cholecalciferol	1.7	N.A.
PXR	NR1I2	SR12813	161	6.6
CAR	NR1I3	CITCO	443	N.A.
RXRα	NR2B1	9-*cis*-RA	40	N.A.
RXRβ	NR2B2	9-*cis*-RA	48	N.A.
RXRγ	NR2B3	9-*cis*-RA	38	N.A.
ERα	NR3A1	17-β-Estradiol	0.074	N.A.
ERβ	NR3A2	17-β-Estradiol	0.25	N.A.
ERRα	NR3B1	Diethylstilbestrol	11900	N.A.
ERRγ	NR3B3	4-Hydroxy-tamoxifen	3200	N.A.
GR	NR3C1	Dexamethasone	0.63	N.A.
MR	NR3C2	Aldosterone	0.21	N.A.
PR	NR3C3	Progesterone	3.2	N.A.
AR	NR3C4	Dihydrotestosterone	0.39	N.A.

N.A., not applicable; TR, thyroid hormone receptor; LXR, liver X receptor; FXR, farnesoid X receptor; VDR, vitamin D receptor; PXR, pregnane X receptor; CAR, constitutive androstane receptor; ER, estrogen receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; PR, progesterone receptor; AR, androgen receptor; RA, retinoic acid; GW7647, 2-(4-(2-(1-cyclohexanebutyl)-3-cyclohexylureido)ethyl)-phenyl-thio)-2-methyl-propionic acid; GW501516, 2-[2-methyl-4-([4-methyl-2-[4-(trifluoromethyl)phenyl)-1,3-thiazol-5-yl]methylsulfanyl]phenoxy]acetic acid; GW4064, 3-[2-[2-chloro-4-[[3-(2,6-dichlorophenyl)-5-(1-methylethyl)-4-isoxazolyl]methoxy]phenyl]ethenyl]-benzoic acid; T0901317, *N*-(2,2,2-trifluoroethyl)-*N*-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-benzenesulfonamide; CITCO, 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime; SR12813, [[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]ethenylidene]bis-phosphonic acid tetraethyl ester.

[a] Antagonistic.

(*S*)-DROL were delivered in dimethylformamide (DMF) so that the final volume of DMF did not exceed 0.5% of each sample's total volume. All binding data were corrected for background and self-absorption of excitation and emission light. Using Prism 3.02 (GraphPad Software, Inc., San Diego, CA), we calculated the apparent K_d values by nonlinear regression for a single-binding site with the equation $Y = B_{max} \times \{X/(K_d + X)\}$, where B_{max} is the maximal binding and K_d is the concentration of ligand required to achieve half-maximal binding.

Results

DROL Is a Ligand for Serum Retinol-Binding Protein RBP4. A crucial factor that governs the delivery of retinoids in vivo is the serum protein complex of RBP4 and transthyretin. Secretion of RBP4 from the liver and its excretion by the kidney depends on the presence of ligand (Kanai et al., 1968; Ronne et al., 1983; Soprano and Blaner, 1994). RBP4 interacts with a membrane protein stimulated by retinoic acid gene 6 expressed in target tissues (Kawaguchi et al., 2007; Isken et al., 2008). RetSat is expressed at high levels in liver and adipose tissue, the two major tissue stores of atROL and major secretors of RBP4. To examine whether DROL could be a ligand of RBP4, we used fluorescence titration. This method employs the well established energy transfer known to exist between tryptophan residues that are located in the vicinity of the binding site on RBP and bound retinol (Goodman and Leslie, 1972). As shown in Fig. 2A, incubation of (*R*)-DROL with RBP4 led to an exponential decay in protein fluorescence. This decay displayed a saturable binding isotherm (Fig. 2B), providing average binding K_d values of 110 ± 11 and 140 nM ± 14 nM for (*R*)-DROL and (*S*)-DROL, respectively. These K_d values also indicate a similar affinity of DROL and atROL for RBP4 based on atROL's reported K_d of 150 nM for human RBP4 (Cogan et al., 1976). Therefore RBP4 could act as a carrier of (*R*)-DROL in blood, transporting it from biosynthetic tissues to target organs.

