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Phytol Metabolites Are Circulating Dietary Factors that Activate the Nuclear Receptor RXR

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RXR is a nuclear receptor that plays a central role in cell signaling by pairing with a host of other receptors. Previously, *9-cis*-retinoic acid (9cRA) was defined as a potent RXR activator. Here we describe a unique RXR effector identified from organic extracts of bovine serum by following RXR-dependent transcriptional activity. Structural analyses of material in active fractions pointed to the saturated diterpenoid phytanic acid, which induced RXR-dependent transcription at concentrations between 4 and 64 μM. Although 200 times more potent than phytanic acid, 9cRA was undetectable in equivalent amounts of extract and cannot be present at a concentration that could account for the activity. Phytenic acid, another phytol metabolite, was synthesized and stimulated RXR with a potency and efficacy similar to phytanic acid. These metabolites specifically displaced [^{3}H]-9cRA from RXR with K_i values of 4 μM, indicating that their transcriptional effects are mediated by direct receptor interactions. Phytol metabolites are compelling candidates for physiological effectors, because their RXR binding affinities and activation potencies match their micromolar circulating concentrations. Given their exclusive dietary origin, these chlorophyll metabolites may represent essential nutrients that coordinate cellular metabolism through RXR-dependent signaling pathways.

INTRODUCTION

Nuclear receptors are transcription factors that regulate gene expression in response to lipophilic ligands such as steroid hormones (Yamamoto, 1985). Ligand binding increases the receptor affinity for hormone-responsive DNA elements (HREs) near target genes that promote specific transcriptional control (Glass, 1994). A large family of receptors coordinates cell physiology through these hormone-regulated gene networks (Evans, 1988). The kindred includes structurally related "orphan" nuclear receptors whose activators are unknown (O'Malley, 1989, 1990). Although most orphan receptor genes were isolated by low-stringency hybridization techniques (Arriza *et al.*, 1987), some, like COUP and HNF-4, initially were described as transcriptional regulators for specific genes (Wang *et al.*, 1989; Sladek *et al.*, 1990).

Activators for orphan receptors have been found by testing compounds in cells transfected with the corre-

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[1] Abbreviations used: ATRA, *all-trans* retinoic acid; FBS, fetal bovine serum; 9cRA, *9-cis* retinoic acid; RAR, retinoic acid receptors.

sponding receptor and HRE-linked reporter genes (Giguere *et al.*, 1986; Green and Chambon, 1987). Aldosterone, retinoic acid, and ecdysone are some of the ligands matched with receptors via these "cis-trans" assays (Arriza *et al.*, 1987; Giguere *et al.*, 1987; Petkovich *et al.*, 1987; Koelle *et al.*, 1991). The nanomolar affinities of these ligands contrast with the micromolar amounts of fatty acids or prostaglandin J_2 required to activate PPARα and PPARγ, respectively (Gottlicher *et al.*, 1992; Keller *et al.*, 1993; Forman *et al.*, 1995b; Kliewer *et al.*, 1995). Similarly, metabolites of farnesyl pyrophosphate (farnesoids) are needed at micromolar levels to induce FXR (Forman *et al.*, 1995a). Fatty acids and farnesoids have been argued as candidate physiological effectors for PPAR and FXR (Keller and Wahli, 1993; Weinberger, 1996). Although many genes regulated by PPAR are linked to fatty-acid metabolism and fatty acids have been detected in PPAR-inducing chromatographic fractions from human plasma (Banner *et al.*, 1993), direct interactions of fatty acids with PPARs have not yet been demonstrated.

RXR is a unique member of this orphan receptor family that facilitates many signaling pathways by heterodimerizing with receptors activated by thyroid hormones, retinoids, vitamin D, fatty acids, and farnesoids (Manglesdorf and Evans, 1995). RXR partners also include the orphan receptors COUP (Kliewer *et al.*, 1992), NGFIb/nurr1 (Forman *et al.*, 1995c; Perlmann and Jansson, 1995), and UR/LXR subfamily members (Song *et al.*, 1994; Teboul *et al.*, 1995; Willy *et al.*, 1995). The variety of these interactions suggests that RXR performs a key regulatory role in cell physiology.

Surveys of chemical compounds revealed *all-trans*-retinoic acid (ATRA)[1] as an RXR inducer (Manglesdorf *et al.*, 1990). However, ATRA did not bind RXR with high affinity, supraphysiological levels were required for activity, and receptors for retinoic acid (RAR) had already been identified (Giguere *et al.*, 1987; Petkovich *et al.*, 1987). Thus, it was proposed that ATRA might be metabolized to a more active form (Manglesdorf *et al.*, 1990). Indeed, ATRA isomerizes to *9-cis*-retinoic acid (9cRA), which activates RXR with a greater potency (Heyman *et al.*, 1992; Levin *et al.*, 1992), but activation of RXR and RAR by 9cRA limits its physiological specificity (Allegretto *et al.*, 1993). Identification of RXR-specific synthetic "retinoids" and methoprene acid (Lehmann *et al.*, 1992; Boehm *et al.*, 1994, 1995; Harmon *et al.*, 1995), coupled with an inability to detect 9cRA in rat serum (Kojima *et al.*, 1994), may argue for the existence of other endogenous RXR-selective terpenoids.

An important question emerging from receptor ligand searches is the following: How are the pharmacological and physiological activators to be distinguished? Receptor specificity is one measure, but matching the ligand potency with its abundance in biological tissues is equally critical. That is, the intracellular concentrations of ligands must be within the ranges of receptor binding affinities and activation potencies. For example, T_3 and T_4 circulate in human plasma at 2 and 100 nM, respectively (Scully *et al.*, 1980), but T_4 activates the thyroid hormone receptor with a 50-fold reduced potency, as compared with T_3 (Shih *et al.*, 1991). Consequently, a low-affinity receptor ligand such as T_4 should not be dismissed as nonphysiological on the basis of potency alone, provided that its total effector activity is similar to that of a less abundant but more active one like T_3.

Circulating levels of steroid and thyroid hormones, retinoids, and vitamin D in animal sera are within the ranges of their receptor-activation potencies. Therefore, orphan receptor activators have been sought from biological tissues such as plasma (Shih *et al.*, 1991; Banner *et al.*, 1993) as well as by a survey of chemical compounds. Chromatographic separation methods can be used to compare the candidate chemically synthesized receptor activators with those extracted from biological tissues to assess their physiological significance. Here we report that a search for RXR activators from bovine serum only revealed ones chromatographically distinct from 9cRA. These phytol metabolites are compelling candidates for physiological RXR effectors, because they satisfy the above criteria for molecules circulating at concentrations potentially relevant for RXR binding and activation.

MATERIALS AND METHODS

Reagents

Fatty acids and other chemicals for enzyme assays were purchased from Sigma Chemical (St. Louis, MO). Cell culture reagents were obtained from Life Technologies (Gaithersburg, MD).

Cell Culture and Transfections

CHO K1 cells were cultured at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle medium (DMEM)/F12 supplemented with 5% fetal bovine serum (FBS), penicillin (50 units/ml), and streptomycin (50 μg/ml). Dextran-coated charcoal was used to adsorb lipids from FBS in extract addition experiments (Samuels *et al.*, 1979). Transfection assays were performed with N,N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES)/calcium chloride (Chen and Okayama, 1988). Briefly, cells were plated at 30% confluence 1 d before transfection. DNA (3.5 μg) in 200 μl of the DNA/calcium phosphate coprecipitate mixture was added to cells growing in 2-ml media/well of a 6-well tissue culture plate (Falcon, Oxnard, CA). Cells were incubated for 7–8 h at 37°C and washed twice with phosphate-buffered saline (PBS) before ligand or extract additions in fresh media containing 5% charcoal-adsorbed FBS. Methanol ($\leq$ 2% final concentration in media) was used for dissolving extracts and ligands. Liposome-mediated transfection of DNAs into CV-1 cells was performed with *N*-[1-(2,3)-dioleoyloxylpropyl-*N*,*N*,*N*-trimethylammonium methyl sulfate (DOTAP) according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN). Liposomes were removed after 2 h, and cells were subsequently treated for 40 h with DMEM-FBS alone or with the indicated compounds. Transfected cells were seeded at 7000 cells per well of a microtiter plate. For the GAL-hRXRα plate, 300 ng TK-$(UAS_G)_4$-LUC, 500 ng CMX-β-gal, and 100 ng CMX-GAL4-hRXRα

plasmid DNAs were added per 10^5 transfected CV-1 cells. For the TK-(CRBPII)-LUC plate, 300 ng TK-(CRBPII)-LUC, 500 ng CMX-β-gal, and CMX-hRXRα were added per 10^5 transfected CV-1 cells.

Enzyme Assays

Transfected cells incubated with extracts or ligands for 24 h were washed with PBS two times and then harvested by scraping into 1.2 ml of isotonic buffer (150 mM NaCl, 40 mM Tris-HCl, pH 8, and 1 mM EDTA). Cells were centrifuged briefly (5000 × *g*, 30 s), and the cell pellets were resuspended in 50 μl of 0.25 M Tris-HCl, pH 8. Cells were then subjected to three freeze/thaw cycles (dry ice-ethanol/37°C) before a final centrifugation step (10,000 × *g*, 3 min). Different volumes of supernatants were used to measure CAT activity (20 μl; Seed and Sheen, 1988) or β-galactosidase activity (2 μl; Herbomel *et al.*, 1984). Luciferase activity was measured as described in Berger *et al.* (1992).

Bovine Serum Extraction

FBS (Life Technologies) or serum from bovine blood (freely grazing steer raised on silage at North Carolina State University School of Veterinary Medicine) were extracted with chloroform and methanol solvents (Bligh and Dyer, 1959). Briefly, 10 ml of serum was mixed with 37.5 ml of chloroform and methanol (2:1) and vigorously shaken for 15 min. The mixture was centrifuged at 2000 × *g* for 20 min. To the supernatant was added 12.5 ml each of water and chloroform to separate the phases. The mixture was centrifuged at 9000 × *g* for 15 min, and the chloroform phase was collected. Alternatively, serum was saponified (2 M KOH at 70°C for 30 min) and twice extracted with diethyl ether. Next, the aqueous solution was acidified with concentrated HCl and then extracted with ether again. Chloroform, methanol, or ether was removed by rotary evaporation under vacuum (Buchi Rotavapor R-124 or Speed Vac SC210A; Savant, Farmingdale, NY).

