Retinyl esters are hydrolyzed in early endosomes of J774 macrophages

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Abstract **The aim of the current study was to identify the subcellular compartment(s) responsible for the hydrolysis of chylomicron remnant-retinyl esters, in J774.1 cells. The cells were incubated with medium containing chylomicron remnant [^{3}H]retinyl ester. Subcellular fractionation was used to separate early endosomes from late endosomes and lysosomes. About 26% and 80% of the total [^{3}H]retinyl esters taken up by the J774 cells were hydrolyzed after 10 min and 60 min of chase, respectively. In the early endosomes, there was a 4-fold increase of radioactivity (nearly all radioactivity associated with retinyl esters) during the first 10 min of chase. The radioactivity in early endosomes was reduced by 43% from 10 min to 60 min and remained stable from 60 to 180 min of chase. From 10 to 60 min the amount of retinol in early endosomes increased from 44% to 82%, indicating an efficient hydrolysis of retinyl esters. Less than 10% and 5% of the total cell-associated radioactivity was found in the late endosomes and lysosomes during the entire chase period. In the chase medium, 84% of the total amount of retinoid released during 180 min was present already after 10 min. The percentage of retinol in the medium increased from 25% to 82% during incubation from 10 to 180 min. These data suggest that retinyl esters are endocytosed together with the chylomicron remnant particle and hydrolyzed in the early endosomes in this cell model.—** Hagen, E., A. M. Myhre, T. E. Tjelle, T. Berg, and K. R. Norum. **Retinyl esters are hydrolyzed in early endosomes of J774 macrophages.** ***J. Lipid Res.*** **1999.** 40: **309–317.**

Supplementary key words chylomicron remnants • retinyl ester • macrophages • vitamin A • retinyl ester hydrolase • endocytosis • early endosomes

Alimentary vitamin A enters the blood as retinyl esters in chylomicrons. After lipoprotein lipase (LPL)-mediated hydrolysis of chylomicrons, the retinyl esters follow the remnant particle, mainly to the liver, but also to extrahepatic tissue, including the bone marrow (1). Major biological activity of vitamin A is mediated through its active metabolite, retinoic acid, which binds to nuclear receptors and thereby influences transcription of several genes (2–7). Formation of retinoic acid from retinyl esters requires hydrolysis of retinyl esters to retinol followed by oxidation of retinol to retinoic acid.

Thus, the hydrolysis of retinyl esters is an important step in the metabolism of retinoids and has been reported to take place in many different tissues: such as liver, eye, intestinal mucosa, lung, kidney, and lachrymal gland (8–16). A neutral, bile salt-dependent retinyl ester hydrolase activity was reported in earlier studies. This enzyme is synthesized in the pancreas and the liver and has been shown to be identical to carboxyl ester lipase (17). It is secreted by the pancreas to the intestinal lumen and probably takes part in the hydrolysis of dietary retinyl esters (9, 10, 13, 18). The function of carboxyl ester lipase in hepatic retinoid metabolism is not clearly understood. In addition to carboxyl ester lipase, a number of tissues contain membrane-bound bile salt-independent, retinyl ester hydrolase activities (16, 18, 19). Bile salt-independent retinyl ester hydrolase activities have been found in plasma membrane, endosomes, endoplasmatic reticulum, cytosol, and lysosomes (8, 9, 11, 13, 16, 20–26), but is specifically enriched in the plasma membrane endosome fraction of the liver (10). This localization would allow the enzyme to play a role in the initial hydrolysis of retinyl esters in chylomicron remnants. In most of the above-mentioned studies cell free systems were used to assay retinyl ester hydrolase activity (27). The physiological importance of these hydrolases could therefore be questioned.

In vivo studies by Harrison, Gad, and Ross (28) demonstrated that retinyl esters in chylomicrons were hydrolyzed in the plasma membrane/endosome fraction of rat liver, indicating that these compartments contain physiologically important retinyl ester hydrolase(s) in the liver. Lipoprotein lipase (LPL) may also hydrolyze retinyl esters in chylomicron remnants (29).

Retinoic acid is important for normal differentiation of white blood cells and other cells and is a potential anticancer drug (30). Retinoic acid already is used in the treatment of acute promyelocytic leukemia (31–33). Retinyl palmitate

Abbreviations: CMR, chylomicron remnants; LPL, lipoprotein lipase; HRP, horseradish peroxydase; HPLC, High Performance Liquid Chromatography; OA, ovalbumin; LPDS, lipoprotein-deficient serum; ER, endoplasmic reticulum; oa-Au, colloidal gold coated with ovalbumin.

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has also been shown to have effects on promyelocytic and non-lymphocytic leukemia (34, 35). The metabolism of retinyl esters in bone marrow-derived cells has not been studied previously, in spite of the well-documented fact that retinoids have important functions in these cells. Several studies show that bone marrow-derived cells have the ability to take up retinyl esters in chylomicron remnants (36–41). In a report by Sklan et al. (42), retinol and retinoic acid were detected in leukocytes isolated from human plasma.