Dihydroretinoids Are Selective Agonists of RAR. Because DROL is a ligand for RBP4 that serves as the major transport mode for atROL, it is likely that DROL will be delivered in vivo to the same target tissues as atROL. To examine whether dihydroretinoids can activate nuclear receptors, we first used a heterologous expression system for nuclear receptors and luciferase-based reporter assay to implement a cell-based pharmacological screen for 25 of the 48 known human nuclear receptors. We tested whether (*R*)-DROL activated nuclear receptors to the same extent as some of the most effective known agonists available for each receptor. HEK 293 cells were transiently transfected with a construct containing the ligand-binding domain of the nuclear receptor fused to the DNA-binding domain of the yeast GAL4 protein and a reporter construct consisting of firefly

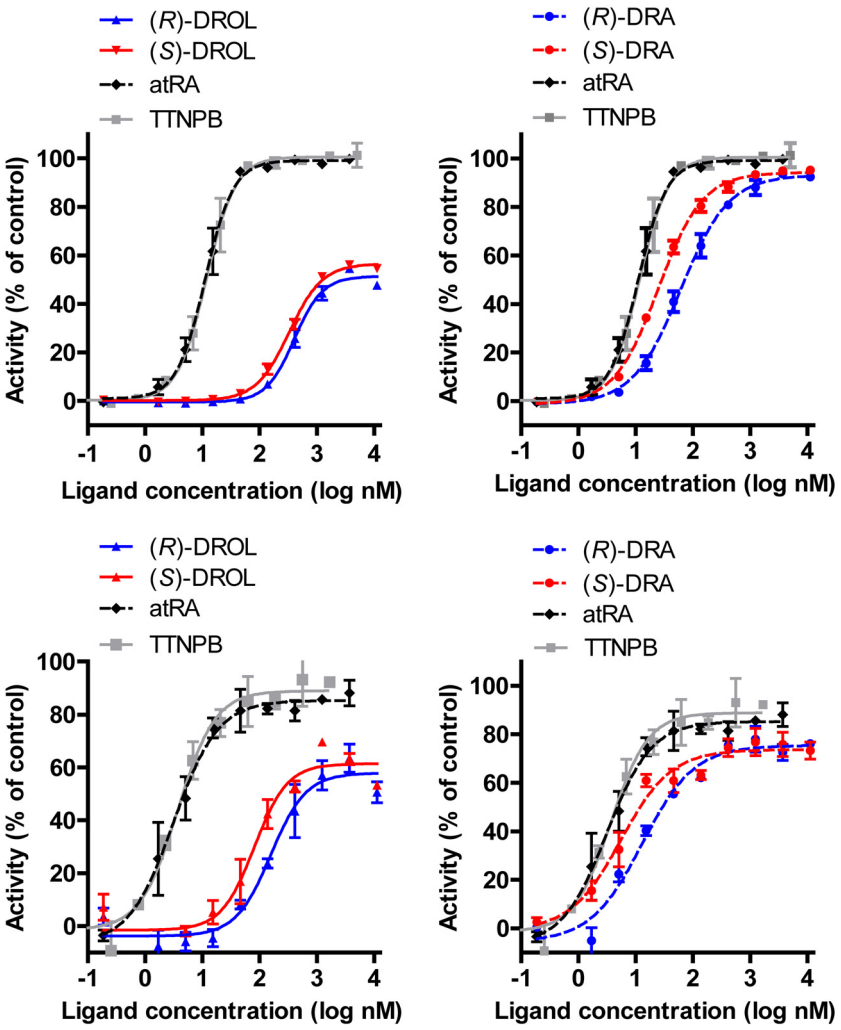

Fig. 3. Activation of human RARβ and RARγ by dihydroretinoids in cell-free cofactor binding assays. The compounds (*R*)-DROL, (*S*)-DROL, (*R*)-DRA, (*S*)-DRA, atRA, and TTNPB were used to activate human RARα (top) and RARβ (bottom) in vitro. The assay relies on the interaction of a biotinylated peptide derived from SRC-1 with a fusion protein of RARα or RARβ and GST. Association of RARs with SRC-1 was detected by FRET with anti-GST antibody coupled to Europium and streptavidin coupled to allophycocyanin. Assays performed in duplicate employed 12 dilutions of each compound or control vehicle alone.

