Lecithin:Retinol A cyltransferase Is Responsible for Amidation of Retinylamine, a Potent Inhibitor of the Retinoid Cycle*

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Lecithin:retinol acyltransferase (LRAT) catalyzes the transfer of an acyl group from the sn-1 position of phosphatidylcholine to all-trans-retinol (vitamin A) and plays an essential role in the regeneration of visual chromophore as well as in the metabolism of vitamin A. Here we demonstrate that retinylamine (Ret-NH2), a potent and selective inhibitor of 11-cis-retinal biosynthesis (Golczak, M., Kuksa, V., Maeda, T., Moise, A. R., and Palczewski, K. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 8162–8167), is a substrate for LRAT. LRAT catalyzes the transfer of the acyl group onto Ret-NH2 leading to the formation of N-retinylpalmitamidate, N-retinylstearamidate, and N-retinylmyristamidate with a ratio of 15:6:2, respectively. The presence of N-retinylamidates was detected in vivo in mice supplemented with Ret-NH2. N-Retinylamidates are thus the main metabolites of Ret-NH2 in the liver and the eye and can be mobilized by hydrolysis/deamidation back to Ret-NH2. Using two-photon microscopy and the intrinsic fluorescence of N-retinylamidates, we showed that newly formed amides colocalize with the retinyl ester storage particles (retinosomes) in the retinal pigment epithelium. These observations provide new information concerning the substrate specificity of LRAT and explain the prolonged effect of Ret-NH2 on the rate of 11-cis-retinal recovery in vivo.

In vertebrates, the retinoid cycle is essential for regeneration of the chromophore 11-cis-retinal, which is an integral part of rhodopsin and cone visual pigments (1). All-trans-retinol is generated by the photosomeration of 11-cis-retinal bound to opsin in photoreceptor cells or supplemented from circulation. It is trapped in the retinal pigment epithelium (RPE)1 in the form of insoluble fatty acid esters in subcellular structures known as retinosomes (2, 3). The enzyme responsible for the esterification of all-trans-retinol in the small intestine, liver, and eye, lecithin:retinol acyltransferase (LRAT) (4, 5), was cloned (6), and its function has been proven (2, 7). Later in the retinoid cycle the 11-cis configuration of the retinol is restored by enzymatic isomerization (8).

The key step in the transformation of all-trans-retinal to 11-cis-retinal is the isomerization reaction. Recently a candidate protein approach and expression cloning demonstrated that RPE65 exhibits Fe2+-dependent isomerization activity (9–11). We have suggested that the regeneration of the chromophore might occur through a retinyl carbocation intermediate (12) and demonstrated that isomerization is inhibited by positively charged retinoids (13). This mechanism would be consistent with Fe2+-catalyzed alkyl cleavage of the retinyl esters. Retinylamine (Ret-NH2) potently and selectively inhibits the isomerization step of the retinoid cycle in vitro and in vivo, whereas modifications of the amino group lead to loss of inhibitory potency. Surprisingly Ret-NH2 has a long lasting effect, and when mice were treated with a single dose of the inhibitor, its inhibitory effect on the cycle was observed for several days (13).

Inhibition of the retinoid cycle may have implications in averting light damage to photoreceptors in some instances or preventing the accumulation of toxic condensation side products of bleached chromophore, all-trans-retinal, in the RPE. In Stargardt disease, a disease associated with mutations in the photoreceptor-specific ATP-binding cassette transporter (ABCR) (14) or elongation of the very long chain fatty acid-like 4 protein (ELOVL 4) (15, 16), the accumulation of all-trans-retinal is thought to be responsible for the formation of a component of a lipofuscin pigment called A2E. This fluorophore is accumulated during the course of the disease and, to a lesser extent, with age in normal individuals. It exerts a toxic effect on retinal cells, causing retinal degeneration and consequent loss of vision (17, 18). In contrast with other less potent inhibitors such as 13-cis-retinoic acid or its metabolically active intermediates (19), Ret-NH2 does not activate the transcrip
tion of genes (13), making it a safer candidate for therapeutic application.

However, a total inhibition of 11-cis-retinoid production would resemble Leber congenital amaurosis (LCA), an autosomal recessive rod-cone dystrophy that presents itself at birth or the first few months of life. Rod and cone photoreceptor functions of the LCA patients are either absent or severely compromised at birth as evidenced by extinguished or barely detectable photopic and scotopic electroretinograms (20). LCA is highly heterogeneous; at least nine subtypes of LCA have been identified (see www.sph.uth.tmc.edu/RetNet/disease.htm) and linked to many unrelated genes. For enzymes of the retinoid cycle, numerous mutations in the RPE65 gene have been linked to LCA (21–24). Null mutations in the human LRAT and RDH12 genes lead to recessive early onset retinal dystrophy, a phenotype very similar to LCA (25–27). Thus, a reduced but not blocked regeneration of rhodopsin could be beneficial for some forms of retinal dystrophies, particularly for a low dose and long lasting inhibitor.

