# Structure Elucidation of Retinoids in Biological Samples Using Postsource Decay Laser Desorption/Ionization Mass Spectrometry after High-Performance Liquid Chromatography Separatio…

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# Cell proliferation inhibition and alterations in retinol esterification induced by phytanic acid and docosahexaenoic acid

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**Abstract We investigated the effects of two natural dietary retinoid X receptor (RXR) ligands, phytanic acid (PA) and docosahexaenoic acid (DHA), on proliferation and on the metabolism of retinol (vitamin A) in both cultured normal human prostate epithelial cells (PrECs) and PC-3 prostate carcinoma cells. PA and DHA inhibited the proliferation of the parental PC-3 cells and PC-3 cells engineered to overexpress human lecithin:retinol acyltransferase (LRAT) in both the absence and presence of retinol. A synthetic RXR-specific ligand also inhibited PC-3 cell proliferation, whereas *all-trans* retinoic acid (ATRA) did not. PA and DHA treatment increased the levels of retinyl esters (REs) in both PrECs and PC-3 cells and generated novel REs that eluted on reverse-phase HPLC at 54.0 and 50.5 min, respectively. Mass spectrometric analyses demonstrated that these novel REs were retinyl phytanate (54.0 min) and retinyl docosahexaenoate (50.5 min). Neither PA nor DHA increased LRAT mRNA levels in these cells. In addition, we demonstrate that retinyl phytanate was generated by LRAT in the presence of PA and retinol; however, retinyl docosahexaenoate was produced by another enzyme in the presence of DHA and retinol.**—Tang, X-H., M-J. Suh, R. Li, and L. J. Gudas. **Cell proliferation inhibition and alterations in retinol esterification induced by phytanic acid and docosahexaenoic acid. *J. Lipid Res.* 2007.** 48: **165–176.**

**Supplementary key words** mass spectrometry • postsource decay • retinoid metabolism • lecithin:retinol acyltransferase

Vitamin A (retinol) and its natural metabolites and synthetic derivatives, known as retinoids, play important roles in regulating cell proliferation, differentiation, and embryonic development (1–3). The physiological activities of retinoids are primarily carried out via nuclear receptors that transcriptionally activate certain retinoid-responsive genes. Two types of retinoid receptors have been identified, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), each with three subtypes, α, β, and γ (4–6). RAR/RXR heterodimers bind to specific DNA sequences known as retinoic acid response elements (7). Ligand binding of RARs and RXRs regulates the transcription of retinoic acid-responsive genes. The actions of RARs and RXRs on gene transcription also require a highly coordinated interaction with a large number of coactivators and corepressors (8).

The appropriate intracellular retinoid concentrations are maintained by the activities of several metabolic enzymes (9–11). *All-trans* retinol (ROH; vitamin A) is the precursor of other natural retinoids. Retinol dehydrogenases oxidize ROH to *all-trans* retinaldehyde, and then *all-trans* retinaldehyde is further metabolized to *all-trans* retinoic acid (ATRA) by retinaldehyde dehydrogenases (11). Currently, four members of the retinaldehyde dehydrogenase family have been identified, retinaldehyde dehydrogenases 1, 2, 3, and 4 (12). ROH is converted to *all-trans* retinyl esters primarily by lecithin:retinol acyltransferase (LRAT), and retinyl esters (REs) are hydrolyzed to retinol by ester hydrolases (9, 13–15). REs are regarded as the storage forms of retinol (9). ATRA is oxidized to more polar metabolites, such as 4-oxo-retinoic acid, by a cytochrome P450 family member (CYP26) (16–19).

Retinoids are required for the appropriate differentiation of normal rodent prostate epithelial cells (20). Epidemiological data have implicated retinoids in the prevention of human prostate cancer (21). An inverse correlation between serum levels of retinoids and prostate cancer incidence has been reported (22, 23). Moreover, human prostate cancer tissues contain five to eight times less ATRA than normal prostate or benign prostatic hyperplasia (24). We have shown that vitamin A (retinol) metabolism is altered in some types of human cancer, such as oral cavity cancer, skin cancer, and breast cancer (25–27).

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Abbreviations: ARAT, acyl-coenzyme A:retinol acyltransferase; ATRA, *all-trans* retinoic acid; DGAT1, acyl-coenzyme A:diacylglycerol acyltransferase 1; DHA, docosahexaenoic acid; HPRT, hypoxanthine guanine phosphoribosyl transferase; LDI, laser desorption ionization; LRAT, lecithin:retinol acyltransferase; MS, mass spectrometry; PA, phytanic acid; PrEC, normal human prostate epithelial cell; PSD, postsource decay; RAR, retinoic acid receptor; RE, retinyl ester; ROH, *all-trans* retinol; RP, retinyl palmitate; RXR, retinoid X receptor.

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In addition, our laboratory showed that both the esterification of ROH to REs and the levels of LRAT, an enzyme involved in vitamin A esterification and storage, were greatly decreased in human prostate cancer cell lines and in patient tumor samples (28).

Huang et al. (29) have shown that mice with both RXRα alleles specifically disrupted in prostate epithelium develop prostate hyperplasia. Conversely, RXR selective retinoids demonstrated efficacy in the prevention of prostate cancer in a rodent model (30). RXR selective retinoids were shown to inhibit the clonal growth of prostate cancer cells (31). Recently, a selective RXR agonist was shown to prevent and overcome multidrug resistance in human prostate cancer PC-3 cells (32). However, the mechanisms by which the inhibition of cell proliferation is achieved are still not clear. Therefore, it is important to explore the effects of RXR ligands on the metabolism of retinol, including the esterification of retinol.

Some unsaturated fatty acids have been shown to be physiological RXR ligands (33, 34). Phytanic acid (PA) and docosahexaenoic acid (DHA) are dietary ligands of RXRs (35–38). PA (3,7,11,15-tetramethylhexadecanoic acid) is a branched-chain fatty acid generated by the oxidation of the phytol side chain of chlorophyll in mammals (39). Because humans cannot release phytol from chlorophyll, PA in the human body comes from dairy products and ruminant fats in the diet (40). DHA is a long-chain ω-3 polyunsaturated fatty acid that is present at high levels in fish oils. ω-3 fatty acids have various physiological functions (41). DHA inhibits cell proliferation and induces apoptosis of breast cancer cells (42) and human colon cancer cells (43). Merendino et al. (44) found that DHA also induced apoptosis of human pancreatic cancer cells.

With respect to prostate cancer, dietary studies in humans have shown that both fish oils and DHA have protective properties (45). DHA can inhibit the growth of androgen-responsive LNCaP cells (46) and androgen-unresponsive PC-3 cells and DU-145 cells (47). Lampen, Meyer, and Nau (48) reported that PA and DHA enhanced the induction of CYP26A1 expression and retinoic acid oxidation by RAR ligands in cells. However, the effects of RXR ligands on the metabolism of retinol have not been examined.

Therefore, we examined the effects of a synthetic RXR ligand (BMS 188649) (49) and these RXR natural ligands, PA and DHA, on the proliferation of human PC-3 prostate cancer cells (androgen-unresponsive prostate cancer cells). We also investigated the effects of these drugs on retinol metabolism in normal human prostate epithelial cells (PrECs), PC-3 cells, and PC-3 cells engineered to overexpress stably human LRAT. We found that both PA and DHA inhibited the proliferation of PC-3 cancer cells and PC-3 cells engineered to overexpress stably human LRAT. Furthermore, both drugs, combined with ROH, generated novel REs through different mechanisms in cultured PrECs and in PC-3 cells. Our results indicate that LRAT catalyzes the esterification of ROH and PA to retinyl phytanate, whereas retinyl docosahexaenoate generated in the presence of DHA and ROH is produced by an enzyme other than LRAT.

