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# Acyl-CoA: retinol acyltransferase (ARAT) and lecithin:retinol acyltransferase (LRAT) activation during the lipocyte phenotype induction in hepatic stellate cells

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**Abstract**

We have examined retinol esterification in the established GRX cell line, representative of hepatic stellate cells, and in primary cultures of ex vivo purified murine hepatic stellate cells. The metabolism of [$^3$H]retinol was compared in cells expressing the myofibroblast or the lipocyte phenotype, under the physiological retinol concentrations. Retinyl esters were the major metabolites, whose production was dependent upon both acyl-CoA:retinol acyltransferase (ARAT) and lecithin:retinol acyltransferase (LRAT). Lipocytes had a significantly higher esterification capacity than myofibroblasts. In order to distinguish the intrinsic enzyme activity from modulation of retinol uptake and CRBP-retinol content of the cytosol in the studied cells, we monitored enzyme kinetics in the purified microsomal fraction. We found that both LRAT and ARAT activities were induced during the conversion of myofibroblasts to lipocytes. LRAT induction was dependent upon retinoic acid, while that of ARAT was dependent upon the overall induction of the fat storing phenotype. The fatty acid composition of retinyl-esters suggested a preferential inclusion of exogenous fatty acids into retinyl esters. We conclude that both LRAT and ARAT participate in retinol esterification in hepatic stellate cells: LRAT's activity correlates with the vitamin A status, while ARAT depends upon the availability of fatty acyl-CoA and the overall lipid metabolism in hepatic stellate cells. 

*Keywords:* Vitamin A; Esterification; ARAT; LRAT; Hepatic stellate cells; Liver

## 1. Introduction

Vitamin A (all-trans retinol and its active metabolites) is important in vision, cellular growth and differentiation as well as in controls of reproduction and development. Since mammals do not synthesize retinoids, they depend exclusively upon the alimentary supply of retinol or its precursors [1]. In order to sustain the essential retinoid-dependent functions, mammals have to be able to absorb and metabolize high quantities of retinoids from dietary sources. They can subsequently store retinoids and release retinol into plasma, in which the homeostasis of circulating retinol is tightly regulated, being maintained close to 2 $\mu$M despite the large fluctuations in the daily vitamin A intake [2].

Liver plays the major role in vitamin A metabolism and storage. Hepatocytes uptake chylomicron-associated vitamin A from diet and transfer retinol to hepatic stellate cells (HSC, Ito cells, hepatic fat-storing cells), which store it as long-chain fatty acid retinyl esters in their cytoplasmic lipid droplets [3]. Under normal circumstances, retinyl esters are the most abundant molecular form of vitamin A in the body, 60–80% being stored in HSC [4]. Since HSC represent less than 1% of the total liver tissue volume, their capacity to metabolize very high and fluctuating quantities of retinol, to esterify and accumulate it, to hydrolyze it and subsequently release it in form of retinol into the plasma, are thus all

*Abbreviations:* ARAT, Acyl-CoA:retinol acyltransferase; BHT, Butylated hydroxytoluene; BSA, Bovine serum albumin; CRBP, Cellular retinol binding protein; DLPC, Dilaurylphosphatidylcholine; DMEM, Dulbecco's modified Eagle's medium; DMSO, Dimethyl sulfoxide; FBS, Fetal bovine serum; HPLC, High performance liquid chromatography; HSC, Hepatic stellate cells; LRAT, Lecithin:retinol acyltransferase; PBS, Phosphate-buffered saline solution; PMSF, Phenymethylsulphonyl fluoride.

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critical for plasma retinol homeostasis and for maintenance of hepatic retinoid stores [5].

HSC can deal with high concentrations of intracellular retinoids [6]. Cellular retinol-binding protein type I (CRBP-I) specifically binds intracellular retinol with great affinity. It captures retinol from cytosol and helps maintaining the low concentrations of potentially membrane-disruptive free retinol [7]. The CRBP-retinol complex is the preferential substrate for oxidizing enzymes involved in retinoic acid synthesis, it is relevant for retinol esterification, and it can protect retinoids from non-specific enzymes and non-enzymatic cell reactants [8,9]. Consequently, the retinol content in HSC depends both upon the retinoid-binding proteins and the functional presence of specific enzymes that participate in its metabolism [10,11].

Retinol esterification is a key reaction that regulates retinol intracellular availability. To date, two retinol-esterifying enzyme activities have been described-lecithin:retinol acyltransferase (LRAT) and acyl-CoA:retinol acyltransferase (ARAT). They are both associated with microsomes in different cell types including HSC. These two enzymes can be distinguished in vitro by substrate affinities and differential sensitivity to inhibitors. LRAT requires a fatty acyl group from the sn-1 position of lecithin as acyl donor, and PMSF or apo-CRBP inhibit its activity. ARAT depends upon acyl-CoA for retinyl ester synthesis. The former uses both free retinol and CRBP-bound retinol as substrate, while the latter catalyzes esterification of free retinol only [3,12,13]. In tissues that do not synthesize CRBP, such as mammary gland, ARAT is the only physiologically active enzyme that esterifies retinol [14]. In tissue where CRBP is present, it is generally considered that the major contribution to retinol ester synthesis is due to the LRAT activity, since the cell content of free retinol is low under normal physiological conditions, and ARAT has a $K_m$ value much higher than LRAT [9,15]. The ARAT physiological activity is easily studied in cells that lack CRBP, but this task is more difficult in cells that have it, since CRBP-bound retinol is denied to ARAT, and under low or standard retinol concentrations the cell CRBP content exceeds the retinol.

In view of the high fluctuation of retinol content in the normal diet, the retinol esterification activity present in HSC cells must be highly sensitive to systemic variations in order to maintain the retinol homeostasis [16]. Vitamin-A deficiency causes a decrease in retinyl ester levels in HSC [17,18]. In addition, situations that are associated with the activation of HSC and their conversion from the lipocyte phenotype (adequate for retinyl ester storage in fat droplets) into the myofibroblast phenotype (essentially involved in extracellular matrix production) cause the hydrolysis of retinyl esters [19,20]. These conditions include liver fibrosis, exposure to ethanol, xenobiotics and various drugs [21]. In vitro, the conversion of quiescent HSC to dividing myofibroblast-like cells is also correlated to the loss of large fat-droplets, decreases of retinyl ester contents and increases of extracellular matrix accumulation [20,22]. Conversely, vitamin-A repleted animals show a lipocyte hyperplasia, they have a high capacity to convert retinol into retinyl esters, and the high retinol status is associated with the induction of CRBP-I and LRAT [23,24].