Here we provide evidence that Ret-NH2 is converted to pharmacologically inactive retinylamidates in vitro and in vivo in the liver and RPE. We demonstrate that the enzyme responsible for the amidation is LRAT and that the amidation products are stored in retinosomes in the RPE.

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3 The abbreviations used are: RPE, retinal pigment epithelium; Ret-NH2, retinylamine; LCA, Leber congenital amaurosis; LRAT, lecithin:retinol acyltransferase; RAN, N-retinylacetamidate; RPN, N-retinylpalmitamidate; HPLC, high performance liquid chromatography; MS, mass spectrometry; HFX, human embryonic kidney; bis-Tris, 2-bis(2-hydroxyethyl)aminomethane; 2-(hydroxyethyl)amine; 3,3,3-triiodo-LHP, 1,2-dihydroxyhexanoic acid; Alkylamine, amino-2-(hydroxymethyl)propane-1,3-diol; DHPC, 1,2-dihexanoyl-sn-glycerol-3-phosphocholine; DGT, acetyl-CoA-thiolyglyceral acyltransferase; WT, wild type; ABCR, ATP-binding cassette transporter, retina-specific.

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An equilibrium maintained between retinylamide and free Ret-NH₂ results in the long lasting and highly potent inhibitory effect of Ret-NH₂.

**EXPERIMENTAL PROCEDURES**

**Animals**—All procedures using mice were approved by the Washington University Animal Care Committees and conformed to recommendations of the American Veterinary Medical Association Panel on Euthanasia and recommendations of the Association of Research for Vision and Ophthalmology. Animals were maintained in complete darkness, and all manipulations were done under dim red light using an Eastman Kodak Co. number 1 safelight filter (transmittance, >560 nm). Typically, 6–12-week-old mice were used in all experiments. *Lrat*−/− mice were obtained and genotyped as described previously (7). Animals were gavaged with 1 mg of Ret-NH₂, N-retinylacetamide (RAN), and N-retinylpalmitamide (RPN) dissolved in 150 μl of vegetable oil 18 h prior to analysis. For gavage with radioactive retinoids, the total radioactivity per single dose did not exceed 10 μCi.

**Materials**—Fresh bovine eyes were obtained from a local slaughterhouse (Schenk Packing Co., Stanwood, WA). Preparation of bovine RPE microsomes was performed according to methods described by Stecher et al. (28). 11,12-[3H]all-trans-retinol was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO).

**Retinoid Preparations**—All retinoids were purified by normal phase HPLC (Beckman Ultrasphere-Si; 5 μm; 4.5 × 250 mm; detection at 325 nm; flow rate, 2 ml/min), and their concentrations were determined spectrophotometrically in EtOH. Absorption coefficients for Ret-NH₂ and N-retinylamides were assumed to be equal to those of retinol and retinyl esters (29, 30).

**Chemical Synthesis**—Ret-NH₂ was prepared according to the method described by Golczak et al. (13). N-Retinylacetamide and N-retinylpalmitamide were prepared by reacting Ret-NH₂ with an excess of acetic anhydride and palmitoyl chloride, respectively, in anhydrous dichloromethane in the presence of N,N-dimethylaniline at 0°C for 1 h. After the reaction was completed (as judged by HPLC), water was added, and the product was extracted with hexane. The hexane layer was washed with saturated NaCl solution, dried with anhydrous magnesium sulfate, filtered, and evaporated in a SpeedVac. Radioactive Ret-NH₂ and N-retinylamides were synthesized starting from 11,12-[3H]all-trans-retinol, which was first oxidized with MnO₂ (CH₂Cl₂, 20 °C, 4 h) to 11,12-[3H]all-trans-retinal. Ret-NH₂ and retinylamides were then prepared using methods described previously (13). N-Retinylheptanamide was prepared by dicyclohexylcarbodiimide-promoted coupling reaction. First dicyclohexylcarbodiimide was reacted with heptanoic acid in dichloromethane. Ret-NH₂ in dichloromethane was added. The reaction mixture was incubated at room temperature for 3 h, extracted, and washed as described above. Mass spectrometry (MS) of synthesized retinoids was performed using a Kratos Analytical Instruments HV-3 direct probe mass spectrometer and electron-impact ionization.

