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Hepatic Stellate Cells Uptake of Retinol Associated With Retinol-Binding Protein or With Bovine Serum Albumin

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Abstract Retinol is stored in liver, and the dynamic balance between its accumulation and mobilization is regulated by hepatic stellate cells (HSC). Representing less than 1% total liver protein, HSC can reach a very high intracellular retinoid (vitamin-A and its metabolites) concentration, which elicits their conversion from the myofibroblast to the fat-storing lipocyte phenotype. Circulating retinol is associated with plasma retinol-binding protein (RBP) or bovine serum albumin (BSA). Here we have used the in vitro model of GRX cells to compare incorporation and metabolism of BSA versus RBP associated [^{3}H]retinol in HSC. We have found that lipocytes, but not myofibroblasts, expressed a high-affinity membrane receptor for RBP–retinol complex (KD = 4.93 nM), and both cell types expressed a low-affinity one (KD = 234 nM). The RBP–retinol complex, but not the BSA-delivered retinol, could be dislodged from membranes by treatments that specifically disturb protein–protein interactions (high RBP concentrations). Under both conditions, treatments that disturb the membrane lipid layer (detergent, cyclodextrin) released the membrane-bound retinol. RBP-delivered retinol was found in cytosol, microsomal fraction and, as retinyl esters, in lipid droplets, while albumin-delivered retinol was mainly associated with membranes. Disturbing the clathrin-mediated endocytosis did not interfere with retinol uptake. Retinol derived from the holo-RBP complex was differentially incorporated in lipocytes and preferentially reached esterification sites close to lipid droplets through a specific intracellular traffic route. This direct influx pathway facilitates the retinol uptake into HSC against the concentration gradients, and possibly protects cell membranes from undesirable and potentially noxious high retinol concentrations. J. Cell. Biochem. 90: 792–805, 2003.

Key words: vitamin-A; liver; retinyl ester; fat-storing cell; lipocyte; RBP; membrane transport; receptor; affinity

Retinoids (vitamin-A and its metabolites) are required for and control multiple physiological activities such as vision, reproduction, morphogenesis, cell proliferation, and differentiation. Vertebrates do not synthesize retinoids, and they depend entirely upon dietary supplies of either retinoids or their metabolic precursors such as carotenes. Consequently, under normal nutritional status, vertebrates store large quantities of the absorbed vitamin-A, in order to grant the availability of retinol during periods of low dietary intake [Ong et al., 1994].

Liver is the major organ of retinol storage and metabolism, containing 60–80% of retinoids in the body, mostly in the form of retinyl esters. Liver parenchymal cells (hepatocytes) initially take up the newly absorbed vitamin-A from the diet, which is transported by blood circulation in chylomicron remnants. They transfer subsequently retinol to hepatic stellate cells (HSC, Ito-cells, or lipocytes), which store vitamin-A in large cytoplasmic lipid droplets. HSCs are thus specific retinoid-storing liver cells, and they

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take a leading part in the retinoid metabolism and homeostasis. The dynamic balance between the accumulation and mobilization of systemic liver retinol deposits is primarily regulated in HSCs, and they can occasionally reach a very high intracellular retinoid concentration since they represent less than 1% of the total liver protein [Hendriks et al., 1985; Blaner et al., 1990; Blomhoff and Wake, 1991].

Retinol is the predominant retinoid in the bloodstream where it circulates bound to a specific retinol-binding protein (RBP) with high-affinity. The concentration of the RBP–retinol holo-complex in blood is regulated within the physiological range (~1.4 μM) by HSCs through the controlled uptake and release of retinol [Napoli, 1999]. HSCs contain high levels of cellular proteins involved in retinoid metabolism, such as cytosolic retinol-binding protein (CRBP) and retinol-esterifying membrane-bound enzymes. These proteins mainly optimize the intracellular transport of retinol and the maintenance of retinyl ester deposits [Blomhoff et al., 1985; Ross, 1993]. HSCs can normally deal with wide fluctuations of dietary retinol uptake, although rare cases of acute retinol intoxication after high ingestion of retinol have been reported [Berg, 1996; Russell, 2000]. Chronic uptake of high pharmacological doses of retinoids, and excessive ingestion of vitamin-A-rich nutritional supplements that are considered to prevent cancer, can lead to cirrhosis and severe hepatic dysfunction [Okuno et al., 1997; Leo and Lieber, 1999].

Despite extensive in vivo studies on retinol metabolism, the molecular mechanisms of retinol uptake by HSCs remain to be elucidated. RBP is potentially involved in the transfer of retinol from hepatocytes into HSCs. This implies the presence of specific membrane-receptors able to interact with the holo-RBP and facilitate retinol uptake from the extracellular environment [Blomhoff et al., 1988, Senoo et al., 1993]. Conversely, it was proposed that retinol, which is hydrophobic and occurs in a dynamic equilibrium with RBP, readily partitions into the lipid phase of membranes and diffuses into the intracellular environment [Ross, 1993; Noy, 2000]. Both hypotheses are supported by experimental data and are still a matter of debate.

We have developed the experimental model of the GRX cell line, originally established from fibro-granulomatous inflammatory reactions in mouse liver. This cell line was identified in previous studies as hepatic myofibroblasts, derived from HSC. Similar to HSCs in situ, GRX cells are characterized by concomitant expression of smooth muscle α-actin, desmin, and glial acidic fibrillary protein (GFAP) [Borojevic et al., 1985; Boloukhère et al., 1993; Guma et al., 2001; Mermelstein et al., 2001]. While GRX cells express the myofibroblast phenotype under standard culture conditions, they can be induced to convert into the vitamin-A-storing lipocyte phenotype by retinoids [Margis and Borojevic, 1989; Borojevic et al., 1990]. In the present study, we have used this model to analyze and compare the incorporation and the metabolism RBP-bound versus albumin-bound [^{3}H]retinol in this model of HSC.

MATERIALS AND METHODS

Cell Cultures and Phenotype Induction

The GRX cell line was obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University of Rio de Janeiro, RJ, Brazil). GRX cells were routinely maintained in the Dulbecco's medium (DMEM; Sigma, St. Louis, MO) supplemented with 5% fetal bovine serum (Cultilab, Campinas, SP, Brazil), and 2 g/L HEPES buffer 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 μM all-trans-retinol (Sigma), dissolved in ethanol (0.1% final concentration) as previously described [Margis and Borojevic, 1989]. After this incubation period, the majority of GRX cells expressed the fat-storing phenotype, with large lipid droplets in their cytoplasm. The concentration of retinol in the stock solution was determined by ultraviolet absorption at 325 nm, using the molar extinction coefficient (ε) of 52.770 cm^{-1} M^{-1}. The retinoid stock solution was stored under nitrogen, in a light-protected container at −20°C. Intracellular lipid droplets were identified with the lipid-soluble stain Oil-Red-O (Sigma).