We found it interesting to study the hydrolysis of retinyl ester in a bone marrow-derived cell line. A physiological approach to study the metabolism of retinyl esters is to use chylomicron remnants radioactively labeled with retinyl esters as a tool. It was of crucial importance to have a cell line that accepted high concentrations of chylomicron remnant [^{3}H]retinyl esters, making it possible to study the intracellular metabolism. The J774 cell line was chosen because it is derived from bone marrow and because it is well characterized with respect to uptake of CMR (43–45). As chylomicron remnants are taken up by receptor-mediated endocytosis in J774 cells (44), the main aim of the present study was to describe the hydrolysis of retinyl ester in the subcellular compartments: early endosomes, late endosomes, and lysosomes.

MATERIALS AND METHODS

Chemicals

Phosphate-buffered saline (PBS) was obtained from Gibco Limited (Paisly, UK) and Dubelcco's Modified Eagle's Medium (DMEM) was from Bio Whittaker (Walkersville, MD). Retinol, holotransferrin, fetal calf serum, KBr, horseradish peroxydase (HRP) type IV, and essentially fatty acid-free albumin were from Sigma Chemicals Co. (St. Louis, MO), [^{3}H]retinol was purchased from NEN (Boston, MA). Gentamycin (Garamycin «Schering-Plough»), midazolam (Dormicum 1 mg/mL «Roche») and fluanisone 10 mg/mL and fentanyl 0.2 mg/mg (Hypnorm "Janssen" Beerse, Belgium) were obtained from a local pharmacy. Insta-gel II plus was obtained from Packard Instruments (Groningen, Netherlands). Sucrose, hexane, methanol, methyl-tert-butyl-ether, and acetonitrile were from E. Merck (Darmstadt, Germany). Percoll for self-generating gradients was purchased from Pharmacia AB (Stockholm, Sweden). Lipoprotein lipase isolated from bovine milk was a generous gift from G. Bengtsson Olivecrona and T. Olivecrona, Umeå, Sweden.

Preparation of lipoproteins

[^{3}H]retinyl ester chylomicron remnants. Male Wistar rats (mean weight 300 g) were fed with EWOS-ALAB (EWOS A/B, Södertelje, Sweden) brood stock feed for rats and mice, containing 12,000 IU vitamin A/kg (i.e., 12.6 μmol/kg). Anesthesia was induced by injecting a mixture of Dormicum/Hypnorm (1:1) s.c. One mL retinyl palmitate (23.330 IU vitamin A = 24,5 μmol) in ground nut oil containing 250 μCi carrier-free [^{3}H]retinol was mixed with 7 mL 10 mm taurocholic acid and sonicated for 40 sec. The lipid emulsion was injected through a duodenal catheter during the first 4 h after the operation. Intestinal lymph was obtained from lymph fistula in Wistar rats while the rats were kept in a Bollman restraining cage (46).

Post-heparin plasma was obtained after an intravenous injection of 150 μL heparin (100 I.E./mL) per 100 g body weight. The rat was exsanguinated after 10 min and plasma was isolated by centrifugation (15 min at 3,500 rpm). Chylomicron remnants were prepared from rat intestinal lymph by incubation with post heparin plasma and bovine serum albumin (BSA) for 70 min at 37°C in a shaking water bath. Each mL contained 0.6 mL 20% w/v fatty acid-free serum albumin in PBS, 0.2 mL post heparin plasma, and 0.2 mL lymph (12 mmol triacyl glycerol). The LPL activity in the incubation mixture was stopped by adding KBr to a density of 1.019 g/mL. Chylomicron remnants were isolated by ultracentrifugation (35,000 rpm for 20 h at 10°C, d 1.019 g/mL), and dialyzed against 20 mm phosphate buffer, pH 7.40, containing 0.85% NaCl and 2 mm EDTA for 24 h. The final preparation was stored for a maximum of 3 weeks in the dark at 4°C and used in experiments within 20 days, usually within 3 days. Mean content of RE was 200 nmol/mL, mean radioactivity was 5×10^6 dpm/mL in the final preparation. Mean specific radioactivity was 1.5×10^3 dpm/nmol retinyl ester. Mean protein content was 8.9 (±4.5) mg/mL and mean triglyceride content was 30.9 (±10.1) mmol/l.

Human lipoprotein-deficient serum (LPDS) was prepared from blood collected from normal fasting subjects. Serum was adjusted to a density of 1.23 g/mL and centrifuged for 48 h at 40,000 rpm. The lipoprotein-containing supernatant was removed and the lipoprotein-deficient plasma dialyzed was against 20 mm phosphate buffer containing 0.85% NaCl and 2 mm EDTA for 24 h and stored in the freezer at −20°C.

HPLC of retinoids

Retinol and retinyl esters were separated by HPLC. The retinoids were extracted from medium, cell lysate, and subcellular fractions using hexane as described previously (47) with some modification. Cells were fixed in ethanol and sonicated before extraction. Both medium and cells were extracted 3 times with hexane. Silanized tubes were used to prevent the retinoids from adhering to the glass. The samples were initially redissolved in 100 μL methanol, but the recovery was below 40% if the retinyl ester content was high. By redissolving the ester-containing samples in 100 μl methyl-tert-butyl-ether:methanol (1:1), the recovery increased to >80%. Fifty μL was injected to the HPLC column with a mobile phase of acetonitrile–water–methyl-tert-butyl-ether in a gradient starting with 96:4:0, changing to 20:2:78 (v/v/v) at a flow rate of 1 mL/min. The mobile phase was continuously degassed with helium.