In primary cultures of HSC isolated from normal rodents or from animals fed on a vitamin A rich diet, lipocytes convert rapidly into proliferating myofibroblasts, loosing their lipid droplets and the stored retinol [5]. Studies on HSC metabolism and retinol storage under the high retinol status require models in which the lipocyte phenotype can be induced and maintained for long periods, such as the GRX cell line model [25]. In a previous study on GRX cells, we have shown that HSC can respond efficiently to a very broad range of extracellular retinol status, and their esterification capacity was not saturated even in high retinol concentrations. We have concluded that GRX cells expressing the lipocyte phenotype had a very high capacity of retinol esterification [26].

Previous investigations from other laboratories have reported that the LRAT activity was modulated as a function of the retinol status, whereas the basal ARAT activity did not respond specifically to the retinol esterification demand in primary culture of HSC [27]. Although the $V_{max}$ of LRAT from tissues including liver as well as from isolated HSC is low, it may be sufficient to esterify retinol in the range of standard concentrations of vitamin A [15]. Under these conditions, ARAT has been considered to be of low significance in physiological retinol metabolism. However, in tissues where LRAT is not active (such as mammary gland), and in those where the low $V_{max}$ of LRAT can be limiting under a high retinol status (such as HSC), ARAT activity may be relevant. Under these conditions, an induction of ARAT is to be expected, and appropriate cell culture models should shed light on the relative relevance of the ARAT and LRAT activities. Since holo-CRBP denies retinol to ARAT, we have now established experimental conditions in which the absence of CRBP leaves free the esterifying activity of ARAT, which can use unbound retinol and endogenous or exogenous fatty acids as substrates.

The aim of the present study was to investigate the cellular retinoid distribution in HSC, its esterification and storage in the conditions representative of a moderate and high exogenous retinol availability, under which the retinol metabolism is essentially directed towards the synthesis of retinyl-esters. We have established experimental conditions that led us to characterize both the ARAT and LRAT activities under different extracellular retinol status, and analyzed the activation of these enzymes during the induction of HSC to convert their phenotype from the myofibroblast to the lipocyte one phenotype, such as occurs in vivo under the high retinol input.

## 2. Materials and methods

### *2.1. Cell cultures and GRX lipocyte induction*

The GRX cells, representative of murine HSC cells [28], were obtained from the Rio de Janeiro Cell Bank (PAB-

CAM, Federal University of Rio de Janeiro, RJ, Brazil). They were routinely cultivated in Dulbecco's medium (Sigma, St Louis, MO, USA) supplemented with 5% fetal bovine serum (Cultilab, Campinas, SP, Brazil), at 37°C, pH 7.4, under 5% $CO_2$ atmosphere. Under these conditions, GRX cells expressed the myofibroblast phenotype. In order to induce the lipocyte phenotype, confluent cell monolayers were incubated for 8 days in the same medium supplemented with 5 $\mu$M all-trans-retinol (Sigma), dissolved in ethanol (0.1% final concentration). The concentration of retinol in the stock solution was determined by ultra-violet absorption at 325 nm, using the molar extinction coefficient ($\epsilon$) of 52.770 $cm^{-1}M^{-1}$. Alternatively, parallel cultures were maintained in the standard medium supplemented with 5 $\mu$M all-trans-retinoic acid (Sigma) or 0.13 mM indomethacin, shown previously to be equally potent inductors of lipocyte phenotype in GRX cells [26,29]. The concentration of retinoic acid was assayed at 350 nm, $\epsilon$ = 45.000 $cm^{-1}M^{-1}$. Both retinoid stock solutions were stored under nitrogen, in a light-protected container at -20°C. Intracellular lipid droplets were identified with the lipophyllic stain Oil-Red-O (Sigma).

### 2.2. Isolation and purification of primary murine HSC

C3H/HeN mice were obtained from the colony bred at the Federal University of Rio de Janeiro. Mice were fed a commercial diet ad libitum. The diet was supplemented with pro-vitamin A-containing fresh vegetables in order to reach high but physiological vitamin A levels (10 mg $\beta$-carotene/Kg). HSC were isolated from livers of 8–12 months old mice, by a pronase-collagenase non-recirculating perfusion technique, previously described [30]. Briefly, mice were anesthetized with diethyl ether, and cannulated into de portal vein. The liver was perfused in situ at 37°C with calcium-free Gey's balanced salt solution (BSS), and subsequently with 0.055% pronase-E and 0.055% collagenase in BSS. The Glisson's capsule was disrupted, cells and partially digested tissues were harvested, resuspended in the same enzyme solution and incubated at 37°C for 30 min. The liver cell suspension was filtered over nylon gauze and cells were harvested by centrifugation at 450 *g* for 10 min. The HSC fraction was purified from the other nonparechymal cells on a two-layer discontinuous Nycodenz density gradient. Viability of isolated fractions was estimated with Trypan Blue, being always over 90%. The fat-storing hepatic cell suspension was monitored under a phase-contrast microscope and had the purity up to 80%. Isolated cells were frozen rapidly in liquid nitrogen and stored at -70°C prior to further analysis.

### 2.3. Retinoid characterization

GRX cells expressing the lipocyte or myofibroblast phenotype were incubated for 6 h (isotopic steady state) in the medium containing 2 $\mu$M [$^3$H]retinol (specific activity = 1 $\mu$Ci/nmol), and the radiolabeled cellular retinoids were analyzed by reverse-phase HPLC. Total retinoids were extracted from $2x10^6$ cells with hexane/ethanol (3:1, v/v) containing 0.1 mg/ml butylated hydroxytoluene (BHT). Three hexane-phases of repeated extractions were pooled, dried under nitrogen stream, dissolved in 100 $\mu$l methanol, and analyzed by chromatography. Retinyl acetate was added as internal standard in order to monitor the extraction yields. Analytical HPLC was carried out by a Shimadzu chromatograph and a Rheodyne injection valve with a 25 $\mu$l loop. A Spherisorb ODS II 5 $\mu$M reverse-phase column (250x4 mm) was used. Retinyl esters were analyzed by the method described by Got et al. [31]. Retinoids were identified and quantified by comparison with standards obtained from Sigma. For quantification of radiolabeled [$^3$H]retinoids, 0.5 ml fractions of HPLC mobile-phase were collected and the radioactivity was monitored by scintillation counting (35–45% counting efficiency).

### 2.4. Subcellular fractions

Myofibroblast and lipocyte GRX cells were harvested, washed twice with potassium phosphate buffer, and the subcellular fractions were prepared by differential centrifugation following the method described by Harrison et al. [4]. Briefly, cells were disrupted at 4°C with a Potter-Elvejhem homogenizer in ice-cold buffer (0.25 mol/L sucrose, 0.01 mol/L $K_2HPO_4$, pH 7.25). The resulting homogenate was centrifuged successively to yield the nuclear fraction (N fraction–1000 *g* for 10 min), the mitochondrial-lysosomal fraction (ML fraction-10.000 *g* for 20 min), the microsomal fraction (100.000 *g* for 1 h) and the high-speed supernatant (cytosol fraction). Pellets were washed twice under the original conditions. Aliquots of the original homogenate and all the fractions were immediately frozen at -70°C. Several markers were used to monitor the homogeneity of fractions: DNA, alkaline phosphatase, 5′-nucleotidase, glucose 6-phosphatase and lactate dehydrogenase. Protein concentrations were determined by the modified Lowry's method [32].