**Inducible Expression of LRAT Protein in HEK Cells**—Mouse LRAT cDNA was cloned as described elsewhere (7). For expression, the LRAT coding region was amplified using the primers 5′-GCCACCATGAA-GAACCAAATGCTGGAAGCT-3′ and 5′-ACATACTGTTGAC-CTGTGGACTG-3′. The PCR product was ligated into the pCR-Blunt II-TOPO vector (Invitrogen) and then subcloned into the EcoRI site of pcDNA4/TO. N-Acetylgalactosaminyltransferase I-negative HEK293 S cells (31), obtained from Dr. G. Khorana (Massachusetts Institute of Technology, Boston, MA), were transfected with the TetR expression plasmid pcDNA6-TR(blaR), and blasticidin-resistant colonies were selected and cloned. Cells were cultured in Dulbecco’s modified Eagle’s
medium, 10% fetal calf serum, and Zeocin and blasticidin antibiotics, and maintained at 37 °C, 5% CO2, and 100% humidity. TetR-expressing HEK cells were transfected with the pcDNA4/TO-LRAT construct and selected with Zeocin. Stable clones were verified for expression of LRAT protein using the anti-LRAT monoclonal antibody (7).

**LRAT Activity Assay**

The reaction was carried out in 10 mM bis-Tris propane buffer, pH 7.5, 1% bovine serum albumin. All-trans-retinol or Ret-NH2 was delivered in 0.8 N, N-dimethylformamide to the final concentration of 20 μM. The reaction was initiated by adding 20 μL of bovine RPE microsomes or 50 μL of LRAT-expressing HEK cell lysate (150 mg of protein). The total volume of the reaction mixture was fixed at 200 μL. The reactions were incubated at 37 °C for the required times and then stopped by adding 300 μL of methanol followed by the same volume of hexane. Retinoids were extracted and analyzed on a Hewlett Packard 1100 series HPLC system equipped with a diode array detector. A normal phase column (Beckman Ultrasphere-Si, 5 μm, 4.5 × 250 mm) and a step gradient of ethyl acetate in hexane at a flow rate of 2 mL/min were used to elute N-retinylamides (10% ethyl acetate for 23 min and then 40% ethyl acetate up to 40 min). To detect Ret-NH2, retinoid separation was performed in 99.5% ethyl acetate with the addition of 0.5% of 7 N ammonia in methanol.

**Mouse Retinoid Extraction and Analysis**—Two mouse eyes or 0.5 g of mouse liver were homogenized in a glass-to-glass homogenizer using 3 mL of 50% methanol in 20 mM bis-Tris buffer, pH 7.4. Retinoids were extracted with 4 mL of hexane. The organic phase was collected, dried down in a SpeedVac, and redissolved in 400 μL of hexane. In the case of the liver extract, 10 and 100 μL of retinoid solution were injected on an HPLC column for the detection of N-retinylamides and Ret-NH2, respectively. For samples from mouse eyes, 100 μL were analyzed for N-retinylamides and Ret-NH2. Separation conditions used for retinoid analysis were the same as described above. To determine the radioactivity distribution among retinoids found in the liver of animals gavaged with 11,12-[3H]all-trans-Ret-NH2 or 11,12-[3H]N-all-trans-retinylamide, products corresponding to the retinyl esters, retinol, retinylamides, and Ret-NH2 were collected during an HPLC run. Fractions containing retinoids were dried down in a SpeedVac and redissolved in 300 μL of N,N-dimethylformamide. The radioactivity of each fraction was examined by scintillation counting and normalized to total radioactivity of the sample injected on the column.

**Two-photon Vitamin A Imaging**—Two-photon excitation microscopy was performed using a confocal/two-photon laser scanning

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**FIGURE 2. Identification of the biosynthetic product of the conversion of Ret-NH2.**

A, the HPLC-purified biosynthetic products of the Ret-NH2 reaction are compared with RPN for their retention time by normal phase HPLC. Elution times for the most abundant biosynthetic product (peak 2) and RPN (b) are identical for the examined conditions. The absorbance spectra for the two compounds are identical with a maximum absorbance at 325 nm (c). ε, electron impact MS analysis of the biosynthetic product (peak 2) and RPN are identical with the parent ion at 523 m/z and two main fragments at 268 and 155 m/z (b, 2). MS of two other products labeled 1 and 2 (A, a) revealed a fragmentation pattern characteristic for retinoids (268 and 155 m/z) and masses 551 and 495, respectively (b, 1 and 3). Taking into consideration elution times, spectral properties, and masses, the products correspond to all-trans-N-retinylamides composed of C18 and C14 acyl groups. mAU, milliabsorbance units.
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FIGURE 3. Identification of N-retinylamides in tissues of Ret-NH₂-supplemented mice. WT mice were gavaged with 1 mg of Ret-NH₂ 18 h prior to analysis. Retinoids were extracted from the liver and eyes and examined by HPLC. Based on the conditions and elution time, N-retinylamides (products 2, 2', 3, 3', and 4) were identified in the liver and eyes. Products 5 and 5' correspond to isomers of Ret-NH₂ (insets). The peak labeled 7 is all-trans-retinol. Comparison of the amount of Ret-NH₂ and N-retinylamides found in the tissues indicates that the majority of Ret-NH₂ is converted into amides. mAU, milliabsorbance units.

