The metabolism of vitamin A is a highly regulated process that generates essential mediators involved in the development, cellular differentiation, immunity, and vision of vertebrates. Retinol saturase converts all-trans-retinol to all-trans-13,14-dihydroretinol (Moise, A. R., Kuksa, V., Imanishi, Y., and Palczewski, K. (2004) J. Biol. Chem. 279, 50230–50242). Here we demonstrate that the enzymes involved in oxidation of retinol to retinoic acid and then to oxidized retinoic acid metabolites are also involved in the synthesis and oxidation of all-trans-13,14-dihydroretinoic acid. All-trans-13,14-dihydroretinoic acid can activate retinoic acid receptor/retinoid X receptor heterodimers but not retinoid X receptor homodimers in reporter cell assays. All-trans-13,14-dihydroretinoic acid was detected in vivo in Lrat−/− mice supplemented with retinyl palmitate. Thus, all-trans-13,14-dihydroretinoic acid is a naturally occurring retinoid and a potential ligand for nuclear receptors. This new metabolite can also be an intermediate in a retinol degradation pathway or it can serve as a precursor for the synthesis of bioactive 13,14-dihydroretinoid metabolites.

Metabolites of vitamin A (all-trans-retinol, all-trans-ROL)3 play essential roles in vision, immunity, cellular differentiation, control of gene expression, and development in vertebrates. For example, 11-cis-retinaldehyde (11-cis-RAL) is the chromophore of visual pigments in the photoreceptor cells (1), whereas all-trans-retinoic acid (all-trans-RAl) and its 9-cis isomer are important regulators of gene expression via retinoic acid receptors (RAR) and retinoid X receptors (RXR) (2). Other active metabolites of ROL include 14-hydroxy-4,14-retro-ROL (3), anhydroretinol (4), and 13,14-dihydroxy-ROL (5), which regulate growth and cellular survival. The ring-oxidized metabolites 4-oxo-ROL, 4-oxo-RAL, and 4-oxo-RA can activate RAR and RXR receptors and have been implicated in the embryonic development of Xenopus (6–8). Bioactive retinoids continue to be discovered; however, many of the enzymes involved in retinoid metabolism have not been identified.

The oxidation of ROL is both a major metabolic pathway for the synthesis of RAL and RA and a catabolic pathway for the clearance of pharmacological doses of ROL by conversion to polar metabolites that are easier to secrete (9). The enzymes involved in the synthesis and degradation of RA have been extensively described. ROL and RAL can be interconverted by microsomal short-chain dehydrogenase/reductase (SDR) (10, 11) and by class I, III, and IV medium-chain alcohol dehydrogenases (ADH) (12). Irreversible oxidation of RAL to RA is carried out by retinal dehydrogenase (RALDH) types 1–4 (13–18). Cytochrome P450 enzymes CYP26A1, CYP26B1, and CYP26C1 carry out the catabolism of RA to 4-hydroxy-RA, 4-oxo-RA, and 18-hydroxy-RA (19–22).

We recently described a novel enzyme that carries out the saturation of the C13-14 bond of all-trans-ROL to generate all-trans-13,14-dihydro-ROL (all-trans-DROL) (23). The enzyme, ROL saturase (RetSat), is found in many tissues, with the highest levels in the liver, kidney, and intestine. RetSat was shown to convert all-trans-ROL to all-trans-DROL, which was detected in several tissues of unsupplemented animals (23). Shirley et al. (24) have described the conversion of 9-cis-RA to 9-cis-13,14-dihydro-RA (9-cis-DRA) in rats, and others have described 9-cis-4-oxo-13,14-dihydroretinoic acid as a major metabolite in the liver of mice supplemented with ROL palmitate (25). The metabolic pathway responsible for the production of 13,14-dihydroretinoids has not been investigated.