## MATERIALS AND METHODS

### Materials

Radiolabeled retinol (*all-trans* 11,12-[$^3$H]) was purchased from New England Nuclear/Dupont (Boston, MA). The specific RXR agonist BMS 188649 was from the Bristol-Myers Squibb Pharmaceutical Research Institute (Buffalo, NY), as described previously (50, 51). DHA was purchased from Sigma Chemical Co. (St. Louis, MO) and Cayman Chemical Co. (Ann Arbor, MI). Betaflow and mono-flow scintillation fluids were purchased from National Diagnostics (Atlanta, GA). All primers for PCR were from MWG-Biotech, Inc. (High Point, NC). TRIzol reagent and Superscript III reverse transcriptase were from Invitrogen (Carlsbad, CA). The SYBR Green I Quantitect kit was purchased from Qiagen (Valencia, CA). All other chemicals used in this research, unless specified, were purchased from Sigma Chemical Co.

### Cell culture

PrECs were purchased from Cambrex (Walkersville, MD) and were cultured according to the instructions of the manufacturer. PC-3 human prostate carcinoma cells were from the American Type Culture Collection (Manassas, VA) and were cultured in RPMI 1640 supplemented with 10% fetal calf serum. PC-3 cells that stably overexpress human LRAT (line RL-PC-3/LRAT-4, generated in our laboratory) were maintained in the same medium as the PC-3 parental cells. For radiolabeling studies, RT-PCR, and Western blot analyses, PrECs and PC-3 cells were cultured in a mixture of 50% prostate epithelial cell growth medium and 50% RPMI 1640 supplemented with 10% fetal calf serum only during the periods of drug treatment, because retinoids are not stable in serum-free medium.

### Stable transfection of the human LRAT in PC-3 cells

An 800 bp PCR fragment of the human LRAT cDNA was cloned into the *Eco*RI and *Xho*I sites of the pcDNA3.1/V5-His A vector (Invitrogen). DNA sequencing confirmed that no mutations were introduced during the PCR cloning process and that the cDNA contained the correct sequence. Electroporation was performed as described previously (52). PC-3 cells were electroporated with 20 μg of pcDNA3.1/V5-His A-LRAT at 200 mV and 960 μF. After 48 h, cells were selected with G418 (300 μg/ml active drug; Sigma) for 2 weeks. Several independent cell colonies were picked, expanded, frozen, and tested for LRAT activity. These stably transfected colonies exhibited ~5- to 10-fold more LRAT activity, as measured by retinol esterification in living cells, than that in the parental PC-3 cells (data not shown).

### Cell proliferation assays

PC-3 and RL-PC-3/LRAT-4 cells that stably overexpress human LRAT were seeded on 24-well plates in triplicate at a density of 10,000 cells/ml/well. The next day, cells were cultured in the presence of 1 μM ATRA, 5 μM RXR agonist (BMS 188649), or different concentrations of PA or DHA in the absence or presence of 1 μM ROH. Fresh drugs were added every 2 days. After 72 and 120 h of culture in the presence or absence of the drugs, the cells were counted in triplicate using a Coulter counter.

### [$^3$H]retinol radiolabeling, retinoid extraction, and HPLC

PrECs and PC-3 cells were plated in 60 mm$^2$ dishes at a density of ~$4 \times 10^5$ cells/dish. PrECs and PC-3 cells were treated with various drugs in the presence of 100 nM [$^3$H]retinol for 6, 12, and 24 h, and cell samples were harvested. The metabolism of [$^3$H]retinol was determined by HPLC analyses of retinoids extracted from harvested cells. Alternatively, cells were plated in

150 mm dishes and cultured with or without drugs in the presence or absence of 1 μM nonradiolabeled retinol for 48 h. Cell samples were then harvested and retinoids were extracted. Cell numbers were counted in duplicate dishes, and the metabolism data were normalized to the cell number.

The extraction of retinoids and the HPLC conditions were as described previously (25). For the radiolabeled samples, nonradiolabeled retinoid standards [ATRA, ROH, and retinyl palmitate (RP)] were added to the radiolabeled samples before extraction. For nonradiolabeled samples, standards only were run as a separate sample in each experiment. For extraction, 350 μl of acetonitrile-butanol (50:50, v/v) and 0.1% butylated hydroxytoluene were added to a total volume of 0.5 ml of cell suspension in PBS. The mixtures were vortexed thoroughly for 60 s. After the addition of 300 μl of a saturated (1.3 kg/l) $K_2HPO_4$ solution and thorough mixing, the samples were centrifuged for 10 min at 12,000 *g* at room temperature. The upper organic layers were collected and transferred to injector vials for automated HPLC analysis. Retinoids from nonradiolabeled samples were extracted in the same manner except that only one standard, retinyl acetate, was added to each sample. (Human cells do not synthesize retinyl acetate, so retinyl acetate is not one of the endogenous retinoids.)

The HPLC analysis was performed using a Waters Millennium system (Waters Corp., Milford, MA) to separate the various retinoids. Samples were applied to an analytical 5 μm reverse-phase C18 column (Vydac, Hesperia, CA) at a flow rate of 1.5 ml/min (25). Retinoids were identified by HPLC based on two criteria: an exact match of the retention times of unknown peaks with those of authentic retinoid standards, and identical ultraviolet light spectra (220–400 nm) of unknowns against spectra from authentic retinoid standards during HPLC by the use of a photodiode array detector.

### Mass spectrometric analysis of retinoids

To identify REs generated during the drug treatments, mass spectrometry (MS) was used as described previously (53). PC-3 cells that stably overexpress exogenous human LRAT (RL-PC-3/LRAT-4) were plated in 150 $mm^2$ dishes and cultured with various drugs in the presence or absence of 1 μM nonradiolabeled retinol for 48 h, and the cell samples were then harvested. The extracted retinoids were separated by HPLC, and appropriate fractions were collected. The HPLC fractions were dried by SpeedVac and redissolved in 5 μl of absolute ethanol. One microliter of the redissolved sample was spotted onto the surface of a stainless-steel target. Mass spectrometry was performed on a Voyager-DE PRO time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a nitrogen laser emitting at 337 nm. Mass spectra were obtained in the positive ion mode at an acceleration voltage of 20 kV by accumulating 300 laser shots. Laser power was adjusted to slightly above the threshold to obtain good resolution and signal-to-noise ratios. Laser desorption ionization (LDI) mass spectra were present in the mass range *m/z* 250∼700. In the postsource decay (PSD) experiments, the precursor ion of interest was isolated, using a timed ion selector, for further structural characterization. Data Explorer 4.0 software, provided by Applied Biosystems, was used for data acquisition and processing. Calibration of mass spectra was done using the standards ROH and RP as external calibrants.

### Real-time RT-PCR

Cells were plated in 35 mm dishes and were treated with fresh PA or DHA, respectively, in the presence or absence of 1 μM ATRA or ROH for 6 and 24 h, and total RNA was extracted by using TRIzol reagent. Total RNA (3 μg per sample) was reverse-transcribed to cDNA using Superscript III reverse transcriptase at 53°C for 1 h. Real-time PCR was performed using gene-specific oligonucleotide primers. These primers were designed to generate cDNA fragments that cross an intron-exon boundary in the genomic DNA. The cDNA generated from 30 ng of total RNA was used for real-time PCR. Real-time PCR was performed on a DNA Engine Opticon system (MJ Research, Boston, MA) with a SYBR Green I Quantitect kit. For every primer set, a series of dilutions of a sample with the highest gene expression, from semiquantitative RT-PCR, was used to generate a standard curve. The conditions for the PCR were as follows: 95°C for 10 min to activate the DNA polymerase, followed by 50 cycles of 94°C for 30 s, primer annealing at 58°C for 30 s, and product extension at 72°C for 30 s. After each cycle, fluorescence was read at 80°C. The primer sequences were as follows. For human LRAT, forward primer, 5′-TGG AAC AAC TGC GAG CAC TTC GTG-3′; reverse primer, 5′-GCA GGA AGG GTA GTG TAT GAT ACC-3′. For human hypoxanthine guanine phosphoribosyl transferase (HPRT), a constitutively expressed enzyme, forward primer, 5′-TGC TCG AGA TGT GAT GAA GG-3′; reverse primer, 5′-TCC CCT GTT GAC TGG TCA TT-3′. HPRT was used as a loading control. We used the University of California, Santa Cruz In-Silico PCR program (http://genome.ucsc.edu/cgi-bin/hgPcr) to ensure that the PCR primers were not homologous with pseudogene sequences.