Purification of Human RBP

RBP was purified from human serum by ammonium sulfate precipitation, followed by hydrophobic interaction chromatography on Phenyl Sepharose CL-4B and gel filtration on Sephadex G-50 Fine (both from Pharmacia

LKB, Uppsala, Sweden) as previously described [Berni et al., 1985]. The total protein content of chromatographic fractions containing RBP was determined by the modified Lowry's method [Markwell et al., 1985]. The purity of the isolated RBP was estimated to be 98% by SDS–polyacrylamide gel electrophoresis followed by Coomassie Blue staining [Laemmli, 1970]. The holo-RBP was quantified by measuring its absorbency at 330 nm, using a molar extinction coefficient (ε) value of 40.740 cm^{-1} M^{-1}. The purified RBP consisted of a mixture of holo- and apo-RBP; approximately 90% RBP was in the holo-form, as judged by the A_{330}:A_{280} ratio [Blaner and Goodman, 1990]. The solution of RBP was stored under liquid nitrogen in 50 mM Tris, 200 mM NaCl, pH 7.0.

Formation of [^{3}H]Retinol–RBP Complex

Apo-RBP was obtained after extraction of retinol from the holo-protein using the procedure described by Heller and Hoerwitz [1973]. Preparations of [^{3}H]retinol-RBP were done by incubation of apo-RBP with 2-fold molar excess of [^{3}H]retinol (specific activity 35.7 Ci/mmol; New England Nuclear, Boston, MA) overnight at 4°C and purified by gel filtration on Sephadex G-25 (Pharmacia LKB) as previously described [Heller and Hoerwitz, 1973]. The specific activity of the complex ranged $3.2–4.8 \times 10^4$ cpm/μg. The same procedure was used to label fatty acid-free BSA with [^{3}H]retinol.

Binding Assay of [^{3}H]Retinol to GRX Cells

Myofibroblast and lipocyte confluent cultures, grown in plastic dishes (Nunc, Roskilde, Denmark), were washed twice with 2 ml ice-cold phosphate buffered saline (PBS). Cultures were serum-starved in DMEM for 4 h at 37°C, washed twice with PBS, and chilled at 4°C. Monolayers were incubated with the binding assay buffer 50 mM Tris-HCl, pH 7.4, containing 0.1% ovalbumin, 150 mM NaCl, and increasing concentrations of either BSA- or RBP-bound [^{3}H]retinol. The [^{3}H]retinol-binding was monitored by incubation of monolayers with the retinol-labeled carrier-proteins alone (total binding) or supplemented with 100-fold excess of unlabeled holo-proteins (non-specific binding). After incubation on a gyratory shaker (50 rotations per min), for 30 min or whenever indicated, the binding assay buffer was removed. Monolayers were washed 3-times with 2 ml ice-cold PBS, and the cells were harvested by dissolution in 1 ml of 0.1 N NaOH. Aliquots of 500 μl were removed for scintillation counting of the cell-associated radioactivity. The specific binding was calculated by subtraction of the non-specific binding of retinol-labeled carrier-proteins from the total binding. The curves generated by the specific data were transformed into plots of the ratio of cell-bound to free radiolabeled proteins versus cell-bound labeled proteins according to the Scatchard [1949]'s method.

In order to monitor displacement of the bound compounds, lipocytes were loaded in serum-free medium with [^{3}H]retinol-BSA or [^{3}H]retinol-RBP for 1 h at 4°C, when they reached the steady state equilibrium (10 nM, 30 Ci/nmol). The supernatant was discarded and cells were exposed to high concentrations of different unlabeled competing protein-complexes at 4°C for 10 min. After removal of the chase medium, cells were washed twice with a large volume of ice-cold PBS and processed for determination of cell-associated radioactivity. In parallel experiments, the complex-binding to cell monolayers was analyzed by incubation of lipocytes loaded with [^{3}H]retinol–protein complexes with heparin (1000 IU/ml), 1.5 M NaCl, 0.3% pronase, 1% Nonidet P40 (Sigma), or 10 mM methyl-β-cyclodextrin (MBCD, Sigma). The supernatants were discarded and the remaining radioactivity was quantified.

Cellular Uptake of [^{3}H]Retinol and [^{3}H]Retinoid Metabolism

GRX cells expressing the lipocyte or the myofibroblast phenotype were plated in a 24-well dishes (2×10^5 cells/cm^2) washed with 2 ml ice-cold PBS, and incubated in serum-free culture medium for 4 h. The medium was substituted by the one containing 1.5–2 μM BSA- or RBP-bound [^{3}H]retinol (isotopic steady state, specific activity 1 μCi/2 nmol). After incubation at 37°C, cells were placed on ice and washed with 2 ml ice-cold PBS. They were harvested, collected by centrifugation, the PBS was removed, and the cell pellet was stored under a nitrogen atmosphere at −20°C, protected from light until the high performance liquid chromatography (HPLC)-analysis and radioactivity-quantification by liquid scintillation spectrometry.

The effect of drugs which block the catabolism of endocytosed proteins or interfere with the intracellular traffic was monitored in lipocyte monolayers pre-treated (1 h, 37°C) with the

endocytosis inhibitors. The uptake of BSA- or RBP-bound [^{3}H]retinol was monitored in a medium containing 1.5–2 μM retinol for 1 h at 37°C. Supernatants were discarded, and we monitored the ^{3}H label incorporated in the total cell content, and in retinol or retinyl-esters fractions. The inhibitors that reduced endocytosis were used initially at the highest concentration described in the previously published studies. Monolayers were treated with the following endocytosis inhibitors (all from Sigma): cytochalasin (25 μg/ml) [Gottlieb et al., 1993], colchicine (50 μM) [Fielding and Fielding, 1995], monesin (100 μM), nigericin (5 μM) [Dinter and Berger, 1998], sodium azide (1 mM) [Fielding and Fielding, 1995] or ρ-chloromercuribenzene sulfonate (ρ-CMBS; 5 μM) [Sundaram et al., 1998], chloroquine (1 mM) [Fielding and Fielding, 1995], ammonium chloride (10 mM), potassium depletion of the medium, and a hypertonic medium [Cuppers et al., 1994].

HPLC Analysis of Cell Extracts Labeled with [^{3}H]Retinoids

Total retinoids were extracted from 2×10^5 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 μl methanol, and analyzed by chromatography. Retinyl acetate was added as internal standard in order to monitor the extraction yields. Radiolabeled cellular retinoids were analyzed by reverse-phase HPLC following the method described by Got et al. [1995]. Analytical HPLC was carried out on a Spherisorb ODS II 5 μM reverse-phase column (250 × 4 mm). Retinoids were identified 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.