In the current study, we used lecithin:ROL acyltransferase (LRAT)-deficient mice to examine the metabolism of ROL palmitate, all-trans-RA, and all-trans-DROL in vivo, with special attention to the formation of C13–14-saturated retinoids. The pathway was reconstituted in vitro using recombinant enzymes and cells transfected with individual retinoid processing enzymes. Finally, we demonstrated that all-trans-DRA can activate transcription in reporter cell assays through RAR/RXR heterodimers but not RXR homodimers.
Materials and Methods

Metabolism of Retinoids in Vivo—All animal experiments employed procedures approved by the University of Washington and conformed to recommendations of the American Veterinary Medical Association Panel on Euthanasia and recommendations of the Association of Research Ophthalmologists. Animals were maintained in a 12-h light and 12-h dark cycle. All manipulations were done under dim red or infrared light (>560 nm). Most experiments used 6–12-week-old mice. Lrat–/– mice were genotyped as described previously (26). Animals were maintained on a control chow diet up to 1 h prior to oral gavage. The appropriate amount of all-trans-ROL palmitate, all-trans-DROL, or all-trans-RA was dissolved in vegetable oil and administered by oral gavage to rats or mice for analysis.

Analysis of Retinoids—Liver (1 g) from retinoid gavaged or naive mice was homogenized in 2 ml of 137 mM NaCl, 2.7 mM KCl, and 10 mM sodium phosphate (pH 7.4) for 30 s using a Polytron homogenizer. 10 μl of 5 mM NaOH was added to 3 ml of the ethanolic extract, and the nonpolar retinoids were extracted using 5 ml of hexane. The extraction was repeated, and the organic phases were combined, dried under vacuum, resuspended in hexane, and examined by normal phase HPLC using a normal phase column (Beckman Ultrasphere Si 5 μm, 4.6 × 250 mm). The elution condition was an isocratic solvent system of 10% ethyl acetate in hexane (v/v) for 25 min at a flow rate of 1.4 ml/min at 20 °C.

In the case of liver samples the extraction efficiency was 95% or better. Mass spectrometry analysis of polar retinoids extracted from tissue samples. As a control for the metabolism of 13,14-dihydroretinoids, previously prepared ethyl 13,14-dihydroretinoate was reduced with disobutyl aluminum hydride at -78 °C. All-trans-4-oxo-DRA has the following UV-visible absorbance spectrum in ethanol, λmax = 328 nm and shoulder at λ = 256 nm, and in hexane, λmax = 314 nm and shoulder at λ = 252 nm.

Cloning and Expression Constructs—Total embryo and liver RNA was obtained from Ambion (Austin, TX) and reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen) and oligo(dT) primers according to manufacturer's protocol. Embryo cDNA was used to amplify the cDNAs of specific genes using Hotstart Turbo PfU polymerase (Stratagene, La Jolla, CA) and the following primers: RA-LDH1, forward 5′-CACCCGAATTTGCTTCGTCCACAAAC-3′ and reverse 5′-GCTGGCTTCTTTAGGATTTCTC-3′; RA-LDH3 forward CACCTGGCAACAGTGGATAGCT and reverse 5′-GCTGGTCTTCACAGGGTGTCT-3′; CYP26B1, forward 5′-CACCACGCGGTCAGAAATCAGGCTGC-3′ and reverse 5′-GCTGGAACAGAGATGACATC-3′; and CYP26C1 forward 5′-ACACCTTCTGCGCATGTTCCT and reverse 5′-CAGAAGGGCTAGAC-3′. The full-length cDNA of RA-LDH2 (MG: 76772, IMAGE: 30471325), RA-LDH4 (MG: 46977, IMAGE: 4223059), and CYP26A1 (MG: 138650, IMAGE: 4210893) was obtained from the Mammalian Gene Collection (MGC). These clones were used as templates to amplify the respective cDNAs using Hotstart Turbo PfU polymerase (Stratagene) and the following primers: RALDH2, forward 5′-CACCAGGAAGATGACATG-3′ and reverse 5′-GCTGGGAACAGAGATGACATC-3′; and RALDH4, forward 5′-CACCTTTGATCAAGGGCCTTC-3′ and reverse 5′-GATTATATGGAATATGTTTATTATCAGTAA-3′; and CYP26A1, forward 5′-CACCACGCGGTCAGAAATCAGGCTGC-3′ and reverse 5′-GATATCTCTTCGGATGTTATTTAAT-3′. The cDNAs for RALDH1, -2, -3, and -4 and CYP26A1, -B1, and -C1 were cloned in the pcdNA3.1 Directional TOPO vector. The pcdNA3.1 Directional TOPO vector under the control of the CMV promoter expresses a recombinant protein fused with a 6× histidine tag peptide (GKIPNPLGLDST) and a Hisα tag (Invitrogen). Both strands of the expression constructs were sequenced to ensure no mutations were present.