### Statistical analysis of the data

All experiments were repeated at least three times. Means and SEM were calculated using Microsoft Excel. The differences among various experimental groups were analyzed by Dr. Kathy Zhou, a statistician at the Weill Medical College of Cornell University, using two-way ANOVA and Tukey tests for multiple comparisons. Differences with $P < 0.05$ were considered statistically significant.

## RESULTS

### PA and DHA inhibit the proliferation of PC-3 cells

First, the effects of natural and synthetic RXR ligands on the proliferation of PC-3 and RL-PC-3/LRAT-4 cells were examined. Cells were plated on 24-well plates in triplicate and were cultured in the presence of various drugs. After 72 and 120 h, ATRA did not have a marked effect on the proliferation of PC-3 cells, whereas the synthetic RXR agonist (BMS 188649) inhibited the proliferation of these cells by >40% at 72 h (data not shown) and 120 h of treatment (**Fig. 1A**). ROH (1 μM) did not inhibit the proliferation of either cell line at 72 h (data not shown) and 120 h of treatment (Fig. 1B, C). However, both PA (50 μM) and DHA (5 and 20 μM) suppressed the proliferation of both cell lines at 72 h (data not shown) and 120 h of treatment, but the presence of 1 μM ROH did not alter the inhibitory effects of these drugs (Fig. 1B, C). PA or DHA treatment resulted in inhibition of the proliferation of PC-3 cells by 34% or 68.5%, respectively ($P < 0.05$) at 72 h (data not shown) and 120 h (Fig. 1B, C). The inhibitory effect of 20 μM DHA on the proliferation of the RL-PC-3/LRAT-4 cells was greater than that on the parental PC-3 cells at 72 h (data not shown) and 120 h of treatment (Fig. 1C). We conclude that the combination of ROH and either PA or DHA did not result in greater inhibition of cell proliferation than did PA or DHA alone.

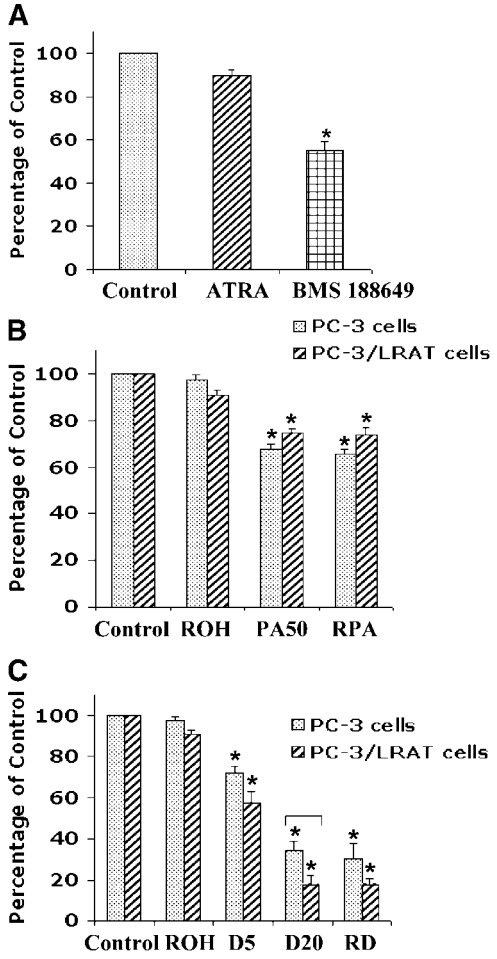


**Fig. 1.** Effects of phytanic acid (PA), docosahexaenoic acid (DHA), and the retinoid X receptor (RXR) agonist BMS 188649 on the proliferation of PC-3 prostate carcinoma cells. Androgen-unresponsive PC-3 prostate carcinoma cells and RL-PC-3/LRAT-4 cells were seeded on 24-well plates. The next day, cells were treated with 1 μM *all-trans* retinoic acid (ATRA), 5 μM of the RXR agonist BMS 188649, 1 μM *all-trans* retinol (ROH), or different concentrations of PA and DHA in the absence or presence of 1 μM ROH. After 120 h, cells were counted using a Coulter counter. A shows the proliferation of PC-3 cells only, and B and C show the proliferation of both parental PC-3 and RL-PC-3/LRAT-4 cells. A: Effects of ATRA and BMS 188649. B: Effects of 50 μM PA in the absence or presence of 1 μM ROH. C: Effects of 5 μM DHA alone and 20 μM DHA in the absence or presence of 1 μM ROH. D5 and D20, DHA at 5 and 20 μM; RD, ROH + 20 μM DHA; RPA, ROH + 50 μM PA. Means ± SEM are shown from three independent experiments, and the data were analyzed by ANOVA. * $P < 0.05$, compared with control. The bars under the bracket in C show statistically significant differences ($P < 0.05$) between the two cell lines at the indicated dose of DHA.

### PA and DHA increase intracellular RE levels

We examined retinol metabolism in cultured PrECs and PC-3 prostate carcinoma cells by HPLC. PrECs and PC-3 cells were cultured in medium containing 100 nM [$^3$H]retinol in the absence or presence of 50 μM PA, 20 μM DHA, or 1 μM of the synthetic RXR agonist BMS 188649 for 12 h. Retinoids in the cells and medium were extracted and separated by HPLC as described in Materials and Methods. In the presence of 100 nM [$^3$H]retinol, the levels of [$^3$H]REs in the PrECs were 3.0 ± 0.12-fold higher than those in the PC-3 cells at 12 h (**Fig. 2A, B**). In the HPLC scan of the PrECs, the predominant RE was the peak at the retention time of 53 min (Fig. 2A).

Treatment with PA or DHA plus 100 nM [$^3$H]retinol resulted in the production of novel [$^3$H]RE peaks (retention times of 54.0 and 50.5 min, respectively, in HPLC diagrams, marked with asterisks) and an increase in RP (Fig. 2A, B, **Tables 1, 2**). We obtained similar results after either a 6 or a 24 h treatment (data not shown). In contrast, the synthetic RXR agonist BMS 188649 did not have any effect on retinol metabolism (Fig. 2A, B). We further investigated the production of REs in both PrECs and PC-3 cells cultured in the presence of a higher concentration of nonradiolabeled ROH (1 μM) for 48 h, either in the presence or absence of PA or DHA. In the absence of exogenously added ROH, there were almost no REs detected in either cell line (Fig. 2C, D, control). The concentrations of REs in PrECs were 6.9 ± 0.24-fold higher than those in the PC-3 cells when the cells were cultured in the presence of 1 μM ROH alone (Fig. 2C, D). In the HPLC scan of the PrECs, the predominant RE was eluted at 53 min. Similar to the results shown in Fig. 2A, B, a 48 h treatment with 50 μM PA or 20 μM DHA plus 1 μM retinol increased RE levels (Tables 1, 2) in both PC-3 cells and PrECs. In addition, cells cultured in the presence of retinol and PA or DHA induced the production of novel RE peaks (Fig. 2C, D).