The HPLC system consisted of a Waters 600E pump and control system from Waters Associates (Milford, MA) monitoring at 326 nm. A computerized integration system from Shimadzu was used (Shimadzu Class LC 10 system, Shimadzu, Japan). The column was a Supelcocil LC-8 column 25o mm × 4.6 mm (SU 5-8297) from Supelco (Bellafonte, PA, USA).

Radioactivity was determined in 2-min elution fractions from the HPLC column. The fractions were mixed with 10 mL Insta Gel II plus and counted in a Packard 1900 TR liquid scintillation analyzer (Packard Instruments, Meriden, CT).

Cells

Murine macrophage-like cell line J774A.1 (ATCC TIB 67) was obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained in RPMI with 10% fetal calf serum containing gentamycin sulfate (0.06 mg/mL) and 2 mm l-glutamine. The cell were incubated in 5% CO_2 at 37°C in humidified atmosphere.

Incubation procedure and cell homogenization

Approximately 4 days before the experiment, the cells were seeded out on 60-mm dishes and grown to confluence. Immediately before the experiment J 774 cells were washed 3 times with

ice-cold PBS, and incubated on ice with chylomicron remnant [^{3}H]retinyl ester for 1 h. Lipoprotein lipase (1 μg/mL) was added to the medium to enhance the binding of chylomicron remnants to the J774 cells. After pre-incubation, the medium was removed and the cells were washed extensively in PBS. Control cells (0-time) were removed at this time point and homogenized, see below. The remaining cells were incubated further with DMEM supplemented with 10% LPDS at 37°C in humidified atmosphere for 10 min, 60 min, or 180 min. After incubation, the cells were washed extensively in ice-cold PBS, harvested by a cell lifter, and centrifuged (1000 *g* for 5 min). The pellet was washed and resuspended in homogenization buffer (0.25% sucrose 3 mm imidazole, 0.5 mm EDTA), homogenized with a syringe until the cells were broken, but the nuclei still were intact as seen by light microscopy. Aliquots of the cell homogenate were taken for HPLC analysis, protein quantification, and determination of radioactivity.

The samples were protected from light and kept on ice or in a centrifuge at 4°C during the procedure. All the handling of the samples was performed in a refrigerated room. In some experiments the cells were preincubated with ^{125}-I-labeled transferrin, a specific marker of early endosomes, for 20 min at 37°C.

Subcellular centrifugation

Initial experiments were done to explore whether the hydrolysis of retinyl esters took place in cytosol or organelles. In these experiments subcellular centrifugation was performed as follows. Cells were scraped off the dishes and homogenized (as described). Nuclei and remaining intact cells were pelleted at 3,500 rpm for 10 min. A crude fraction of organelles was separated from the cytosol by centrifugation for 1 h at 17,000 rpm in a microfuge. Retinol and retinyl esters were determined in samples from the nuclei, the organelle fraction, and the cytosol.

Subcellular fractionation of the organelle fraction was done by a modification of the method of Tjelle et al. (48). Briefly, the postnuclear fraction was placed at the top of a 17% Percoll gradient with 2 mL 64% sucrose at the bottom and centrifuged for 1 h at 18,000 rpm. Two main bands were seen after the centrifugation, a low-density band containing early endosomes and a high-density band containing late endosomes and lysosomes (48). The gradients were fractionated by cutting the tube above the upper band, below the upper band, above the lower band, and below the lower band (**Fig. 1**). The fractions were transferred to silanized Schliff tubes using silanized Pasteur pipettes. An aliquot from each fraction was removed to measure total radioactivity. The remaining fraction was mixed with ethanol, frozen overnight, and extracted for HPLC analysis.

The pellet consisted of non-homogenized cells and nuclei and was resuspended in 1 mL homogenization buffer and transferred to a silanized Schliff tube; an aliquot was taken for determination of radioactivity.

To verify separation between early and late endosomes/lysosomes, the cells were incubated with ^{125}I-labeled ovalbumin, pulsed for 5 min at 4°C, and chased for 45 min at 37°C. In this setting ovalbumin will be transferred to late endosomes or lysosomes. Horseradish peroxidase (HRP) was used as a marker of early endosomes. The cells were incubated for 5 min at 37°C with HRP. Sixty % of the ovalbumin was recovered in the late endosomes/lysosomes, whereas 20% was recovered in the early endosomes fraction. Seventy % of the HPR was recovered in the top or in the early endosome fraction and less than 10% in the late endosome/lysosome fraction. In some experiments, late endosomes were isolated from lysosomes using colloidal gold coated with ovalbumin (oa-Au) to achieve density shift between late endosomes and lysosomes. In these experiments the cells were incubated for 2 h with oa-Au, then washed with PBS before new medium was added to the cells. The next day the cells were incubated with chylomicron remnant [^{3}H]retinyl ester as described above. After homogenization, lysosomes were isolated as described by Tjelle et al. (48). The remaining homogenate was treated as described above to separate early endosomes from late endosomes.