luciferase driven by a GAL4-controlled promoter. Cells were treated with a test compound and monitored for firefly luciferase expression. Results of the screen of (*R*)-DROL versus 25 human nuclear receptors are listed in Table 1. The screen revealed that (*R*)-DROL or its metabolites activated RARβ and RARγ at the same maximal level as the control agonist TTNPB, but, as expected, with much higher EC_{50} values of just below 2 μM (compared with EC_{50} values of 0.6 and 0.4 nM for TTNPB with RARβ and RARγ, respectively). (*R*)-DROL showed low efficacy in activating pregnane X receptor and RARα and in antagonizing the PPARα activity. (*R*)-DROL antagonized PPARα at a minimal effective concentration of 3 μM and low potency, 1.8-fold repression of basal activity. Repression of PPARα was not observed using DRA (not shown). Using the highest concentration of (*R*)-DROL that did not elicit cellular toxicity, we observed RARα and pregnane X receptor activation at 40 and 20%, respectively, of the maximal response induced by reference compounds. All other combinations of test compounds with nuclear receptors, including PPARs, did not show activity above threshold. The results of this nuclear receptor screen fit well with previously published studies that focused on the activity of dihydroretinoids in activating RARs (Moise et al., 2005, 2008). Therefore, by examining a much larger set of nuclear receptors, we showed that dihydroretinoids have a very narrow transcriptional profile in activating RARs.

TABLE 2

Activation of RARs by dihydroretinoids in cell-free cofactor binding assays

The compounds (*R*)-DROL, (*S*)-DROL, (*R*)-DRA, (*S*)-DRA, atRA, and TTNPB were used to activate human RARα and RARβ in vitro. Activation was assayed by monitoring the interaction of liganded RAR with SRC-1 by FRET. Assays were carried out as described under *Materials and Methods*.

Ligand	EC_{50}	
	RARα	RARβ
	nM	
(*S*)-DROL	328	81
(*R*)-DROL	402	157
(*S*)-DRA	25	5.6
(*R*)-DRA	61	13.1
atRA	11.2	3.1
TTNPB	11.8	3.7

DRA Is a Potent Activator of RAR in Vitro. Because data from the nuclear receptor screen suggested that (*R*)-DROL or its metabolites can activate RARβ and RARγ, we next employed an in vitro biochemical assay to monitor directly the interaction between test ligands and nuclear receptor. Binding of agonists to nuclear receptors induces a conformational change in a helical motif, referred to as AF-2, that allows RARs to couple to coactivator proteins that mediate their transcriptional effects. Members of the steroid receptor coactivator (SRC-1) family are among the best molecularly characterized nuclear receptor coactivators. SRC-1 is required for ligand-dependent transcription of transiently transfected and chromosomally integrated reporter genes by the estrogen receptor and RAR (Llopis et al., 2000). Coactivators interact with liganded nuclear receptors through a central domain that contains three copies of a conserved recognition motif with the consensus sequence LXXLL (Ding