High Performance Liquid Chromatography

Pure chemical standards or bovine serum extracts were resuspended in 80% methanol and injected into a 1 ml Rheodyne sample loop connected to a Beckman System Gold high-performance liquid chromatography unit (HPLC). The LC system consisted of an RP18 guard column (15 × 3.2 mm, RP18; Alltech, Deerfield, IL) linked to a separation column (4.6 × 25 cm, Econosphere C18, 5 μ particle size; Alltech) and a Gilson FC 203B fraction collector (Middleton, WI). UV absorbance was monitored with a Beckman diode array detector module 168. The sample was eluted with an 80% methanol/20% 10 mM ammonium acetate mobile phase for 5 min, after which a linear gradient (80–100% methanol, 20 min) was applied and held at 100% methanol for 10 min. Fractions were collected, dried, and dissolved in DMEM/F12 containing 5% dextran-coated charcoal-absorbed FBS for measurement of CAT activity in the cis-trans assay.

Silica Gel Chromatography

Pure phytanic acid or a chloroform extract of bovine serum was loaded on a silica gel column (4 cm wide × 10 cm height) and eluted with 20% ethyl acetate/80% hexane. In all, 8-ml fractions were collected in 13 × 100-mm glass test tubes, dried by rotary evaporation, resuspended in media containing charcoal-adsorbed FBS, and tested in the cis-trans assay as described.

Mass Spectroscopy

Gas chromatography/mass spectrometry (GC/MS). The trimethylsilyl (TMS) derivative of serum fraction 23 and the phytanic acid standard were prepared by reacting 5 μl of each sample with 10 μl of *N,O*-bis(trimethylsilyl)trifluoroacetamide (Supelco, Bellefonte, PA). Reaction mixtures were heated at 70°C for 15 min. An aliquot (0.5 μl) of the reaction mixture was injected onto a Quadrex methylphenyl 5 capillary GC column (30 × 0.25 mm ID, 0.25 mm film) in a Hewlett-Packard 5880 gas chromatograph equipped with a J&W on-column splitless injector and connected to a Finnigan 700 Ion Trap Detector. Full-scan data were obtained over the mass range 40–650 daltons, at a scan rate of 2 s/scan. We used the following temperature program: initial temperature 40°C (1 min hold); programmed to 300°C at 10°/min; hold at 300°C for 30 min.

Fast atom bombardment. A VG ZAB-4F magnetic sector instrument was used to obtain fast atom bombardment (FAB) data at an accelerating voltage of 8 kV. An Ion Tech atom gun and xenon atoms were used to bombard the sample. The samples were introduced into the mass spectrometer via a coaxial continuous-flow FAB interface. This interface uses a coaxial arrangement of fused silica capillaries to independently deliver the FAB matrix (glycerol) and the analytes. The instrument was scanned from 1000 to 100 daltons at 5 s/decade to acquire the full-scan negative ion data.

Electrospray/ionization MS. Measurements were made on a Fisons-VG Quattro BQ triple-quadrupole mass spectrometer equipped with a pneumatically assisted electrospray ion source operating at atmospheric pressure. The HPLC fractions containing biologically active material and phytanic acid were reconstituted in acetonitrile and mixed with equal volumes of the LC mobile phase (80% acetonitrile/20% water containing 1% ammonium hydroxide). Samples were introduced by loop injection into the mobile phase at a flow rate of 8 μl/min, and spectra were acquired in the negative ion continuum-mode scan rate. The mass scale was calibrated with polyethylene glycol with an average molecular weight of 400 atomic mass units (amu). Theoretical isotope distributions were computed with Fisons Instruments Opus software.

Synthesis of Phytenic Acid

Phytenic acid was prepared from phytol (Sigma) by adapting a two-step MnO_2 oxidation procedure (Corey *et al.*, 1968). Phytol was oxidized to phytal by using activated MnO_2 (Aldrich Chemical, Milwaukee, WI) to give an ~60:40 mixture of geometric isomers by nuclear magnetic resonance (NMR). The isomers were partially separated by chromatography on silica gel, with 5% ethyl acetate in hexane as eluent. The partially purified phytal isomers were each further oxidized to the corresponding methyl ester by treatment with MnO_2·NaCN·methanol. Chromatography on silica gel with 2% ethyl acetate in hexane gave the individual isomers. Saponification of the methyl esters by KOH in 70:30 methanol/water yielded the free acids. The major isomer was assigned trans stereochemistry on the basis of comparison of the ^1H NMR chemical shifts of the methyl and methylene groups attached to the double bond. As examples, for the trans methyl ester, the chemical shift of the methyl group is 2.11 parts per million (ppm), and the chemical shift for the methylene group is 2.07 ppm. For the cis isomer, the methyl group is relatively shielded by the carbonyl group (1.84 ppm), and the methylene group is relatively deshielded (2.56 ppm).

Hormone Binding

[^{3}H]-ATRA or [^{3}H]-9cRA binding to baculovirus-expressed RAR(α, β, γ) or RXR(α, β, γ) polypeptides was measured as described previously (Allegretto *et al.*, 1993). Receptor genes expressing these recombinant proteins were all of human origin except RXRβ and RXRγ, which were derived from the mouse. The assay buffer consisted of 8% glycerol, 120 mM KCl, 8 mM Tris-HCl, 5 mM CHAPS, 4 mM dithiothreitol, and 0.24 mM phenylmethylsulfonyl fluoride, final pH 7.4 (room temperature). The final volume for binding assays was 250 μl, which contained 10–40 μg of protein extract plus 5 nM of [^{3}H]-ATRA for RARs or 10 nM [^{3}H]-9cRA for RXRs, plus varying concentrations of competing ligands. Incubations were performed at 4°C until equilibrium was achieved. Nonspecific binding is defined as that binding remaining in the presence of 1 μM of the appropriate unlabeled retinoid isomer. At the end of the incubation,

50 μl of 6.25% hydroxylapatite was added in the appropriate wash buffer (100 mM KCl, 10 mM Tris-HCl, and either 5 mM CHAPS [RXRs] or 0.5% Triton X-100 [RARs]) to bind the receptor–ligand complexes. Mixtures were vortexed and incubated at room temperature for 30 min and centrifuged, and the supernatants were removed. Hydroxylapatite pellets were washed two more times with wash buffer, and the receptor–ligand complexes were determined by liquid scintillation counting of the pellets. After correcting for nonspecific binding, IC_{50} values were determined. The IC_{50} value is defined as the concentration of competing ligand required to decrease specific binding by 50%, which is determined graphically from a computer-based log–logit plot of the data (Cheng and Prusoff, 1973).

RESULTS

RXR Effector Activity from Bovine Serum

We initially attempted to identify activators from bovine serum (Shih *et al.*, 1991) for an orphan receptor called OR6, which binds to an AGGTCA direct repeat HRE separated by 4 bp (DR4), but only in the presence of RXR (Umesono *et al.*, 1991). CHO cells were transfected with a DR4-linked CAT reporter plasmid DNA along with an OR6 expression vector, and CAT activity was measured. A lipid extract of FBS was added (Bligh and Dyer, 1959), but this had no effect on CAT activity (our unpublished observations). Although the extract stimulated activity eightfold when RXR was added, RXR alone showed a similar effect (our unpublished observations). These results suggested that the bovine serum activator was mediating its effects through RXR.

Therefore, the serum effector was compared with 9cRA, a previously described RXR effector from liver (Heyman *et al.*, 1992). The chloroform extract of serum was separated by reverse-phase HPLC, and the eluted fractions were tested for RXR effector activity. Unexpectedly, the RXR activator had a retention time (R_t) between 19 and 22 min (Figure 1), which did not coincide with the elution profile for a 9cRA standard (R_t = 7 min). Because 9cRA is chemically similar to ATRA, we added a tracer amount of [^{3}H]-ATRA (1 nM) to a serum sample to ask whether retinoic acid could be extracted by this method. Nearly all of the radioactivity (83%) was found in the chloroform fraction, thus supporting the utility of the Bligh and Dyer method for extracting retinoids (our unpublished observations).

RXR Effector Activity Is Distinct from 9cRA

Because the retinoic acid in serum may have been resistant to extraction by the Bligh and Dyer method, a procedure specifically used to isolate retinoids was used here to characterize the RXR effector (Kojima *et al.*, 1994). We also wanted to exclude vitamin supplements as potential sources for exogenous retinoids that are sometimes given to donor herds. Therefore, serum from a freely grazing steer was saponified and

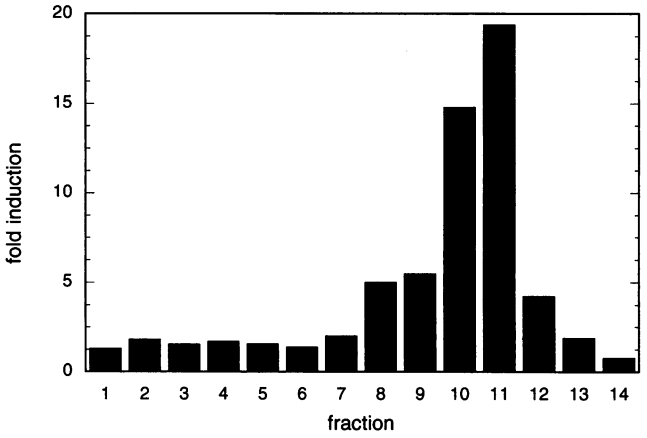

Figure 1. Identification of an RXR effector activity from bovine serum. RXR effector activity profile from chloroform extract of FBS fractionated by reverse-phase HPLC. The chloroform fraction from a Bligh and Dyer extract of 20 ml of FBS was separated by reverse-phase HPLC methods, as described in MATERIALS AND METHODS. Two-minute fractions were pooled and tested for RXR effector activity with the cotransfection assay. Subconfluent (30%) CHO cells were transfected with 1.25 μg of SV-$(DR4)_3$-CAT reporter plasmid (Umesono *et al.*, 1991), 0.25 μg of human pRS-OR6 or 0.25 μg of CMX-mouse RXRα, 1.25 μg of pCH111, and 0.5 μg pGEM4 DNAs. The pCH111 plasmid (Yao *et al.*, 1993) expressing β-galactosidase was included to correct for differences in transfection efficiency. Normalized CAT activity was plotted against column fraction assayed. A 9cRA standard had a retention time of 7 min via this method. The experiment was performed three times with similar results. Note that the coefficient of variation for CAT activity measurements is typically <15%.

ether extracted, and then the aqueous phase was acidified and extracted with ether again. [^{3}H]-ATRA in a parallel sample was quantitatively extracted by ether (95%) from the acidified aqueous solution, marking this as another effective means for retinoid isolation. In contrast, RXR-inducible CAT activity was found only in the ether extract of the basic solution (our unpublished observations). This material was separated by the HPLC conditions described above (Figure 1), and the 1-min fractions were collected and tested for RXR effector activity. An RXR-specific activator (R_t = 23–24 min, Figure 2A) was identified that eluted later than ATRA or 9cRA (R_t = 8.8 and 7.5 min, respectively). Therefore, both saponified and nonsaponified serum extracts contained an RXR activator with chromatographic properties distinct from 9cRA.