### LRAT mRNA levels in PrECs and PC-3 cells after treatment with PA or DHA plus retinol

To determine whether PA or DHA treatment changed the levels of LRAT mRNA in the PrECs or PC-3 carcinoma cells, we treated cells for 6 or 24 h with various drugs, followed by the isolation and reverse transcription of RNA. Real-time PCR was performed to detect LRAT mRNA. We found that LRAT mRNA levels in the untreated, control PrECs were 12.9 ± 3.1-fold higher than those in the PC-3 carcinoma cells. After a 6 h treatment, LRAT mRNA levels were increased by 1 μM retinol plus 20 μM DHA ($P = 0.08$), and 1 μM retinol also slightly increased LRAT expression in both cell lines. However, other drug treatments (50 μM PA, 20 μM DHA, and 1 μM retinol plus 50 μM PA) did not change LRAT mRNA levels (**Fig. 3A**). After a 24 h treatment with different drugs (1 μM retinol, 50 μM PA, 20 μM DHA, 1 μM retinol plus 50 μM PA, and 1 μM retinol plus 20 μM DHA), LRAT mRNA levels were not altered in either the PrECs or PC-3 cells (Fig. 3B).

### LRAT activity generates the novel RE peak in cells treated with PA plus retinol

The novel RE peak at 54.0 min generated by treatment of PrECs with retinol plus PA was greater than that in PC-3 cells (Fig. 2C, D). This is consistent with earlier data from our laboratory showing that LRAT protein levels are higher in PrECs than in PC-3 cells (28). To determine whether the novel RE peak observed in cells treated with PA plus ROH resulted from an LRAT-mediated esterifica-

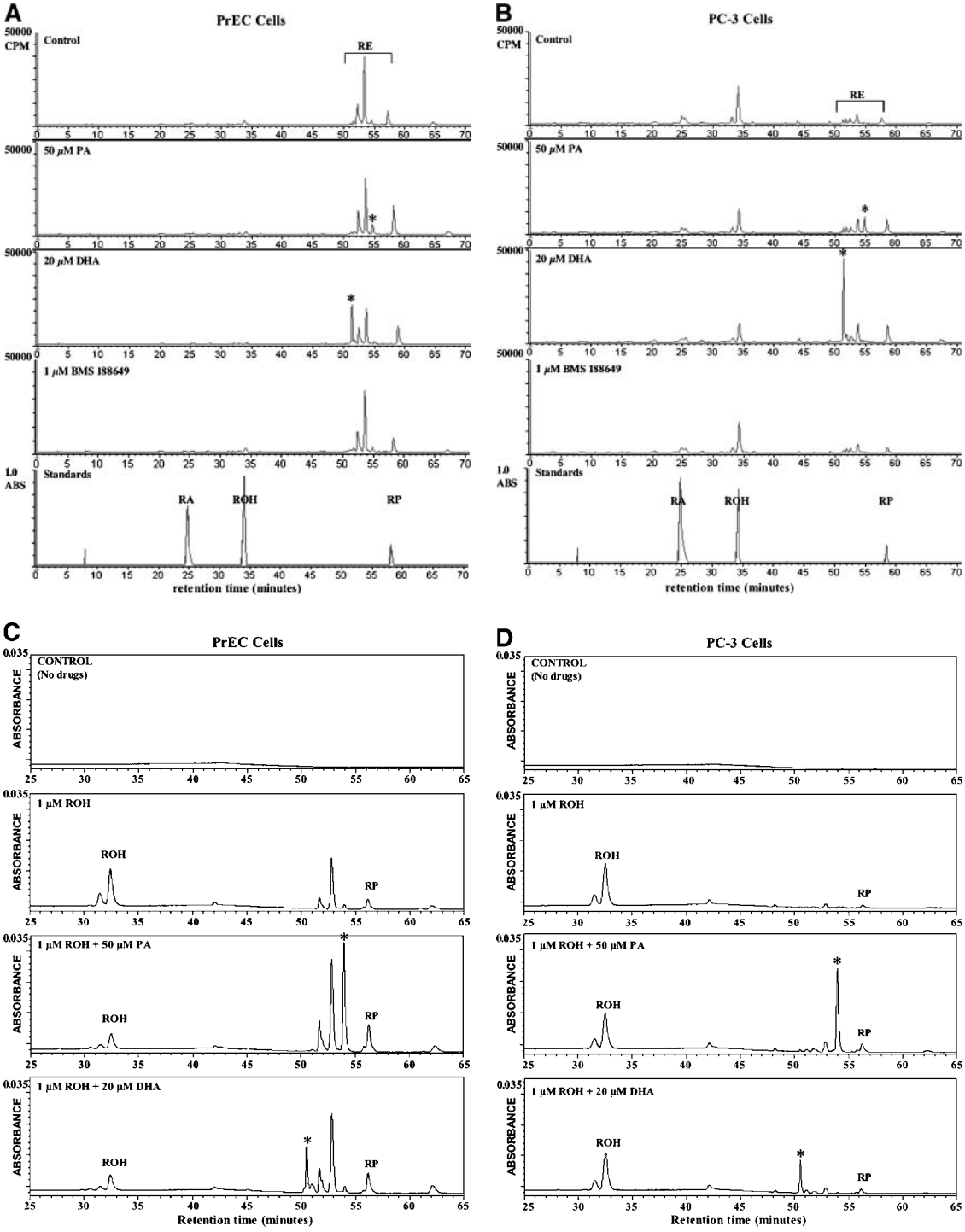


**Fig. 2.** Effects of PA or DHA on the metabolism of [$^3$H]retinol or nonradiolabeled retinol in normal human prostate epithelial cells (PrECs) and in human prostate cancer PC-3 cells. A, B: Cells were plated in 60 mm dishes and treated with PA (50 μM), DHA (20 μM), or BMS 188649 (5 μM) for 12 h in the presence of 100 nM [$^3$H]ROH, and retinoid metabolism was analyzed by HPLC. In A (PrEC) and B (PC-3), all of the y axes (cpm) are the same scale. C, D: Cells were plated in 15 cm dishes and treated with PA (50 μM) or DHA (20 μM) plus 1 μM retinol for 48 h. The metabolism of retinol was determined by HPLC analyses at 340 nm of retinoids extracted from harvested cells. In C (PrEC) and D (PC-3), all of the y axes (absorbance) are the same scale. ABS, absorbance; RA, *all-trans* retinoic acid; RE, retinyl esters; RP, retinyl palmitate. Results from one representative experiment of three are shown. The asterisks indicate the novel RE peak.

tion reaction, we measured the levels of this novel RE peak (at the retention time of 54.0 min) in both PC-3 cells and in PC-3 cells that were stably transfected with an expression construct containing the full-length human LRAT cDNA downstream of the cytomegalovirus promoter (RL-PC-3/LRAT-4 cell line). Culture for 48 h in the presence of 50 μM PA plus 1 μM ROH resulted in the appearance of this novel RE peak at 54.0 min in both parental PC-

TABLE 1. Changes in $^{3}$H-labeled and nonradiolabeled RP in cells cultured in the presence of PA and ROH (the area of RP in PC-3 cells cultured in the presence of retinol only is referred to as 1)

| Cells | Treatments | $^{3}$H-labeled RP from cells cultured in the presence of 100 nM [$^{3}$H]ROH or 100 nM [$^{3}$H]ROH and 50 μM PA for 12 h | Nonradiolabeled RP from cells cultured in the presence of 1 μM ROH or 1 μM ROH and 50 μM PA for 48 h |
|---|---|---|---|
| PrECs | Retinol | 2.07 ± 0.16 | 4.46 ± 0.46 |
| | Retinol + PA | 4.51 ± 0.23[a] | 14.98 ± 1.83[b] |
| PC-3 cells | Retinol | 1 | 1 |
| | Retinol + PA | 2.07 ± 0.23[a] | 4.49 ± 0.59[b] |

PA, phytanic acid; PrEC, normal human prostate epithelial cell; ROH, *all-trans* retinol; RP, retinyl palmitate. Each experiment was performed three times.