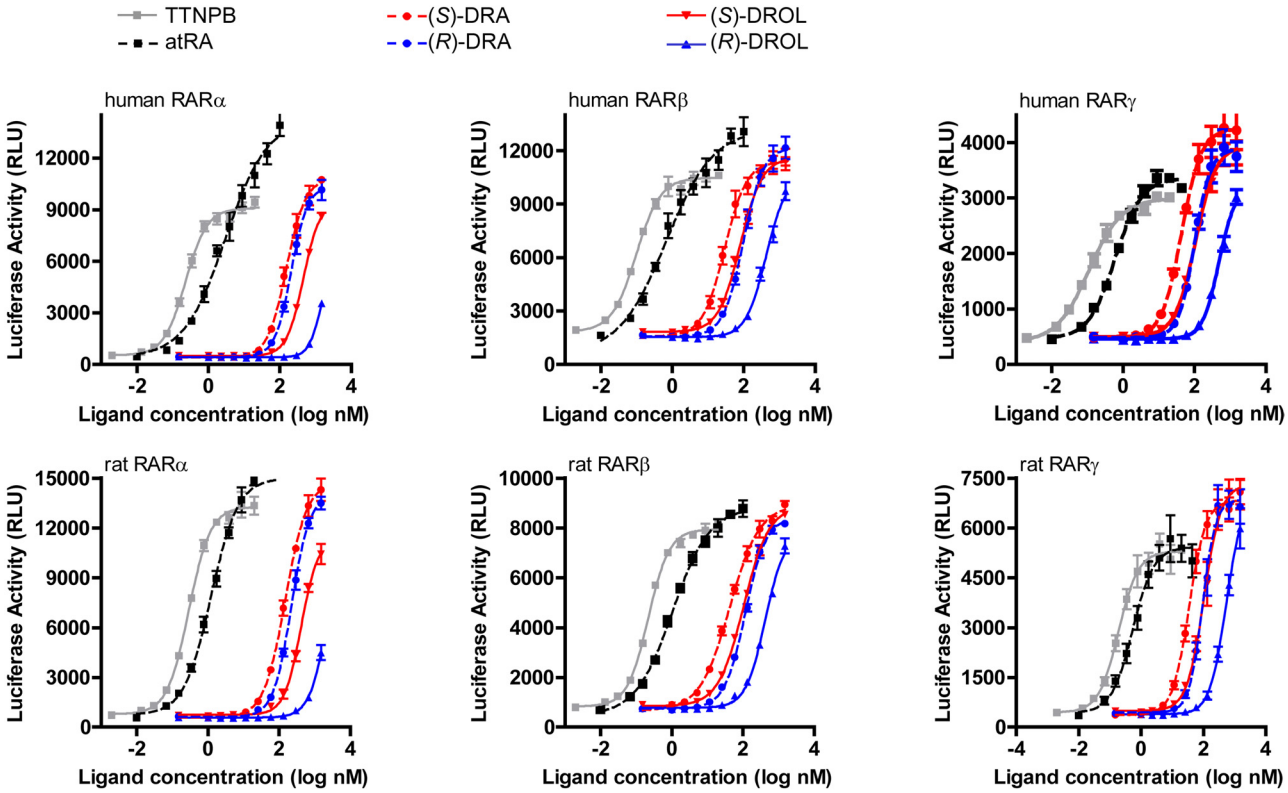

Fig. 4. Effects of dihydroretinoids on the transcriptional activity of the RAR. The ligand-binding domain of RAR was fused to the GAL4 DNA-binding domain. (*R*)-DROL, (*S*)-DROL, (*R*)-DRA (*S*)-DRA, atRA, and TTNPB were used to activate human (top) and rat (bottom) RARα-, RARβ- and RARγ-GAL4 fusion proteins in cells transfected with a *P. pyralis* luciferase reporter construct driven by a promoter containing 5×GAL4 sites. A second *R. reniformis* luciferase reporter driven by a constitutive promoter was used as an internal control and to normalize the intensity signal. Assays were performed in triplicate with 11 dilutions of each compound plus vehicle alone.

et al., 1998). In the absence of ligand, RARs can bind to corepressors, such as nuclear receptor corepressor (*N*-CoR) proteins and silencer proteins, like silencing mediator for retinoid and thyroid receptor (SMRT), which lead to condensation of chromatin and sequestration of promoter elements that inhibits transcriptional activity.

We used (*R*)-DROL, (*S*)-DROL, (*R*)-DRA (*S*)-DRA, atRA, and TTNPB to study the interaction between the ligand-binding domain of human RARα and RARβ with SRC-1. As RetSat and dihydroretinoids are found at the highest levels in the liver (Moise et al., 2004, 2005), we focused our study on the RARα and RARβ isoforms that are more predominant in this organ. Results of the FRET screen of the interaction of RARα or RARβ with SRC-1 in the presence of dihydroretinoids are depicted in Fig. 3 and Table 2. It is noteworthy that both (*R*)-DRA and (*S*)-DRA were comparable with atRA in their potency to activate RARα and RARβ in vitro. The EC_{50} values of (*R*)-DRA and (*S*)-DRA for RARβ were 13.1 and 5.6 nM, respectively. For comparison, the EC_{50} values of atRA and TTNPB for RARβ were 3.1 and 3.7 nM, respectively. In addition, both (*R*)-DRA and (*S*)-DRA approached full efficacy in activating RARα and RARβ at 84 to 94% of the level of induction seen with reference compounds atRA and TTNPB. Instead, (*R*)-DROL and (*S*)-DROL activated RARα and RARβ at 51 to 69% of the level of induction seen with reference compounds (Fig. 3).