It was conceivable that retinoids were destroyed by this rigorous extraction method. Therefore, DNAs for the human retinoic acid receptor (RARα) and βRARE-CAT reporter were transfected into cells to permit detection of the RAR activators ATRA and retinol (Giguere *et al.*, 1987; Sucov *et al.*, 1990). Activities coincident with 9cRA, ATRA (R_t = 7. 5 and 8.8 min), and retinol (R_t = 20 min) were confined to the acidified extract (Figure 2B); none

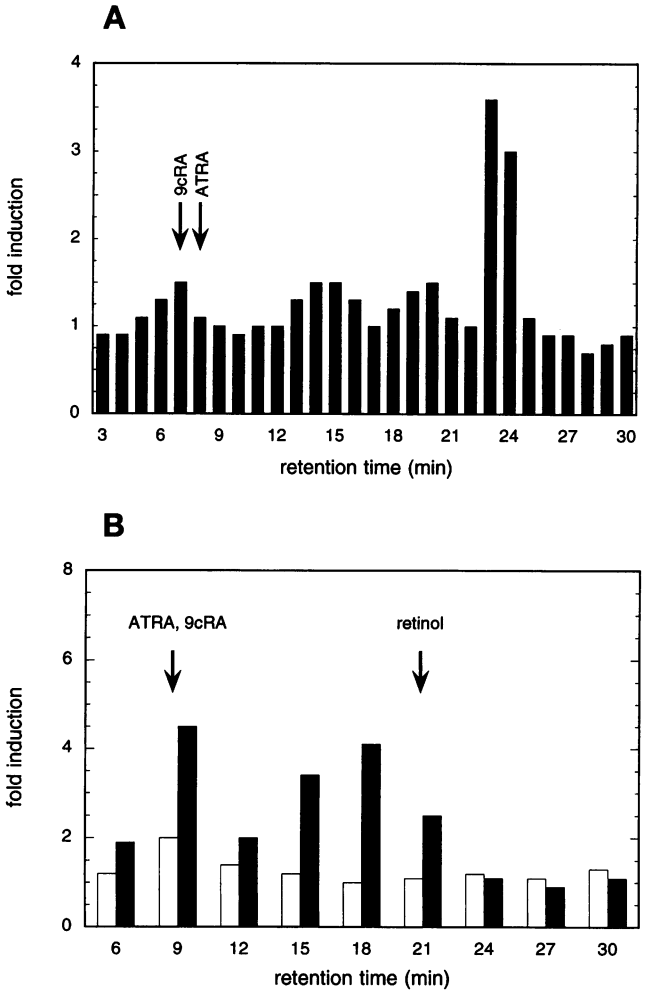

Figure 2. RXR and RAR activators in bovine serum can be extracted by saponification and ether extraction. Bovine serum (10 ml) was saponified (2 M KOH, heated at 70°C for 30 min) and extracted with diethyl ether. The aqueous phase was acidified, and ether was extracted again. Basic and acidic ether extracts were dried and fractionated separately by reverse-phase HPLC, as described in MATERIALS AND METHODS. (A) Separation of RXR activators from bovine serum by reverse-phase HPLC. One-minute fractions were collected and dried, and a portion (5%) was taken up in a medium containing 5% charcoal-adsorbed FBS for testing by cis-trans assay, as described in Figure 1. (B) Characterization of RAR activators from bovine serum by reverse-phase HPLC. Three-minute fractions were tested by cotransfecting an SV-$(\beta RARE)_2$-CAT reporter plasmid and a plasmid DNA expressing the human RARα receptor into CHO cells, essentially as described in Figure 1. Symbols: shaded bars, acidic extract; closed bars, basic extract. Fold induction values are relative to control samples containing methanol vehicle. A control sample in B containing 200 nM ATRA showed a 4.9-fold induction by comparison.

was found in the ether extract of the basic solution in which the RXR effector activity was observed. In addition, a broad range of activity more polar (R_t < 20 min) than retinol was seen. This material may correspond to hydroxylated retinol metabolites, such as 4-oxo-retinol, the acid derivative of which was shown to activate RAR (Pijnappel *et al.*, 1993). Nevertheless, although peaks of activity cannot be assigned, it is clear that RAR and RXR activators have distinct pH-dependent partitioning characteristics in ether. Moreover, the functional integrity of RAR activators is maintained during extraction. By inference, 9cRA should have been found in the acidic fraction, but no corresponding RXR effector activity was detected here (our unpublished observations). These results suggest that the bovine serum activator is distinct from 9cRA, but they do not exclude the possibility that 9cRA may still be an intracellular signal in the liver or kidney, where it was originally described (Heyman *et al.*, 1992).

Fatty Acids Copurify with RXR Effector Activity

To characterize the molecular structure of the RXR activator, the active fraction of the basic ether extract (R_t = 23 min) and two adjacent inactive ones (R_t = 22 and 25 min) were analyzed by various mass spectrometric techniques. Negative ion electrospray spectra, obtained by flow-injection analyses of these fractions, contained ions of m/z 283 and 311 (Figure 3A). The abundance of the m/z 311 ion corresponded to the RXR activities in these fractions (Figure 2A), whereas the abundance of the m/z 283 ion did not follow the RXR activities. Relative isotopic abundance measurements for these negative ions predicted the molecular formulas $C_{18}H_{36}O_2$ and $C_{20}H_{40}O_2$ for the molecular weight 284 and 312 Da components, which are consistent with the elemental compositions of stearic acid and phytanic acid, respectively (our unpublished observations). The same two prominent $(M\text{-}H)^-$ ions, m/z 283 and 311, were also observed by negative-ion fast atom bombardment mass spectrometry (our unpublished observations).

GC/MS analysis of the TMS-derivatized saponified sample showed a peak corresponding in mass to the $(M\text{-}CH_3)^{+\cdot}$ fragment ion (m/z 369) of the TMS derivative of phytanic acid, as well as a low-abundance peak corresponding to the molecular ion (m/z 384). The full-scale mass spectrum and the retention time of this component were in agreement with those of the TMS derivative of authentic phytanic acid (Figure 3B), run under identical conditions. Cochromatography of the sample and the phytanic acid standard gave a single peak in the reconstructed ion chromatogram for m/z 369, as well as for other characteristic ions.

Phytanic Acid Is the Serum RXR Activator

A single chromatographic step was deemed unlikely to have separated the RXR activators from other serum components. Nonetheless, the above results prompted us to examine a collection of fatty acids for RXR activation. Although linoleic, oleic, stearic, farne-

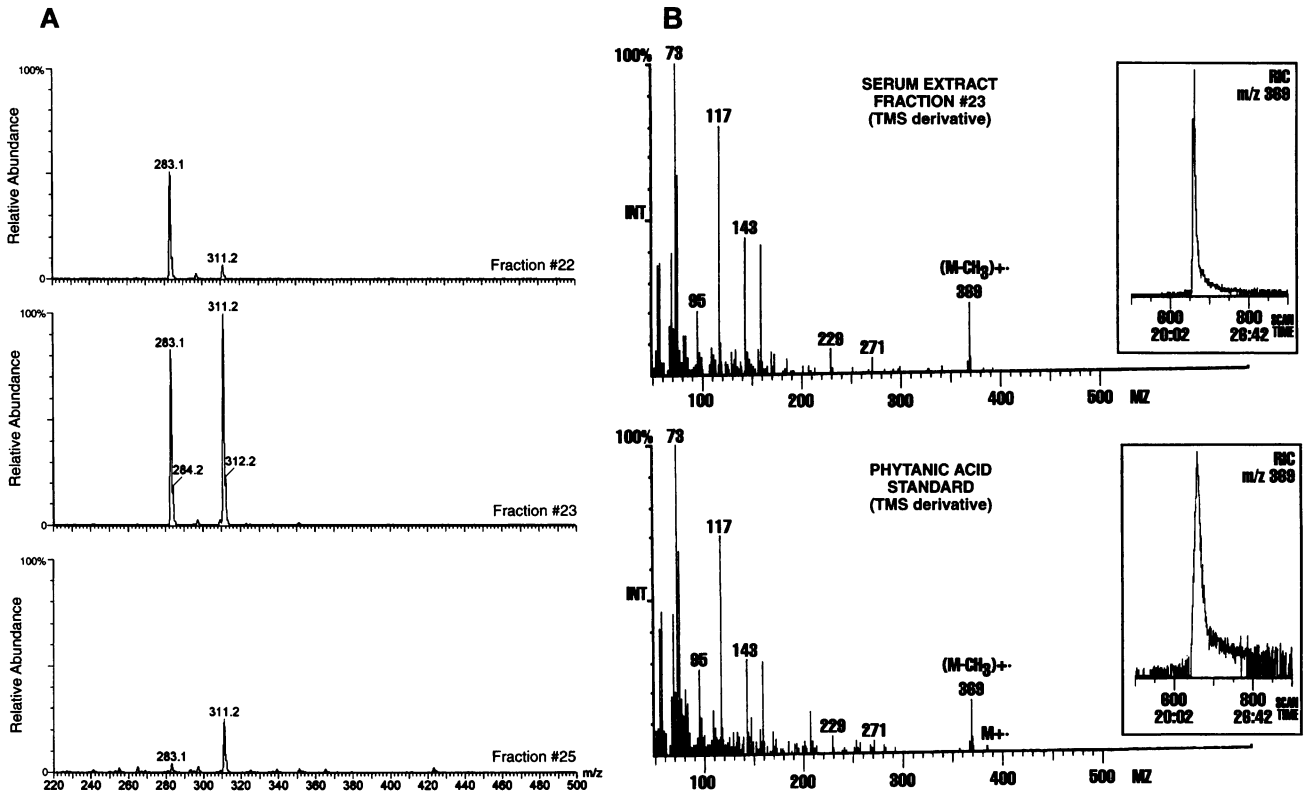

Figure 3. Fatty acids copurify with RXR effector activity. (A) Comparison of molecular ion abundances in HPLC samples near RXR effector activity peak. The material remaining from the HPLC-separated fractions (22, 23, 25) in Figure 2A was used for electrospray-ionization mass spectrometry in the negative ion continuum mode (see MATERIALS AND METHODS). Molecular ions (m/z) are plotted against their relative abundances in each sample. (B) Electron impact mass spectra of the major component of the RXR-active fraction from bovine serum extract and authentic phytanic acid standard, obtained from GC/MS analyses of the TMS-derivatized samples. Insets show the reconstructed ion chromatograms for m/z 369, the $(M\text{-}CH_3)^+$ ion of the TMS derivative of phytanic acid.