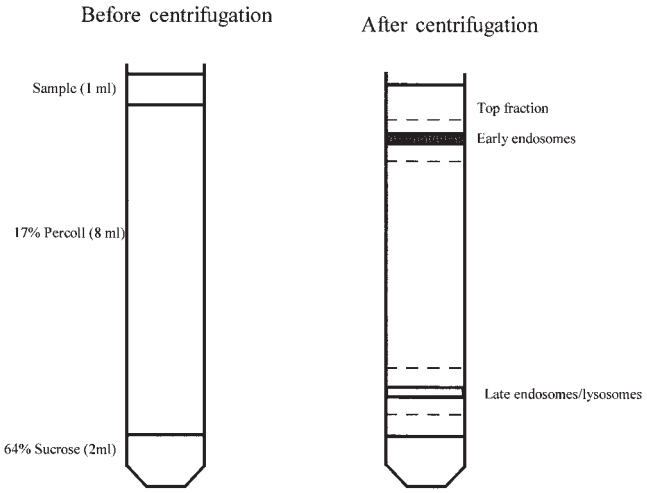

Fig. 1. The subcellular fractionation gradients before and after centrifugation, with sites of cutting indicated. J774 cells were incubated with chylomicron remnant [^{3}H]retinyl esters as described under Materials and Methods. The postnuclear supernatant was loaded on top of a 17% Percoll gradient with 64% sucrose at the bottom and centrifuged 1 h at 18,000 rpm. The dotted lines indicate the sites of tube cutting. Radioactivity in the fractions was determined together with HPLC analysis of retinol and retinyl esters as described in Materials and Methods.

RESULTS

Initially, experiments were set up to investigate whether the presence of newly endocytosed CMR would alter the density of the early endosomes, or whether the early endosomes loaded with CMR were destroyed during homogenization.

We therefore preincubated J774 cells with ^{125}I-labeled transferrin, a specific marker of early endosomes (48, 49). Endocytosed CMR did not alter density of early endosomes significantly (data not shown).

Next, experiments were done to investigate whether the hydrolysis of retinyl esters took place in cytosol and/or organelles. At time zero (after the 1-h pulse with CMR) 0.18 nmol retinoid/mg protein was present in cytosol. From 5 to 15 min of chase, the amount of retinoid decreased from 0.11 nmol/mg protein to 0.075 nmol/mg protein and a further decrease to 0.042 nmol/mg cells protein was observed after 60 min (**Fig. 2A**).

The percentage distribution between retinol and retinyl ester in cytosol changed from 10:90 to 20:80 during the first 15 min of chase, and after 60 min 40% was retinol and 60% retinyl ester (Fig. 2B). In the pellet, representing the organelles, respectively, 0.12, 0.083, and 0.084 nmol retinoid/mg protein was present after 0 min, 5 min, and 15 min of chase (Fig. 2A). After 60 min the amount of ret-

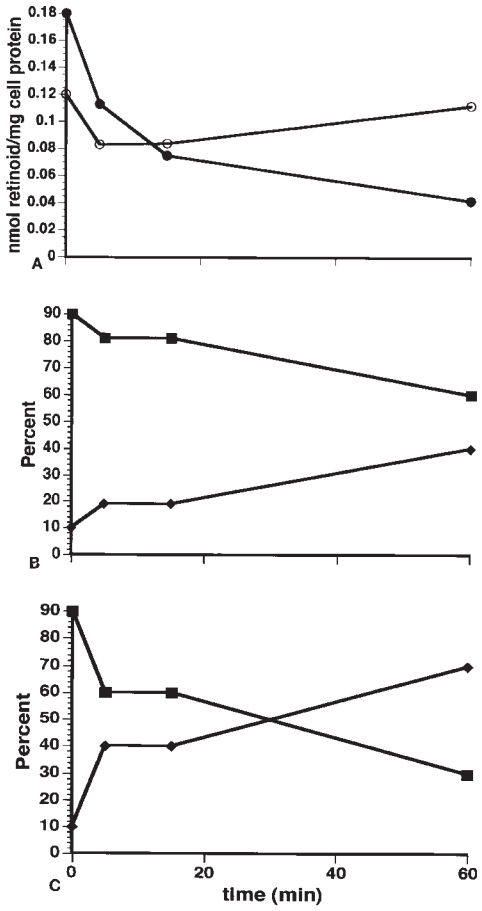

Fig. 2. A: Total amount of retinoid (calculated from measurement of an aliquot) in cytosol (●) and organelles (○) in J774 cells incubated with CMR [^{3}H]retinyl ester 1 h at 0°C (0-time) and further for the indicated time intervals in medium without chylomicron remnants at 37°C. Subcellular centrifugation was performed to separate cytosol from organelles as described under Materials and Methods. B and C: show the percentage distribution between retinol (◆) and retinyl ester (■) in the cytosol and organelles, respectively. Retinol and retinyl esters were quantified on the basis of their percentage distribution related to the calculated amount of total retinoids in the respective compartments. The figure shows results from one typical experiment. Two plates of cell were homogenized and added together at each time point.