DRA Weakly Activates RAR in Vivo. Previous studies of RAR activation by (*R*)-DRA or (*S*)-DRA did not distinguish between different RAR isoforms, in that these studies employed a modified F9 teratocarcinoma reporter cell line that expresses all three RARs (Zelent et al., 1989). Given the potent activation of RARα and RARβ by (*R*)-DRA and (*S*)-DRA seen in vitro, we reexamined whether (*R*)-DROL, (*S*)-DROL, (*R*)-DRA, or (*S*)-DRA would show potency and selectivity for human or rat RARα, RARβ, or RARγ in cell-based assays. We used all three isoforms of RAR from these two distant species to allow us to distinguish whether any effects seen were subtype-specific. The results of the cell-based assay of activation of RARs are shown in Fig. 4 and Table 3. Both (*S*)-DRA and (*R*)-DRA act as full agonists of RAR activation with maximal efficacies ranging from 98 to 138% of the activity manifested by atRA reference compounds. However, the potency of both (*S*)-DRA and (*R*)-DRA for RARs in cell-based assays was far lower than atRA or TTNPB. For example, (*S*)-DRA and (*R*)-DRA had EC_{50} values of 43.4 and ~101 nM, respectively, for human RARγ. Meanwhile, atRA and TTNPB had EC_{50} values of 0.6 and 0.1 nM, respectively.

TABLE 3
Cell-based assays of the RAR activation by dihydroretinoids
The compounds (*R*)-DROL, (*S*)-DROL, (*R*)-DRA, (*S*)-DRA, atRA, and TTNPB were used to activate human RARα, RARβ, and RARγ in cell-based transcription assays. Activation was assayed by monitoring the expression of a luciferase reporter in transfected cells. Assays were carried out as described under *Materials and Methods*.

Ligand	EC_{50}		
	RARα	RARβ	RARγ
		nM	
(*S*)-DROL	458	80	107
(*R*)-DROL	1500	425	482
(*S*)-DRA	156	29.4	43.4
(*R*)-DRA	210	99	101
atRA	2.96	0.68	0.60
TTNPB	0.23	0.10	0.11

We also found that (*S*)-DRA is a more potent activator of RARs than the naturally occurring form, (*R*)-DRA. Conversely, the precursor (*S*)-DROL evidenced more potency than the (*R*)-DROL precursor. The greater potency of (*S*)-DRA than (*R*)-DRA in activating RARs is in agreement with previous observations (Moise et al., 2008). Treatment with (*R*)- or (*S*)-DRA had no effect on the activation of RAR by atRA, which rules out antagonism of atRA activation of RARs by these compounds (Fig. S1).

atRA, but Not (*R*)-DRA, Accumulates in the Nucleus. To explain the low potency of dihydroretinoids in activating RARs in cell culture transactivation assays compared with in vitro cofactor recruitment assays, we first assessed whether (*R*)-DRA was cleared more rapidly than atRA from the culture system employed in the cell-based assay. We incubated HEK 293 cells with exogenous atRA or DRA and assayed the levels of compounds before and after the 16-h incubation period. Our results suggest that (*R*)-DRA was actually more metabolically stable than atRA in the cell culture system used to study RAR activation. Initial concentrations of atRA and DRA were equal, but (*R*)-DRA was present at 60.0 ± 5.5% of its initial levels compared with 41.6 ± 5.6% for atRA after 16 h induction (Fig. 5). This suggests that metabolic stability did not influence RAR activation by dihydroretinoids.

We next analyzed the subcellular distribution of atRA versus DRA. HEK 293 cells incubated with equal concentrations of atRA or DRA accumulated substantially more atRA than DRA in the cytoplasm and nucleus (Fig. 6). In fact, we detected no DRA in nuclear extracts of cells incubated with DRA. These results suggest that impaired nuclear import could contribute to the weaker activity exhibited by DRA in cell-based versus in vitro functional assays.