Subcellular Fractions

GRX cells expressing the lipocyte phenotype were incubated with [^{3}H]retinol-RBP or [^{3}H]retinol-BSA, disrupted, and the subcellular fractions were obtained by differential centrifugation following the method described by Harrison et al. [1987]. Briefly, cells were disrupted at 4°C with a Potter–Elvejhem homogenizer in ice-cold buffer with 0.25 M sucrose, 10 mM K_2HPO_4, 0.4 mM phenyilmethylsulfonyl fluoride (PMSF), 1 mM Ethylenediaminetetraacetic acid (EDTA), pH 7.25. The resulting homogenate was centrifuged successively to yield the nuclear fraction (N fraction −1,000*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), the lipid-droplets (floating fraction), and the high-speedsupernatant (cytosol fraction). Pellets were washed twice under the same conditions. Aliquots of the original homogenate and all the fractions were dissolved in 0.1 N NaOH and radioactivity determined by a liquid scintillation.

Statistical Analysis

All data are expressed as means ± SD. Statistical significance was determined by the Student's unpaired *T*-test (two-tailed). Group differences considered significant for $P > 0.05$.

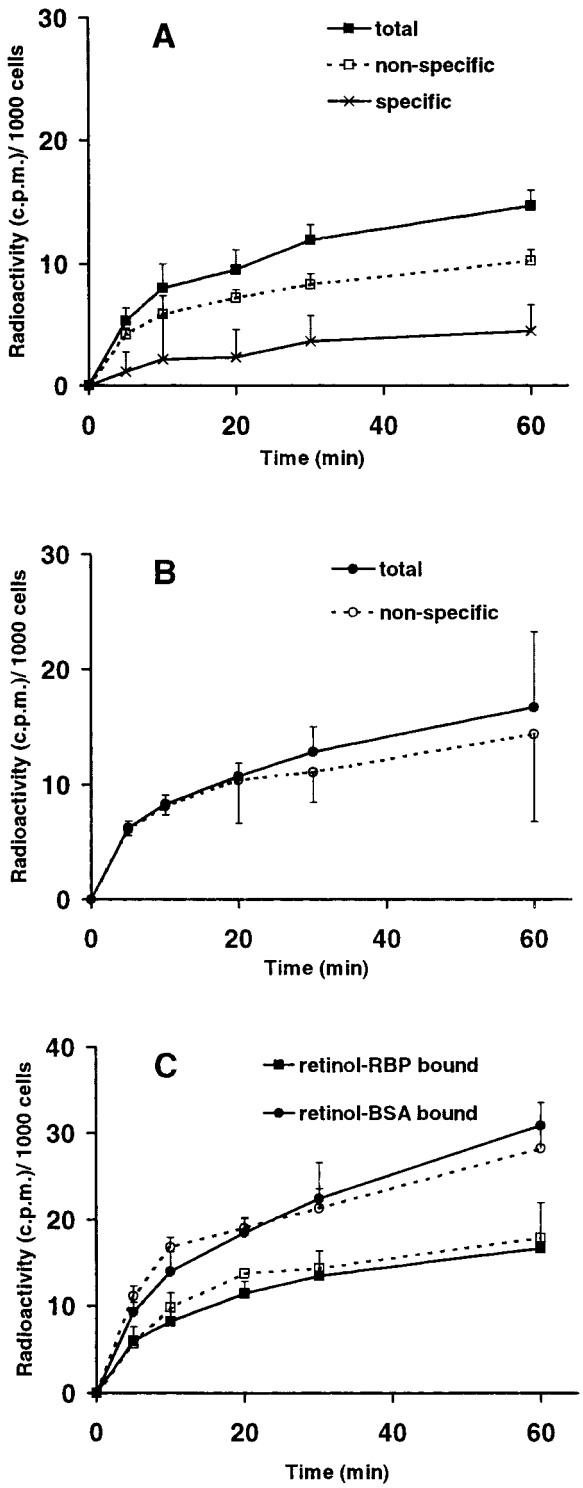
RESULTS

Binding of [^{3}H]Retinol-RBP or [^{3}H]Retinol-BSA to GRX Cells

We first addressed the question of the cell surface binding of [^{3}H]retinol. The free (ethanol dispersed) retinol has a micellar behavior in aqueous solutions, and has a great-affinity for lipid membranes. It was shown to partition non-specifically into several types of cell membranes in vitro [Creek et al., 1989, 1993; Matsuura et al., 1993; Troen et al., 1994; Hodam and Creek, 1998], including the GRX cell line [Vicente et al., 1998]. We have now chosen to use the physiological [^{3}H]retinol–RBP complex, and the [^{3}H]retinol–BSA complex, as a mode of retinol delivery to cells. Previous studies have demonstrated that BSA can efficiently bind retinol, similar to other hydrophobic compounds present in plasma [Futterman and Heller, 1972; Noy and Xu, 1990], and we have shown that it modulates the retinol availability in GRX cell cultures [Vicente et al., 1998], but the physiological role of this binding in vivo has not been established.

GRX cells expressing the lipocyte phenotype (4×10^5 cells) were starved in a serum-free medium and incubated with the binding assay buffer containing [^{3}H]retinol-labeled RBP or BSA (2×10^4 cpm), at 4°C (Fig. 1A,B) or 37°C (Fig. 1C). Serum depletion efficiency was monitored by HPLC; it reduced the presence of RBP in the cell layer to undetectable levels (data not shown). The cell-associated radioactivity

was determined at increasing times of incubation. Non-specific association was determined by the simultaneous incubation with 100-fold molar excess of unlabeled ligand. The difference between the results obtained in absence or presence of the excess of ligand indicated the amount of [^{3}H]retinol specifically associated with cell monolayers. Association of [^{3}H]retinol to monolayers was time- and temperature-dependent (Fig. 1A,B,C, respectively), both for RBP- and BSA-bound retinol.



At 4°C, approximately 30% of the association of [^{3}H]retinol-RBP to lipocytes was specific, and reached an equilibrium after 30 min of incubation (Fig. 1A). This incubation time was chosen for all the further assays. Conversely, the association of [^{3}H]retinol-BSA to lipocytes at 4°C was only slightly inhibited in the presence of 100-fold molar excess of unlabeled ligand, indicating that under these conditions retinol-BSA binding to lipocytes was non-specific, or that it was of very low-affinity (Fig. 1B). Incubation of GRX cells with RBP- or BSA-bound [^{3}H]retinol at 37°C (Fig. 1C) increased the [^{3}H]retinol accumulation. The uptake of radioactivity observed at 37°C was not reduced by the presence of an excess of unlabeled retinol complexes (Fig. 1C-dotted line), indicating that a high capacity for retinol uptake and accumulation under this condition was non-specific.