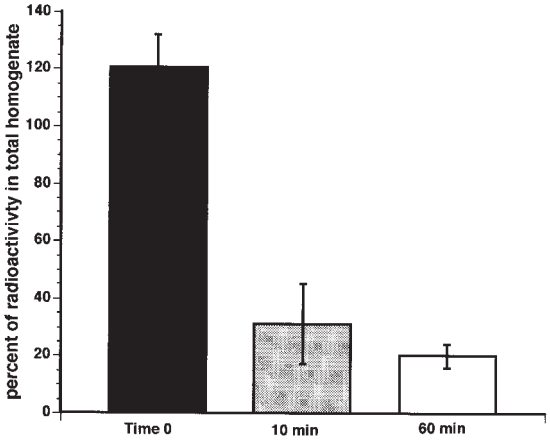

Fig. 3. Radioactivity released during preparation for homogenization; J774 cells were incubated with chylomicron remnant [^{3}H] retinyl ester for 1 h at 0°C. The cells were then washed and either scraped in PBS or incubated further for 10 min or 60 min in medium without chylomicron remnants. Before homogenization, the cells were scraped in PBS, pelleted, resuspended in homogenization buffer, and pelleted once more before homogenization. The figure shows the amount of radioactivity released from the expressed as percent of radioactivity remaining in the homogenate.

inoids increased to 0.112 nmol/mg protein. The percentage distribution between retinol and retinyl ester was 40:60, 40:60, and 70:30 after 5 min, 15 min, and 60 min, respectively (Fig. 2C).

These results suggested that the hydrolysis of retinyl esters took place mainly in the organelles, but there was a surprisingly high amount of retinyl esters in the cytosol fraction at time zero and after 5 and 15 min of chase. As pointed out above, the reason was probably not due to a density shift of CMR-loaded endosomes or to rupture of early endosomes during homogenization. Another possibility was that the cytosol fraction was contaminated with membrane-bound but not internalized CMR. CMR released from the cell surface during homogenization would appear in the cytosol fraction. This fits with the observed results that the amounts of retinyl esters in cytosol were highest at time zero and decreased rapidly during the first 15 min of chase.

Before homogenization, the cells were scraped into PBS and then pelleted by centrifugation; the cells were subsequently resuspended in homogenization buffer and pelleted once more by centrifugation. The amount of radioactivity in the supernatants prepared during the washing procedure was determined. **Figure 3** shows that at time zero the amount of radioactivity in the supernatants exceeded the amount of radioactivity in the total homogenate by a factor of 1.21 ± 0.11 (SD). After 10 and 60 min, the relative amount of radioactivity in the supernatants compared to the total homogenate was 0.31 ± 0.14 and 0.20 ± 0.04 (SD), respectively. These results show that noninternalized chylomicron remnants are easily lost during preparation of the cells for incubations shorter than 10 min, and that this is a plausible explanation for the high amount of retinyl esters in the cytosol at early time points.

To further characterize the hydrolysis of retinyl esters during endocytosis, several experiments were done to separate early endosomes from late endosomes and lysosomes. In chylomicron remnants, the concentration of retinyl esters and the amount of radioactivity differ between the batches. Therefore, the following figures show data from typical experiments. The relative changes in the distribution between retinol and retinyl esters were similar from experiment to experiment.

Total homogenate

The chylomicron remnants were bound to the cells during 1 h incubation at 0°C. Approximately 0.5–1.5% of the added dose of radioactivity was then associated with the cells.

Figure 4 shows the total amount of radioactivity associ-

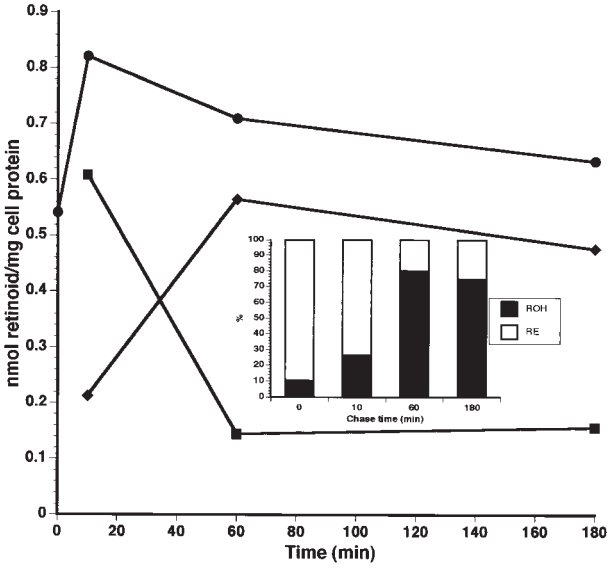

Fig. 4. Total amount of retinoids (●) and distribution between retinol (◆) and retinyl esters (■) in the total homogenate. The inserted panel shows the percentage distribution between retinol (ROH) and retinyl esters (RE) at the different time points. J 774 cells were incubated with chylomicron remnant-[^{3}H]retinyl esters for 1 h at 0°C, washed 6 times with PBS, and chased for the indicated time periods in medium without radioactivity at 37°C. Homogenization was performed as described under Materials and Methods. Radioactivity was measured in 40-μL aliquots from the total homogenate. The absolute amounts of retinoids were calculated on the basis of specific radioactivity in the chylomicron remnant [^{3}H]retinyl esters. Quantification of retinol and retinyl esters was done by HPLC as described. The figure shows the results from one typical experiment. Homogenates from two plates of cells were added together for each time point, error bars is therefore not given.

ated with the cell homogenate after 1 h of incubation on ice and at indicated time points (10, 60, and 180 min) during the chase. During the first 10 min of chase, there was an apparent increase in the amount of cell-associated retinoids. During the next 170 min a slight decrease in cell-associated retinoids was observed. In quantitative terms, 0.54 nmol retinoid was bound per mg cell protein at the start of the chase (time 0). After 10 min chase there was an increase to 0.82 nmol retinoid/mg cell protein and then an almost linear decrease to 0.634 nmol retinoid/mg cell protein the next 170 min.