Mouse Rxr-α was cloned using the primers 5′-GGGATGATGAGTATGTCGACAAG and 5′-AGCGATCCGCGCTGCTGCTCTC from reverse transcription mouse cDNA. The Rxr-α open reading frame was amplified into the Directional TOPO (Invitrogen) using the primers 5′-GGGATGATGAGTATGTCGACAAG and 5′-AGCGATCCGCGCTGCTGCTCTC from reverse transcription mouse cDNA. The Rxl-α open reading frame was amplified into the Directional TOPO (Invitrogen) using the primers 5′-GGGATGATGAGTATGTCGACAAG and 5′-AGCGATCCGCGCTGCTGCTCTC from reverse transcription mouse cDNA. The Rxl-α open reading frame was amplified into the Directional TOPO (Invitrogen) using the primers 5′-GGGATGATGAGTATGTCGACAAG and 5′-AGCGATCCGCGCTGCTGCTCTC from reverse transcription mouse cDNA. The Rxl-α open reading frame was amplified into the Directional TOPO (Invitrogen) using the primers 5′-GGGATGATGAGTATGTCGACAAG and 5′-AGCGATCCGCGCTGCTGCTCTC from reverse transcription mouse cDNA. The Rxl-α open reading frame was amplified into the Directional TOPO (Invitrogen) using the primers 5′-GGGATGATGAGTATGTCGACAAG and 5′-AGCGATCCGCGCTGCTGCTCTC from reverse transcription mouse cDNA. The Rxl-α open reading frame was amplified into the Directional TOPO (Invitrogen) using the primers 5′-GGGATGATGAGTATGTCGACAAG and 5′-AGCGATCCGCGCTGCTGCTCTC from reverse transcription mouse cDNA. The Rxl-α open reading frame was amplified into the Directional TOPO (Invitrogen) using the primers 5′-GGGATGATGAGTATGTCGACAAG and 5′-AGCGATCCGCGCTGCTGCTCTC from reverse transcription mouse cDNA. The Rxl-α open reading frame was amplified into the Directional TOPO (Invitrogen) using the primers 5′-GGGATGATGAGTATGTCGACAAG and 5′-AGCGATCCGCGCTGCTGCTCTC from reverse transcription mouse cDNA.
Identification of All-trans-DROL and Its Metabolites in the Liver of Lrat\(-/-\) Mice Gavaged with All-trans-ROL Palmitate—ROL absorption in mammals is an active process driven by esterification and hydrolysis cycles. Esterification of ROL is carried out mainly by the LRAT enzyme (30). In the absence of LRAT, the equilibrium between ROL and ROL esters is shifted in favor of free ROL. Mice deficient in LRAT expression (Lrat\(-/-\) mice) are severely impaired in their ROL uptake and storage capacity (26). Wild type mice, on the other hand, convert most of the ingested ROL to esters, which sequester ROL from circulation and metabolism. Thus, we chose to study the saturation and oxidation of all-trans-ROL to 13,14-dihydroretinoid metabolites in Lrat\(-/-\) mice.

Given their similar chemical properties, it is not surprising that all-trans-DROL and all-trans-ROL follow parallel metabolic pathways. Two different groups of Lrat\(-/-\) mice were dosed with either 10⁶ units of all-trans-ROL palmitate/kg body weight or 10⁵ units of all-trans-ROL palmitate/kg body weight, and their livers were examined for polar and nonpolar retinoid metabolites at 3 h post-gavage. Reverse phase HPLC analysis of polar hepatic retinoids indicated the presence of all-trans-RA (Fig. 1, A and B, peak 5) and all-trans-DRA (Fig. 1, A and B, peak 4), as well as a cis-DRA isomer (Fig. 1, A and B, peak 2).