### 2.5. ARAT activity assays

The basal approach to measure the ARAT activity was the net retinyl ester synthesis, based on the method described by Ross [33]. All-trans-retinol dispersed in DMSO was added to duplicate reaction assay mixtures, containing 150 mM $K_2HPO_4$ buffer, 20 $\mu$M BSA and 100 $\mu$g microsomal protein, pH 7.4. Palmitoyl-CoA or lauroyl-CoA (Sigma) was added in some reactions in a final 60 $\mu$M concentration. The reaction mixture was incubated at 37°C for 10 min in a water bath. Every test mixture was accompanied by a control, in which the microsomal proteins had been heat-inactivated. At the end of incubation, 1 ml cold ethanol was added to stop the reaction, and the resulting mixture was extracted twice with hexane. The extracts were pooled, the solvent was removed under nitrogen stream and the major

retinyl esters (retinyl palmitate and retinyl oleate) were analyzed by HPLC, as described above. The amount of newly synthesized retinyl esters was determined from the difference between incubations with fresh thawed and heat-inactivated samples.

In some experiments, microsomal suspension was treated with 0.5 M hydroxilamine hydrochloride (Sigma) before incubation with retinol. The microsomes were incubated with hydroxilamine during 20 min at 37°C, washed and recentrifuged to remove the reagent excess.

### 2.6. LRAT activity assays

LRAT activity was monitored with 5 $\mu$M [$^3$H]retinol (60 dpm/pmol) using the phosphatidylcholine contained within the membranes as the endogenous fatty acyl donor. The reaction mixture contained [$^3$H]retinol, 100 $\mu$g microsomal protein and 20 $\mu$M bovine serum albumin (BSA) in 0.2 M $K_2HPO_4$ buffer, pH 7.2. After 5 min, the reaction was interrupted with 1 ml ice-cold ethanol containing 100 $\mu$g BHT. [$^3$H]retinyl esters were separated from [$^3$H]retinol substrate using a 10% deactivated alumina column (Sigma) [34]. Heat-inactivated microsomes were unable to synthesize radiolabeled retinyl esters. When indicated, 60 $\mu$M dilaurylphosphatidylcholine (DLPC) was added to reaction mixture. Inhibition of the reaction by 3 mM PMSF (Sigma) was monitored in order to distinguish the LRAT and ARAT activities.

## 3. Results

### 3.1. [$^3$H]retinoid composition of GRX cells expressing the lipocyte or the myofibroblast phenotype

The GRX cells had stellate or elongate fibroblastoid morphology. Under standard culture conditions, GRX myofibroblasts were highly proliferative, showed low level of contact inhibition like all the cells of the smooth muscle lineage, and after confluence grew into the typical "hills and valleys" pattern. In long term confluent cultures, rare GRX myofibroblasts converted spontaneously into lipocytes with intracellular fat droplets. In the standard culture medium the only source of retinoids was FBS, in which the vitamin A content was never above 100 nM. Under these conditions, the vitamin A content in the myofibroblasts was below the limit of detection by HPLC. When confluent GRX cultures were exposed for 8 days to 5 $\mu$M retinol, the cells decreased the growth rate, the polygonal cells became predominant and acquired gradually the lipocyte phenotype accumulating the cytoplasmic fat droplets. After 8 days of induction, the majority of cells showed numerous refringent fat droplets that never fused into a single fat drop such as observed in adipocytes. In the presence of other lipocyte-inducing conditions, 5 $\mu$M retinoic acid or 0.13 mM indomethacin, the GRX cells followed the pattern of modifications similar to that observed in retinol-induced cultures, as previously described [26,29].

In order to investigate the distribution and metabolism of [$^3$H]retinol in GRX cells under physiological retinol concentrations, we used cultures expressing the myofibroblast or the lipocyte phenotype (8 days culture under standard conditions or retinol-treatment, respectively), and incubated them with 2 nmol/ml retinol. Myofibroblasts and lipocytes were trace-labeled with 2 $\mu$Ci [$^3$H]retinol in serum-free medium for 6 hr, and retinoid metabolites were monitored in lipid extracts by reverse phase HPLC (Fig. 1A and 1B). Several radioactive peaks were characterized by co-elution with known standards and by their behavior under different extraction and purification conditions. Typical radio-chromatograms from HPLC eluates are shown in the Fig. 1(C and D). [$^3$H]Retinoids obtained from the cells included mainly retinol and several acyl species of long chain fatty acid esters. In lipocytes, the acyl composition of [$^3$H]retinyl esters included palmitate/oleate (forming a single peak), linoleate and stearate. In myofibroblasts, the observed esters were essentially retinyl palmitate/oleate. A smaller content of retinol-derived carboxylic acids was also identified in GRX cells and their metabolism will be described separately [V.A. Fortuna, in preparation].

Qualitatively, the overall [$^3$H]retinoid composition of lipocytes was similar to myofibroblasts, but we observed great quantitative differences (Fig. 2). Although lipocytes already had large endogenous stores of unlabeled retinyl esters acquired during the induction program, they showed high [$^3$H]retinol incorporation into the cellular retinoid pool. When the extracellular retinol concentration was close to the physiological one, the total cell [$^3$H]retinoid uptake in myofibroblasts (9660 dpm/million cells) was approximately one-third of that in lipocytes (28370 dpm/million cells).

As shown in the Fig. 2, [$^3$H]retinyl esters were the most abundant [$^3$H]retinoids in GRX cells, and their higher content was the major difference between the two cell phenotypes. [$^3$H]Retinyl esters content in lipocytes was five-fold larger than in myofibroblasts. The relative retinyl ester content in lipocytes reached 46% of total retinoids, while in myofibroblasts it was only 25%. Simultaneously, unesterified retinol was 11% of total retinoids in lipocytes and 14% in myofibroblasts. Lipocytes and myofibroblast could thus uptake the same relative quantity of retinol, despite the lower absolute retinoid content in the latter ones. This result indicated that retinyl ester synthesis and storage were limited in myofibroblasts but not in lipocytes, even in retinol concentrations close to the plasma levels used throughout the labeling period. The lower unesterified retinol content in myofibroblasts is in agreement with our previous results reporting the lower CRBP-I level [26].