[a] $P < 0.01$.

[b] $P < 0.05$.

3 cells and RL-PC-3/LRAT-4 cells. However, the level of this novel RE peak at 54.0 min (**Fig. 4A, B**, asterisks) in the RL-PC-3/LRAT-4 cells was much greater (intracellular concentration, 9.33 ± 0.58 μM) than in the parental PC-3 cells (intracellular concentration, 0.74 ± 0.15 μM). This suggests that this novel peak is synthesized by LRAT. This novel peak at 54.0 min exhibits a maximum absorbance at 328 nm, which indicates that it is an RE (data not shown).

To confirm whether the tentatively assigned peak was an RE, total retinoids extracted from the RL-PC-3/LRAT-4 PC-3 cells were saponified by treatment with saturated KOH in methanol and subsequently separated by HPLC (53). All of the RE peaks, including the novel peak, disappeared, whereas the level of the ROH peak was greatly increased (data not shown). These results confirmed that this novel peak at 54.0 min is an RE.

### LRAT activity does not participate in the generation of the novel RE peak in cells treated with DHA plus retinol

The intensities of the retinol ester (RE) peak at 50.5 min, generated by culture in the presence of DHA plus retinol, were similar in both PrECs and PC-3 cells (Fig. 2C, D). We examined the potential role of LRAT in the synthesis of the novel RE peak at 50.5 min using parental PC-3 and RL-PC-3/LRAT-4 cells. A 48 h treatment with 20 μM DHA plus 1 μM ROH induced a novel RE peak at 50.5 min in both cell lines. The levels of this peak in both cell lines were similar (intracellular concentration, 1.70 ± 0.27 μM in parental PC-3 cells vs. 1.81 ± 0.24 μM in RL-PC-3/LRAT-4 PC-3 cells) (Fig. 4A, B), indicating that the novel RE peak is not synthesized by LRAT. The novel peak, generated by culture in the presence of DHA plus ROH, exhibited a maximum absorbance at 328 nm (data not shown). In a similar manner, saponification of the total extracted retinoids was carried out and confirmed that the novel peak was an RE (data not shown).

TABLE 2. Changes in $^{3}$H-labeled and nonradiolabeled RP in cells cultured in the presence of DHA and ROH (the area of RP in PC-3 cells cultured in the presence of retinal only is referred to as 1)

| Cells | Treatments | $^{3}$H-labeled RP from cells cultured in the presence of 100 nM [$^{3}$H]ROH or 100 nM [$^{3}$H]ROH and 20 μM DHA for 12 h | Nonradiolabeled RP from cells cultured in the presence of 1 μM ROH or 1 μM ROH and 20 μM DHA for 48 h |
|---|---|---|---|
| PrECs | Retinol | 2.07 ± 0.16 | 4.46 ± 0.46 |
| | Retinol + DHA | 3.87 ± 1.24[a] | 9.88 ± 1.65[a] |
| PC-3 cells | Retinol | 1 | 1 |
| | Retinol + DHA | 2.15 ± 0.56[a] | 2.64 ± 0.33[b] |

DHA, docosahexaenoic acid. Each experiment was performed three times.

[a] $P < 0.05$.

[b] $P < 0.01$.

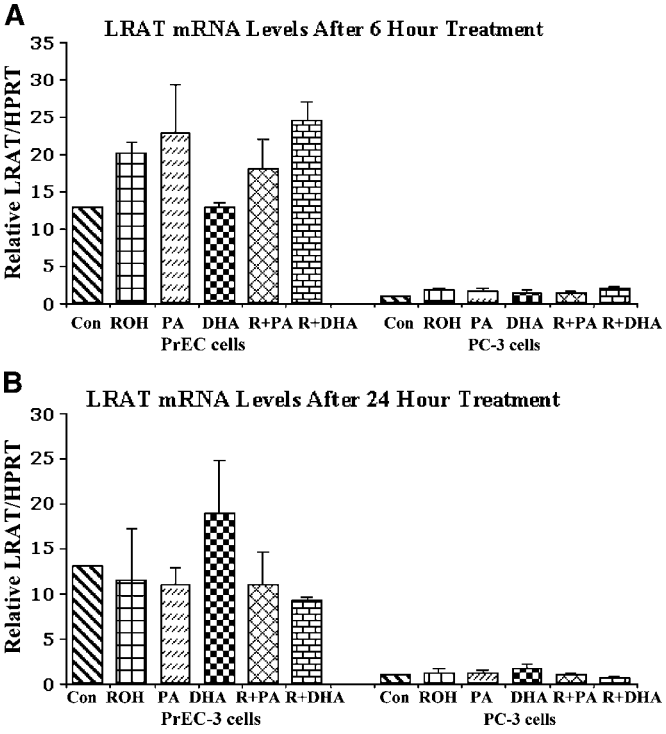


**Fig. 3.** Effects of PA and DHA on lecithin:retinol acyltransferase (LRAT) mRNA levels in PC-3 cells and PrECs. Cells were treated with various drugs for 6 h (A) or 24 h (B), and total RNA was isolated and used for reverse transcription. The cDNA from 30 ng of total RNA was used for real-time PCR. LRAT mRNA levels were normalized to hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA. LRAT mRNA levels in the control PC-3 cells were designated as 1. In these experiments, the concentrations of ROH, PA, and DHA were 1, 50, and 20 μM, respectively. Con, control; R+PA, ROH + PA; R+DHA, ROH + DHA. Experiments were repeated three times. Means ± SEM are shown from three independent experiments, and the data were analyzed using two-way ANOVA and Tukey tests for multiple comparisons.

### Use of mass spectrometry to identify the novel RE peaks

Because of the limited number of commercially available retinoid standards, MS was used to identify and characterize the novel RE peaks at 50.5 and 54.0 min. Using LDI-MS in the positive ion mode, REs show a characteristic fragment molecular ion $[M]^{+\bullet}$ at *m/z* 269, which fits one known fragmentation pathway of REs during LDI-MS: the elimination of the fatty acyl chain (54).