We also observed another polar DROL metabolite, which eluted earlier than all-trans-DRA, on reverse phase HPLC (Fig. 1, A and B, peak 1) and had the same absorbance spectrum as all-trans-DRA standard (Fig. 1E). This metabolite was not chemically characterized; however, based on its polar character, it could represent a taurine or glucuronide DRA conjugate. The spectra and elution profiles of synthetic all-trans-DRA and all-trans-DRA isolated from liver matched (Fig. 1F). All-trans-DRA was synthesized according to procedures published previously (23) and was characterized by ¹H NMR (supplemental Table II).

We examined the nonpolar hepatic retinoid metabolites by normal phase HPLC. At 3 h post-gavage with ROL palmitate, the livers of the examined mice contained high levels of all-trans-ROL (Fig. 1, C and D, peak 11), whereas all-trans-DROL (Fig. 1, C and D, peak 8) was found at 280–330-fold lower levels (Table I). The absorbance spectra and elution profile of all-trans-DROL matched the synthetic standard prepared according to published procedures (23) and characterized by ¹H NMR (Fig. 1, C, D, and G, and supplemental Table II).

Another nonpolar 13,14-dihydroretinoid metabolite (Fig. 1, C and D, peak 6) that was present at higher levels than DROL was identified in the liver of mice gavaged with all-trans-ROL palmitate. The spectra of this compound also matched that of all-trans-DROL (Fig. 1G). The compound does not coelute with cis-DROL isomers and has a different UV-visible absorbance maximum than cis-DROL isomers (not shown). We were able to esterify the compound, whereas NH₂OH treatment had no effect on its elution profile (not shown). Thus, we conclude that the functional group of the compound eluting as peak 6 (Fig. 1, C and D) is alcohol. Electron-impact mass spectrometry analysis of the collected fraction corresponding to peak 6 indicates the presence of a compound with an m/z of 274 (Fig. 1F).

Following gavage of Lrat\(-/-\) mice with synthetic all-trans-DROL, we observed significant levels of all-trans-DRA and all-trans-4-oxo-DRA. These were identified based on their chromatographic profile, m/z, and absorbance spectra, which matched those of synthetic standards (supplemental Fig. 8A and inset spectra). All-trans-4-oxo-DRA was synthesized according to the scheme depicted in supplemental Fig. 7 and was
characterized by $^1$H NMR (supplemental Table II). The livers of mice gavaged with DROL were also found to contain low levels of C19-ROL (supplemental Fig. 8B, peak 4, and inset spectrum).

This is in contrast to the high levels of C19-ROL observed in all-trans-ROL palmitate gavaged mice. It has been reported that rats can convert exogenously administered 9-cis-RA to 9-cis-DRA and its taurine conjugate (24). We have shown that RetSat does not saturate all-trans-RA (23) or 9-cis-RA. This would suggest that another pathway is responsible for saturation of the C13–14 bond of RA to produce DRA. In the current study, we found no evidence of all-trans-DRA or all-trans-4-oxo-DRA formation in the livers of $Lr a t^{−/−}$ mice gavaged with all-trans-RA at 3 h post-gavage (supplemental Fig. 9). A compound different from all-trans-DRA (supplemental Fig. 9, marked with *) with a maximum absorbance of 257 nm eluted before the expected elution time of all-trans-DRA. This would suggest that 13,14-dihydroretinoid metabolites can only be derived from all-trans-DROL after saturation of all-trans-ROL by RetSat, emphasizing the key

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**TABLE I**

| Compound identified | $10^6$ IU/kg body weight-dose level of all-trans-ROL palmitate | $10^6$ IU/kg body weight-dose level of all-trans-ROL palmitate |
|---------------------|-------------------------------------------------------------|-------------------------------------------------------------|
| All-trans-RA        | $9,400 \pm 300$                                              | $320 \pm 240$                                               |
| cis-DRA             | $190 \pm 17$                                                 | $10 \pm 2$                                                  |
| Fig. 1, A and B, peak 1 | $460 \pm 50$                                               | $37 \pm 9$                                                  |
| All-trans-ROL       | $28,000 \pm 300$                                             | $7,000 \pm 1,200$                                           |
| All-trans-DROL      | $100 \pm 18$                                                 | $21 \pm 4$                                                  |
| Fig. 1, C and D, peak 6 | $1,800 \pm 370$                                           | $200 \pm 8$                                                 |
role played by RetSat at this branch of vitamin A metabolism. We also found no evidence of C19-ROL in the livers of Lrat−/− mice gavaged with all-trans-RA at 3 h post-gavage (not shown).