### 3.2. Retinol esterification

The quantitative differences in the retinoid composition of the GRX phenotypes indicated differences in the retinyl

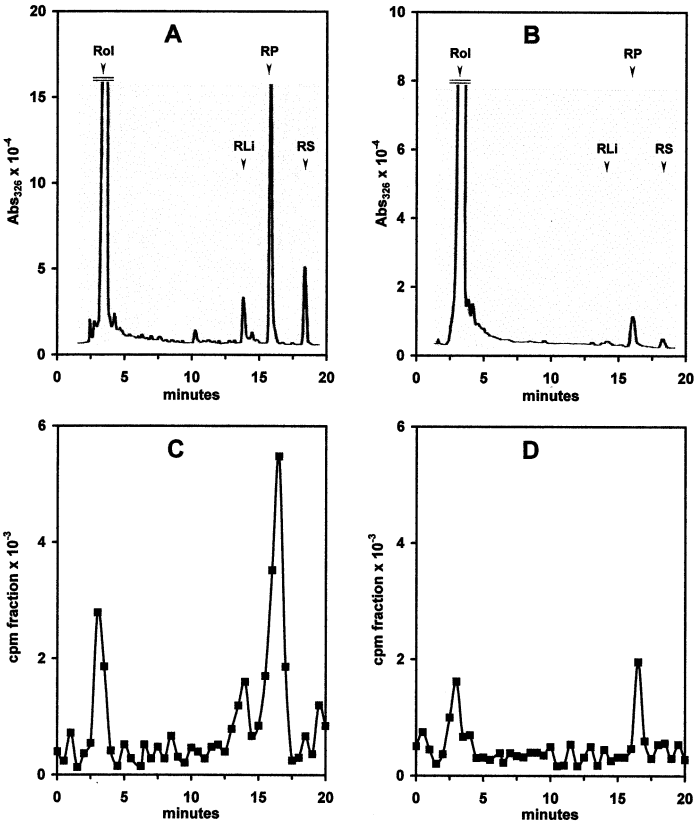


Fig. 1. Chromatographic analysis of retinyl esters extracted from GRX cells expressing the lipocyte (A, C) or the myofibroblast (B, D) phenotype. Total retinoids from GRX cultures incubated for 6 h with [$^3$H]retinol were purified and separated by HPLC (A, B). The radioactivity was monitored in fractions of the mobile-phase (C, D). Identical results were obtained in two different experiments, with duplicate HPLC analysis. The elution positions of known standards (Rol, retinol; RLi, retinyl linoleate; RP, retinyl palmitate plus retinyl oleate; RS, retinyl stearate) are indicated.

ester synthesis. In further experiments we monitored the ARAT or LRAT activities and investigated the properties of retinol esterification in GRX homogenates. In agreement with previous reports [4,35], we observed that GRX microsomes were the subcellular fraction containing the major part of the esterification capacity (data not shown), and this fraction was chosen for further studies. ARAT and LRAT activities in microsomal preparations from different tissues and cultured cells were reported to synthesize in vitro a considerable quantity of retinyl esters from unbound [$^3$H]retinol [4,12]. We therefore incubated GRX microsomes (300 $\mu$g) with 5 $\mu$M [$^3$H]retinol (1 $\mu$Ci/2 nmol) for 30 min at 37°C. The synthesized retinoids were extracted for HPLC analysis and the main retinyl ester peaks recovered for radioactivity counting. No retinyl-ester peaks were observed in the absence of protein or retinol. Under the used basal conditions, GRX lipocyte microsomes converted [$^3$H]retinol mostly to retinyl palmitate and oleate (40 pmol), and in lesser amounts to [$^3$H]retinyl stearate and linoleate (Fig. 3A). This pattern was similar to [$^3$H]retinyl ester composition observed in cultures of GRX lipocytes, trace-labeled for 6 hr in serum free-medium (Fig. 1A), and reflected the endogenous pool of fatty acids available for retinol esterification.

When lipocyte microsomes were supplemented with 60 $\mu$M dilauroylphosphatidylcholine (DLPC), an exogenous substrate for LRAT, [$^3$H]retinyl laurate (16.2 pmol) was produced besides the basal [$^3$H]retinyl esters (Fig. 3B-upper trace). Preincubating the microsomes with 3 mM PMSF, a potent LRAT inhibitor, decreased both the basal synthesis of [$^3$H]retinyl esters using the endogenous acyl donor (82%) and the [$^3$H]retinyl laurate synthesis from exogenous DLPC (100%) (Fig. 3B–lower trace). The ability to use DLPC as an acyl donor and free retinol as a substrate, as well the sensitivity of the esterification to PMSF, confirmed that LRAT activity was present in GRX lipocyte microsomes and mostly accounted for the basal esterification. Conversely, it is noteworthy that myofibroblast microsomes could not produce [$^3$H]retinyl laurate from DLPC (Fig. 3D–lower trace), despite of the low but constant production of endogenous [$^3$H]retinyl palmitate/oleate esters (6.1 pmol).

On the other side, ARAT activity was demonstrated when lipocyte microsomes were incubated with 5 $\mu$M [$^3$H]retinol supplemented with 60 $\mu$M lauroyl-CoA. We observed that both [$^3$H]retinyl laurate (75.3 pmol) as well as [$^3$H]retinyl palmitate/oleate (53 pmol) were produced (Fig. 3C–upper trace). Under the same conditions, myofibroblast

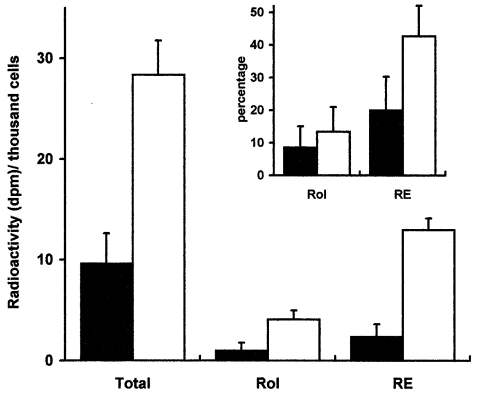


Fig. 2. [$^3$H]Retinoid composition of GRX lipocytes and myofibroblasts. Lipocyte (open bars) and myofibroblast (filled bars) cultures were incubated with 2 $\mu$M [$^3$H]retinol and cellular [$^3$H]retinoid content was analyzed by HPLC. Radioactivity was quantified in fractions that migrated as retinol (Rol) and retinyl esters (RE). The inset represents the relative contribution of retinol and retinyl esters to the total retinoid content. Results represent data from two different experiments done in triplicate. Mean values and standard deviations (SD) are represented.