FIGURE 4. LRAT transfers heptanoate from DHPC to all-trans-retinol and Ret-NH₂. A, incubation of RPE microsomes with all-trans-retinol in the presence of DHPC leads to the formation of retinyl ester containing a shorter acyl group (retinyl heptanoate), which was confirmed by coelution with synthetic standard and MS spectrometry. B, in the presence of Ret-NH₂, similar experimental conditions promote formation of N-retinylheptanamide as proved by comparison of retention time and MS pattern with a synthetic standard. Similar substrate specificity observed in both cases suggests that LRAT is responsible for this reaction. mAU, milliabsorbance units.

microscope (LSM 510 MP-NLO; Carl Zeiss MicroImaging, Inc., Thornwood, NY) with LSM510 software version 3.0. Detailed methods were described in previous publications (2, 3). For localization of Ret-NH₂ in the RPE cells, eyecup preparations were exposed to 0.25 mM Ret-NH₂ caged with 100 mM (2-hydroxypropyl)-β-cyclodextrin for 15 min and washed briefly with Ames’ medium (Sigma) for 3 min. Ames’ medium was equilibrated with argon to purge O₂ from the solution.
RESULTS

Ret-NH₂ Is Converted into N-Retinylamides upon Incubation with RPE Microsomes—HPLC analysis of the retinoids extracted from the standard isomerization assay (12) showed no significant changes (within 5%) in the amount of Ret-NH₂. When Ret-NH₂ was incubated with RPE microsomes in the absence of all-trans-retinol, the level of Ret-NH₂ dropped significantly with time concomitantly with the appearance of a new peak that eluted with the front of the HPLC column (Fig. 1A, top panel). Putative products of Ret-NH₂ conversion were separated in 10% ethyl acetate/hexane, revealing the presence of three different compounds (Fig. 1A, bottom panel). Based on the elution time from a normal phase HPLC column and the shape and maximum of UV absorbance spectra, these products were retinoids less polar than Ret-NH₂ (Fig. 1A). As measured by the disappearance of Ret-NH₂, the observed reaction progress was much slower than the esterification of all-trans-retinol driven by LRAT (Fig. 1B).

The unknown compounds 1, 2, and 3 were separated and purified by collecting the appropriate fractions from a normal phase HPLC column (Fig. 1A). The purity of biosynthetic products was examined by HPLC (Fig. 2A, a) prior to MS analysis. The biosynthetic compounds have an m/z of 551, 523, and 495 with respect to their order of elution from an HPLC column (Fig. 2B, a, b, and c). These observed masses correlate with the masses of N-retinylamides possessing C₁₈, C₁₆, and C₁₄ acyl groups within their structure. To collect more evidence, the MS pattern of synthetic N-retinylpalmitamide was compared with the most abundant biosynthetic product (Fig. 2A, a, peak 2). The molecular ion peak and MS fragmentation patterns of both compounds are identical. Additionally comparison of elution time from the HPLC column and UV absorbance spectra revealed no differences between synthetic and biosynthetic products (Fig. 2A, b and c). Thus, we conclude that RPE microsomes converted Ret-NH₂ into three main amides: RPN, N-retinylstearamide, and N-retinylmyristamide produced in a ratio of 15:6:2, respectively.

N-Retinylamides Can Be Detected in Tissues of Mice Gavaged with Ret-NH₂—The potential application of Ret-NH₂ as an inhibitor of the retinoid cycle in vivo led us to investigate whether N-retinylamides can be detected in mouse tissue. Mice were gavaged with 1 mg of Ret-NH₂ 18 h prior to retinoid analysis. The presence of N-retinylamides in the liver and eye extracts could be easily detected by normal phase HPLC chromatography (Fig. 3). N-Retinylamides were recognized based on
comparison of their UV absorbance spectrum and elution time. Interestingly, the amount of Ret-NH₂ found in the examined tissues was smaller than the amount of N-retinylamides, 4 and 15 nmol, respectively, in the livers and 102 and 140 pmol found in the eyes (Fig. 3, insets). These results suggest that Ret-NH₂ is efficiently converted into N-retinylamides in vivo.