The levels of all-trans-RA, all-trans-DRA, and the compounds eluting as peak 1 in Fig. 1, A and B, and as peak 6 in C and D, are indicated in Table I and reflect the different starting levels of ingested ROL palmitate. The levels of all-trans-DRA are much lower (30–50-fold) than those of all-trans-RA, which could indicate that saturation by RetSat is a limiting step. The low levels of all-trans-DROL in comparison with all-trans-ROL also support this explanation. The levels of all-trans-DROL and all-trans-DRA may also be low because of further processing to shorter chain or to other more oxidized metabolites.

Characterization of the Metabolic Pathway of All-trans-DROL to All-trans-DRA—Given that all-trans-DRA is detected in vivo as a metabolite of all-trans-DROL, we decided to examine its possible mode of synthesis using reconstituted enzyme systems. To oxidize all-trans-DROL to the corresponding aldehyde all-trans-DRAL, we used ADH purified from horse liver (EC 1.1.1.1), which is active toward both primary and secondary alcohols. All-trans-DROL and all-trans-ROL were incubated with purified enzyme and the appropriate cofactors. Following the reaction the samples were treated with NH₂OH, extracted into the organic phase, and examined by normal phase HPLC. All-trans-RAL or all-trans-DRAL oximes were identified by comparison with synthetic standards (23). ADH efficiently carried out the conversion of all-trans-ROL to all-trans-RAL and of all-trans-DROL to all-trans-DRAL in the presence of NAD and NADP cofactors (Fig. 2, A and B) and not in their absence (not shown). The boiled enzyme did not exhibit any activity toward either substrate. Next, photoreceptor-specific RDH (prRDH) and RDH12 were tested for ability to catalyze the oxidation of all-trans-DROL to all-trans-DRAL. Both prRDH and RDH12 were active in converting all-trans-ROL to all-trans-RAL, but not in the absence of NADPH.

FIG. 2. Oxidation of all-trans-ROL and all-trans-DROL to the respective aldehydes. Purified ADH (Sigma) catalyzed the oxidation of all-trans-DROL to all-trans-DRAL (A) and all-trans-ROL to all-trans-RAL (B) in the presence of NAD and NADP. Control reactions using boiled enzyme were negative and show that the conversion is enzymatic. Retinoids were extracted and analyzed by normal phase HPLC. The products of the reaction were syn- and anti-all-trans-DRAL oximes (A) and syn- and anti-all-trans-RAL oximes (B). The experiment was performed in triplicate and repeated.
all-trans-RAL but much less so in converting all-trans-DROL to all-trans-RAL (results not shown).

Conversion of all-trans-DRAL to DRA is mediated by RALDH enzymes. Mouse RALDH1–4 cDNAs were cloned and fused at their C terminus with a tag containing a V5 epitope and His6 stretch. Glycosylation-deficient HEK-293S cells were transiently transfected with the tagged constructs of RALDH1–2, -3, or -4 under the control of the CMV promoter. These cells allow the reproducible, high level expression of recombinant proteins (27). The cell homogenate of transfected cells was supplemented with NAD, NADP, and ATP cofactors and with all-trans-RAL or all-trans-DRAL substrates. RALDH2 and -3 both efficiently converted all-trans-RAL and all-trans-DRAL into all-trans-RA and all-trans-DRA, respectively (Fig. 3, A and B). The products all-trans-RA and all-trans-DRA were identified based on their elution time, absorbance spectra, and comparison with authentic standards (Fig. 3, A and B, peak 1, identified as RA) and peak 6 (identified as DRA) are shown in middle and bottom panels on the right, respectively. The experiment was performed in duplicate and repeated three times. Similar results were obtained with cells transfected with RALDH3 tagged at the C terminus with V5-His6 tag.
be more active than others, as seen for mouse RALDH2 ($K_m = 0.66 \mu M$ for all-trans-RAL) versus mouse RALDH1 ($K_m = 11.6 \mu M$ for all-trans-RAL) (31, 32). Untransfected cells also exhibited significant activity toward both all-trans-RAL and all-trans-DRAL (Fig. 3, gray line chromatogram), suggesting endogenous RALDH activity in HEK-293S cells.