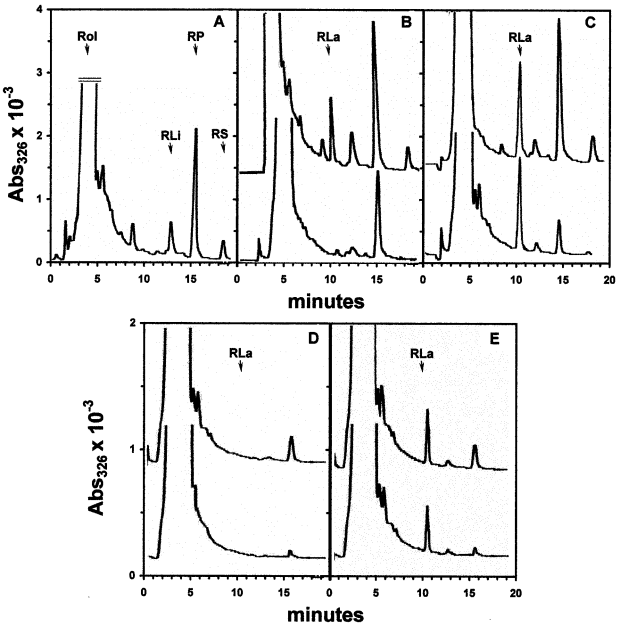


Fig. 3. Chromatographic analysis of LRAT and ARAT esterification activities in the microsomal fractions from GRX lipocytes or myofibroblasts. (A) Lipocyte microsomes incubated with retinol produced retinyl linoleate (RLi), retynil palmitate plus retynil oleate (RP) and retynil stearate (RS). Microsomes isolated from lipocytes (B-C) or myofibroblasts (D-E) were assayed for esterification activity in the absence (upper tracing) or presence (lower tracing) of 3 mM PMSF. Dilauroylphosphatidylcholine (DLPC) (in B-D) or lauroyl-CoA (in C-E) (60 $\mu$M) were added as fatty acid donors, in order to monitor the LRAT or ARAT activity, respectively, producing the retinyl laurate (RLa). Reactions were incubated for 20 min at 37°C, retinoids were extracted and analyzed by HPLC. Data are representative of two independent experiments.

microsomes also produced [$^3$H]retinyl laurate (21 pmol) and [$^3$H]retinyl palmitate/oleate (7.4 pmol) (Fig. 3D–upper trace). PMSF-treatment of microsomes greatly reduced the endogenous esterifying activity of both lipocytes (85%) and myofibroblasts (82%), but it had no effect on ARAT activity that used lauroyl-CoA as the acyl donor (Fig. 3C–lower trace).

Taken together, these results show that both ARAT and LRAT were present in GRX microsomes, but the LRAT was essential for the basal esterification in lipocyte microsomes, producing the physiological major retinyl esters (Fig. 3A).

### *3.3. Characteristics of retinyl ester synthesis in GRX microsomes*

In order to determine the optimal assay conditions of retinol esterification in vitro, we incubated GRX lipocyte microsomes with free retinol in the presence or not of various exogenous substrates. Initial incubations with 60 $\mu$M retinol and 100 $\mu$g protein for each fraction were run at 37°C, during increasing periods of time, in the presence of acyl CoA. The Fig. 4A shows that new retinyl ester production occurred in a time-dependent pattern and was constant for 10 min. The linearity of the reaction was observed with the microsomal protein increase up to 300 $\mu$g in reaction mixture (data not shown). No enzyme activity was observed when microsomes were heat-inactivated at 90°C for 10 min.

In order to study the effects of retinol and fatty acyl-CoA on rates of esterification, we incubated the microsomes with different substrate concentrations and analyzed the produced retinyl esters. Retinol esterification increased more than 6-fold above the basal level when microsomes were incubated with 60 $\mu$M palmitoyl-CoA, and more than 3-fold when incubated with 60 $\mu$M lauroyl-CoA (Fig. 4B). The increased yield of esters were accounted for by a specific increase of the ester species corresponding to the added fatty acyl CoA over the retinyl esters produced in the basal reaction. This strongly indicates that GRX microsomes contained the ARAT activity.

The acyl CoA-dependent activity increased with respect to the added substrate within the concentration range investigated, and the maximal rate of esterification was observed above 60 $\mu$M retinol (Fig. 4B). The kinetic parameters calculated from the X-axis intercepts for Lineweaver-Burk plots revealed the apparent $K_m$ = 14,7 $\mu$M and $V_{max}$ = 526 pmol/mg/min of retinyl palmitate-oleate formed (Fig. 4C).

A basal esterification was consistently observed when retinol and buffer alone were incubated with microsomes. As shown in the Fig. 4B, this basal reaction ranged from 40 to 70 pmol/mg/min for microsomes from lipocytes. In contrast to the saturating retinol concentration for acyl-CoA-supplemented reactions, the basal esterification reached a zero-order kinetic at lower retinol values. The basal reaction under study had a typical Michaelis-Menten kinetic, and the reaction parameters were the apparent $K_m$ = 4,1 $\mu$M and $V_{max}$ = 74,1 pmol/mg/min (Fig. 4B). Therefore, the micro-

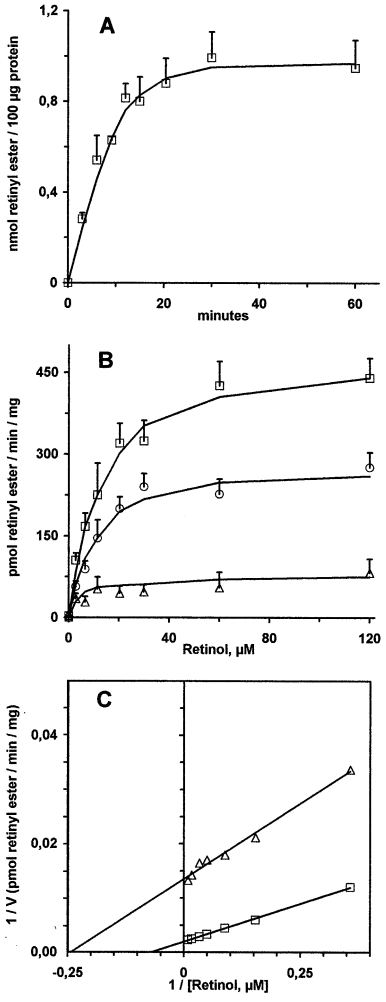


Fig. 4. (A) Time course of retinyl ester synthesis. Lipocyte microsomes (100 $\mu$g) were incubated with 60 $\mu$M retinol and 60 $\mu$M palmitoyl-CoA. The produced retinyl esters were extracted and quantified. Data represent mean values of a typical experiment done in triplicate, and standard deviations. (B) Rate of retinyl ester synthesis as a function of retinol concentration. Lipocyte microsomes were incubated with increasing retinol concentrations in the absence (open triangles) or presence of either 60 $\mu$M palmitoyl-CoA (open squares) or lauroyl-CoA (open circles). The produced retinyl esters were extracted and quantified. Mean values of two different experiments done in triplicate and standard deviations are shown. (C) Linewaver-Burke plots for the esterification in basal conditions (open triangles) or in reactions supplemented with 60 $\mu$M palmitoyl-CoA (open squares). The line equation obtained by linear regression analysis for the basal condition data was $y = 0{,}0556x + 0{,}0135$, $R^2 = 0{,}98$, and for the supplemented incubation data was $y = 0{,}028x + 0{,}0019$, $R^2 = 0{,}99$. From these equations, apparent $K_m$ and $V_{max}$ values were calculated to be 4,1 $\mu$M and 74,1 pmol/min/mg, respectively, for the basal conditions, and 14,7 $\mu$M and 526 pmol/min/mg, respectively, for the incubation supplemented with palmitoyl-CoA.

somal acyl-CoA supplemented esterification seemed to be different from the basal esterification, both in terms of the capacity and the affinity.