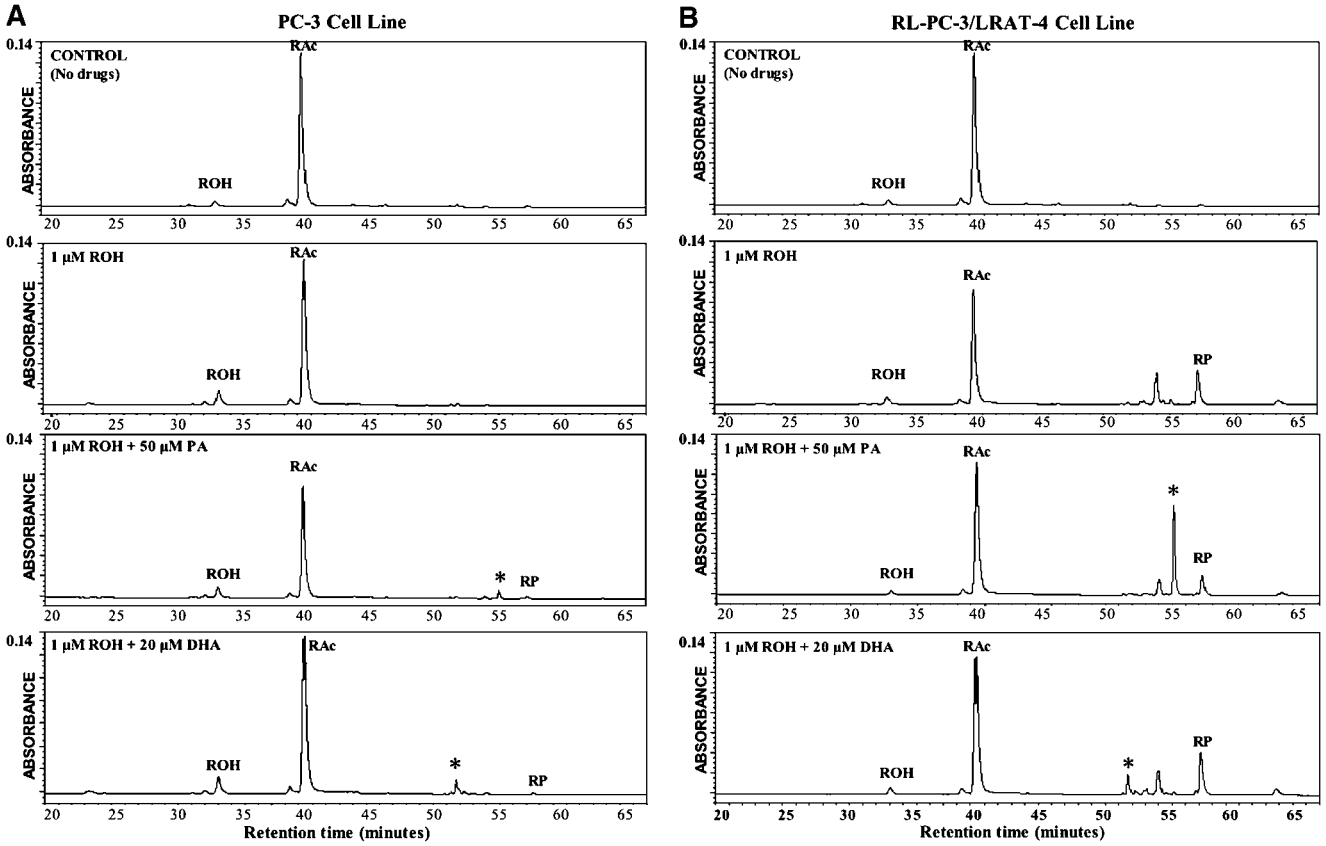


**Fig. 4.** Role of LRAT in the synthesis of a novel RE generated by cells cultured in the presence of PA or DHA plus retinol. Parental PC-3 cells (A) and RL-PC-3/LRAT-4 cells (B) were cultured in 15 cm dishes in the presence of PA (50 μM) or DHA (20 μM) plus 1 μM ROH for 48 h. Intracellular retinoids were extracted and analyzed by HPLC at 340 nm. Before retinoid extraction, retinyl acetate was added to each sample. RAc, retinyl acetate. This experiment was performed four times with similar results. The asterisks indicate the novel RE peak.

A known RP, collected after HPLC separation of retinoids extracted from RL-PC-3/LRAT-4 PC-3 cells, was first tested to demonstrate that this technique was suitable for the identification of new REs. Radical molecular ion $[M]^{+\bullet}$ without the loss of intact molecular mass information as well as characteristic fragment ions were detected from the RP: *m/z* 269.2, 480.5, and 524.5 (**Fig. 5B**). The fragment ion at *m/z* 269.2 is from REs by elimination of the fatty acyl chain, which is an indicator of REs. The fragment ion at *m/z* 480.5 was the decarboxylated ion, and it resulted from another known fragmentation pathway: decarboxylation through a cyclic transition state (54). Furthermore, the PSD mass spectrum of the selected molecular ion at *m/z* 524.5 was obtained in the positive ion mode. Based on the retinyl moiety generated from RP, PSD fragment ions were assigned. PSD fragment ions result mainly from two pathways: the breaking of alternative polyene bonds and the opening of a nonaromatic six carbon structure ring in the retinyl moiety. Especially, the characteristic PSD fragment ions at *m/z* 93, 105, 119, 123, 132, 144, 175, 253, and 268 can provide unique structural information for potentially characterizing novel REs. A detailed explanation and assignment of the mechanism of PSD fragment ions have been provided elsewhere (53). In addition, we infer the information of a fatty acid from the mass difference between a molecular ion and the characteristic PSD fragment ion at *m/z* 268. Therefore, the PSD approach developed here clearly reveals structural information about RP (Fig. 5C) and is suitable for the identification and characterization of the novel REs (53).

The mass spectra of the novel RE peak from the RL-PC-3/LRAT-4 cells cultured in the presence of PA plus ROH are shown (**Fig. 6**). LDI-MS analysis of the HPLC fraction corresponding to the novel RE peak at 54.0 min showed three peaks at *m/z* 269.2, 580.5, and 536.5, but these peaks were not detected in the mass spectrum of the HPLC fraction at 54.0 min from the same cells cultured in the presence of ROH alone (Fig. 6B, C). The characteristic fragment ion at *m/z* 269 indicates the presence of retinoids. The molecular ion observed at *m/z* 580.5 corresponds to retinyl phytanate (Fig. 6A), and the peak at *m/z* 536.5 is a satellite ion from decarboxylation. For further characterization of this peak at *m/z* 580.5, PSD LDI-MS analysis was carried out to obtain the structural information of the novel RE (Fig. 6D). The PSD fragmentation pattern of the ion putatively ascribed to retinyl phytanate was matched with the known fragmentation patterns of RE (53). Furthermore, based on the fact that

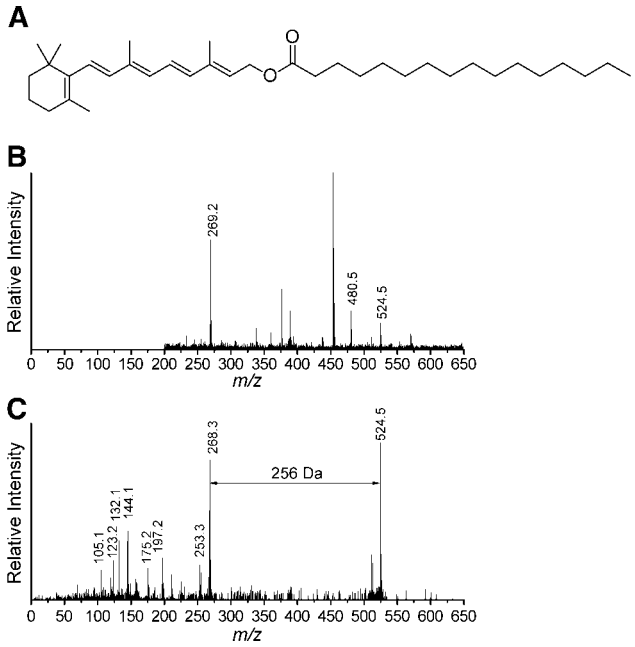


**Fig. 5.** Identification of the RP peak on the HPLC scan to validate the mass spectrometry methods. RL-PC-3/LRAT-4 cells were cultured in the presence of 1 μM ROH for 48 h. The HPLC fraction (from the extracted retinoids) at the retention time of RP (57.2 min) was analyzed using mass spectrometry. A: Molecular structure of RP. B: Mass spectrum of the HPLC fraction at the retention time of RP. C: Postsource decay (PSD) structural analysis of the peak of *m/z* 524.5 in B.