**Oxidation of All-trans-DRA**

The level of RA is tightly controlled by both spatially and temporally regulated synthesis and degradation. RA catabolism is carried out by cytochrome P450 enzymes CYP26A1, -B1, and -C1. It is important to determine whether DRA could also be catabolized in a similar manner. HEK-293S cells were transfected with expression constructs of CYP26A1, -B1, and -C1 fused at their C termini with a V5 epitope and His$_6$ stretch. Transfected and untransfected cells were incubated with all-trans-RA or all-trans-DRA substrate in culture because CYP26A1, -B1, and -C1 activity was adversely affected by homogenization of cells. Oxidized metabolites of all-trans-RA and all-trans-DRA were present in CYP26A1-transfected cells but not in untransfected cells (Fig. 4, A and B). These metabolites, which could include all-trans-4-oxo-(D)RA, all-trans-4-hydroxy-(D)RA, all-trans-5,8-epoxy-(D)RA, and all-trans-18-hydroxy-(D)RA, were identified as polar compounds eluting shortly after the injection spike (Fig. 4, peaks 1 and 2 and peaks 7–9, and inset spectra). One of the oxidized all-trans-DRA compounds was identified as all-trans-4-oxo-DRA because it matched the elution profile and absorbance spectrum of a synthetic standard (Fig. 4, lower right, inset panel). Peaks 10 and 11 represent cis- and all-trans-DRA, respectively. The experiment was performed in duplicate and repeated three times. Similar results were obtained with cells transfected with CYP26B1 and -C1.

![Fig. 4. Oxidation of all-trans-RA and all-trans-DRA.](image-url)
Conversion of All-trans-DROL to All-trans-DRA in RPE—
Retinoid metabolism occurs in many embryonic and adult tissues. Thus, it is important to determine whether the entire pathway of synthesis of all-trans-DRA can be reconstituted with tissue extracts. All-trans-DROL (supplemental Fig. 10, peak 2) was efficiently converted to all-trans-DRA (supplemental Fig. 10, peak 1) by microsomes prepared from RPE cells in the presence of dinucleotide cofactors NAD and NADP. All-trans-DRA was identified based on its elution profile and absorbance spectrum in comparison with synthetic all-trans-DRA (supplemental Fig. 10 and inset spectra). RPE microsomes also catalyzed the conversion of all-trans-ROL into all-trans-RA (results not shown), which indicates that adult RPE could be an active all-trans-RA, all-trans-DRA synthesis site. The main ROL oxidizing activity in the RPE is catalyzed by SDR family enzymes. The efficient conversion of all-trans-DROL to all-trans-DRA in the RPE supports the existence of SDR enzymes that can convert all-trans-DROL into all-trans-DRAL. Further studies are required to examine the substrate specificity of the known SDR enzymes from the RPE with respect to all-trans-DROL.

Based on the known all-trans-ROL oxidation pathway and results presented here, we propose that following saturation of all-trans-ROL to all-trans-DROL, all-trans-DROL is oxidized to all-trans-DRA and later to all-trans-4-oxo-DRA and possibly other oxidized metabolites of all-trans-DRA. We showed that the same enzymes involved in the oxidation of ROL to RA are also involved in the oxidation of DROL to DRA as depicted in Scheme 1. All-trans-DROL and other more oxidized metabolites occur naturally and represent a novel and potentially important pathway in the metabolism of vitamin A. This hypothesis is supported by the unequivocal identification of all-trans-DROL and all-trans-DRA in Lrat−/− mice gavaged with all-trans-ROL palmitate.