soic, palmitic, and arachidonic acids (40 μM) were without effect, a similar amount of phytanic acid produced a fivefold induction of CAT activity with the RXR-specific CRBPII-CAT reporter (Figure 4A). Phytanic acid responsiveness was RXR dependent, using both DR4-CAT and CRBPII-CAT reporters (Figure 4B). The dynamic range for phytanic acid activation of mouse RXRα was between 4 and 64 μM, with cellular toxicity exhibited at higher doses. Other compounds with phytyl side chains were tested, including phytol, vitamin E, and vitamin K_1, but these failed to activate RXR when added at 50 μM concentrations (our unpublished observations).

Absorbance profiles depicting the retention times for phytanic acid, its metabolic precursor phytenic acid, and 9cRA have been included for reference (Figure 4C). Note that the retention time for pure phytanic acid (R_t = 22.5 min) coincides with that of the serum RXR activator (Figure 2A). The time difference between the phytanic acid absorbance and the RXR effector activity is due to a 30 sec delay time in this LC system. As further evidence for identity, a phytanic acid standard coeluted with the serum RXR activator when separated by silica gel chromatography with 20% ethyl acetate in hexane as the developing solvent (Figure 4D). Together these results suggest that the RXR activator in serum corresponds to phytanic acid.

Detection of Phytenic Acid

The DR4-CAT reporter plasmid was originally selected for isolating RXR activators from bovine serum in favor of CRBPII-CAT because of its more robust induction in CHO cells (Figure 4A). Despite its RXR dependency, DR4 had not been previously described as an RXR-responsive element, and thus its use here might be judged inappropriate. Therefore, we sought to confirm the presence of phytanic acid in bovine serum extracts by using assays designed with greater RXR specificity. Four copies of the DNA binding site (upstream activating sequence UAS_G) for the yeast GAL4 gene product or a CRBPII response element were separately inserted in the

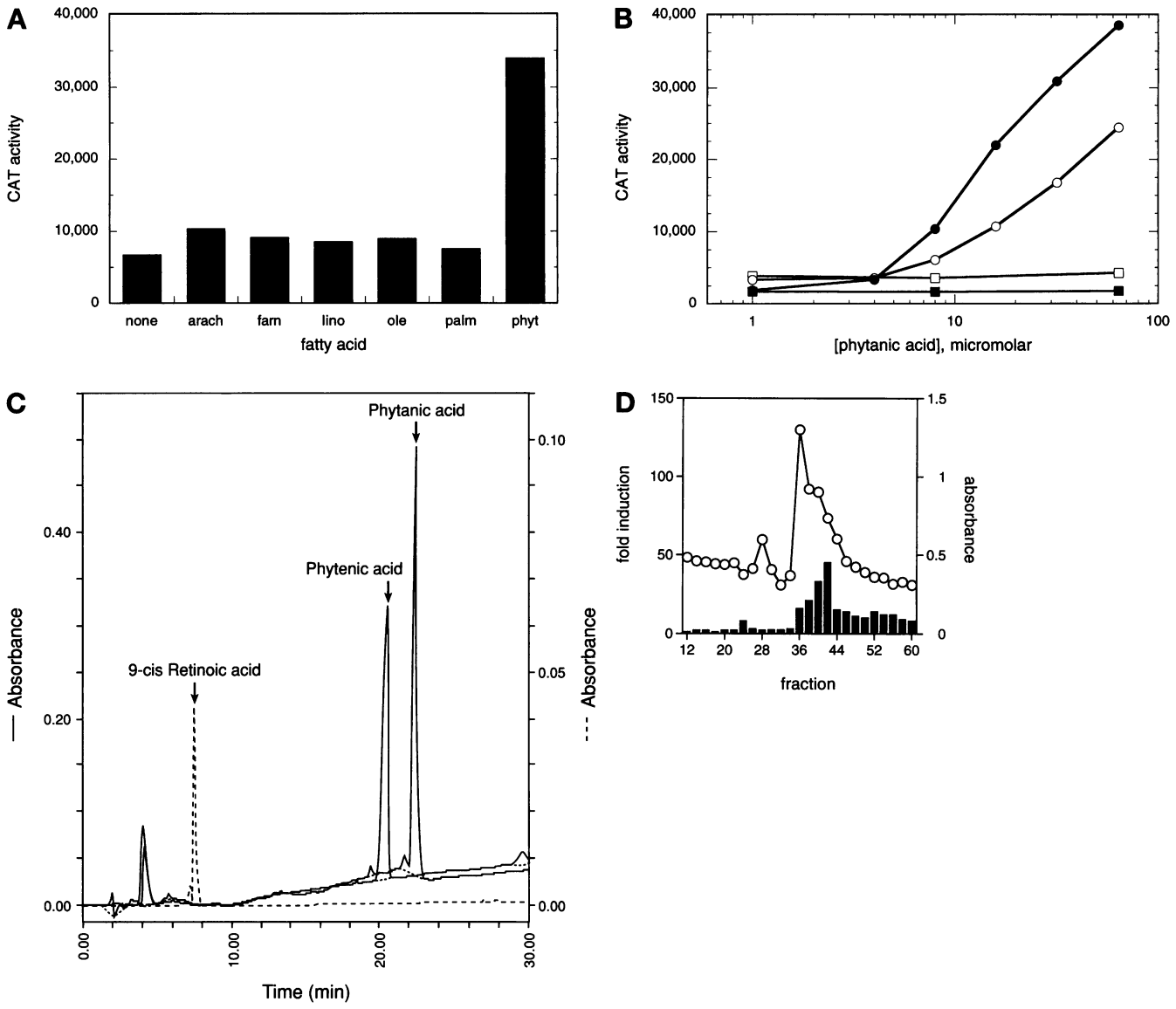

Figure 4. Phytanic acid is the RXR activator from bovine serum. (A) RXR activation by fatty acids. Various fatty acids were tested for RXR-specific activity by using the CRBPII-CAT reporter plasmid DNA (Manglesdorf *et al.*, 1991) and mouse RXRα (Manglesdorf *et al.*, 1992). Arachidonic, farnesoic, linoleic, oleic, palmitic, and phytanic acids (40 μM; Sigma) were added to CHO cells transfected as described in Figure 1. CAT activities are the averages from duplicate transfected plates. (B) Phytanic acid dose–response curves. Phytanic acid dilutions were added to CHO cells transfected with either SV-$(DR4)_3$-CAT [DR4] or SV-(CRBPII)-CAT [CRBPII] reporter plasmids in the absence or presence of mouse RXRα. Open squares: CRBPII, no RXR; open circles: CRBPII, +RXR; closed squares: DR4, no RXR; closed circles: DR4, +RXR. Assays were performed as described in Figure 1. Results are expressed as averages from duplicate plate lysates. (C) Retention time for phytanic acid and 9cRA standards separated by reverse-phase HPLC. One micromole of a phytanic acid standard (Sigma) and 20 nmol of synthetic phytenic acid were injected separately into a sample loop and fractionated as described in MATERIALS AND METHODS. Absorbance was monitored at 220 nm (solid lines) with a Beckman diode array detector module 168. One nanomole of 9cRA was separately injected, and absorbance was monitored at 325 nm (dotted line). Note that the delay time between absorbance measurement and fraction collection is ~30 s. Measured retention times: 9cRA (7.5 min); phytenic acid (20.5 min); phytanic acid (22.3 min). (D) RXR effector activity and phytanic acid coelute by silica gel chromatography. FBS (500 ml) was extracted by the method of Bligh and Dyer. The chloroform-soluble fraction was applied to a silica gel column (40 μ particle size, 4 × 10 cm bed volume) in 2 ml. The column was developed with 500 ml of 20% ethyl acetate in 80% hexane. Eight-milliliter fractions were collected, and the dried material from two adjacent fractions was taken up in methanol; 5% was tested as described in Figure 1. The relative fold induction of CAT activity for each sample compared with control methanol vehicle (solid bars) is presented. An authentic phytanic acid standard (5 mg; Sigma) subsequently was separated, and its absorbance (open circles) was monitored at 220 nm. The experiment was repeated, and a similar profile was obtained.

herpes simplex virus thymidine kinase promoter that was linked to the firefly luciferase gene (Forman *et al.*, 1995c). These reporter plasmids were independently cotransfected into CV-1 cells with CMX-GAL4-RXR (Forman *et al.*, 1995c), a chimeric receptor fusing the GAL4 DNA-binding domain to the human RXRα ligand-binding domain, or with CMX-human RXRα (Yao *et al.*, 1993) as the respective receptor plasmids.

A chloroform extract of FBS (Bligh and Dyer, 1959) was separated by reverse-phase HPLC as described in Figure 2B, but this time the eluate was collected in 0.3 min fractions to afford greater analytical resolution. The material was divided in half, each was added to the two sets of CV-1 cells cotransfected as described above, and normalized luciferase activities were measured. The superimposable profiles contained two peaks of activity (19.0 and 21.6 min; Figure 5) corresponding to the absorbance profiles for phytenic acid and phytanic acid, respectively (R_t = 18.2 and 20.8 min for this particular column). The amounts of serum extract used for these assays were ~10-fold greater than those used earlier (Figure 2, A and B). Thus, the cytotoxicity shown in two adjacent fractions (R_t ~21 min) may have been due to increased amounts of stearic acid that elutes just before phytanic acid (Figure 3A). Nevertheless, phytenic acid now became detectable, but as before (Figure 2A), a peak coincident with 9cRA (R_t = 7 min) was not found. Thus, these data confirm and extend the results previously obtained with the DR4-CAT reporter plasmid to define both phytanic acid and phytenic acid in bovine serum extracts.