In order to find whether the endogenous microsomal acyl-CoA fatty acids sustained the basal reaction, we incubated microsomes with hydroxilamine, which reacts with acyl-CoA thiolesters to form hydroxamates. The washing of

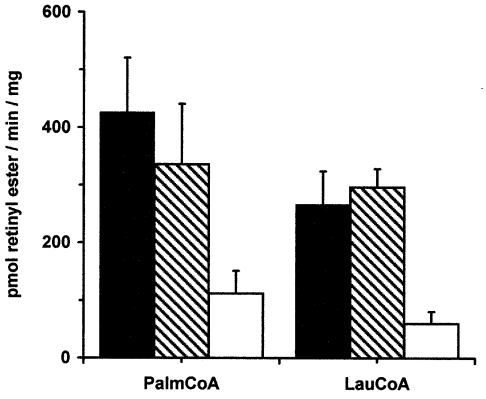


Fig. 5. ARAT activities in GRX microsomes. Retinol-esterifying activities were assayed in microsomes from retinol-induced (filled bars) or indomethacin-induced lipocytes (hatched bars) and myofibroblasts (open bars). Microsomes were incubated with 60 $\mu$M retinol in presence of 60 $\mu$M palmitoyl-CoA or 60 $\mu$M lauroyl-CoA. Results (mean values and standard errors) are representative of four experiments.

microsomes with hydroxilamine did not change the basal esterification (72.3 $\pm$ 17 pmol retinyl ester/mg/min), neither the increased effect on esterification by acyl-CoA fatty acid supplements (434 $\pm$ 28 pmol of retinyl ester/mg/min). Retinol esterification incubations in the presence of 0.5 M hydroxilamine plus fatty acyl-CoA were carried out to check the reagent efficiency, and no retinyl ester synthesis occurred. These results strongly suggested that endogenous acyl CoA-fatty acids bound to or trapped within lipocyte microsomal vesicles did not support basal retinol esterification and provide evidence that the retinol esterification observed in the absence of acyl-CoA was due to LRAT activities and another source of fatty acids.

### *3.4. ARAT activity in the two GRX phenotypes*

Once the optimal assay conditions were established, we investigated the ARAT activity in GRX microsomes. Experiments were conducted with microsomes from myofibroblast or lipocyte phenotypes (both retinol- and indomethacin-induced lipocytes). GRX cells treated with indomethacin expressed the lipocyte phenotype but, different from the retinol-induced lipocytes, they had no endogenous retinoids. Therefore, the microsomes from indomethacin-induced lipocytes were representative of cells in which lipid synthesis and accumulation were enhanced, but the genes dependent upon retinoids were not induced. All the assays were done under conditions that conferred the linear enzyme reaction rates. We observed that ARAT activity was present in myofibroblast microsomes at low levels (111.8 $\pm$ 39.5 pmol/mg/min) when compared to the very high specific activity of ARAT in both retinol-induced (426 $\pm$ 145 pmol/mg/min) or indomethacin-induced lipocyte microsomes (335,6 $\pm$ 105.0 pmol/mg/min) (Fig. 5). Although ARAT activities were higher with palmitoyl CoA than lauroyl CoA, they showed similar patterns of activity in all the GRX phenotypes.

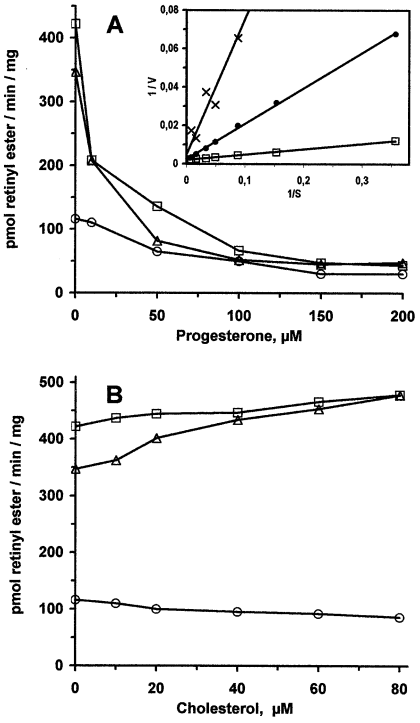


Fig. 6. Progesterone and cholesterol effect on ARAT activity. Microsomes from myofibroblasts (open circles), retinol-induced (open squares) and indomethacin-induced lipocytes (open triangles) were pre-incubated with progesterone (A) or cholesterol (B) for 5 min at 37°C. Esterification activities were assayed after a subsequent incubation with 60 $\mu$M retinol plus 60 $\mu$M palmitoyl-CoA. The inset shows a Linewaver-Burke analysis for ARAT activity from lipocyte retinol-induced microsomes in absence (open square), presence of 10$\mu$M (closed circles) or 100 $\mu$M (crosses) progesterone. The line equation obtained by linear regression analysis for the lipocyte retinol-induced microsomes in absence of progesterone was $y = 0{,}028x + 0{,}0019$, $R^2 = 0{,}99$. In the presence of 10 $\mu$M progesterone the equation was $y = 0{,}185x + 0{,}0024$, $R2 = 0{,}99$ and in the presence of 100 $\mu$M the line equation was $y = 0{,}707x + 0{,}0046$, $R2 = 0{,}99$. From the equations, apparent $K_m$ and $V_{max}$ values were calculated to be 14,7 $\mu$M, and 76,7 pmol/min/mg, respectively, for the non-supplemented condition, 76,7 $\mu$M and 416 pmol/min/mg, respectively, for the incubation supplemented with 10$\mu$M progesterone, and 153,7 $\mu$M and 217,4 pmol/min/mg, respectively, for the incubation supplemented with 100$\mu$M progesterone. Results of a typical experiment are shown.