Ret-NH₂ Is Amidated Due to LRAT Activity—To identify the enzyme that is responsible for amidation, we tested whether LRAT can utilize Ret-NH₂ as a substrate. First we carried out qualitative analysis of retinyl esters and N-retinylamides formed in an excess of 1,2-diheptanoyl-sn-glycero-3-phosphocholine (DHPC). DHPC, present in millimolar concentration in the reaction mixture containing RPE microsomes and all-trans-retinol, serves as a donor of the C7 acyl group that is efficiently transferred onto all-trans-retinol. Consequently retinyl heptanoate was produced and was identified based on its mass spectrum and elution profile from normal phase HPLC in comparison with a synthetic standard (Fig. 4A). The same experiment performed in the presence of Ret-NH₂ led to the formation of an amide containing a short acyl chain whose mass and elution time from the normal phase HPLC column perfectly matched the properties of N-retinylheptanamide (Fig. 4B). Considering transfer of the heptanoyl group from DHPC onto both all-trans-retinol and Ret-NH₂, we conclude that LRAT could be responsible for both enzymatic activities.

In addition, the production of N-retinylamides in LRAT-expressing HEK cells was investigated. Prior to the experiments, LRAT-expressing HEK cells were examined for expression and activity of the enzyme by immunoblotting and standard assays (Fig. 5B, a and b). Cells were harvested and homogenized, and the lysate was incubated in the presence of Ret-NH₂. The products of the reaction were analyzed by normal phase HPLC. This analysis revealed striking differences between the retinoid composition extracted from untransfected and LRAT-expressing cells. In the presence of LRAT, products corresponding to N-retinylamides were observed (Fig. 5A, top panel) that correlate with the decrease of Ret-NH₂ to below detection limits (Fig. 5A, bottom panel). In the control cells, the unreacted substrate was observed, and no amides were formed (Fig. 5A). Quantification of the retinoids found in the examined samples is shown in Fig. 5B, c. Additionally incubation of the cell lysate with Ret-NH₂ led to an elevation of all-trans-retinol that was observed directly by HPLC in untransfected cells. In the case of LRAT-expressing cells, the amount of retinyl esters increased. Thus, as an alternative to amidation, Ret-NH₂ can be also deaminated to all-trans-retinol.

FIGURE 6. LRAT KO mice reveal an absence of N-retinylamides present in tissues. Lrat / / mice were gavaged with 1 mg of Ret-NH₂. Eighteen hours later, retinoids from liver and eyes were extracted and separated by HPLC. Lrat / / mice reveal a lack of N-retinylamide production (top panel, arrows indicate theoretical position of RPN). Instead, an increased level of Ret-NH₂ is detected compared with the WT mice (bottom panel). In the case of Lrat / / mice an elevation of all-trans-retinol level is observed. The detected retinyl esters are most likely formed via the activity of enzymes other than LRAT. mAU, milliabsorbance units.
To further investigate the role of LRAT in Ret-NH₂ acylation in vivo, we designed assays using Lrat⁻/⁻/⁻ mice. Following gavage of Lrat⁻/⁻/⁻ mice with synthetic Ret-NH₂, we observed a complete lack of N-retinylamide formation in the examined livers (Fig. 6, top) concomitant with the detection of intact Ret-NH₂ (Fig. 6, bottom). Together these observations demonstrate that LRAT utilizes Ret-NH₂ as an acceptor of acyl groups. Additionally analysis of the chromatograms obtained from in vivo studies indicated that Ret-NH₂ was transformed into vitamin A. This transformation was confirmed by the increased level of all-trans-retinol found in Lrat⁻/⁻/⁻ mice gavaged with Ret-NH₂ as well as the elevation of retinyl esters despite the absence of LRAT (Fig. 6, top). Without LRAT, retinol esterification most likely occurs due to the action of acyl CoA:diacylglycerol acyltransferase (DGAT) enzymes (32, 33) or the recently described product of the GS2 gene (34).

**N-Retinylamides Can Be Hydrolyzed Back to Ret-NH₂ in Vivo**—In contrast to Ret-NH₂, N-retinylamides do not inhibit the formation of 11-cis-retinol in vitro (13). We showed that Ret-NH₂ has a long lasting affect on visual recovery (more than 48 h), which is surprising considering the efficient conversion of Ret-NH₂ into N-retinylamides observed in vivo. It may be that once synthesized, N-retinylamides do not remain intact but are slowly hydrolyzed back to Ret-NH₂. To address this hypothesis, WT mice were gavaged with 1 mg of RAN 18 h prior to HPLC analysis of retinoids extracted from liver. Based on the retention time from a normal phase HPLC column, RAN and C₁₈, C₁₆, and C₁₄ N-retinylamides were identified (Fig. 7A). The presence of

**FIGURE 7.** RAN is hydrolyzed back to the Ret-NH₂ and converted into RPN in vivo. WT mice were gavaged with 1 mg of RAN prior to extraction and separation of retinoids. The chromatogram shown in A (top) reveals concomitance of RAN and RPN in examined livers. Bottom chromatogram indicates elution time of chosen standards. B, distribution of radioactivity among main retinoids found in the WT mouse livers as a result of oral gavage with 11,12-[³H]all-trans-Ret-NH₂, RAN, or RPN. mAU, milliabsorbance units; ROL, all-trans-retinol.