Characterization of the Transactivation Activity of All-trans-
DRA—All-trans-RA binding to RAR and 9-cis-RA binding to RAR or RXR can control the expression of genes containing RA-response element (RARE) sequences within their promoter region. RARE elements are composed of direct repeats (DR) of the canonical sequence PuG(G/T)TCA separated by one to five nucleotides. Activated RAR/RXR heterodimers can associate with RARE composed of DR separated by five nucleotides (DR5), which are found in the promoter region of many genes including the RARβ gene (33).

We studied whether DRA could also control gene expression through RAR activation by using a DR5 RARE-reporter cell line. The F9 teratocarcinoma cell line expresses endogenous RAR and RXR and is exquisitely sensitive to the effects of RA. This cell line has been transfected with lacZ under the control of a minimal promoter and upstream DR5 elements (29). F9-RARE-lacZ cells were treated with different doses of all-trans-RA and all-trans-DRA for 24 h, after which the cells were harvested, and the β-galactosidase activity was evaluated by X-gal staining (Fig. 5, top panels). All-trans-DRA transactivation of DR5-induced β-galactosidase expression was observed at higher concentrations than the equivalent effect produced by RA. All-trans-RA and all-trans-DRA induction activity was quantified by using the soluble substrate o-nitrophenyl β-D-galactopyranoside. The colorless substrate was cleaved by β-galactosidase to yellow colored o-nitrophenol, whose absorbance was measured at 420 nm using a spectrophotometer (Fig. 5).
HEK-293S cells cotransfected with a DR1-lacZ reporter construct and mouse RXRα. In combination with a previous report on the identification of all-trans-DROL as the product of RetSat (23), this study characterized the enzymatic pathway responsible for the formation of all-trans-DRA from all-trans-ROL. Saturation of the C13,14 bond of all-trans-ROL by RetSat produces all-trans-DROL, which is oxidized to the corresponding retinaldehyde, all-trans-DRAL, by ADH-1 and possibly by SDR family RDHs present in the RPE. All-trans-DRAL is oxidized to all-trans-DRA by RALDH1–4. All-trans-DRA can be oxidized to all-trans-4-oxo-DRA in mice gavaged with all-trans-DROL and in vitro by cytochrome P450 enzymes CYP26A1, -B1, and -C1, suggesting a possible pathway for its degradation (Scheme 1). All the substrates and products of reactions and metabolites isolated from mouse tissues were identified by comparing their UV-Visible absorbance spectra and chromatographic profile with authentic synthetic standards characterized by NMR and mass spectrometry. Contrary to a previous report indicating the conversion of 9-cis-RA to 9-cis-DA (24), we found no evidence of in vivo conversion of all-trans-RA into all-trans-DRA. Thus, all-trans-DRA can only be derived from oxidation of all-trans-ROL, and RetSat is the sole known enzyme responsible for catalyzing the key step in all-trans-DRA formation. These findings indicate that saturation of all-trans-ROL by RetSat is an active and possibly important step in the metabolism of retinoids in vivo.

Synthesis and Degradation of All-trans-DRA—Many of the ADH and SDR families and some RALDHs are expressed in the retina and RPE (17, 39–43). We demonstrate in the current study that a pathway of conversion of all-trans-ROL into all-trans-DRA exists and is efficient in RPE microsomes (supplemental Fig. 10). This implies that all-trans-DRA synthesis can occur in the same tissues where all-trans-RA synthesis occurs and that all-trans-DRA could have a concentration gradient in different tissues. This gradient will be determined by the availability of synthetic and catabolic enzymes as well as the availability of primary substrate, i.e. all-trans-ROL.

RA bioavailability is tightly regulated by the balance between itsbiosynthesis and catabolism (44). The cytochrome P450-type enzymes, which include ubiquitously expressed CYP26A1, -B1, and -C1 (19, 20, 22, 45), oxidize RA to 4-OH-RA, 4-oxo-RA, 18-OH-RA, and 5,8-epoxy-RA. Thus, CYP26 enzymes are involved in limiting spatial and temporal levels of RA, and in concert with ADH, SDR, and RALDH they guard a desirable level of RA, protecting against fluctuations in the nutritional levels of ROL. As shown here, CYP26A1, -B1, and -C1 enzymes also metabolize all-trans-DRA. This could also contribute to a temporal and spatial gradient of DRA in vivo.