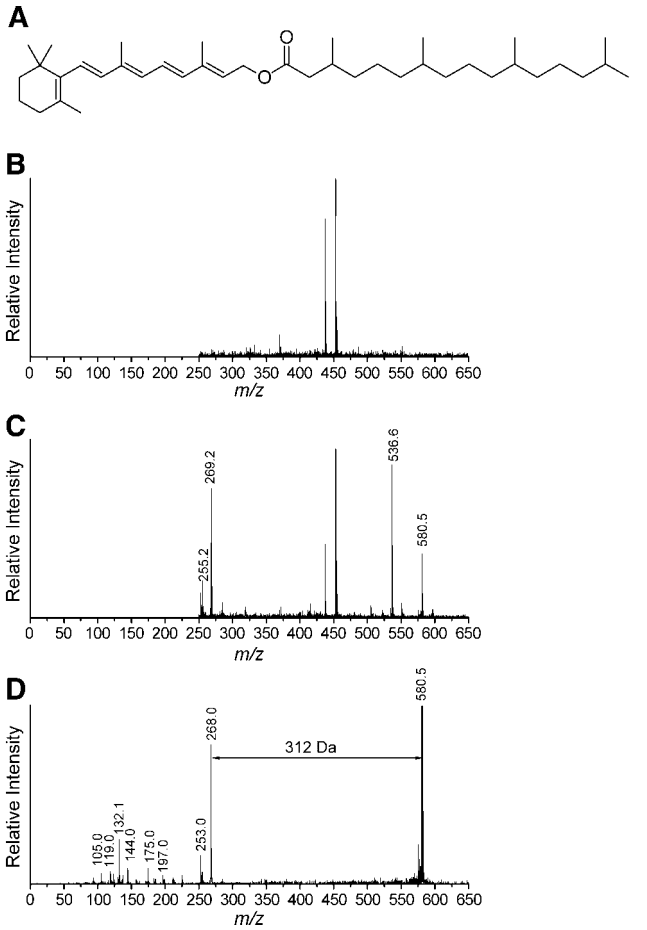


**Fig. 6.** Identification of the novel RE peak generated by culture of PC-3 cells that overexpress human LRAT in the presence of PA. RL-PC-3/LRAT-4 cells were cultured in the presence of 1 μM ROH or 1 μM ROH and 50 μM PA for 48 h. Then, the HPLC fraction at 54.0 min was collected and analyzed by mass spectrometry. A: Molecular structure of retinyl phytanate. B, C: Mass spectra of the 54.0 min fraction from cells treated with ROH only (B) or with ROH and PA (C). D: PSD structural analysis of the peak of *m/z* 580.5 in C. This experiment was performed five times with the same results.

the mass difference between a molecular ion and the characteristic PSD fragment ion at *m/z* 268, we deduced that the 312 Da from the PSD mass spectrum indicates a PA. Therefore, these results indicate that the novel RE peak at 54.0 min in the HPLC chromatograms (Figs. 2, 4) is the retinyl phytanate.

Based upon the strategy outlined above, the novel RE peak eluting at 50.5 min from PrECs, PC-3 cells, and RL-PC-3/LRAT-4 cells cultured in the presence of DHA plus ROH (Figs. 2, 4) was identified by using MS. There are three peaks at *m/z* 269.2, 552.5, and 596.5, which correspond to the diagnostic indicator of retinoid, the $[M\text{-}44]^{+\bullet}$ satellite ion, and the radical molecular ion, respectively (**Fig. 7C**). However, these ions were not observed in the mass spectrum of the HPLC fraction that elutes at 50.5 min from the same cells cultured in the presence of ROH alone (Fig. 7B). Moreover, we performed a PSD LDI-MS analysis of the molecular ion peak at *m/z* 596.5 to confirm the novel RE as the retinyl docosahexaenoate (Fig. 7D). Although we could not assign an ion at *m/z* 585 observed at the PSD mass spectrum because of an abnormal fragmentation, the characteristic PSD fragment ions at *m/z* 119, 123, 132, 144, 175, 253, and 268 as well as the information of fatty acid (328 Da) are enough to confirm that the selected molecular ion at *m/z* 596 was a RE. These results indicate that the novel RE peak from cells cultured in the presence of DHA plus ROH is the retinyl docosahexaenoate.

## DISCUSSION

### Effects of RXR ligands on the proliferation of human prostate cancer cells

The use of RXR-specific ligands is a potentially promising approach for the prevention and treatment of human prostate cancer (31, 32). Consistent with these reports, we found that a synthetic RXR agonist, BMS 188649, but not ATRA, inhibited the proliferation of the PC-3 human prostate carcinoma cells by 44% (Fig. 1A). Moreover, two RXR natural ligands, PA and DHA (35–38), also inhibited the proliferation of the parental PC-3 cells and PC-3 cells that stably overexpress human LRAT (RL-PC-3/LRAT-4 cells) (Fig. 1B, C). This inhibition occurred at concentrations not much greater than the ranges of the levels of PA and DHA in human serum, which are up to 30 and 12.7 μM, respectively (55, 56). These results confirmed previous reports that DHA inhibits human prostate cancer cell pro-

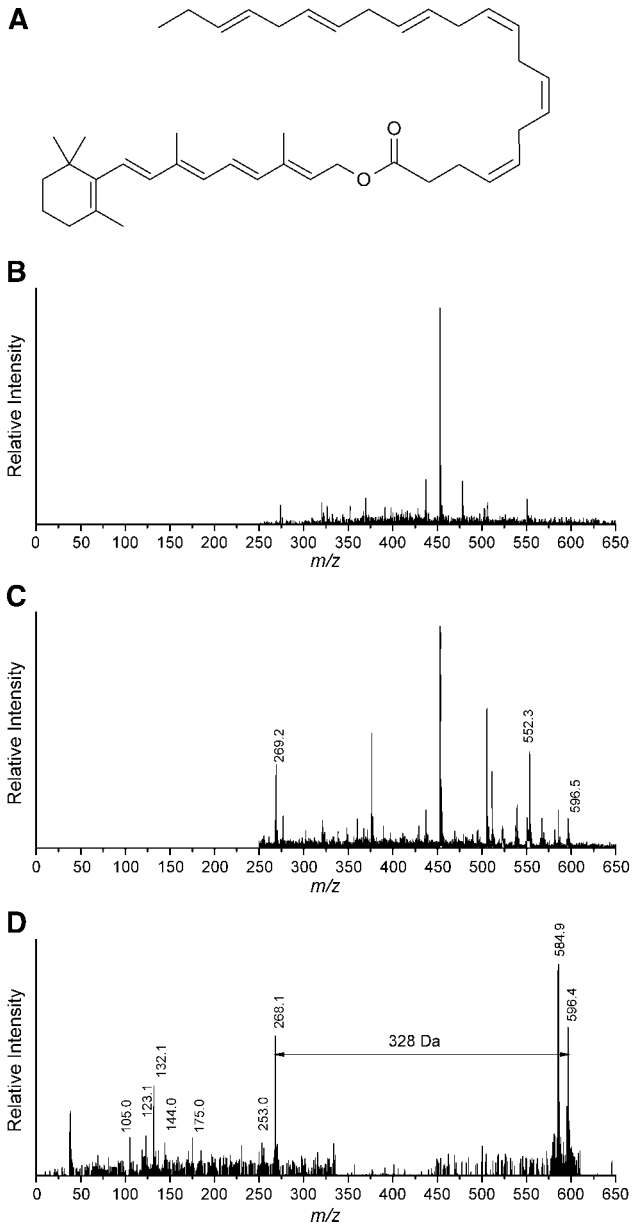


**Fig. 7.** Identification of the novel RE peak generated by culture of PC-3 cells that overexpress human LRAT in the presence of DHA. RL-PC-3/LRAT-4 cells were cultured in the presence of 1 μM ROH or 1 μM ROH and 20 μM DHA for 48 h. Then, the HPLC fraction at 50.5 min was collected and analyzed using mass spectrometry. A: Chemical structure of retinyl docosahexaenoate. B, C: Mass spectra of the 50.5 min fraction from cells treated with ROH only (B) or with ROH and DHA (C). D: PSD mass spectrum of the selected molecular ion of the peak of *m/z* 596.5 in C. This experiment was performed three times with the same results.

liferation (30, 31, 46, 47). More importantly, our results indicate that PA could be a useful nutrient for dietary preventive and therapeutic approaches to human prostate cancer. Previous reports show that PA and DHA can induce apoptosis and have cytotoxic effects (57–60); therefore, the proapoptotic and cytotoxic activities of PA or DHA probably are among the causes of their inhibitory effects on human prostate cancer cell proliferation. In addition, some clinical data have suggested that the serum levels of PA are positively associated with prostate cancer risk (61), although in that study the sample size was small and the difference in the serum PA levels between the unaffected controls and the prostate cancer patients was small as well (0.08 ± 0.03 vs. 0.10 ± 0.06 mg/100 ml).