Receptor-Binding Assays and the Scatchard's Analysis

In order to quantify the capacity of GRX cells expressing the lipocyte or the myofibroblast phenotype to interact specifically with RBP-bound [^{3}H]retinol, cell monolayers were incubated at 4°C with increasing concentrations of [^{3}H]retinol-RBP alone or supplemented with 100-fold excess of unlabeled holo-RBP, for 30 min. The concentration-dependent curve of [^{3}H]retinol-RBP binding demonstrated the presence of a finite number of saturable binding sites for both cell types (Fig. 2A,B). The Scatchard's analysis (Fig. 3A,B) of the specific binding produced curvilinear plots for lipocytes, while linear plots were observed for myofibroblasts. Curves obtained for lipocytes were compatible with the existence of two classes of

Fig. 1. Time and temperature dependence of [^{3}H]retinol-binding to GRX cells. Lipocyte monolayers were incubated in serum-free medium at 4°C (**A** and **B**) or at 37°C (**C**). A: [^{3}H]retinol–retinol-binding protein (RBP) binding, B: [^{3}H]retinol-bovine serum albumin (BSA) binding. Cell-associated radioactivity was monitored with (dashed line) or without (filled line) addition of 100-fold molar excess of unlabeled ligands. Results represent mean values and standard errors of three different experiments done in triplicate. Group differences were rejected as not significant for $P > 0.05$. If an error bar is not visible, it is smaller than the symbol.

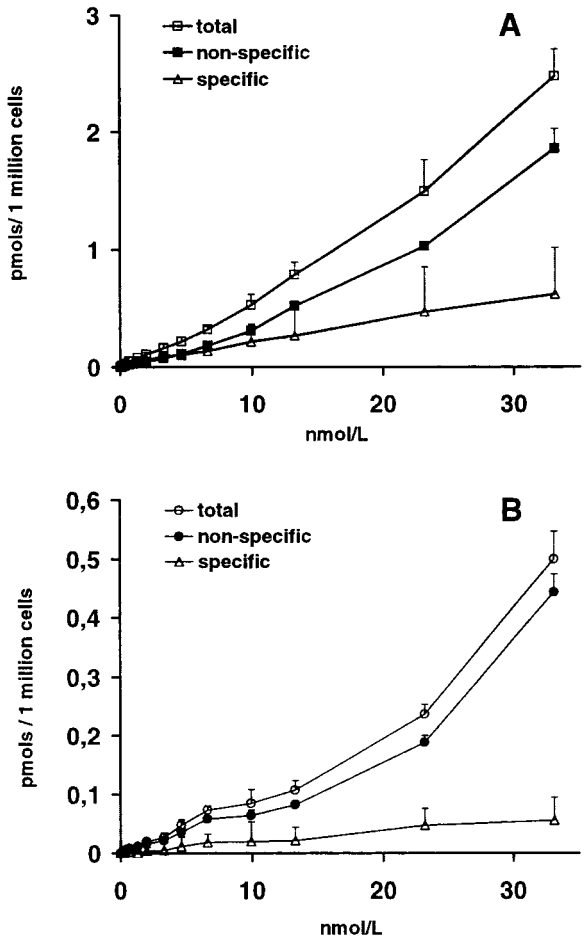


Fig. 2. Concentration-dependence of [^{3}H]retinol-RBP binding to lipocytes (**A**) and myofibroblasts (**B**). Cell monolayers were incubated at 4°C with increasing concentrations of [^{3}H]retinol-RBP for 30 min. Nonspecific binding was determined for each retinol-RBP concentration in the presence of 100-fold excess of unlabeled holo-RBP. Mean values of three different experiments done in triplicate and standard errors are shown. Group differences were rejected as not significant for $P > 0.05$. If an error bar is not visible, it is smaller than the symbol.

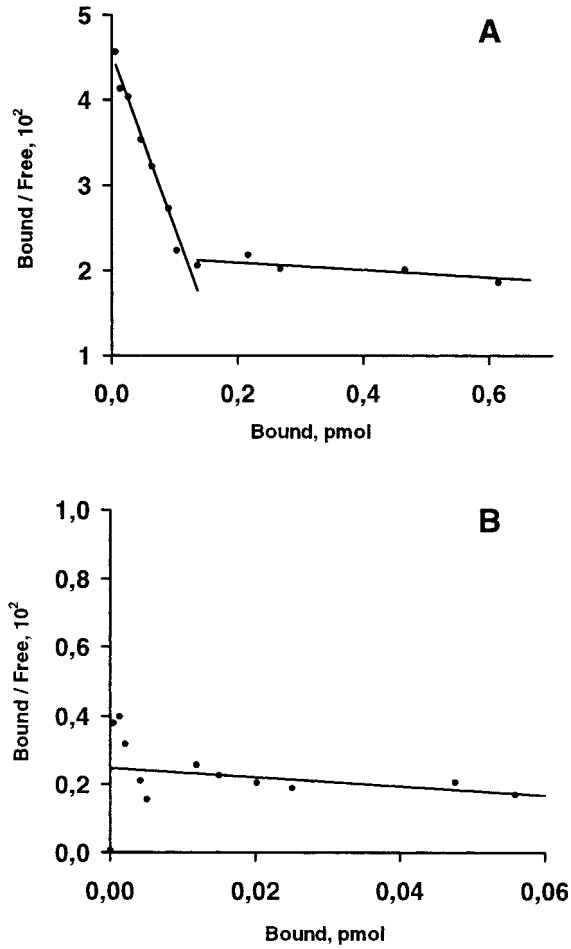


Fig. 3. Scatchard's analysis of specific binding. [^{3}H]retinol-RBP specific binding data to cell-surface of lipocytes (**A**) and myofibroblasts (**B**) were plotted and the continuous curves were calculated by using the best fit parameter values, which were for A: $Kd_{high} = 4.9$ nM, $Bmax_{high} = 0.223$ pmol/10^6 cells; $Kd_{low} = 234$ nM, $Bmax_{low} = 508$; for B: $Kd = 246$ nM, $Bmax = 0.552$ pmol/10^6 cells.

binding sites, 2–5% being in the high-affinity state (Kd = 4.93 nM) and 95–98% in the low-affinity state (Kd = 234 nM). Binding parameters derived from the Scatchard's plot indicated a mean of 3×10^6 binding sites/cell for the pool of low-affinity sites, whereas the pool of high-affinity sites was 0.134×10^6/cell. The analysis of the dissociation constants (Kd) indicates that the high-affinity binding of retinol-RBP to lipocytes was 47-times higher than that of the low-affinity binding, and a density of binding sites/cell was 22-times lower. Conversely, the Scatchard's plot indicated that myofibroblasts had a reduced number of low-affinity binding sites (Kd = 246 nM; 0.33×10^6 binding sites/cell). In conclusion, lipocytes and myofibroblasts are different both in capacity and affinity of the retinol-RBP binding sites.