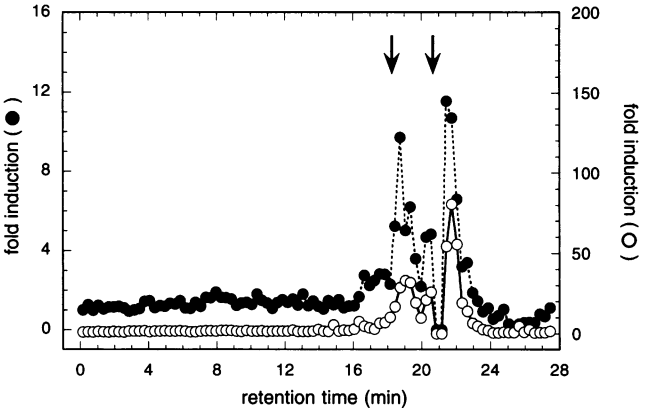

Figure 5. Detection of phytenic acid in bovine serum extracts. FBS (40 ml) was extracted by the method of Bligh and Dyer (1959) (see MATERIALS AND METHODS). The dried chloroform extract was dissolved in 400 μl of methanol and separated into 0.3-min fractions by the reverse-phase HPLC conditions described in Figure 1. Two plates of CV-1 cells (700,000 cells per microtiter plate) were transfected with either TK-(CRBPII)-LUC reporter and human RXRα receptor (closed circles) or TK-$(UAS_G)_4$-LUC reporter and GAL4-hRXRα chimeric receptor (open circles) plasmid DNAs by liposome-mediated transfer, as described in MATERIALS AND METHODS. Each plate was incubated with one-half of the fractionated extracts for 40 h. Portions of the cell lysates were used to assay luciferase, β-galactosidase, and cytotoxic activities as described (Berger *et al.*, 1992). Single-point luciferase measurements were performed, which typically exhibit 10% variations in this assay. Fold induction is expressed as the relative luciferase activity in the presence of fractionated extracts as compared with untreated cells. The cytotoxicity in two fractions at 21 min is denoted by zero inductions. Duplicate control wells to which the FXR activator JH III (40 μM) was added showed onefold inductions; those to which the RXR-specific ligand LG69 (100 nM; Boehm *et al.*, 1995) was added produced 25-fold and 110-fold inductions by using CRBPII-CAT or GAL4-CAT reporters, respectively. Elution positions for phytenic acid and phytanic acid (corresponding to absorbance peaks measured at 220 nm) were R_t = 18.2 and 20.8 min, respectively, and are denoted by arrows.

Phytol Metabolites Bind and Activate RXR

These and other phytol metabolites derived from the phytyl chain of chlorophyll (Figure 6A) were compared for dose-dependent stimulation of RXR, using CRBPII-CAT as the reporter plasmid. Synthetic phytenic acid, consisting of 40% cis and 60% trans isomers, was tested along with phytanic acid, pristanic acid, and 9cRA. The dose responses for RXR activation by phytenic and phytanic acids were similar, having EC_{50} values of 15 μM, whereas pristanic acid, a phytanic acid metabolite, stimulated with a lower potency and efficacy (Figure 6B). By comparison, 9cRA induced activity at a concentration ~200-fold lower than that for either of phytanic acid or phytenic acid. Testing of the separated isomers of phytenic acid at 32 μM revealed that the trans-isomer induced RXR effector activity 4.5-fold, which paralleled that of phytanic acid, whereas the cis isomer was nearly inactive (our unpublished observations).

Direct interaction of phytanic acid with RXR was measured by displacing [^{3}H]-9cRA bound to baculovirus-expressed RXR proteins with unlabeled 9cRA or phytol and its metabolites (Allegretto *et al.*, 1993). Phytenic acid and phytanic acid competed one-half of the [^{3}H]-9cRA binding to human RXRα with a K_i of ~2.3 and 4.4 μM, whereas pristanic acid did so only at 15.1 μM (Table 1). The K_i for phytol was 67.2 μM, suggesting that it is an ineffective 9cRA competitor. Moreover, the binding affinities of phytanic acid and phytenic acid for the individual RXRs were similar. In contrast, phytol metabolites did not displace [^{3}H]-ATRA from RAR even at 100 μM, which demonstrates their binding specificity. Finally, the binding affinity of 9cRA for RXR is 200 times greater than that of phytanic acid or phytenic acid (Table 1), which is similar to their relative potencies for RXR activation (Figure 6B). We conclude that the transcriptional effects of phytol metabolites are specifically transduced by directly binding to RXR.

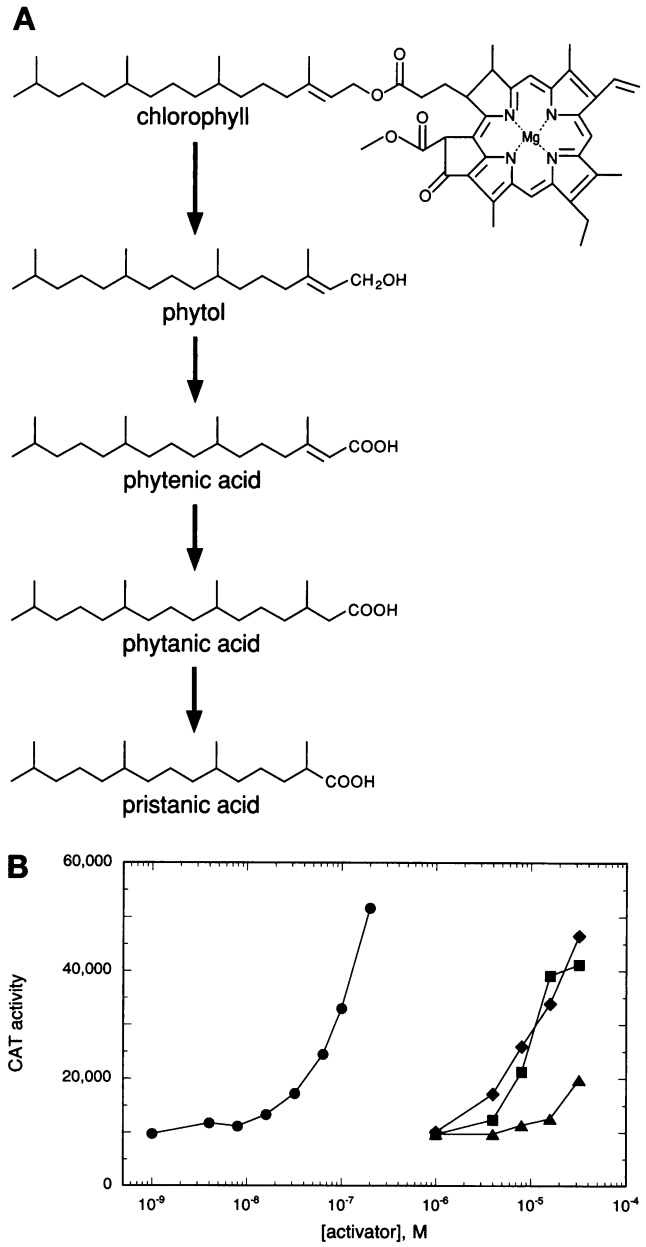

Figure 6. Various chlorophyll metabolites activate RXR. (A) Metabolic pathway from chlorophyll to pristanic acid. First, the phytol ester is hydrolyzed, which is followed by oxidation to phytenic acid. Phytenic acid is then hydrogenated to phytanic acid and α-hydroxylation; oxidation leads to pristanic acid. Pristanic acid is finally metabolized by fatty acid β-oxidation pathways. The trans isomers of phytol and phytenic acid are illustrated here. (B) RXR effector activity induced by phytol metabolites and 9cRA. Increasing amounts of 9cRA, phytanic acid, phytenic acid (40% cis/60% trans isomer mixture), and pristanic acid were added to cells transfected with the RXR-specific CRBPII-CAT reporter plasmid and mouse RXRα. CAT activity was measured from duplicate wells in an assay configured similarly to that described in Figure 1. Average values for CAT activity from duplicate transfected plates are plotted against increasing activator concentrations. Symbols: circles, 9cRA; diamonds, phytenic acid; squares, phytanic acid; triangles, pristanic acid.

DISCUSSION

Refsum's Disease

The diterpenoid structure of phytanic acid (Sonneveld *et al.*, 1962; Lough, 1964) suggested that it might be synthesized from mevalonate, but neither endogenous biosynthetic routes nor intestinal microbes contribute to circulating pools in mammals (Steinberg, 1965, 1967). Phytol metabolites in animal tissues are exclusively derived from the phytyl side chain of chlorophyll. Phytanic acid may be elevated 50-fold and constitute >20% of the fatty acids in patients with Refsum's disease, an inherited metabolic disorder characterized by an α-hydroxylase gene defect that prevents phytanic acid conversion to pristanic acid (Figure 5A; Steinberg, 1983). The neuropathological signs in these patients may be caused by demyelination induced by α-oxidation of phytanic acid in nerve cells that maintain a preference for long-chain ($> C_{20}$) fatty acid substrates (Steinberg, 1983). Tonic RXR stimulation by these grossly elevated levels of phytanic acid may also play a specific pathological role, but rats given 1% dietary phytol showed no changes in nerve functions (Steinberg *et al.*, 1966). Because these diets raised phytanic acid levels to 30% of total fatty acids just as in Refsum's disease, targeted disruption of the α-hydroxylase gene may provide a better approach for understanding the pathological effects.

Phytol Metabolites as Transcriptional Signals

Phytanic acid and phytenic acid levels in normal human serum are 6 μM and 2 μM, respectively (Avignan, 1966). Like other fatty acids, 70% of the phytanic acid probably exists as triacylglycerol or phospholipid esters that are rapidly oxidized and that vary with dietary conditions (Mohrhauer and Holman, 1963; Mize *et al.*, 1966, 1969). Although the estimated free phytanic acid (2 μM) is only at the threshold for RXR stimulation (Figure 4B), equipotent phytenic acid may also contribute to the RXR effector pool (Mize *et al.*, 1966). In addition, the charcoal-treated serum used in this bioassay may have adsorbed some of the added phytanic acid, thereby reducing its effective concentration. Phytol is unlikely to be an RXR effector because at 50 μM it neither bound nor activated RXR, whereas at higher concentrations it showed cytotoxic effects. Finally, the limited effector activity shown by pristanic acid suggests that other phytanic acid metabolites, such as α-hydroxyphytanic acid or ω-carboxylated derivatives, may also be RXR inducers (Billimoria *et al.*, 1982).