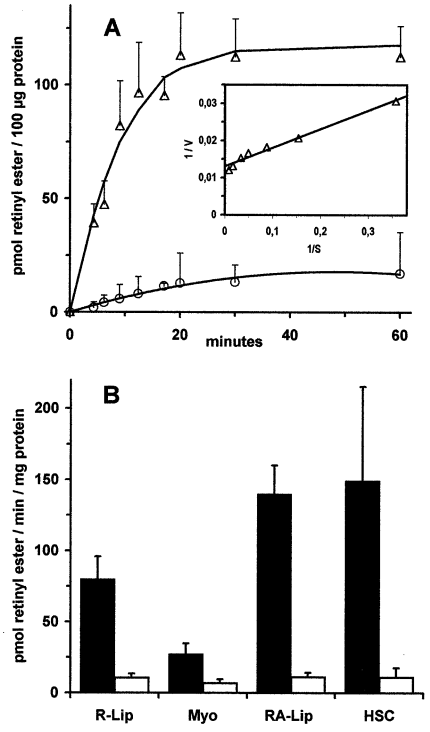


Fig. 7. LRAT activities in microsomes isolated from cultured GRX cells and from primary Hepatic Stellate Cells (HSC). (A) Microsomes from retinol-induced GRX lipocytes were incubated for 5 min with 3 mM PMSF (open circles) or vehicle (ethanol) (open triangles). Retinyl ester synthesis was monitored after a subsequent incubation with 5$\mu$M [$^3$H]retinol for the indicated periods. The inset shows Linewaver Burke plot for LRAT activity. The line equation obtained by linear regression analysis for the basal condition data was $y = 0{,}0566x + 0{,}0131$, $R^2 = 0{,}98$. From these equations, apparent $K_m$ and $V_{max}$ values were calculated to be 4,1 $\mu$M and 74,1 pmol/min/mg, respectively. Results (mean values +/− SD) are representative of three experiments done in duplicates. (B) Microsomes from primary hepatic stellate cells (HSC), GRX myofibroblasts (Myo), GRX retinol-induced (R-Lip) or retinoic acid-induced (RA-Lip) lipocytes were pretreated with 3 mM PMSF (open bar) or vehicle (filled bar), followed by incubation with 5 $\mu$M retinol as above. Results (mean values and standard errors) represent four independent experiments.

Enzyme activities of different microsomes were also measured in the presence of progesterone, one of the polar steroids that inhibit both microsomal ARAT and acyl-CoA: cholesterol acyltransferase (ACAT), or cholesterol that inhibits retinol esterification due to the ACAT activity [33, 36]. Progesterone inhibited the esterification of retinol in both lipocyte and myofibroblast microsomes in a dose-dependent manner, eliminating 90% of specific activity at the highest concentrations (Fig. 6A). In contrast, the highest cholesterol concentration tested elicited only non-significant modifications of the retinol esterification rate of lipocyte and myofibroblast microsomes (Fig. 6B). These results showed that lipocyte microsomes had a high ARAT specific activity, and that a considerable increase of the enzyme activity occurred during the induction of the lipocyte phenotype program in GRX cells, associated with their increased capacity to accumulate and synthesize lipids.

### 3.5. LRAT activity in the two GRX phenotypes

During the experiments in which retinyl esters were produced in vitro from all the studied microsomes, a basal esterification independent of acyl-CoA occurred at low levels. In order to monitor the LRAT activity, an increase in the sensitivity of detection of newly synthesized retinyl esters was necessary. Lipocyte microsomes were incubated with 5 $\mu$M [$^3$H]retinol without addition of acyl-CoA. We detected a membrane-associated [$^3$H]retinyl ester production using an endogenous acyl donor, which was 90% inhibited by 3 mM PMSF, confirming the presence of LRAT (Fig. 7A). This activity reached a plateau after 20 min of incubation at 37°C. The initial LRAT specific activity was higher in

retinol-induced lipocytes (79.8 ± 16 pmol/min/mg) than in myofibroblasts (27.3 ± 7.4 pmol/min/mg). These results showed that LRAT activity in GRX microsomes correlated with the retinoid status in cell cultures; they are in agreement with the induction of LRAT activity by vitamin A in vivo, where its activity is pronounced in microsomes from vitamin A sufficient rats and barely detectable in vitamin A deficient animals [15,37].

### *3.6. Retinoic acid-mediated induction of LRAT in the two GRX phenotypes and in ex vivo obtained murine hepatic stellate cells (HSC)*

LRAT activity has been described in several rat and human tissue homogenates assayed in vitro [1,9,35]. In order to validate our observation on cultured GRX cells, HSC were isolated from middle-to-old age vitamin A-sufficient mice, and their microsomes were prepared ex vivo and assayed for LRAT activity. In the assay conditions described above, murine primary HSC showed a high LRAT activity, sensitive to PMSF (Fig. 7B). The LRAT activity was in fact higher in primary HSC microsomes than in those obtained from GRX cell induced in vitro to express the lipocyte phenotype. Admitting that the HSC LRAT activity in vivo corresponds in behavior and properties to GRX LRAT, it is likely that retinol esterification in vivo was increased and positively regulated in the vitamin A-sufficient mice.

The retinoid-dependent induction of gene expression programs associated with cell differentiation is mediated by retinoic acid. In turn, retinoic acid is recognized to be a potent inducer of retinoid metabolism, including LRAT activity in rat liver [23,37]. Since the studied middle-to-old age mice were fed with a sufficient vitamin A diet, conditions that are correlated to an increased LRAT activity [15], we questioned whether the differences between GRX and primary HSC microsomes were related to a better retinoic acid production in vivo than in vitro. In order to investigate retinoic acid effects on GRX LRAT activity, we induced GRX lipocyte by incubation with 5 $\mu$M retinoic acid. In these cells, the full induction of the lipocyte phenotype occurs, but the cells have only a very low cellular retinol content and do not store retinyl esters. These conditions permitted a better assay of LRAT activities, since there is no endogenous retinyl esters present during the in vitro reactions. Noteworthy, the LRAT activity in microsomes of lipocytes induced with retinoic acid were higher than those observed in retinol-induced lipocytes, and very similar to microsomes of primary murine HSC cultures (Fig. 7B). These results are in agreement with retinol metabolism observed in rat HSC, and with the retinoic acid effect on liver LRAT [35,38]. Therefore, the LRAT activity in retinol-treated GRX cells was representative of a retinoic acid induction, reflecting the sufficient vitamin A status, and myofibroblast activity corresponded to a low vitamin A status, or induction of fibrogenic activity and myofibroblast proliferation associated with hepatic inflammatory reactions [17,18].

Taken together, these results suggest that ARAT and LRAT activities have distinct patterns of induction during the execution of the lipocyte differentiation program, and contribute to the differences in the retinyl ester content of lipocyte and myofibroblast GRX phenotypes.

## 4. Discussion

We have analyzed the retinol metabolism and esterification in HSC, using the model of the established GRX cell line. This model is representative of a small hepatic population of perisinusoidal stellate cells that can be induced to express the lipocyte phenotype, when they store high quantities of intracellular retinyl esters and supply retinol to the circulating plasma [1,9]. We have shown that the lipocyte induction is accompanied by a net increase in the production of retinyl esters. Accordingly, the two major retinyl esterification activities ARAT and LRAT were present and both were modulated by the lipocyte conversion. Since, to our knowledge, only the LRAT induction has been described, we have searched to distinguish the catalytic activities of the two enzymes. In order to separate the additional controls involving the cellular uptake of retinol and the differences in the CRBP-retinol content in the cytosol, we have done the major part of this study using the purified microsomal subcellular fraction of the studied cells, which was shown to harbor the major retinol esterification activity in ours as well in other cellular models [4,12,39].