Comparison of [^{3}H]Retinol Bound to RBP or BSA Delivered to GRX Cells

Independently of the mode of delivery, [^{3}H]retinol was rapidly taken up by GRX cells at 37°C (Fig. 1C). In order to monitor whether retinol metabolism was dependent upon the protein-ligand, lipocytes and myofibroblast were trace-labeled in a serum-free medium containing physiological concentrations (1.3 μM) of [^{3}H]retinol-RBP or -BSA, at 37°C for 1 and 12 h (specific activity 1 μCi/2 nmol). The culture

medium was removed, the total [^{3}H]retinoids were extracted and analyzed by HPLC. In accordance with the previous reports on retinol metabolism in other cells [Matsuura et al., 1993; Troen et al., 1994], GRX cells could uptake and esterify [^{3}H]retinol to [^{3}H]retinyl esters independently whether bound to RBP (Fig. 4A,C) or to BSA (Fig. 4B,D). The total cellular [^{3}H]retinoids extracted from lipocytes and myofibroblasts showed the HPLC elution profiles similar to those previously described [Vicente et al., 1998, Fortuna et al., 2001]. Retinol and retinyl esters were the major retinoids formed during the studied incubation period (data not shown). We did not find any significant radioactivity coeluting with all-trans or cis-isomers of retinoic acid standards, suggesting that retinoic acid was not a relevant metabolite from retinol during this incubation period, or that it was rapidly converted to other compounds. After

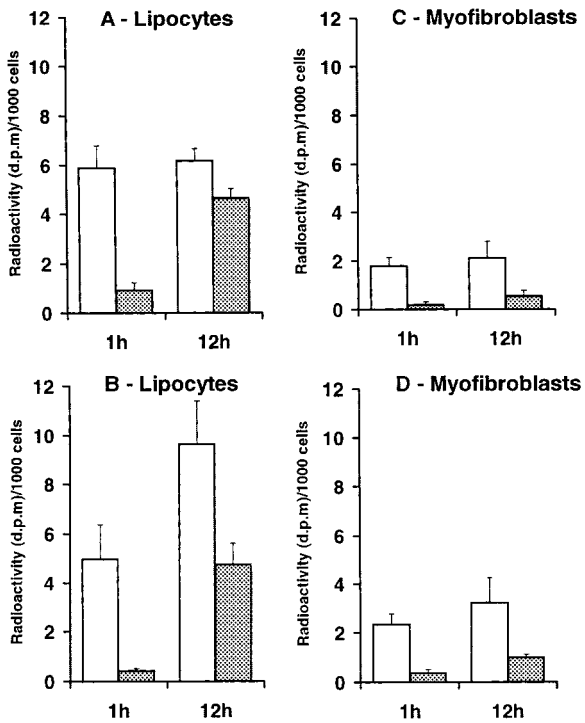


Fig. 4. Accumulation of retinoids in GRX lipocytes (**A** and **B**) and myofibroblasts (**C** and **D**). Lipocytes and myofibroblasts were trace-labeled with 1.5 μM [^{3}H]retinol-RBP (A and C) or [^{3}H]retinol-BSA (B and D) at 37°C (specific activity 1 μCi/2 nmol). After different incubation times, cell cultures were extracted and the total [^{3}H]retinoid content was analyzed by high performance liquid chromatography (HPLC). Radioactivity was quantified in fractions that migrated as retinol (open bars) and retinyl esters (full bars). Results represent mean values and standard errors of four independent experiments done in triplicate. Group differences were rejected as not significant for $P > 0.05$. If an error bar is not visible, it is smaller than the symbol.

1 h, the major labeled compound found in lipocytes was the unesterified [^{3}H]retinol, which accounted for 85–92% of the recovered radioactivity (Fig. 4A,B). When lipocytes were incubated with [^{3}H]retinol for 12 h, the relative proportion of retinyl esters increased to 43 and 33% in cells incubated with [^{3}H]retinol-RBP or -BSA, respectively. In myofibroblasts (Fig. 4C,D), the total amount of unesterified [^{3}H]retinol plus accumulated [^{3}H]retinyl esters was 3- to 4-fold less than in lipocytes. This result is in agreement with our previous studies, and is potentially related either to the low level of the carrier-retinol binding sites described above, or to the high CRBP expression and induction of retinol-esterifying enzymes during the lipocyte differentiation of GRX cells [Vicente et al., 1998; Fortuna et al., 2001]. Myofibroblasts exposed for 1 h to labeled ligands showed unesterified [^{3}H]retinol as the main cellular retinoid (85–90%), and the accumulation of retinyl esters in myofibroblasts reached 20–23% when incubated for 12 h with either [^{3}H]retinol-RBP or -BSA. The rate and the extent of retinol esterification were similar when the retinol was delivered to the cells bound to RBP or BSA.

Dislodging of [^{3}H]Retinol Bound to Lipocytes

The binding of [^{3}H]retinol-RBP or -BSA to lipocyte membranes was further characterized by testing conditions that could dislodge the cell-associated radioactivity. These studies were done incubating lipocytes with the [^{3}H]retinol–BSA or [^{3}H]retinol–RBP complexes at 4°C, until steady-state equilibrium. The release of the ligand was done using the molar excess of the unlabeled ligand. When we used [^{3}H]retinol-RBP, the excess of unlabeled holo–RBP complex or apo-RBP could release the membrane-bound radioactivity, while BSA was unable to displace it (Table I). Conversely, the assayed proteins could not displace the cell-associated radioactivity when [^{3}H]retinol-BSA was the ligand. In accordance with previous studies, our results indicated that [^{3}H]retinol was associated to lipocytes at 4°C in a retinol-carrier protein-dependent way [Sivaprasadarao and Findlay, 1988; Shingleton et al., 1989; van Bennekum et al., 1993]. The association of retinol to lipocytes from the RBP complex was specific, since it was only displaced by ligand in molar excess. We conclude that retinol delivered from RBP complex involved protein recognition

TABLE I. Displacement of Retinol Associated With Lipocytes Cell-Surface

Displacement condition	[^{3}H]Retinol-RBP	[^{3}H]Retinol-BSA
PBS	80.1 (±16.9)	81.6 (±3.8)
4 μM Holo-RBP	11.8 (±2.6)	85.7 (±6.7)
4 μM ApoRBP	20.8 (±3.6)	80.3 (±8.5)
20 μM ApoBSA	73.2 (±7.6)	88.4 (±9.9)
1,000 IU heparin/ml	103.4 (±9.7)	85.2 (±12.2)
1.5 M NaCl	79.1 (±3.0)	99.1 (±5.7)
0.3% Pronase	94.8 (±27.3)	87.2 (±6.1)
1% Nonidet P40	18.1 (±3.3)	13.5 (±0.1)
10 mM MBCD	30.3 (±4.9)	40.9 (±6.4)

Data represent the percentage of [^{3}H]retinol that remained associated after the displacement. Results represent mean values of two experiments done in quadruplicate and standard errors.

at a specific binding sites (receptors) located at the cell surface, which could distinguish the [^{3}H]retinol–RBP complex from other retinol–protein complexes. This binding did not involve internalization of RBP–retinol complex or retinol partition into the membrane during the experimental procedure, since it was sensitive to the presence of soluble competing molecules in the extracellular environment.