The EC_{50} values for RXR activation by phytol metabolites were estimated assuming that the dose–response maxima were reached at 64 μM (Figure 3A). These nonsaturating dose–response curves are

Table 1. Competition of phytol and metabolites for [^{3}H]-ATRA and [^{3}H]-9cRA binding to RXRs

	K_i					
Compound	RARα	RARβ	RARγ	RXRα	RXRβ	RXRγ
ATRA[a]	18.2 ± 2.1	17.3 ± 1.8	14.6 ± 1.8			
9-cis RA[a]				10.2 ± 1.5	22.1 ± 2.3	19.8 ± 0.6
Phytol[b]	> 100	70 ± 30	> 100	67.2 ± 32.8	41.9 ± 0.2	47.1 ± 12.6
Pristanic acid[b]	> 100	74.8 ± 25.3	88 ± 12	15.1 ± 8.6	13.3 ± 3.3	25.6 ± 17.2
Phytanic acid[b]	> 100	> 100	> 100	4.4 ± 0.7	4.1 ± 0.2	3.6 ± 0.7
Phytenic acid[b]	> 100	> 100	> 100	2.3 ± 0.4	3.7 ± 1.1	2.4 ± 0.4

[a] Values are in nM and represent the mean ± SEM of two determinations.

[b] Values are in μM and represent the mean ± SEM of two determinations, except for phytanic and phytenic acid binding to RXRs, where n = 3.

Binding assays were performed as previously described (Allegretto *et al.*, 1993).

probably due to cellular toxicity in which, above 64 μM, the limits for fatty-acid binding to serum albumin were exceeded (Herndon *et al.*, 1969; Spector *et al.*, 1969). Alternatively, some of the natural isomers of phytanic acid (Baxter and Milne, 1969) may inhibit RXR binding. Integration of the effector activities produced by each of these isomers in the tested sample of phytanic acid may thus give rise to the nonsaturable activity profile. Nevertheless, given that their plasma levels approximate their RXR binding affinities and activation potencies, phytanic acid and phytenic acid remain compelling candidates for humoral RXR effectors.

The units of RXR effector activity caused by phytanic acid were only crudely assessed in our experiments, but the activity caused by the injected serum sample (Figure 2A) can be accounted for by the peak of activity found in fractions 23 and 24. The contribution of phytanic acid to the total serum activity can be estimated on the basis of its concentration (5 mg/100 ml) in bovine plasma (Avignan, 1966). Five percent (0.5 ml) of the 10 ml-extracted serum sample was assayed for RXR effector activity by using the DR4-CAT reporter plasmid (Figure 2A). Thus, the estimated phytanic acid (0.025 mg or 80 nmol) in fractions 23 and 24 (Figure 2A) in 4 ml of media is 20 μM, which approximates the EC_{50} value in the dose–response curve. Importantly, the induction in this experiment was submaximal, evidence for which is given by the threefold increase (Figure 2A) as compared with the 16-fold maximum induction seen in Figures 1 and 4B. Because phytanic acid and phytenic acid constitute the only RXR-inducing molecular species in serum (Figure 5), both seem to define the bulk of activity.

Distinct Humoral Diterpenoid Activators for RAR and RXR

Circulating ATRA levels are 6 nM (Napoli *et al.*, 1985; Tang and Russel, 1990), which are sufficient for RAR stimulation (Giguere *et al.*, 1987) but not for RXR activation (Manglesdorf *et al.*, 1990; Allegretto *et al.*, 1993). In contrast, retinol binds RAR 35 times less potently than ATRA (Repa *et al.*, 1993), and 1000 times more retinol is required for RAR activity (Giguere *et al.*, 1987). Although the circulating retinol in human plasma (2 μM) may seem more than adequate for RAR induction (Miller *et al.*, 1984), its effective concentration may be limited by retinol-binding proteins such as CRBPII (Ong, 1987). The RAR activators from the acidified ether extracts of serum described here may support both ATRA and retinol as circulating effectors (Figure 2B). Activators more polar than retinol could be hydroxylated metabolites such as 14-hydroxy-4,14-*retro*-retinol (Buck *et al.*, 1991) and 13,14-dihydroxy-retinol that would likely have retention times less than that of retinol (Derguini *et al.*, 1995). Description of the ATRA metabolites 4-oxo-retinoic acid and 3,4-didehydro-retinoic acid as RAR inducers may also support this hypothesis (Allenby *et al.*, 1993; Pijnappel *et al.*, 1993). Further fractionation of these extracts might help to establish the identities of these RAR activators.

At the same time, our findings seem to diminish support for retinoids as circulating RXR activators in bovine serum. A peak of RXR effector activity corresponding to 9cRA was not detected with our methods, a result that agrees with its reported absence in normal rat serum (Kojima *et al.*, 1994). Based on the dose response curve in Figure 6B as well as from others (Heyman *et al.*, 1992; Pijnappel *et al.*, 1993), 50 nM 9cRA should have been detectable with our assay system. Because no peak of RXR effector activity corresponding to 9cRA was observed with a 20-ml extract sample (Figure 5), 9cRA must be present in bovine serum at concentrations lower than 0.5 nM as tested in the 200-μl well.

Our data do not exclude the possibility that 9cRA may be an intracellular signal. Support for 9cRA as a physiological RXR effector comes from a cochromato-

graphing 350-nm absorbance peak detected in mouse liver and kidney extracts (Heyman *et al.*, 1992). Although 9cRA binds and activates RXR with high affinity and potency (Heyman *et al.*, 1992; Levin *et al.*, 1992), the fractions corresponding to this absorbance peak were not tested for RXR activation. Endogenous levels of 9cRA were estimated as 4 ng/g (13 nM) of liver and 30 ng/g (100 nM) of kidney by measuring the areas of absorbance peaks (Heyman *et al.*, 1992). By using the estimate for 9cRA in bovine serum (0.5 nM) calculated above, humoral 9cRA must be 25 times lower than that found in liver. If ATRA serves as the precursor for 9cRA, this cellular metabolite must accumulate against a considerable concentration gradient. Nevertheless, a cellular enzyme may catalyze this isomerization, and, thus, both humoral phytol metabolites and 9cRA synthesized in cells may cooperate to mediate physiological effects through RXR. Molecular structure determination of the RXR effectors extracted from other animal tissues may help to resolve this issue.

Candidate Essential Fatty Acids

Chlorophyll is best recognized as an energy transducer in plants that captures sunlight for oxygen, sugar, and lipid synthesis and thereby establishes the foundation for animal food chains. Phytol metabolites may now strengthen this link between heterotrophs and autotrophs by integrating the dietary state of the animal with RXR-dependent signaling systems to balance the lipid stores in adipose tissue against cellular needs. Insights into the functions of phytol metabolites may emerge from comparisons with linoleic acid and other unsaturated fatty acids that are important dietary factors synthesized by plants (Burr and Burr, 1929, 1930; Aaes-Jorgensen, 1961).

Although they serve as important precursors for prostaglandin synthesis (Bergstrom, 1966), unsaturated fatty acids may also share equally crucial roles as receptor signals. For example, linoleic and arachidonic acids activate PPARα with a potency of 30 μM (Gottlicher *et al.*, 1992; Banner *et al.*, 1993; Keller *et al.*, 1993). Linoleic acid may contribute as much as 20% (40 μM) of the total fatty acids (200 μM) in human or rat sera (Swell *et al.*, 1961; Scully *et al.*, 1980). Although these levels are within PPARα activation range, other ligands have been noted for PPARγ, such as prostaglandin J_2 (Forman *et al.*, 1995b; Kliewer *et al.*, 1995). The distinct pharmacological characteristics noted for different PPARs (Kliewer *et al.*, 1994) may be specified by unique subsets of dietary or endogenously synthesized fatty acids and their oxidized and cyclized metabolites. A strategy similar to that outlined here could also guide the isolation of PPAR effectors from tissue extracts.

Phytanic acid is obtained only from dietary sources and is rapidly oxidized just like other fatty acids, but its specific nutritional requirement is unknown. Abundant sources of phytanic acid in human diets are milk, cheese, and especially butter (Lough, 1977). The caloric value of phytanic acid is only fractionally that of linoleic acid because of their abundance differences, and thus its contribution to cellular energy reserves must be low. It remains uncertain whether pathological states will develop in animals fed phytanic acid-deficient diets. This might be expected, given the number of signaling pathways converging with RXR. Potential pathological signs could overlap those produced by deficiencies of linoleic acid, thyroid hormones, vitamins A and D, or other ligands whose receptors cooperate with RXR. Preparation of diets lacking phytol and its metabolites will be critical for these nutritional studies. Like the fat-soluble vitamins A and E, it may also be necessary to maintain animals on phytanic acid-deficient diets for prolonged times to deplete stored forms of the fatty acid.

It may be of interest to note that linoleic acid deficiency retards animal growth and that butter efficiently restores the weight lost in rats given fat-free diets (Burr and Burr, 1930; Aaes-Jorgensen, 1961). Although linoleic acid has been shown to be one active component, phytanic acid may represent another of their postulated "vitamine F" growth-promoting substances (Evans and Burr, 1928). Phytanic acid could also serve as a growth factor for cells in culture, because linoleic acid replacement of serum albumin and its bound fatty acids has been shown to increase their plating efficiency in serum-free media (Ham, 1963).