**FIGURE 8.** N-Retinylamides inhibit recovery of visual chromophore in vivo. Mice were gavaged with 2 mg of Ret-NH₂, RAN, or RPN for 12 h before bleaching for 20 min at 150 cd/m² watt in a Ganzfeld chamber. Regeneration of 11-cis-retinal was allowed for 6 h in the dark, and retinoids were extracted from the eye and separated by normal phase HPLC. Analysis of the chromatograms indicates an effect comparable to Ret-NH₂ of N-retinylamides on the regeneration of 11-cis-retinal for the applied doses and experimental conditions.
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**FIGURE 9. Effect of Ret-NH₂ administration on retinosomes.** A, retinosomes before rhodopsin activation in control mouse. B, retinosomes imaged 30 min after photobleaching of rhodopsin in control mouse. C, retinosomes imaged 20 h after photobleaching in control mouse. D, retinosomes before rhodopsin activation in a mouse administrated with 1 mg of Ret-NH₂. E, retinosomes imaged 30 min after photobleaching in a mouse administrated with 1 mg of Ret-NH₂. F, retinosomes imaged 20 h after photobleaching in a mouse administrated with 1 mg of Ret-NH₂. G, fluorescence intensities (left) and all-trans-retinyl esters were quantified. 1, control mice before photobleaching of rhodopsin. 2, control mice 30 min after photobleaching. 3, control mice 20 h after photobleaching. 4, mice gavaged with 1 mg of Ret-NH₂ and dark-adapted. 5, mice gavaged with 1 mg of Ret-NH₂ 30 min after photobleaching. 6, mice gavaged with 1 mg of Ret-NH₂ 20 h after photobleaching. Scale bar, 20 μm. Error bar, ±S.E. au, arbitrary units.

N-retinylamides that were different from those administrated suggest that RAN is hydrolyzed to Ret-NH₂, which is then converted primarily into RPN. Alternatively, but in our opinion less likely, is the explanation that amides can undergo a transacylation reaction.

To follow the metabolites of Ret-NH₂ and its amides, radiolabeled retinoids were used. WT mice were gavaged with 11,12-[³H]Ret-NH₂, RAN, or RPN, and after 18 h the main retinoids present in the liver were separated by HPLC, collected, and examined by scintillation counter. The distribution of radioactivity among retinoids is shown in Fig. 7B. Because Ret-NH₂ and RAN are converted mostly to RPN, it is no surprise that the radioactivity profiles for each compound look similar. Interestingly, besides RPN, most of the radioactivity was found in the fractions corresponding to the retinyl esters, reflecting the deamidation of Ret-NH₂ observed earlier. These findings led us to conclude that retinyl esters and RPN are the main metabolites of Ret-NH₂ in vivo.

The data described above encouraged us to use non-inhibitory N-retinylamides as precursors of Ret-NH₂ (a potent inhibitor of retinoid isomerases). For this purpose WT mice were gavaged with 2 mg of RAN or RPN, exposed to strong light, and left for recovery of the rhodopsin chromophore. The quantity of 11-cis-retinal in the eyes was then examined by HPLC. The experimental data revealed that animals supplemented with N-retinylamides, like with free Ret-NH₂ or its salt, exhibited a much slower recovery of 11-cis-retinal compared with untreated animals (Fig. 8).

**Two-photon Imaging of Ret-NH₂ and N-Retinylamide in the RPE Cells** —RPE cells, where the enzymatic process of 11-cis-retinal regeneration occurs, are targets for the biological activity of Ret-NH₂. Using a two-photon microscopy technique (2, 3), we investigated the subcellular localization of Ret-NH₂ and N-retinylamides within mouse RPE cells. Albino mice gavaged with Ret-NH₂ revealed increased basal fluorescence intensity compared with control animals. This was attributed to the accumulation of retinyl esters and N-retinylamides in the eye (Fig. 9, A and D; see Fig. 3 for quantification). The distribution of fluorescence correlated with the retinoid intrinsic fluorescence pattern described previously (2) that may suggest that N-retinylamides colocalize with retinosomes. Bleaching of rhodopsin in the mouse eye led to temporary accumulation of retinyl esters (35) and, consequently, to a further increase in fluorescence within the RPE layer (Fig. 9, B and E). In the presence of Ret-NH₂ the high level of fluorescence persisted as a result of retinoid cycle inhibition (Fig. 9, C and F) in contrast with its short duration in untreated mice.