Based on the assumption that the retinoids either are stored in the cells or processed and secreted out from the cells, one should expect that the radioactivity in the total homogenate either would be stable or decrease during the chase. The increased amount of radioactivity observed the first 10 min of chase was therefore somewhat unexpected. However, the reason was probably that more radioactivity was lost during preparation for homogenization at time zero than at later time points, as shown in Fig. 3.

The amounts of retinol and retinyl esters in total homogenate are shown in Fig. 4, and the relative amounts of retinol and retinyl esters at each time point are shown in the inserted panel. During the first 10 min of chase the ratio between amounts of retinol and retinyl esters did not change much, but total amounts of both retinol and retinyl esters increased. There was a rapid decrease in retinyl esters and a corresponding increase in retinol from 10 min to 60 min, reflecting an effective hydrolysis of retinyl esters. During the last 120 min of chase there was a decrease in retinol from 0.57 nmol/mg cell protein to 0.48 nmol/mg cell protein and a small increase in retinyl esters, from 0.14 to 0.16 nmol/mg cell protein. At time 0 the percentage distribution between retinol and retinyl esters was 10:90. During the chase the values were 26:74, 80:20, and 75:25 after 10, 60, and 180 min respectively. In summary, the results show effective hydrolysis of retinyl esters from 10 to 60 min of chase and a tendency towards reesterification during the last 120 min.

Chase medium

The changes in the concentration of [^{3}H]retinol and [^{3}H]retinyl esters in cells incubated with chylomicron remnant [^{3}H]retinyl esters were reflected in the incubation medium (presented in **Fig. 5**). Most of the [^{3}H]retinoids were released during the first 10 min of incubation. From 10 to 60 min an additional increase from 0.30 to 0.36 nmol/mg cell protein was observed during the chase. The substantial release of retinoids during the first 10 min of the chase was probably due to release of membrane-bound chylomicron remnants to the chase medium. This is supported by the fact that 75% of the radioactivity found in the medium after 10 min was retinyl esters. As shown in Fig. 5, there was a rapid decrease in the amount of retinyl esters from 10 to 60 min of chase (from 0.23 to 0.12 nmol retinoid/mg cell protein) and a slower decrease from 60 to 180 min (from 0.12 to 0.07 nmol reti-

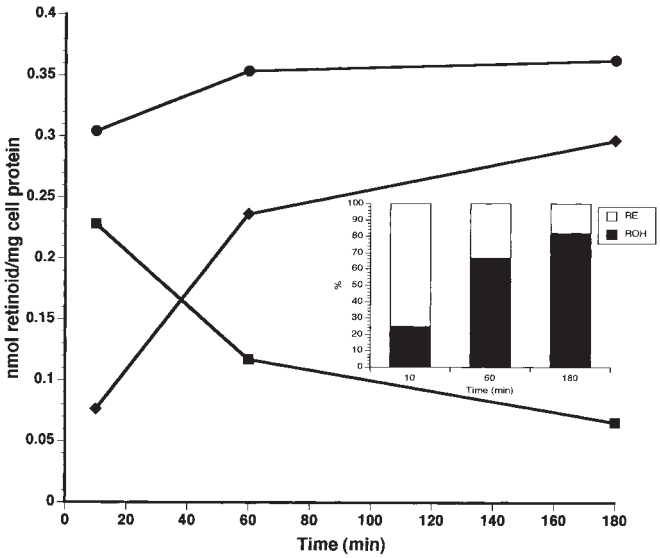

Fig. 5 Total amount of retinoids (●) and the percentage distribution of retinol (◆) and retinyl esters (■) in the chase medium after 10, 60, and 180 min of chase. The inserted panel shows the percentage distribution of retinol (ROH) and retinyl esters (RE), at the indicated time points. Cells were incubated as described in legend to Fig. 5. Radioactivity was measured in aliquots of the medium. Aliquots were mixed with ethanol and extracted for HPLC as described under Materials and Methods.

noid/mg cell protein). The retinol increased in a corresponding manner (0.08, 0.24, and 0.30 nmol retinoid/mg cell protein after 10, 60, and 180 min, respectively). The percentage distribution between retinol and retinyl esters was 25/75, 68/32, and 82/18 after 10, 60, and 180 min, respectively. The data clearly show an effective hydrolysis of retinyl esters. No certain conclusion can be made whether the hydrolysis of retinyl esters took place on the cell surface or whether the chylomicrons were internalized, the retinyl esters hydrolyzed inside the cell, and the retinol secreted out of the cell. To determine whether hydrolysis of retinyl esters took place inside the cell, subcellular fractionation was performed and retinol and retinyl ester were determined in the subcellular compartments.