Dietary Lipids as Nutritional Signals

Phytol metabolites, unsaturated fatty acids, retinol, and farnesoids may form a unique class of micromolar cellular metabolites that also serve as signals for RXR and some of its receptor partners. Linoleic acid and other unsaturated fatty acids are candidate physiological PPAR effectors that regulate the genes for enzymes involved in lipid metabolism (Keller and Wahli, 1993). The carotenoid metabolite retinol may be an important RAR signal, as discussed above. Similarly, metabolites of farnesyl pyrophosphate (FPP) have been postulated to regulate isoprenoid synthesis through FXR interactions (Forman *et al.*, 1995a; Weinberger, 1996). FPP in liver (0.4 μM) (Bruenger and Rilling, 1988) is slightly lower than the micromolar farnesoids needed for FXR induction, but activation of a farnesoid metabolic shunt could increase the pool of FXR effectors in some dietary states. Critical evaluation of this hypothesis will require measurements of cellular farnesoid levels and their FXR-binding potentials. Finally, phytol metabolites may be nutritional signals linking the animal's dietary state with a variety

of endocrine and intracrine signaling pathways through the nuclear receptor RXR. As a model, we propose that these dietary lipids may coordinate gene expression for fatty-acid biosynthetic and oxidative enzymes by PPAR and RXR interactions. In conjunction with farnesoids and FXR, these networks could regulate the flux of acetyl CoA from intermediary metabolism to meet cellular lipid needs during variable dietary conditions.

Defining Physiological Receptor Ligands

The fat-soluble vitamins retinol, cholecalciferol, α-tocopherol, and phylloquinone were isolated more than 60 years ago when nutritionally-deprived animals served as the physiological assays guiding their purification (Evans and Bishop, 1923). The cis-trans assay now provides a modern means to identify hormones and vitamins for orphan receptors like RXR. Our proposal for phytol metabolites as physiological effectors rests on the findings that their circulating levels may be relevant for RXR binding and activation. Moreover, these were the only activators found in bovine serum. It may be premature to attach physiological significance to these chlorophyll metabolites at this time. However, our results minimally suggest that the absolute potency of an activator is less important than its potency relative to its natural abundance. Measurements of steady-state intracellular levels of phytol metabolites in various tissues during different nutritional states could be compared with their RXR-binding properties to further test this hypothesis.

To more firmly establish their physiological relevance, these and other orphan receptor ligands must also comply with a broader set of postulates analogous to those identifying the etiologic roles of bacteria for animal diseases. Physiological effects mediated by phytanic acid must first be described. Animals raised on phytanic acid-depleted diets may offer one way to identify these cell functions specifically controlled by RXR. These assayable end points might then lead to the purification of phytol metabolites from tissue extracts, just as uterine cell changes in ovariectomized mice guided the isolation of estrogenic substances (Allen and Doisy, 1923).

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REFERENCES

Aaes-Jorgensen, E. (1961). Essential fatty acids. Physiol. Rev. *41*, 1–41.

Allen, E., and Doisy, E.A. (1923). An ovarian hormone. Preliminary report on its localization, extraction and partial purification, and action in test animals. J. Am. Med. Assoc. *81*, 819–821.

Allegretto, E.A., McClurg, M.R., Lazarchik, S.B., Clemm, D.L., Kerner, S.A., Elgort, M.G., Boehm, M.F., White, S.K., Pike, J.W., and Heyman, R.A. (1993). Transactivation properties of retinoic acid and retinoid X receptors in mammalian cells and yeast. J. Biol. Chem. *268*, 26625–26633.

Allenby, G., Bocquel, M.-T., Saunders, M., Kazmer, S., Speck, J., Rosenberger, M., Lovey, A., Kastner, P., Grippo, J.F., Chambon, P., and Levin, A.A. (1993). Retinoic acid receptors and retinoid X receptors: interactions with endogenous retinoic acids. Proc. Natl. Acad. Sci. USA *90*, 30–34.

Arriza, J.L., Weinberger, C., Cerelli, G., Glaser, T.M., Handelin, B.L., Housman, D.E., and Evans, R.M. (1987). Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. Science *237*, 268–275.

Avignan, J. (1966). The presence of phytanic acid in normal human and animal plasma. Biochim. Biophys. Acta *166*, 391–394.

Banner, C.D., Gottlicher, M., Widmark, E., Sjovall, J., Rafter, J.J., and Gustafsson, J.-A. (1993). A systematic analytical chemistry/cell approach to isolate activators of orphan nuclear receptors from biological extracts: characterization of peroxisome proliferator-activated receptor activators in plasma. J. Lipid Res. *34*, 1583–1591.

Baxter, J.H., and Milne, G.W.A. (1969). Phytenic acid: identification of five isomers in chemical and biological products of phytol. Biochim. Biophys. Acta *176*, 265–277.

Berger, T.S., Parandoosh, Z., Perry, B.W., and Stein, R.B. (1992). Interactions of glucocorticoid analogues with the glucocorticoid receptor. J. Steroid Biochem. Mol. Biol. *41*, 733–738.

Bergstrom, S. (1966). The prostaglandins. Recent Prog. Horm. Res. *22*, 153–175.

Billimoria, J.D., Clemens, M.E., Gibberd, F.B., and Whitelaw, M.N. (1982). Metabolism of phytanic acid in Refsum's disease. Lancet *1*(8265), 194–196.

Bligh, E.G., and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. *37*, 911–917.

Boehm, M.F., McClurg, M.R., Pathirana, C., Manglesdorf, D., White, S.K., Hebert, J., Winn, D., Goldman, M.E., and Heyman, R. (1994). Synthesis of high specific activity [^{3}H]-*9-cis*-retinoic acid and its application for identifying retinoids with unusual binding properties. J. Med. Chem. *37*, 408–414.

Boehm, M.F., Zhang, L., Zhi, L., McClurg, M.R., Berger, E., Wagoner, M., Mais, D.E., Suto, C.M., Davies, P.J.A., Heyman, R.A., and Nadzan, A.M. (1995). Design and synthesis of potent retinoid X receptor-selective ligands that induce apoptosis in leukemia cells. J. Med. Chem. *38*, 3146–3155.

Bruenger, E., and Rilling, H.C. (1988). Determination of isopentenyl diphosphate and farnesyl diphosphate in tissue samples with a comment on secondary regulation of polyisoprenoid biosynthesis. Anal. Biochem. *173*, 321–327.

Buck, J., Derguini, F., Levi, K., Nakanishi, K., and Hammerling, U. (1991). Intracellular signaling by 14,-hydroxy-4,14-*retro*-retinol. Science *254*, 1654–1656.

Burr, G.O., and Burr, M.M. (1929). A new deficiency disease produced by the rigid exclusion of fat from the diet. J. Biol. Chem. *82*, 345–367.

Burr, G.O., and Burr, M.M. (1930). On the nature and role of the fatty acids essential in nutrition. J. Biol. Chem. *86*, 587–621.

Chen, C., and Okayama, H. (1988). High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. *7*, 2745–2752.

Cheng, Y.C., and Prusoff, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor, which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem. Pharmacol. *22*, 3099–3108.

Corey, E.J., Gilman, N.H., and Ganem, B.E. (1968). New methods for the oxidation of aldehydes to carboxylic acids and esters. J. Am. Chem. Soc. *90*, 5616–5617.

Derguini, F., Nakanishi, K., Hammerling, U., Chua, R., Eppinger, T., Levi, E., and Buck, J. (1995). 13,14-dihydroxyretinol, a new bioactive retinol metabolite. J. Biol. Chem. *270*, 18875–18880.

Evans, H.M., and Bishop, K.S. (1923). Existence of a hitherto unknown dietary factor essential for reproduction. J. Am. Med. Assoc. *81*, 889–892.

Evans, H.M., and Burr, K.S. (1928). A new dietary deficiency with highly purified diets. III. The beneficial effect of fat in the diet. Proc. Soc. Exp. Biol. Med. *25*, 390–397.

Evans, R.M. (1988). The steroid and thyroid hormone receptor superfamily. Science *240*, 889–895.

Forman, B.M., Goode, E., Chen, J., Oro, A.E., Bradley, D.J., Perlmann, T., Noonan, D.J., Burka, L.T., McMorris, T., Lamph, W.W., Evans, R.M., and Weinberger, C. (1995a). Identification of a nuclear receptor that is activated by farnesol metabolites. Cell *81*, 687–695.

Forman, B.M., Tontonoz, P., Chen, J., Brun, R.P., Spiegelman, B.M., and Evans, R.M. (1995b). 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 is a ligand for the adipocyte determination factor PPARγ. Cell *83*, 803–812.

Forman, B.M., Umesono, K., Chen, J., and Evans, R.M. (1995c). Unique response pathways are established by allosteric interactions among nuclear hormone receptors. Cell *81*, 541–550.

Giguere, V., Hollenberg, S.M., and Evans, R.M. (1986). Functional domains of the human glucocorticoid receptor. Cell *46*, 645–652.

Giguere, V., Ong, E.S., Segui, P., and Evans, R.M. (1987). Identification of a receptor for the morphogen retinoic acid. Nature *330*, 624–629.

Glass, C.K. (1994). Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. Endocrinol. Rev. *15*, 391–407.

Gottlicher, M., Widmark, E., Li, Q., and Gustafsson, J.-A. (1992). Fatty acids activate a chimera of the clofibric acid-activated receptor and the glucocorticoid receptor. Proc. Natl. Acad. Sci. USA *89*, 4653–4667.

Green, S., and Chambon, P. (1987). Oestradiol induction of a glucocorticoid-responsive gene by a chimaeric receptor. Nature *324*, 615–617.

Ham, R.G. (1963). Albumin replacement by fatty acids in clonal growth of mammalian cells. Science *140*, 802–803.

Harmon, M.A., Boehm, M.F., Heyman, R.A., and Manglesdorf, D.J. (1995). Activation of mammalian retinoid X receptors by the insect growth regulator methoprene. Proc. Natl. Acad. Sci. USA *92*, 6157–6160.

Herbomel, P., Bourachot, B., and Yaniv, M. (1984). Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. Cell *39*, 653–662.

Herndon, J.H., Steinberg, D., Uhlendorf, B.W., and Fales, H.M. (1969). Refsum's disease: characterization of the enzyme defect in cell culture. J. Clin. Invest. *48*, 1017–1040.

Heyman, R.A., Manglesdorf, D.J., Dyck, J.A., Stein, R.B., Eichele, G., Evans, R.M., and Thaller, C. (1992). *9-cis* retinoic acid is a high-affinity ligand for the retinoid X receptor. Cell *68*, 397–406.

Keller, H., Dreyer, C., Medin, J., Mahfoudi, A., Ozato, K., and Wahli, W. (1993). Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. Proc. Natl. Acad. Sci. USA *90*, 2160–2164.

Keller, H., and Wahli, W. (1993). Peroxisome proliferator-activated receptors: a link between endocrinology and nutrition? Trends Endocrinol. Metab. *4*, 291–296.