To more accurately track the accumulation and localization of N-retinylamides we switched to a less complex, more reduced system. Visualization of the retinyl esters in the isolated, unstained RPE cells displayed the characteristic pattern of retinosomes located on the peripheries of the RPE cells (Fig. 10A). To record the fluorescence signal from only exogenously delivered Ret-NH₂, internal fluorescence emanating from endogenous retinoids was depleted by UV photobleaching. This led to the decomposition of endogenous retinoids, and consequently, no fluorescence signal could be detected using the given experimental conditions (Fig. 10B). Ret-NH₂ was then applied to the RPE cells and imaged by two-photon microscopy. A significant increase in fluorescence was observed in retinosomes as well as in the cytoplasmic/endoplasmic reticulum area. Analysis of the picture revealed that Ret-NH₂ and N-retinylamides localized to the retinosomes (Fig. 10C). By taking advantage of the pH-dependent fluorescence of Ret-
NH₂ (Fig. 11) it was possible to discriminate between signals from amine and amide. Ret-NH₂ was quenched and washed out in the cytoplasmic/endoplasmic reticulum area by Ames’ medium with 0.1% Triton X-100 at pH 5.5. In this case, retinosomes still retained fluorescence as shown by representative arrows. A–D, scale bars indicate 20 μm. E, relative fluorescence intensities were quantified based on two-photon imaging. 1, fluorescence intensity in retinosomes before UV radiation. 2, fluorescence intensity in RPE cells after UV photobleaching. 3, RPE fluorescence after application of Ret-NH₂. 4, RPE fluorescence after application of Ret-NH₂ and washing by Ames’ medium with 0.1% Triton X-100 at pH 5.5. Error bar, ±S.E. F, HPLC separation of the retinoids found in the eyecup used for retinosome visualization. UV photobleached sample (corresponding to B) reveals no detectable levels of retinoids (top panel, dotted line). Incubation with Ret-NH₂ (sample corresponding to D) leads to the formation of 321 pmol of N-retinylamides, which could be detected after washing with 0.1% Triton X-100 at pH 5.5 (top panel, solid line). At the same time only 18 pmol of Ret-NH₂ were observed in the given sample (not shown). au, arbitrary units.

FIGURE 10. Two-photon imaging of retinylamine and retinylamide in the RPE cells. A, retinyl esters were imaged by two-photon microscopy. Arrows indicate representative localizations of aggregated retinyl esters to retinosomes. B, RPE cells were irradiated by UV illumination (350 nm) and imaged by two-photon microscopy. Retinyl esters were photobleached; hence fluorescent structures are not observed. C, Ret-NH₂ was applied to the RPE cells and imaged by two-photon microscopy. Ret-NH₂/N-retinylamide localized to retinosomes and endoplasmic reticulum. D, Ret-NH₂ was washed out, and fluorescence was quenched by Ames’ medium with 0.1% Triton X-100 at pH 5.5. In this case, retinosomes still retained fluorescence as shown by representative arrows. A–D, scale bars indicate 20 μm. E, relative fluorescence intensities were quantified based on two-photon imaging. 1, fluorescence intensity in retinosomes before UV radiation. 2, fluorescence intensity in RPE cells after UV photobleaching. 3, RPE fluorescence after application of Ret-NH₂. 4, RPE fluorescence after application of Ret-NH₂ and washing by Ames’ medium with 0.1% Triton X-100 at pH 5.5. Error bar, ±S.E. F, HPLC separation of the retinoids found in the eyecup used for retinosome visualization. UV photobleached sample (corresponding to B) reveals no detectable levels of retinoids (top panel, dotted line). Incubation with Ret-NH₂ (sample corresponding to D) leads to the formation of 321 pmol of N-retinylamides, which could be detected after washing with 0.1% Triton X-100 at pH 5.5 (top panel, solid line). At the same time only 18 pmol of Ret-NH₂ were observed in the given sample (not shown). au, arbitrary units.

DISCUSSION

Ret-NH₂ is an important tool for study of the retinoid cycle and is also a potential pharmacological agent. To understand the long lasting effects of Ret-NH₂, we analyzed the metabolism of this compound using in vitro and in vivo assays. The results revealed that Ret-NH₂, which actively and potently inhibits the synthesis of 11-cis-retinal (13), is in equilibrium between its free and amidated forms in the liver and in the eye. LRAT is the enzyme responsible for its amidation (Fig. 12).