The initial cell-association of retinol-RBP to its membrane binding site is expected to be released by agents that disturb the non-covalent protein–protein interactions (highly polar molecules such as heparin, high salt solutions), as well as to be sensitive to proteases [Sivaprasadarao and Findlay, 1988a]. In order to further characterize the interaction of [^{3}H]retinol–protein complexes with GRX cell surface, lipocytes were incubated as above, and the dislodging studies were run for 10 min at 4°C (Table I). We did not observe a release of cell-associated radioactivity by protein–protein disturbing conditions. Conversely, both BSA- or RBP-bound [^{3}H]retinol were fairly well released by the lipid phase disturbing agents (Nonidet P40 and MBCD). These results suggested that the integrity of hydrophobic interactions among membrane lipids, as well as between membranes and proteins (including the retinol–RBP complex) may contribute to the cellular uptake of the [^{3}H]retinol.

Cellular Fate of [^{3}H]Retinol

In order to settle whether [^{3}H]retinol delivered to lipocytes in association with RBP or BSA followed different intracellular metabolic pathways, we studied the subcellular distribution of radioactivity in lipocytes, trace-labeled in serum-free medium with 1.3 μM [^{3}H]retinol-RBP or -BSA at 37°C, for increasing periods (specific activity 1 μCi/2 nmol). After the incubation, the cells were harvested, washed with PBS, and disrupted at 4°C. The total radioactivity of the subcellular fractions was immediately determined in scintillation counter. In parallel, subcellular fractions were submitted to HPLC separation, and we monitored the intracellular [^{3}H]retinoids distribution between cell fractions. The radioactive content of cells incubated with [^{3}H]retinol-RBP was found mainly in the cytosol after 1 h (70%). After 12 h, it accumulated mostly in membranes (61%) and in lipid droplets (24%; Fig 5A). Furthermore, the early finding of [^{3}H]retinol in the cytosol (Fig. 6A) suggested a rapid transfer of the tracer from extracellular aqueous environment to the intracellular cytosolic proteins, in accordance with the published data [Sundaram et al., 1998]. The progressive transfer of the radioactivity from cytosol to microsomes and finally to lipid-droplets (in which it was mainly stored as retinyl esters) suggested the participation of these fractions in the intracellular retinol traffic as intermediate and final compartments, respectively (Fig. 6A). In contrast to the data obtained with the RBP-bound retinol, cells exposed to BSA-bound [^{3}H]retinol showed a high radioactivity content associated with membrane fraction during all the incubation times (Fig. 5B; $P < 0.01–0.001$). The presence of retinyl esters in lipid droplets was negligible when compared to the RBP-delivered retinol ($P < 0.001$). Lipocytes incubated with [^{3}H]retinol-BSA during 12 h had a significant increase of [^{3}H]retinol in the nuclear fraction as compared to [^{3}H]retinol-RBP treated cells ($P < 0.001$).

Effects of Metabolic Inhibitors on the Uptake of RBP- or BSA-Bound [^{3}H]Retinol

In order to address the question of whether the endocytosis of the membrane retinol–protein complexes was a required step for

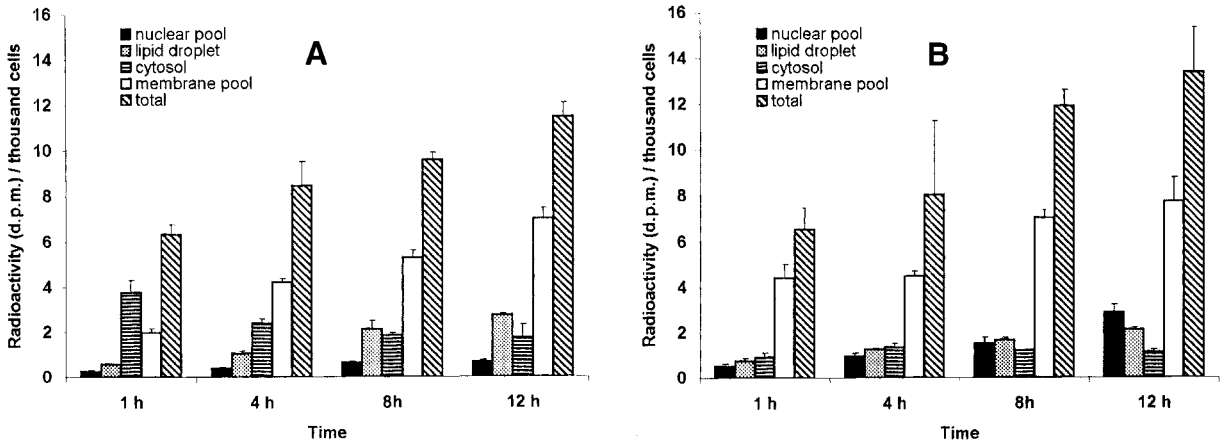


Fig. 5. Intracellular [^{3}H]retinoid distribution—Lipocytes were trace-labeled with 1.3 μM [^{3}H]retinol-RBP (**A**) or free [^{3}H]retinol (**B**) at 37°C (specific activity 1 μCi/2 nmol). Cells were harvested, washed with phosphate buffered saline (PBS), disrupted at 4°C, and fractionated by differential centrifugation. The results represent mean values of four experiments and standard errors. If an error bar is not visible, it is smaller than the symbol.

[^{3}H]retinol uptake, lipocyte monolayers were pre-treated with specific endocytosis inhibitors and then submitted to binding with [^{3}H]retinol-RBP or -BSA. The presence of inhibitors had no dramatic effect on the total cell-associated radioactivity, but they interfered with [^{3}H]retinol and [^{3}H]retinyl ester distribution (Table II). Cytochalasin, a drug that interferes with actin filament organization [Gottlieb et al., 1993], and colchicine that interferes with microtubule polymerization [Fielding and Fielding, 1995] had no clear effect on the uptake or metabolism of [^{3}H]retinol whether bound to RBP or BSA ($P > 0.05$). Monesin (100 μM) and nigericin (5 μM) inhibited mainly the esterification of [^{3}H]retinol ($P < 0.01–0.05$). The sodium salt of ρ-CMBS (5 μM), a specific inhibitor of the putative RBP receptor [Sivaprasadarao and Findlay, 1988], and sodium azide (1 mM) [Fielding and Fielding, 1995], an efficient inhibitor of ATPases that catalyze transmembrane ion transport, decreased significantly esterification but not the [^{3}H]retinol uptake from RBP or BSA complexes ($P < 0.01–0.05$). Other endocytosis inhibitors, such as chloroquine (1 mM), ammonium chloride (10 mM), potassium depletion of the medium and a hypertonic medium, did not influence uptake or metabolism of [^{3}H]retinol bound to RBP or BSA (data not shown). Taken together, these data show that agent that disturbs the classical clathrin-mediated endocytosis did not interfere with retinol uptake.