Several reports indicated that LRAT was the major enzyme involved in retinol esterification in liver, including in the HSC [1,9,35]. This membrane-bound activity catalyzes retinyl ester synthesis from the CRBP-retinol complex or from free-retinol, with the apparent $K_m$ equal or lower than the cytosolic concentrations of substrates [13,39,40]. Having a high affinity for CRBP-retinol complex, the changes in substrate availability were predicted to affect barely its rate of ester formation. Although the $V_{max}$ of LRAT activity from liver or HSC is low, it was assumed to be sufficient to esterify retinol in the range of concentrations of dietary vitamin A [1,35]. Accordingly, we have found that the basal esterifying activity in microsomes was due to LRAT, in both phenotypes, using the endogenous membrane lecithin as a fatty acid donor. As expected, it was much lower in myofibroblasts, which could not use the supplemented DLPC as a fatty acid donor, in contrast to lipocytes that could use it. Yost et al. [39] have reported a similar low level retinol esterification with exogenous DLPC in liver microsomes, despite the fact that LRAT and ARAT were present. The reason for this limitation is at present unknown, but suggest a high efficiency of LRAT-mediated esterification in microsomes from retinol-induced lipocytes. Retinol and retinoic acid treatment in vitro raised the LRAT activity to high levels, close to those observed in microsomes of ex vivo purified HSC from vitamin replete mice, confirming that LRAT was sensi-

tive to the vitamin A status, and was induced via the retinoic acid-dependent signaling pathway.

Addition of fatty acyl-CoA disclosed the latent ARAT activity in microsomes of all the studied cells. Considering that no significant endogenous acyl-CoA was trapped inside microsome vesicles, the rate and extent of retinol esterification by ARAT activity was limited in basal conditions. ARAT was considered not to correlate with the extracellular retinol status [9,33,40]. In liver, the ARAT has a relatively high $V_{max}$, but the $K_m$ is also high in comparison to the typical concentration of cytoplasmic unesterified retinol, suggesting that it should have only a minor role in retinyl ester synthesis under physiological conditions [1,4]. However, in CRBP-null mice the ARAT catalyzed efficiently retinyl ester synthesis in HSC from free retinol [41]. In addition, ARAT may also participate in retinyl ester production in circumstances in which free retinol concentration is higher than that of CRBP, such as hypervitaminosis A [40].

In our model, the ARAT was clearly induced in the lipocyte phenotype. In contrast to LRAT, the ARAT induction was not dependent of retinol, and was equally induced when the lipocyte conversion of myofibroblasts was elicited via retinoic acid pathway or via the indomethacin pathway [29]. The mechanism of the indomethacin effect on lipid synthesis in HSC is unknown, but it is also observed in adipocytes [42]. In both cases, the lipid synthesis is enhanced using endogenous as well as exogenous activated fatty acids [43]. The availability of fatty acyl-CoA is apparently a relevant control point in activation of retinol esterification via ARAT. Chromatographic analysis of GRX retinoids showed that retinyl palmitate and oleate were the predominant retinyl esters, followed by smaller quantities of linoleate and stearate. This composition was also found in ex vivo cultured rodent HSC described by other authors [3,27,41]. We observed that the fatty acid moiety of retinyl esters correlated with the neutral lipid composition of GRX cells and of the serum, rather than with lecithin, since palmitic and oleic acids were the major components of all the major cellular neutral lipid compartments and the serum [43,44]. Yumoto et al. [45] reported similar data for hepatic stellate cell lipids. In our culture conditions, stearic acid was relatively high in serum, in free fatty acid pool and in lecithin of myofibroblasts, and it was lower in lipocytes. Conversely, linoleic acid was present in serum and in free fatty acid pool of myofibroblasts, but not in free acids of lipocytes or in lecithin of either of the two phenotypes. Taken together, these results indicate that extracellular fatty acids are preferentially incorporated and used for retinol esterification in HSC cells, despite the presence of CRBP and potential esterifying activity of LRAT using lecithin as the fatty acid donor. This is in agreement with other studies indicating that retinyl esters reflect the composition of exogenous fatty acids, rather than that of lecithin [27,46]. These data strongly support the hypothesis that ARAT has a relevant role in retinol esterification in animals as well as in vitro cultures of mammalian cells.

Beyond the source of the acyl-donor that distinguished ARAT or LRAT activities, its is quite clear that their kinetic are also different. In lipocyte microsomes, the LRAT and ARAT reactions exhibited different apparent $K_m$ values (4,1 $\mu$M and 14,7 $\mu$M, respectively) for solvent-dispersed retinol. This result agrees with the two activities with different affinities for retinol described for in vivo models [1,9,14, 35]. Within the limits of this difference, LRAT had a higher affinity for retinol than ARAT, as described for liver and HSC microsomes [9,33,35]. We identified $V_{max}$ values almost one order of magnitude higher for ARAT than LRAT. When lipocyte microsomes were incubated with retinol concentrations normally found in cytosol (5 $\mu$M), neither LRAT nor ARAT activities were at their highest esterification rates (40 and 135 pmol retinyl ester/min/mg, respectively, calculated from line equation velocities). This is consistent with the idea that in lipocytes microsomes the retinyl ester synthesis is provided with high efficiency, covering a large range of retinol concentrations.

Considering the cytosolic retinol concentrations described in literature (1–5 $\mu$M), the presence of cytosolic CRBP-bound retinol as a dominant substrate, and the apparent $K_m$ values determined for retinol esterification in GRX microsomes, we consider that ARAT does not predominate in lipocyte cultures under low retinol concentrations. Due to the low affinity of ARAT for retinol as compared to LRAT, ARAT might contribute to the total retinol esterification when both retinol and fatty-acyl CoA contents are increased, such as may occur after the acute ingestion of retinol-rich food, preventing accumulation of unesterified retinol that can cause an acute retinol intoxication. On the other side, under chronic low retinol ingestion, most of HSC express the myofibroblast phenotype, in which we have reported low but constant CRBP expression in myofibroblasts, concomitant with undetectable cellular retinol content [26]. In this case, all the CRBP is present as apo-CRBP. LRAT is inhibited by the presence of high apo-CRBP concentrations, leaving potentially to ARAT the temporary predominant role in retinol esterification.

In conclusion, the results of this study show that both ARAT and LRAT activities participate in retinyl ester storage in GRX cells, and that both respond to the retinol status: LRAT through a direct induction through the retinoic acid pathway, and ARAT through the overall induction of the lipocyte phenotype. Additional controls are expected to have relevant roles, such as CRBP status or the bio-availability of fatty acid donors and retinol in the microsomal environment. These issues are objects of ongoing studies.

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