Transformation of Ret-NH₂ to Retinylamides and Storage in Retinosomes—Ret-NH₂ was amidated 50–100 times more slowly than all-trans-retinol was esterified to retinyl esters (Fig. 1B). C₁₆ was the most abundant form of the amide followed by C₁₈ and C₁₄ (Fig. 2). Several candidate enzymes could be responsible for the amidation process. Examples include fatty acid acyltransferases, which are integral membrane proteins that contain a DHHC-Cys-rich domain and are involved in protein palmitoylation (36, 37), and DGAT enzymes. Recently DGAT1 was found to be involved in retinyl ester synthesis (32, 33). Here we provide evidence that the enzyme responsible for the majority of Ret-NH₂ amidation is LRAT (Figs. 4–6).
Amidation of Retinylamine

![Diagram of Amidation of Retinylamine](Image)

**FIGURE 12. Transformation of Ret-NH₂ in vivo.** Ret-NH₂ can be metabolized in two main ways. It can undergo an amidation reaction governed by LRAT that leads to the formation of N-retinylamides. Alternatively Ret-NH₂ can be deaminated to retinol followed by formation of retinyl esters.

LRAT has a modest substrate specificity in terms of recognition of the β-ionone moiety (38–40). Its efficient utilization of amine as compared with an alcohol group was unexpected. However, if the mechanism of LRAT action involves first the acyl transfer from a lipid to a Cys residue at the active site of the enzyme as has been suggested (41), either all-trans-retinol or Ret-NH₂ could undergo this transacylation as a substrate.

**Oxidation of Ret-NH₂ to Vitamin A**—Using radioactive Ret-NH₂, we found that this compound can also be oxidized to retinol (vitamin A) and in turn be esterified to retinyl esters (Figs. 7 and 12). Thus, the metabolism of Ret-NH₂ involves either the formation of more chemically inert amides or oxidation to vitamin A, making it an attractive compound for in vivo studies of the retinoid cycle and for potential applications to treat human retinal dystrophies.

**Implication of Potential Therapeutic Use of Ret-NH₂**—The use of potent inhibitors of isomerization in vivo after light exposure would prevent or slow down the recovery of the visual pigment chromophore and could have an important protective role in cases of damage causing excessive exposure to light. Because Ret-NH₂ inhibits the regeneration of rhodopsin, which is responsible for causing damage in response to light overexposure (42), it may be able to inhibit retinal damage in such cases. In Stargardt disease (14), associated with mutations in the ABCR transporter, the accumulation of all-trans-retinal has been proposed to be responsible for the formation of a lipofuscin pigment, A2E, which can be toxic toward retinal cells and causes retinal degeneration and consequent loss of vision (17, 18). It was proposed that treating patients with an inhibitor of retinol dehydrogenases, 13-cis-retinoic acid (Accutane, Roche Applied Science) might prevent or slow down the formation of A2E and might have protective properties that could help to maintain normal vision (19, 43). It is not yet clear whether Ret-NH₂ will have a protective effect in either case, but in contrast with 13-cis-retinoic acid, which can spontaneously isomerize to the all-trans isomer and in turn activate the nuclear receptors retinoid X receptor and retinoic acid receptor, Ret-NH₂ does not interact at micromolar concentrations with retinoid X receptor and retinoic acid receptor (13). Thus, Ret-NH₂ thus potentially offers a safer alternative to 13-cis-retinoic acid as a pharmacological agent in preventing or reducing damage due to light exposure and in cases of retinal degenerative disease. Ret-NH₂ is also stored in the eye and liver as retinylamides and released after hydrolysis back to free Ret-NH₂. Importantly, Ret-NH₂ could be used in patients less frequently than 13-cis-retinoic acid.

**Application of Ret-NH₂ in Studies of the Retinoid Cycle**—In the RPE, retinylamides are synthesized from exogenous Ret-NH₂, transported, and stored in retinosomes (Fig. 9). The fact that retinylamides have a long lasting inhibitory effect on the recycling of all-trans-retinol to 11-cis-retinal suggests that retinolamides, as predicted from their resemblance to lipid droplets (2, 3), are active organelles involved in the retinoid cycle. Ret-NH₂ and other amides offer numerous research applications to be explored. A particularly useful application may be the pH dependence of the intrinsic fluorescence of Ret-NH₂ to discriminate the drug from other autofluorescence in the eye. Two-photon microscopy used to study the retina (2, 3) should also provide further useful applications in observing drug accumulation in the eye.

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