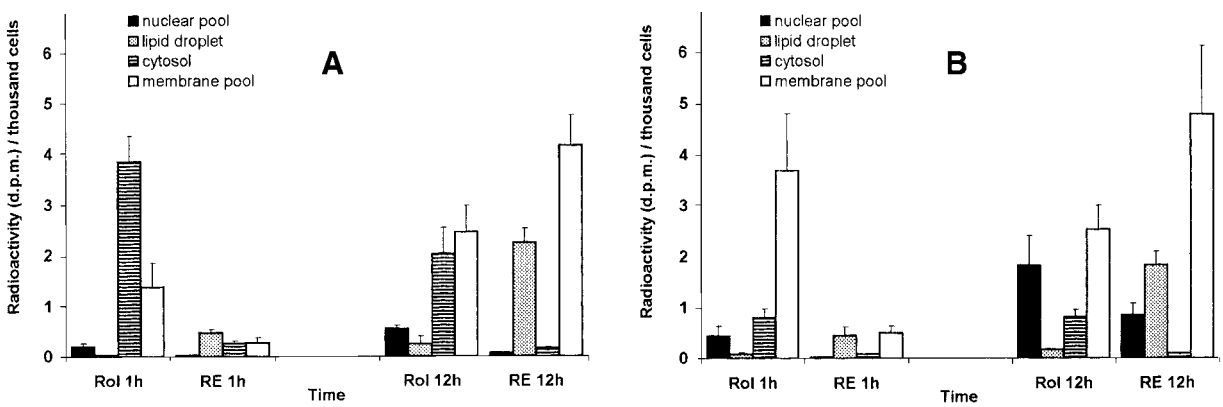


Fig. 6. Retinol (Rol) and Retinyl ester (RE) distribution among cellularc fractions. Subcellular fractions prepared with lipocytes trace-labeled with 1.3 μM [^{3}H]retinol-RBP (**A**) or [^{3}H]retinol-BSA (**B**) at 37°C (specific activity 1 μCi/2 nmol) were analyzed by HPLC and quantified. The results represent four experiments. If an error bar is not visible, it is smaller than the symbol.

TABLE II. Effect of Endocytosis Inhibitors on Retinol Incorporation Into GRX Lipocytes

	[^{3}H]Retinol-RBP			[^{3}H]Retinol-BSA		
Inhibitor	Total	[^{3}H]Retinol	[^{3}H]Retynil esters	Total	[^{3}H]Retinol	[^{3}H]Retynil esters
PBS (none)	100	43 ± 16	52 ± 15	100	51 ± 9	45 ± 10
Cytochalasin D	118 ± 5	64 ± 9	44 ± 7	80 ± 12	48 ± 9	36 ± 10
Colchicine	105 ± 5	52 ± 16	51 ± 8	85 ± 2	36 ± 10	34 ± 4
Monesin	111 ± 11	83 ± 10	20 ± 6	82 ± 6	57 ± 13	17 ± 2
Nigericin	98 ± 8	64 ± 7	27 ± 5	57 ± 13	35 ± 15	15 ± 5
ρ-CMBS	104 ± 2	72 ± 12	34 ± 5	85 ± 18	41 ± 7	19 ± 3
Azide	95 ± 18	59 ± 13	23 ± 6	70 ± 1	40 ± 9	18 ± 5

Results represent the mean values and standard errors of two independent experiments done in triplicate.

However, drugs that modify the intercellular traffic of organelles, such as monesin, nigericin, and azide disturbed the route of retinol transfer to the esterification sites.

DISCUSSION

HSCs play the leading function in uptake, metabolism, storage, and homeostasis of retinoids. Despite a very high intracellular content of retinoids under standard nutritional conditions, they are able to efficiently uptake retinol from plasma. They are at a strategic position in liver. As opposed to other connective tissue cells whose contact with plasma is dependent upon the permeability of the endothelial layer, HSCs have a direct access to the circulating plasma in the Disse's space. Simultaneously, they are in close apposition with hepatocytes that metabolize retinoids and produce both free retinol and RBP. The HSCs ability to selectively uptake and concentrate the circulating retinol, which is in large part bound to RBP, suggests the presence of an efficient receptor system, whose full characterization had remained elusive. Previous studies have suggested an active participation of RBP in delivery of retinol to HSCs [van Bennekum et al., 1993; Troen et al., 1994]. However, it is still controversial as to whether the uptake of retinol through a specific receptor occurs on HSC membranes, and what role such receptor would play in the delivery and further metabolism of retinol in these cells.

We report here studies on the transport of retinol from extracellular carrier proteins into HSCs, and its further cellular metabolism. Our experiments support the existence of a specific site for high-affinity binding of [^{3}H]retinol-RBP on lipocytes, but not on myofibroblasts, based on observation of a competition between RBP-bound radiolabeled and unlabeled retinol. Scatchard's analysis has characterized this site at the lipocyte plasma membrane. The Kd indicated that the receptor was saturated within the normal plasma RBP concentration of 1.3 μM, and that RBP-retinol could be taken up efficiently from much lower concentrations that are normally found in extracellular fluids. The low-affinity binding-site was present on both HSC phenotypes, albeit in much lower quantity on myofibroblasts. We have already shown that the retinoid-mediated induction of the lipocyte phenotype in HSCs provided molecular mechanisms required to deal with high quantity of retinol, namely by induction of the CRBP expression and of the enzymes involved in retinol esterification [Vicente et al., 1998; Fortuna et al., 2001]. The induction of the receptor involved in uptake of retinol is the necessary complement to the previously described molecular mechanisms, showing that the retinoid-mediated induction of the lipocyte phenotype is an integrated program leading to the differentiation of a highly efficient liver cell population dedicated to retinol metabolism. This is also in agreement with observations that HSCs expressing the myofibroblast phenotype are a vitamin-A low-containing hepatic cell population, predominant in situations that require an increased production of extracellular matrix. Vitamin-A-deficient status is one of the conditions that can induce HSC to undergo transition to myofibroblasts [Gressner and Bachem, 1990; Hellemans et al., 1999].