In examining the delivery of dihydroretinoids, we focused on their nuclear import by accessory proteins. Delivery of

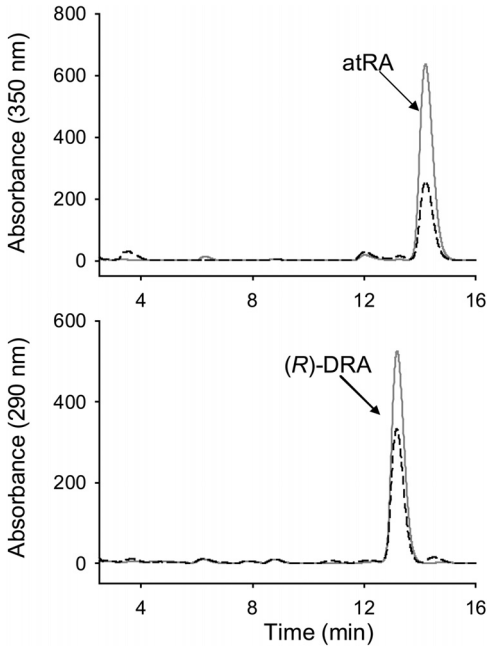

Fig. 5. Retinoid analyses of HEK 293 cells incubated with atRA and (*R*)-DRA. HEK 293 cells were incubated for 16 h with 1 μM atRA (top) or (*R*)-DRA (bottom). Organic extracts of cells and media before (gray solid line) and after (black dashed line) the 16-h incubation were analyzed by reversed-phase HPLC. Compounds were identified by comparing their spectra and elution characteristics with those of authentic standards.

atRA to the nucleus is achieved by accessory proteins CRABPI and CRABPII, soluble proteins that belong to the intracellular ligand-binding protein family (Ong and Chytil, 1975). Both CRABPI and -II remain in the cytosol in the absence of ligand but translocate to the nucleus in its presence. CRABPII delivers the ligand by associating directly with RARs (Delva et al., 1999) and enhancing their activity (Budhu and Noy, 2002). To determine whether DRA, like atRA, can induce nuclear translocation of CRABPII, we expressed CRABPII as a GFP fusion protein and then treated cells with several concentrations of agonist. We found that CRABPII-GFP localized predominantly in the cytoplasm before addition of agonist but translocated into the nucleus in the presence of atRA (Fig. 7). Treatment with DRA also resulted in nuclear localization of CRABPII-GFP but to a lesser extent than with atRA (Fig. 7). This finding indicated that DRA was not delivered to the nucleus by CRABPs as efficiently as atRA to activate RAR-dependent gene expression.

Discussion

Dihydroretinoids Act as Selective RAR Agonists. Several retinoids act as potent ligands for nuclear receptors that control gene expression by binding directly to DNA response elements of target genes. all-*trans* and 9-*cis*-retinoic acid bind to and activate RAR (α, β, or γ), whereas 9-*cis*-atRA also binds to RXR (Giguere et al., 1987; Petkovich et al., 1987; Heyman et al., 1992). DRA shares a similar structure and employs the same synthetic enzymes as atRA (Moise et al., 2005), and its levels are regulated by RAR-controlled genes, such as Cyp26A1 (Moise et al., 2005, 2007). These observations prompted us to examine whether dihydroretinoids could indeed act as ligands of nuclear receptors within concentrations observed in vivo.

Because many nuclear receptors share binding site motifs, we employed a fusion construct of the ligand-binding domain of the respective nuclear receptor with the DNA-binding domain of GAL4. Binding and activation of the nuclear receptor-GAL4 fusion protein by tested ligands was assayed based on the expression of firefly luciferase reporter driven by a GAL4-controlled promoter. This approach allowed us to specifically interrogate each receptor and avoid interference from endogenous nuclear receptors expressed in HEK 293 cells that recognize the same site. Although cell lines derived from liver and adipose tissue would replicate the metabolism of dihydroretinoids more faithfully, we have previously shown that some of the dihydroretinoid metabolites are secreted in the circulation and, as seen here, these metabolites bind RBP4 and could be taken up via protein stimulated by retinoic acid gene 6 by target tissues. At the current stage, it is hard to predict which tissue is the most likely target of dihydroretinoids; therefore, we sought to find the most likely nuclear receptor target of dihydroretinoids regardless of target tissue. (*R*)-DROL was used to screen the library because this retinoid, by virtue of being the direct product of RetSat, is the parent compound of all dihydroretinoids derived from RetSat and can be converted in cells to other more active dihydroretinoids (Moise et al., 2005, 2008). The advantage of this method was that it enabled us to screen a larger group of dihydroretinoid compounds than use of a downstream metabolite. However, we also were limited by the ability of the cell to convert (*R*)-DROL to more active metabolites. Though the strategy produced a much weaker response than if we had used the bioactive compound(s) directly, we still were able to obtain a basic profile of dihydroretinoid activity with (*R*)-DROL, which in turn allowed an investigation of specific receptors in more detail.