Retinoids in subcellular compartments

Earlier reports (28, 50) indicate that retinyl esters in liver may be hydrolyzed in the early endosomes. Subcellular centrifugation was therefore performed in order to separate early endosomes from late endosomes and lysosomes. After centrifugation, 80–90% of the total cell-associated radioactivity was recovered in the top fractions (cytosol and membranes), the early endosomes, and the late endosomes/lysosomes. When the lysosomes were separated from late endosomes, less than 3% was recovered in the lysosomes after chase (results not shown). As demonstrated in **Fig. 6**, there was a small increase in the concentration of retinoids in the top fraction after 60 min of incubation, from 0.11 to 0.15 nmol/mg cell protein at 10 and 60 min, respectively, followed by a decrease to 0.09 nmol/mg protein at 180 min. In the early endosomes the corresponding values were 0.32, 0.18, and 0.17 nmol/mg cell protein. In the late endosomes/lysosomes less than 0.04 nmol retinoid/mg cell protein was found at all three time points.

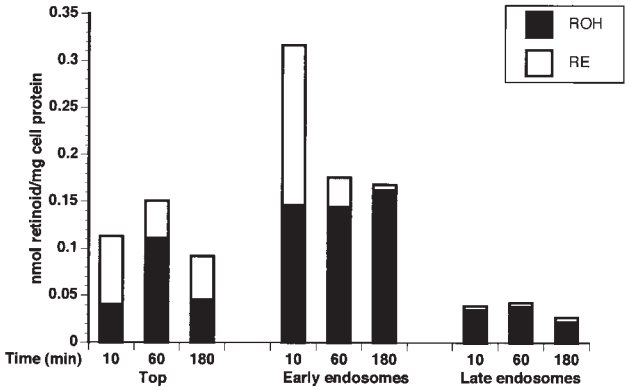

Fig. 6 Intracellular distribution of retinol (ROH) and retinyl esters (RE) in subcellular fractions. The amounts of retinoids in the top fraction (Top), the early endosomes and late endosomes at the different time points are shown. The height of the bars indicates the total amount of retinoids, the black part the retinol and the white part the retinyl esters. J774 cells were incubated as described in legend to Fig. 4. The cells were homogenized and fractionated as described in Materials and Methods. Total radioactivity was measured in aliquots from each fraction. Retinol and retinyl esters were quantified by lipid extraction and subsequent HPLC analysis as described under Materials and Methods. The figure shows results from one typical experiment.

In the early endosomes 56% of the radioactivity was retinyl ester after 10 min, reflecting newly endocytosed chylomicron remnants. After 10 min the percentage of retinol and retinyl esters in the top fraction was 41 and 59%, respectively. Probably this radioactivity mostly represents floating chylomicron remnants from the cell surface or from ruptured early endosomes. The main change in the subcellular distribution of retinoids from 10 to 60 min of chase was an effective hydrolysis of retinyl esters in the early endosomes and a corresponding decrease in the amount of retinol. The loss of retinol corresponded to the increase in radioactivity in the chase medium. There was also a small increase in retinol in the top fraction. From 60 to 180 min the amount of radioactivity was stable in the early endosomes, but the percentage of retinol increased from 82 to 96%. In the top fraction, there was a slight increase in total retinoids from 10 to 60 min (from 0.13 to 0.151 nmol/mg cell protein) and the relative amount of retinol increased from 36 to 74%. A possible interpretation may be that some of the retinol from the early endosomes was transported to the cytosol or to membranes floating to the top fraction. From 60 to 180 min the amount of retinoids in the top fraction was reduced from 0.151 to 0.092 nmol/mg cell protein. The percentage retinol decreased from 74 to 49%. The amount of retinyl esters increased from 0.39 to 0.46 nmol/mg cell protein. This probably means that some of the retinol was reesterified and accumulated as lipid droplets in the cytosol. In the late endosome fraction there were only small amounts of retinoid at all time points, mostly as retinol. These results clearly indicate that late endosomes/lysosomes are not an important site for hydrolysis of retinyl esters.

DISCUSSION

Vitamin A has important functions for normal differentiation of bone marrow-derived cells and retinyl esters transported in chylomicron remnants are a physiologically important source for vitamin A in these cells. J774 cells are macrophage-like cells and were chosen as a model to study retinyl ester hydrolysis because macrophages are active in uptake of CMR (44, 45).

Retinyl ester hydrolases have been studied extensively (10). At least two different forms of hydrolases have recently been described; bile salt-dependent and bile salt-independent retinyl ester hydrolases. The former type has recently been isolated and found to be identical to the pancreatic carboxyl ester lipase (17). Because of its bile salt dependency it is very unlikely to operate in J774 cells.