The present study of [^{3}H]retinol uptake in GRX cells indicated that the two proposed models, namely the direct partitioning into the hydrophobic part of cell membranes and the receptor-mediated uptake, can coexist in HSCs. In agreement with the partitioning model, we observed that the rapid and spontaneous incorporation of [^{3}H]retinol from BSA was not

specific and did not reach saturation under our experimental conditions. Previous studies have shown that BSA binds retinol with a relatively low-affinity, favoring its dissociation and partitioning into the cell membranes [Sivaprasadarao and Findlay, 1988; Noy and Xu, 1990]. Confirming this point, after a short period of incubation, retinol that was incorporated in membranes from [^{3}H]retinol–BSA complex could not be dislodged by apo-BSA or by non-specific treatments, which can release molecules non-covalently bound to membranes. The fact that compounds that interfere with ionic interactions (heparin, NaCl) could not release BSA-delivered [^{3}H]retinol indicates that it was sequestered in the innermost hydrophobic part of the membrane. Detergents that can disrupt protein–protein, protein–membrane, and lipid–membrane interactions released the retinol diffused into membranes.

In contrast to the BSA-bound retinol, the [^{3}H]retinol delivered to cells from RBP could be readily and nearly completely (80%) dislodged by a 3-fold molar excess of physiological unlabeled (holo or apo) RBP. Moreover, [^{3}H]retinol delivered to lipocytes by RBP was not removed by large concentrations of apo-BSA. These observations suggested that [^{3}H]retinol delivered by RBP does not readily diffuse into the hydrophobic phase of the membrane, but it remains sequestered in a specific molecular complex containing RBP.

The existence of specific RBP receptors has been proposed in several models. They can potentially explain why RBP-null mutant has such a strong functionally deficient phenotype, despite the ability of BSA and chylomicrons to transport retinoids in blood [Biesalski et al., 1999]. RBP-deficient mice display reduced blood retinol levels and impaired visual function during the first months of life. However, they are viable and fertile. When maintained on a vitamin-A sufficient diet, they acquire normal vision by 5 months of age, even though their blood retinol level remains low [Quadro et al., 1999; Vogel et al., 2002]. Even the non-hepatic RBP expressed ectopically in muscle can deliver retinol to eyes and restore their function [Quadro et al., 2002]. A striking phenotype of the RBP-null mice is that they possess larger than normal hepatic vitamin-A stores, but are nevertheless dependent on a continuous dietary intake of vitamin-A. Therefore, absence of RBP in target cells that require retinol for the normal function can be compensated by other retinol delivery systems. Conversely, regulation of vitamin-A metabolism, storage and release that occur in HSC, depend upon the efficient delivery of retinol by RBP.

Purification and cloning of RBP receptor has been reported from retinal pigment epithelial cells [Bavik et al., 1996] and placental membranes [Sivaprasadarao et al., 1994]. The RBP 65 kDa protein (Rpe65) receptor expressed in retinal cells, [Nicoletti et al., 1995; Redmond et al., 1998] was recently identified as specific RBP involved in intramembrane transfer of retinoids [Jahng et al., 2003]. Human placental membranes displayed specific interactions with binding proteins making possible the transfer of retinol from extracellular RBP to intracellular CRBP [Sundaram et al., 1998]. Mutations introduced into RBP decreased or abolished its interaction with the receptor without affecting the retinol-binding capacity [Sivaprasadarao and Findlay, 1994].

Properties of the RBP receptor described here in HSC correlate with those of other RBP receptors. The binding of RBP to a specific and saturable site on lipocyte membranes was required for efficient initial steps of the delivery of [^{3}H]retinol and for its transfer to HSC cytosol. Interestingly, [^{3}H]retinol delivered to lipocytes by RBP could be displaced both by excess of unlabeled protein ligand and by detergents. This may indicate that two levels of binding coexist. The first one can be protein–protein interaction involving the holo-RBP and the receptor. The second one may require an efficient interaction of the [^{3}H]retinol-RBP/receptor complex with organized membrane lipids, either through their direct binding to the complex, or providing the appropriate structural molecular support required for its functional binding and internalization, such as lipid rafts or caveolae. Disturbing either of the two steps can abrogate the retinol binding and uptake.

We further studied the kinetics and temperature dependence of the uptake of [^{3}H]retinol from RBP and BSA. Incubation at 4°C diminished the [^{3}H]retinol uptake from both RBP and BSA complexes. These results are in agreement with previous studies that report the temperature-dependence of retinol uptake [van Bennekum et al., 1993]. The increased uptake at 37°C was probably dependent upon plasma membrane recycling. A time-dependent

increase of cell associated radioactivity was observed for both [^{3}H]retinol–protein complexes. Considering that the avidity of BSA for [^{3}H]retinol is 30-fold lower than that of RBP [Noy and Xu, 1990], we would expect higher cell associated radioactivity in [^{3}H]retinol-BSA incubations [Vicente et al., 1998]. However, the steady-state level of [^{3}H]retinol uptake from BSA was only slightly higher than that from RBP. This indicated that the dissociation of [^{3}H]retinol was potentially the rate-limiting step for its uptake from BSA complex, but not for [^{3}H]retinol–RBP complex, confirming the presence of a receptor-mediated process that required the presence of the RBP–retinol complex.

We addressed the question of whether the RBP- or BSA-mediated delivery of [^{3}H]retinol involved specific intracellular pathways, and we found that RBP-bound retinol was handled differently from the retinol–BSA complex. The direct delivery of retinol into the intracellular environment was favored in the former case. Sundaram et al. [1998] studied the transfer of [^{3}H]retinol from serum RBP to CRBP, mediated by a membrane channel, and suggested that the RBP receptor induced the release of [^{3}H]retinol from circulating RBP and facilitated its transfer to the intracellular CRBP in a membrane-dependent manner. This supports the suggestion that retinol influx will occur as long as apo-CRBP is available within the cell [Noy and Blaner, 1991; Blaner and Olson, 1994]. Our results are in agreement with this proposal and indicate a biological significance of retinol channeling, since [^{3}H]retinol from RBP complex was transferred to cytosol and more efficiently stored in lipid droplets. Previous studies indicate that BSA [Schnitzer and Oh, 1994] and RBP [Malaba et al., 1995; Pol et al., 1999] share a common localization in caveolae, small non-coated plasmalemmal vesicles with high receptor concentration that facilitate transmembrane transport of ligands through receptor-mediated potocytosis [Pol et al., 1999]. This model is supported by our observation showing that the retinol uptake from RBP is not sensitive to inhibitors of clathrin-mediated internalization, but it is disturbed by treatments that disorganize caveolae and lipid rafts. We propose that retinol dissociates from the holo-RBP when it is still bound to the peripheral compartment, and it is directly transferred to the intracellular transporter, reaching esterification sites and subsequently the lipid droplets. This influx pathway facilitates the retinol uptake into HSC against the concentration gradients, and protects cell membranes from undesirable and potentially noxious high retinol concentrations. Retinol toxicity after a very high nutritional uptake may correspond to the saturation of the available RBP. Under such conditions, the excess of retinol may circulate bound to BSA, from which it can partition into membranes and disturb their functional organization.

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