The screen of human nuclear receptors with (*R*)-DROL revealed that dihydroretinoids exhibit a narrow transcriptional profile in activating primarily RARs. Both (*R*)- and (*S*)-DRA displayed potencies comparable with atRA in vitro in activating RARα and RARβ; however, in cell-based assays (*R*)- and (*S*)-DRA showed far less potency than atRA (EC_{50} was 43- to 168-fold higher for DRA than atRA). The relatively small difference in the potency of DRA versus atRA in activating RARs in vitro was unexpected given the large difference seen in cell culture assays. We previously proposed that the weaker agonistic activity of DRA versus atRA could be explained by a weaker affinity for the receptor imparted by unfavorable contacts between ligand and the ligand-binding pocket (Moise et al., 2008). Alternatively, it is also possible that atRA is taken up by cells and delivered to RAR with higher efficiency than DRA.

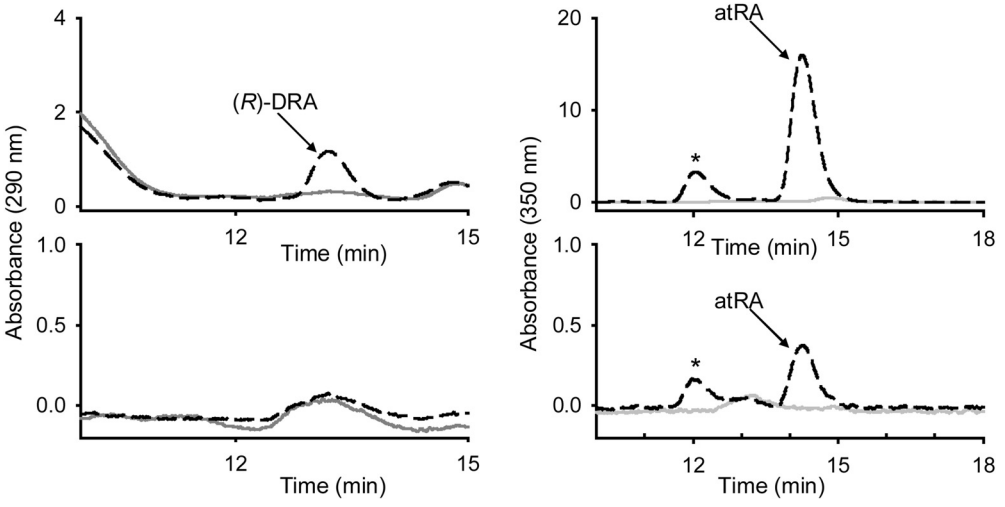

Fig. 6. Retinoid analyses of nuclear and cytoplasmic extracts of HEK 293 cells incubated with atRA and (*R*)-DRA. HEK 293 cells were incubated for 3 h with 1 μM (*R*)-DRA (left) or atRA (right). Nonpolar retinoids found in the nuclear (bottom) and cytoplasmic (top) fractions derived from equal numbers of cells incubated with vehicle alone (gray solid lines) or after incubation with either atRA or DRA (black dashed lines) were analyzed by reverse phase HPLC. Compounds were identified based on comparing their spectra and elution characteristics with those of authentic standards (asterisk-labeled peak corresponds to 13-*cis*-retinoic acid formed during incubation and subcellular fractionation). (*R*)-DRA was not detected in the nuclear fraction because chromatograms of both (*R*)-DRA- and vehicle alone-treated cells overlapped (bottom left).

Cellular Uptake and Nuclear Import Adversely Affect RAR Activation by DRA. To explain the enhanced activity of atRA in cell-based assays we show that even though (*R*)-DRA is more metabolically stable than atRA, it does not accumulate in cells or cell nuclear fractions and does not induce nuclear import of CRABPII to the same extent as atRA. Therefore, we propose that receptor binding, cellular uptake and distribution of retinoids enhance the activation of RAR by its cognate ligand, atRA, as opposed to molecules that are very closely related to it, such as DRA. Our previous studies showed that, in vivo, after supplementation of large doses of retinyl palmitate, hepatic levels of (*R*)-DRA are 30- to 50-fold lower than those of atRA (Moise et al., 2005). Therefore, it is unlikely that DRA can achieve cellular concentrations sufficient to activate RAR in a physiological setting. The current results argue that conversion of all-*trans*-retinol to DROL could act to circumvent the formation of atRA and reduce the activation of RAR in tissues where RetSat is expressed.