Non-bile salt-dependent retinyl ester hydrolase activity has been described in liver and other tissues: lung, lachrymal gland, and retinal pigment epithelial cells (8, 11, 13, 18, 19, 23, 24, 51–53). The bile salt-independent retinyl ester hydrolase activity is more specific towards retinyl esters and was shown to be located in microsomal preparations with only little activity in the soluble fraction. Harrison et al. (28) studied the metabolism of intravenously injected ^{3}H-labeled chylomicrons in rat liver and found that the

retinyl esters were mainly hydrolyzed in early endosomes and/or at the plasma membrane. The radiolabeled retinoid was not transported to the lysosomes, but rather to fractions enriched with endoplasmic reticulum. It was also demonstrated that retinyl ester hydrolase activity was enriched in the early endosome/plasma membrane fraction. Blomhoff et al. (1, 47) found that the retinoid in the liver did not follow the same endocytic route as the endocytosis marker asialofetuin. Subcellular fractionation indicated that the retinol was transported to the endoplasmic reticulum. The present finding in the J774 cells indicates that the hydrolysis and intracellular transport of endocytosed chylomicron remnant retinyl esters are similar to that found in the liver parenchymal cell.

It is well documented that chylomicron remnant retinyl esters are taken up in the bone marrow (54), but they do not seem to be stored there (36, 39, 40). Our data are consistent with secretion of newly formed retinol from the cell. However, most of the retinoids associated with the cells at the start of the chase were still inside the cell after 180 min. There was also a small tendency towards reesterification. This probably means that the J774 cells have the capacity to reesterify retinol. In J774 cells retinol esterification has been reported earlier (55) in cells exposed to medium containing retinol dispersed in dimetylsulfoxide and oleate. Oleate stimulated esterification of retinol without changing the intracellular pool of retinoids. As the chase was done for only 180 min, it is not possible to conclude from these experiments where the retinoids ultimately end up in steady state. In other experiments (T. Gundersen, E. Hagen, K. R. Norum, R. Blomhoff, unpublished results), there is a continuous but declining secretion of retinol to the medium. After 24 h about 50% of the remaining retinoid was retinyl ester in these experiments. Taken together, our data suggest that newly formed retinol is partly secreted out of the cell and partly reesterified and probably stored in lipid droplets in the cytosol. A small percentage of the retinol is metabolized to retinoic acid and other metabolites (T. Gundersen, E. Hagen, K. R. Norum, R. Blomhoff, unpublished results). Probably there is a simultaneous hydrolysis and reesterification of retinyl esters in these cells. As we have only measured the ester:alcohol ratio at given time points, our data do not necessary reflect net hydrolysis, but the result of the balance between hydrolysis and reesterification. In studies by Skrede et al. (36, 38), treatment with vitamin A did not increase the vitamin A content of rabbit bone marrow and white blood cells. In rabbits 20–40% of the total amount of retinoid in bone marrow was found to be esterified, independent of the vitamin A feeding. In view of these results it was not surprising that we observed some reesterification of retinol. This indicates that these cells at least have a short-term capacity for retinoid storage.

The effective redistribution of radioactivity from retinyl esters to retinol in the chase medium is probably due to retinol released from the cells and/or hydrolysis of retinyl ester on the cell surface. The loss of retinol from the total homogenate does indeed indicate secretion of retinol from the cell. However, the total increase in free retinol in the chase medium exceeded the net loss of retinol from the cells. This can either be explained by simultaneous uptake of chylomicron remnants in the cells and secretion of free retinol from the cell or by hydrolysis of retinyl esters in remnants on the cell surface. In a report by Blaner et al. (29) lipoprotein lipase hydrolyzes retinyl esters when incubated with BFC-β2 adipocytes, especially in the presence of apoC-II. Lipoprotein lipase was used in our experiments to increase the binding of chylomicron remnants to the cell surface. In our experiments the temperature was kept strictly at 0°C when LPL was present. The cells were washed 6 times with PBS before the temperature was raised to 37°C. It is therefore unlikely that the added LPL could be responsible for the effective loss of retinyl ester seen in chase medium. Monocyte-derived macrophages and macrophages of human atherosclerotic plaques have been reported to secrete or express LPL (56, 57). J774 cells do secrete small amounts of LPL themselves (58). Thus it cannot be excluded that some of the retinyl ester hydrolysis might have been mediated by LPL on the cell surface. There might also be a retinyl ester hydrolase linked to the plasma membrane itself (28). Further studies are needed to answer these questions.

The binding, uptake and degradation of chylomicron remnants are well documented in J774 cells (43–45). Receptor-mediated endocytosis is thought to be the mechanism for uptake of chylomicron remnant in theses cells. Although a number of receptors have been shown to mediate uptake of chylomicron remnants, no exclusive chylomicron remnant receptor has been identified.

The reliability of the results in the present study is dependent on the method used for subcellular centrifugation to separate early endosomes from late endosomes and lysosomes. The distribution of marker enzymes confirms that the separation was satisfactory. It should therefore be safe to conclude that the chylomicron remnants enter early endosomes after binding to the cell surface.

The main finding in the present study was that an effective hydrolysis of chylomicron remnant retinyl esters took place in the early endosomes of these cells. After being hydrolyzed in the early endosomes, the retinol does not follow the endocytic pathway to late endosomes and lysosomes. During the 180 min that the cells were observed, some of the retinol stayed associated with the early endosomes, some was released out of the cells, and a small amount was reesterified.

The present study confirms that retinyl esters in chylomicron remnants are effectively taken up and metabolized in the bone marrow-derived J774 cells. Net hydrolysis takes place in the early endosomes and maybe on the cell surface. Taken together with other observations, most of the retinol leaves the cell, but a fraction is probably oxidized to retinoic acid and a fraction is esterified for temporary storage.

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