Identification of Chain-shortened ROL Metabolites—In this study we report the identification of an ROL metabolite that contains an alcohol functional group and is saturated at the C13,14 bond and chain-shortened at C-15. Chain-shortened ROL metabolites have been described in early studies that followed the fate of radioactive 14C-labeled RA or all-trans-ROL (46–48). One possible pathway for their synthesis could be through α-oxidation of all-trans-DRA as suggested previously by others (24). The C19-ROL metabolite could be the product of a reduced C19-aldehyde intermediate produced during the α-oxidation of all-trans-DRA (equivalent to the pristinal intermediate of the pristane acid degradation pathway). Only low amounts of C19-ROL were observed in Lrat−/− mice supplemented with all-trans-DROL and with the levels obtained in mice gavaged with all-trans-ROL palmitate. This discrepancy might be accounted for by the fact that endogenous all-trans-DROL has access to a different
Metabolism of 13,14-Dihydroretinoids

reertoire of enzymes than does all-trans-DROL administered by gavage. The definite pathway of synthesis of the C19-ROL could be established by using knock-out animal models deficient in specific enzymes of this pathway.

Potential Role of 13,14-Dihydroretinoids in Vertebrate Physiology—Based on experiments using mice deficient in specific enzymes involved in retinoid metabolism, it was shown that ADH1 and RALDH1 are involved in a protection mechanism in response to pharmacological doses of ROL. Adh1−/− and Raldh1−/− mice were much more sensitive to ROL-induced toxicity than their wild type counterparts (49, 50). It was proposed that conversion of ROL to RA protects against excess levels of dietary ROL. This idea is counterintuitive considering the well known toxic effects of RA. Here we show that ADH1 and RALDH1 are also involved in DROL oxidation to DRA and that all-trans-DRA is a much weaker activator ofRAR- or RXR-mediated transcription compared with all-trans or 9-cis-RA. Thus, it is possible that saturation of the C13,14 bond of all-trans-ROL could be the first step in a degradation pathway, which provides protection against pharmacological doses of all-trans-ROL and circumvents the formation of RA. Our findings show that the combined amounts of hepatic DROL and DROL metabolites amount to less than one-third of the amount of hepatic all-trans-RA at 3 h post-gavage with 106 IU ROL palmitate/kg body weight. This would suggest that saturation by RetSat is a rate-limiting reaction in the metabolic pathway.

Another possibility is that RetSat activity leads to production of novel bioactive 13,14-dihydroretinoids. We identify all-trans-DRA as an activator ofRAR/RXR heterodimer-mediated transcription. The tissue concentration and transactivation profile of all-trans-DRA are both lower than those of all-trans-RA. It is possible that all-trans-DRA and other DROL metabolites could have important transactivation activity in certain physiological circumstances. The local concentration of 13,14-dihydroretinoid ligand might reach higher levels as a result of being trapped by receptors or binding proteins. Given that the local concentration and binding affinity are sufficient, all-trans-DRA could be an important endogenous ligand for RAR or possibly for other nuclear receptors. The finding that the same enzymes that were thought to act specifically in the formation of RA are also responsible for the formation of DRA has to be considered in attempts to rescue with RA the phenotype of knock-out animal models deficient in these enzymes. In one such example, Raldh2−/− mouse embryos cannot be completely rescued by maternal RA supplementation and die prenatally (51). It is interesting to speculate if other retinoid metabolites, including 13,14-dihydroretinoids, in addition to RA may be necessary for a complete rescue of Raldh2−/− embryos. The identification of the all-trans-DRA metabolic pathway is the first step in this process, and more studies are necessary to establish the physiological role of DRA and other DROL metabolites in controlling gene expression.

In summary, we describe a new metabolic pathway for vitamin A that leads to a new class of endogenous bioactive retinoids. We demonstrate that all-trans-ROL saturation to all-trans-DROL followed by oxidation to all-trans-DRA occurs in vivo. All-trans-DRA can activate transcription of reporter genes by binding RAR but does not bind RXR. The oxidative pathway of all-trans-DROL employs the same enzymes as that of all-trans-ROL. We expect that these previously unknown metabolites will help us better understand the vital functions of retinoids in vertebrate physiology.

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