The increased expression of LRAT did not affect the proliferation of PC-3 cells either in the absence or the presence of ROH, although in the presence of ROH the levels of REs in RL-PC-3/LRAT-4 cells were 11.13 ± 1.4-fold greater than those in the parent PC-3 cells (Fig. 4A, B). Also, ROH did not alter the inhibitory effects of PA and DHA on the proliferation of parental PC-3 and RL-PC-3/LRAT-4 cells. Therefore, although in the presence of ROH both PA and DHA increased the levels of REs and can be esterified into novel REs in human prostate cancer PC-3 cells, it seems that REs do not play a major role in inhibiting the proliferation of these cells. However, as shown in Fig. 1C, the extent of the inhibition on the proliferation of the RL-PC-3/LRAT-4 cell line by 20 μM DHA was greater than that in parental PC-3 cells. These data indicate that the overexpressed human LRAT sensitizes PC-3 cells to DHA growth inhibition by an unknown mechanism.

### Changes in retinol metabolism elicited by the RXR natural ligands PA and DHA in PrECs and human prostate cancer cells

Our results (Fig. 2) confirmed that the levels of REs are greatly reduced in PC-3 cells compared with PrECs (28). Furthermore, in both cell lines, culture in the presence of retinol plus PA increased the levels of some REs, such as RP, and resulted in a novel RE peak (Fig. 2). Culture of both cell lines in the presence of retinol plus DHA showed similar results (Fig. 2). However, the synthetic RXR agonist BMS 188649 did not have any effect on RE levels in either cell line (Fig. 2A, B). Although it has been reported that both PA and DHA are natural ligands of RXR (35–38), our data suggest that the changes in the levels of REs caused by culture in the presence of 1 μM retinol plus PA or DHA occur via a mechanism other than the activation of RXRs.

No reports have been published that these two novel REs, retinyl phytanate and retinyl docosahexaonate, exist in vivo in any mammalian tissue. However, 11-*cis* retinyl docosahexaenoate has been reported in the eyes of lobster (*Homarus*) and crayfish (*Procambarus*) (62). Normally, PA is preferentially taken up by the liver, and ~50% of the free fatty acid pool in hepatocytes is PA (56). The major sources of DHA in the human body are diet and conversion from linolenic acid in the liver (primary site), astrocytes, and vascular endothelial cells in the retina and brain. The nervous system, along with sperm, has the highest concentration of DHA (63).

Although the liver is the organ in which up to 80% of the total ROH and REs in vertebrates is stored (64, 65), retinyl phytanate and retinyl docosahexaonate generally cannot be detected in the liver, possibly because of the low levels of PA and DHA in the serum and the

microenvironments in tissues. In human serum, the level of PA is <30 μM and the level of DHA is <12.7 μM (55, 56).

### Role of LRAT in the synthesis of retinyl phytanate and retinyl docosahexaonate

LRAT is regarded as a major enzyme involved in the esterification of ROH in tissues (66, 67). We showed, through the use of a genetically engineered cell line, that LRAT catalyzes the synthesis of retinyl phytanate from ROH and PA (Fig. 4A, B). In contrast, our results suggest that LRAT does not catalyze the production of retinyl docosahexaenoate (Fig. 4A, B).

Acyl-coenzyme A:retinol acyltransferase (ARAT) has been reported to catalyze the synthesis of REs (68), although this enzyme has not been cloned or characterized at the molecular level. It is possible that ARAT catalyzes the synthesis of retinyl docosahexaenoate. Recent reports have shown that acyl-coenzyme A:diacylglycerol acyltransferase 1 (DGAT1), a triacylglycerol synthesis enzyme (69), can catalyze the synthesis of REs in an acyl-CoA-dependent manner (70, 71). Therefore, it is possible that DGAT1 catalyzes the synthesis of retinyl docosahexaenoate. In addition, the theoretical three-dimensional ball-and-stick models of palmitate and PA are similar, but they are different from that of DHA (**Fig. 8**). This suggests that LRAT does not catalyze the synthesis of retinyl docosahexaenoate because of the difference in the chemical structure of DHA.

### Effects of PA or DHA on LRAT mRNA levels

LRAT null mice demonstrate impaired absorption and storage of retinoids (70) and are more susceptible to vitamin A deficiency (72). Therefore, changes in LRAT expression should alter the levels of REs. It has been reported that in rat liver and lung, LRAT expression and vitamin A storage are regulated by vitamin A status and by treatment with ATRA (66, 73). In this study, among the various 6 h drug treatments, retinol plus DHA and retinol alone increased LRAT mRNA levels in the normal PrECs and the PC-3 cell line (Fig. 3). These results are consistent with the reports that RXR-selective agonists alone are inactive in the induction of LRAT mRNA (74). In contrast, retinol has been shown to dramatically induce hepatic LRAT enzymatic activity (75).

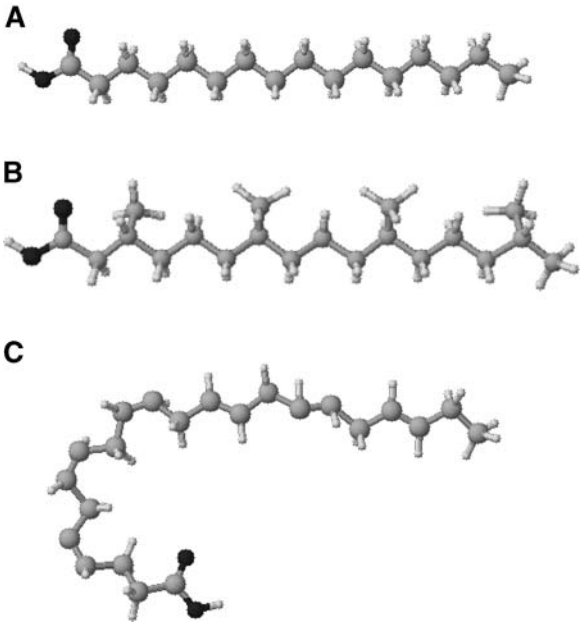


**Fig. 8.** Three-dimensional ball-and-stick models of palmitate (A), PA (B), and DHA (C). Gray, carbon; black, oxygen; white, hydrogen.

### Characterization of novel REs using mass spectrometry

The use of MS to analyze polar retinoids and REs has been reported (53, 54, 76, 77). Our data confirmed that LDI-MS is a suitable approach to detect retinoids, especially REs, in biological samples. However, LDI mass spectra (Figs. 5B, 6B, 6C, 7B, 7C) gave rise to many parent ions because this fraction may have many other coeluting biological compounds (e.g., phospholipids, sphingolipids, glycerides, and fatty acids). We did not try to identify each peak, especially *m/z* 453, because this was not our major focus.

The novel REs were identified and confirmed as retinyl phytanate or retinyl docosahexaenoate (Figs. 6, 7). These results show that PA or DHA can be a substrate for the synthesis of RE. Along with intact molecular mass to identify REs, a PSD LDI-MS approach was used to confirm that the selected ion of interest in the mass spectrum was a RE, based on the PSD fragmentation patterns of a standard RE. Determination of the chemical structures of novel REs is essential for studying the physiological role of these compounds, because once the chemical structure is known, the compounds can be chemically synthesized and used in cellular assays. Thus, an MS approach will be useful in the identification of, and in gaining structural information about, novel retinoids from various sources.

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