Kliewer, S.A., Forman, B.M., Blumberg, B., Ong, E.S., Borgmeyer, U., Manglesdorf, D.J., Umesono, K., and Evans, R.M. (1994). Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. Proc. Natl. Acad. Sci. USA *91*, 7355–7359.

Kliewer, S.A., Lenhard, J.M., Willson, T.M., Patel, I., Morris, D.C., and Lehmann, J.M. (1995). A prostaglandin J_2 metabolite binds peroxisome proliferator-activated receptor γ and promotes adipocyte differentiation. Cell *83*, 813–819.

Kliewer, S.A., Umesono, K., Heyman, R.A., Manglesdorf, D.J., Dyck, J.A., and Evans, R.M. (1992). Retinoid X receptor-COUP-TF interactions modulate retinoic acid signaling. Proc. Natl. Acad. Sci. USA *89*, 1448–1452.

Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P., and Hogness, D.S. (1991). The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. Cell *67*, 59–77.

Kojima, R., Fujimori, T., Kiyota, N., Toriya, Y., Fukuda, T., Ohashi, T., Sato, T., Yoshizawa, Y., Takeyama, K., Mano, H., Masushige, S., and Kato, S. (1994). In vivo isomerization of retinoic acids: rapid isomer exchange and gene expression. J. Biol. Chem. *269*, 32700–32707.

Lehmann, J.M., Jong, L., Fanjul, A., Cameron, J.F., Lu, X.P., Haefner, P., Dawson, M.I., and Pfahl, M. (1992). Retinoids selective for retinoid X receptor-response pathways. Science *258*, 1944–1946.

Levin, A.A., Sturzenbecker, L.J., Kazmer, S., Bosakowski, T., Huselton, C., Allenby, G., Speck, J., Kratzeisen, C., Rosenberger, M., and Lovey, A. (1992). *9-cis* retinoic acid stereoisomer binds and activates the nuclear receptor RXRα. Nature *355*, 359–361.

Lough, A.K. (1964). Blood lipids. 4. The isolation of 3,7,11,15-tetramethylhexadecanoic acid (phytanic acid) from ox-plasma lipids. Biochem. J. *91*, 584–588.

Lough, A.K. (1977). The phytanic acid content of the lipids of bovine tissues and milk. Lipids *12*, 115–119.

Manglesdorf, D.J., Borgmeyer, U., Heyman, R.A., Zhou, J.Y., Ong, E.S., Kakizuka, A., and Evans, R.M. (1992). Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. Genes Dev. *6*, 329–344.

Manglesdorf, D.J., and Evans, R.M. (1995). The RXR heterodimers and orphan receptors. Cell *83*, 841–850.

Manglesdorf, D.J., Ong, E.S., Dyck, J.A., and Evans, R.M. (1990). Nuclear receptor that identifies a novel retinoic acid response pathway. Nature *345*, 224–229.

Manglesdorf, D.J., Umesono, K., Kliewer, S.A., Borgmeyer, U., Ong, E.S., and Evans, R.M. (1991). A direct repeat in the cellular retinol-binding protein type II gene confers differential regulation by RXR and RAR. Cell *66*, 555–561.

Miller, K.W., Lorr, N.A., and Yang, C.S. (1984). Simultaneous determination of plasma retinol, α-tocopherol, lycopene, α-carotene, and

β-carotene by high-performance liquid chromatography. Anal. Biochem. *138*, 340–345.

Mize, C.E., Avignan, J., Baxter, J.H., Fales, H.M., and Steinberg, D. (1966). Metabolism of phytol-U-^{14}C and phytanic acid-U-^{14}C in the rat. J. Lipid Res. *7*, 692–697.

Mize, C.E., Herndon, J.H., Jr., Blass, J.P., Milne, G.W.A., Follansbee, C., Laudat, P., and Steinberg, D. (1969). Localization of the oxidative defect in phytanic acid degradation in patients with Refsum's disease. J. Clin. Invest. *48*, 1033–1040.

Mohrhauer, H., and Holman, R.T. (1963). The effect of dose level of essential fatty acids upon fatty acid composition of the rat liver. J. Lipid Res. *4*, 151–159.

Napoli, J.L., Pramanik, B.C., Williams, J.B., Dawson, M.I., and Hobbs, P.D. (1985). Quantitation of retinoic acid by gas-liquid chromatography–mass spectrometry: total versus all-*trans*-retinoic acid in human plasma. J. Lipid Res. *26*, 387–392.

O'Malley, B.W. (1989). Did eucaryotic steroid receptors evolve from intracrine gene regulators? Endocrinology *125*, 1119–1120.

O'Malley, B.W. (1990). Orphan receptors: in search of a unifying hypothesis of activation. Mol. Endocrinol. *6*, 1359–1361.

Ong, D.E. (1987). Cellular retinol-binding proteins. Arch. Dermatol. *123*, 1693a–1695a.

Perlmann, T., and Jansson, L. (1995). A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1. Genes Dev. *9*, 769–782.

Petkovich, M., Brand, N.J., Krust, A., and Chambon, P. (1987). A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature *330*, 444–450.

Pijnappel, W.W.M., Hendricks, H.F.J., Folkers, G.E., van den Brink, C.E., Dekker, E.J., Edelenbosch, E., van der Saag, P.T., and Durston, A.J. (1993). The retinoid ligand 4-oxo-retinoic acid is a highly active modulator of positional specification. Nature *366*, 340–344.

Repa, J.J., Hanson, K.K., and Clagett-Dame, M. (1993). All-*trans*-retinol is a ligand for the retinoic acid receptors. Proc. Natl. Acad. Sci. USA *90*, 7293–7297.

Samuels, H.H., Stanley, F., and Casanova, J. (1979). Depletion of L-3,5,3′-triiodothyronine and L-thyroxine in euthyroid calf serum for use in cell culture studies of the action of thyroid hormone. Endocrinology *105*, 80–85.

Scully, R.E., McNeely, B.U., and Galdabini, J.J. (1980). Normal reference laboratory values. N. Engl. J. Med. *302*, 37–48.

Seed, B., and Sheen, J.-Y. (1988). A simple phase-extraction assay for chloramphenicol acyltransferase activity. Gene *67*, 271–277.

Shih, W., Mears, T., Bradley, D.J., Parandoosh, Z., and Weinberger, C. (1991). An adenoviral vector system for functional identification of nuclear receptor ligands. Mol. Endocrinol. *5*, 300–309.

Sladek, F.M., Zhong, W., Lai, E., and Darnell, Jr., J.E. (1990). Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. Genes Dev. *4*, 2353–2365.

Song, C., Kokontis, J.M., Hiipakka, R.A., and Liao, S. (1994). Ubiquitous receptor: a receptor that modulates gene activation by retinoic acid and thyroid hormone receptors. Proc. Natl. Acad. Sci. USA *91*, 10809–10813.

Sonneveld, W., Haverkamp Begemann, P., van Beers, G.J., Keuning, R., and Schogt, J.C.M. (1962). 3,7,11,15-tetramethylhexadecanoic acid, a constituent of butterfat. J. Lipid Res. *3*, 351–355.

Spector, A.A., John, K., and Fletcher, J.E. (1969). Binding of long-chain fatty acids to bovine serum albumin. J. Lipid Res. *10*, 56–67.

Steinberg, D. (1983). Phytanic acid storage disease (Refsum's disease). In: The Metabolic Basis of Inherited Disease, ed. J.B. Stanbury, J.B. Wyngaarden, D.S. Frederickson, J.L. Goldstein, and M.S. Brown, New York: McGraw-Hill, 731–747.

Steinberg, D., Avignan, J., Mize, C. (1965). Conversion of U-^{14}C-phytol to phytanic acid and its oxidation in *heredopathia atactica polyneuritiformis*. Biochem. Biophys. Res. Commun. *19*, 783–789.

Steinberg, D., Avignan, J., Mize, C.E., Baxter, J.H., Cammermeyer, J., Fales, H.M., and Highet, P.F. (1966). Effects of dietary phytol and phytanic acid in animals. J. Lipid Res. *7*, 684–691.

Steinberg, D., Mize, C.E., Avignan, J., Fales, H.M., Eldjarn, L., Try, K., Stokke, O., and Refsum, S. (1967). Studies on the metabolic error in Refsum's disease. J. Clin. Invest. *46*, 313–322.

Sucov, H.M., Murakami, K.H., and Evans, R.M. (1990). Characterization of an autoregulated response element in the mouse retinoic acid receptor-type beta gene. Proc. Natl. Acad. Sci. USA *87*, 5392–5396.

Swell, L., Law, M.D., Schools, P.E., Jr., and Treadwell, C.R. (1961). Tissue lipid fatty acid composition in pyridoxine-deficient rats. J. Nutr. *74*, 148–156.

Tang, G., and Russell, R.M. (1990). *13-cis* retinoic acid is an endogenous compound in human serum. J. Lipid Res. *31*, 175–182.

Teboul, M., Enmark, E., Li, Q., Wikstrom, A.C., Pelto-Huikko, M., and Gustafsson, J.-A. (1995). OR-1, a member of the nuclear receptor superfamily that interacts with the *9-cis*-retinoic acid receptor. Proc. Natl. Acad. Sci. USA *92*, 2096–2100.

Umesono, K., Murakami, K., Thompson, C.C., and Evans, R.M. (1991). Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D_3 receptors. Cell *65*, 1255–1266.

Wang, L.-H., Tsai, S.Y., Cook, R.G., Beattie, W.G., Tsai, M.-J., and O'Malley, B.W. (1989). COUP transcription factor is a member of the steroid receptor superfamily. Nature *340*, 163–166.

Weinberger, C. (1996). A model for farnesoid feedback control in the mevalonate pathway. Trends Endocrinol. Metab. *7*, 1–6.

Willy, P.J., Umesono, K., Ong, E.S., Evans, R.M., Heyman, R.A., and Manglesdorf, D.J. (1995). LXR, a nuclear receptor that defines a distinct retinoid-responsive pathway. Genes Dev. *9*, 1033–1045.

Yao, T.-P., Forman, B.M., Jiang, Z., Cherbas, L., Chen, J.D., McKeown, M., Cherbas, P., and Evans, R.M. (1993). Functional ecdysone receptor is the product of the *EcR* and *ultraspiracle* genes. Nature *366*, 476–479.

Yamamoto, K.R. (1985). Steroid receptor-regulated transcription of specific genes and gene networks. Annu. Rev. Genet. *19*, 209–252.