Physiological Relevance of Dihydroretinoids during Adipocyte Differentiation. Given that RetSat is a direct PPAR target and that ablation of RetSat expression results in impaired activation of PPARγ, we were especially interested in the effect of dihydroretinoids on activation of PPARs (Sun et al., 2008; Schupp et al., 2009). Our screen revealed that dihydroretinoids do not activate PPARγ or PPARβ/δ but do show low antagonistic activity for PPARα at high concentrations. It is noteworthy that the screen was conducted with DROL used as a metabolic precursor of dihydroretinoids. Possibly DROL needs to be metabolized to produce a more bioactive compound that could affect PPAR responses, and this metabolic conversion was inefficient in the HEK cell system employed. However, current results are conclusive in that they do not support a direct role of DRA in the activation of PPARs.

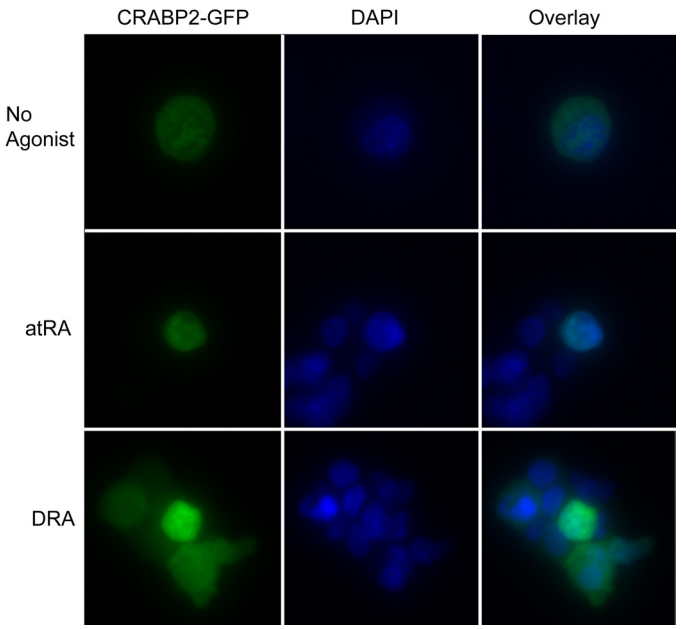

Fig. 7. Effects of atRA and DRA on nuclear translocation of CRABPII-GFP. HEK 293 cells transfected with CRABPII-GFP were treated with vehicle (top), 10^{-7} M atRA (middle) or 10^{-7} M DRA (bottom). Cells were counterstained with 4′,6-diamidino-2-phenylindole (nuclear stain) and imaged by fluorescence microscopy.

Considering the essential role of RetSat during adipocyte differentiation in vitro, the results presented here predict that conversion of atROL to (*R*)-DROL by RetSat does not produce a PPARγ agonist that promotes adipocyte differentiation. Conversely, our results also show that conversion of atROL to (*R*)-DROL does not result in the production of an endogenous RAR activator. Taking into consideration the previous observations that DROL does not compensate for ablation of RetSat expression and that DROL leads to inhibition of adipogenesis through activation of RARs (Moise et al., 2008; Schupp et al., 2009), it is possible that there are other unidentified products of RetSat that have pro-adipogenic effects. An alternate explanation is that RetSat plays a role in converting retinol, a potent inhibitor of adipogenesis, to (*R*)-DROL. (*R*)-DROL is then oxidized to (*R*)-DRA, which is a far less potent agonist of RARs than atRA and, consequently, a weaker inhibitor of adipogenesis. Differentiation between these and other possible scenarios will require further study of RetSat per se and effects of its expression on global retinoid levels.

Acknowledgments

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Address correspondence to: Dr. Alexander R. Moise, Department of Pharmacology & Toxicology, School of Pharmacy, University of Kansas, Lawrence, KS 66045. E-mail: alexmoise@ku.edu