PPARβ regulates vitamin A metabolism-related gene expression in hepatic stellate cells undergoing activation

Karine Hellemans,1,* Krista Rombouts,1 Erik Quartier,† Andrea S. Dittié,‡‡ Andreas Knorr,** Liliane Michalik,†† Vera Rogiers,§ Frans Schuit,7 Walter Wahli,†† and Andrea Geerts*1

Laboratory of Molecular Liver Cell Biology,†† Molecular Pharmacology Unit,† Diabetic Research Center, Department of Toxicology,§ Vrije Universiteit Brussel, 1090 Brussels Belgium; Bayer AG,** Institute for Cardiovascular Research, Wuppertal, Germany; and Institute for Animal Biology,†† University of Lausanne, Switzerland

Abstract Activation of cultured hepatic stellate cells correlated with an enhanced expression of proteins involved in uptake and storage of fatty acids (FA translocase CD36, Acyl-CoA synthetase 2) and retinol (cellular retinol binding protein type I, CRBP-I; lecithin:retinol acyltransferases, LRAT). The increased expression of CRBP-I and LRAT during hepatic stellate cells activation, both involved in retinol esterification, was in contrast with the simultaneous depletion of their typical lipid-vitamin A (vitA) reserves. Since hepatic stellate cells express high levels of peroxisome proliferator activated receptor β (PPARβ), which become further induced during transition into the activated phenotype, we investigated the potential role of PPARβ in the regulation of these changes. Administration of L165041, a PPARβ-specific agonist, further induced the expression of CD36, B-FABP, CRBP-I, and LRAT, whereas their expression was inhibited by antisense PPARβ mRNA. PPARβ-RXR dimers bound to CRBP-I promoter sequences.13 Our observations suggest that PPARβ regulates the expression of these genes, and thus could play an important role in vitA storage. In vivo, we observed a striking association between the enhanced expression of PPARβ and CRBP-I in activated myofibroblast-like hepatic stellate cells and the manifestation of vitA autofluorescent droplets in the fibrotic septa after injury with CCl4 or CCl4 in combination with retinol.—Hellemans, K., K. Rombouts, E. Quartier, A. S. Dittié, A. Knorr, L. Michalik, V. Rogiers, F. Schuit, W. Wahli, and A. Geerts. PPARβ regulates vitamin A metabolism-related gene expression in hepatic stellate cells undergoing activation. J. Lipid Res. 2003. 44: 280–295.

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Vitamin A (vitA) must be provided in the diet in order to maintain growth, health, and life of higher organisms. The formation and hydrolysis of retinyl esters (REs) are key processes in the metabolism of the micronutrient vitA.

Although REs are found in a variety of tissues and cell types, most of the total body retinoid is accounted for by the RE stored in the liver. Hepatocytes take up vitA esters from chylomicron remnants. After hydrolysis of the RE, retinol is transferred to the hepatic stellate cells (Ito cells, fat storing cells, lipocytes). Here, retinol may either be mobilized to the plasma or stored as esters of long-chain fatty acids (LCFAs) (1). The most characteristic ultrastructural feature of hepatic stellate cells is the presence of large vitA-rich lipid droplets in their cytoplasm. Unlike adipocytes, hepatic stellate cells are not involved in energy storage, but they represent a particular cell population specialized in storage of lipid soluble substances, the major one being retinol. These RE reserves form the major endogenous source of retinoids that can be delivered to peripheral tissues for conversion to biologically active forms.

Apart from vitA storage, hepatic stellate cells control the extracellular matrix turnover in the space of Disse and contribute to the control of blood flow through the sinusoidal capillaries (2). Acute and chronic liver injury activates hepatic stellate cells to undergo transition into myofibroblast-like cells (3) that play an important role in inflammation and tissue repair (4). Myofibroblastic hepatic stellate cells display enhanced proliferation and synthesize unbalanced amounts of extracellular matrix proteins, matrix-degrading enzymes, and their inhibitors, resulting in matrix accumulation (5). Activation of hepatic stellate cells is a central event in wound repair and in the pathogenesis of liver fibrosis during chronic liver in-
jerry. Interestingly, the changes observed in primary hepatic stellate cell cultures strongly resemble the phenotypical changes during the in vivo activation process. Therefore, cultured hepatic stellate cells are commonly used as a model to study their role during hepatic tissue repair and fibrogenesis.

During cultivation of hepatic stellate cells, a steady decrease of their vitA reserves is observed (6). Although typically 70% to 80% of the total body vitA reserves are found in the hepatic stellate cells (7, 8), the molecular pathways behind retinol storage and its depletion during hepatic stellate cell activation are still poorly understood. In the past, most attention has been focused on the expression of binding proteins and enzymes directly interacting with retinol, such as retinol binding proteins (RBP), lecithin:retinol acyltransferases (LRATs), Acyl-CoA:retinol acyltransferases (ARATs), and RE hydrolases (9, 10), whereas the expression of proteins involved in the handling of fatty acids remains unstudied. VitA storage in hepatic stellate cells, however, depends on the solubilization of newly formed esters in lipid droplets, which typically consist of about 40% RE, 30% triglycerides, 15% cholesteryl esters, and 4% phospholipids (9, 11). It has been shown that this relative composition is influenced by the dietary retinoid status, but not by low or high triglyceride intake (11). Retinol esterification and storage thus requires a tightly controlled lipid metabolism. Nuclear hormone receptors like the peroxisome proliferator-activated receptors (PPARα, β, and γ) could play a role therein.

PPARs direct the activation of responsive genes by forming heterodimers with retinoid X receptors (RXRs). The PPAR-RXR dimer binds to a consensus peroxisome proliferator responsive response element (PPRE) that is identified as a direct repeat motif of hexamer half-sites, AGGTCA, spaced by one nucleotide (DR1). Imperfect PPREs have been identified in various genes with variations in the binding site and spacer sequence (12). PPARs modulate the expression of various genes implicated in fatty acid (FA) uptake, binding, degradation, and lipoprotein assembly and transport. Target genes include intracellular fatty acid binding proteins (FABPs), acylCoA synthetases (ACSs), and FA translocase (FAT/CD36) (13). Furthermore, PPARs are shown to play an important role in the control of inflammatory responses and inflammation-related disorders (14).

In a previous study, we demonstrated that in liver tissue PPARβ was almost exclusively expressed by the highly specialized lipid-vitA-storing hepatic stellate cells (15), an observation that was suggestive of a role of the receptor in vitA storage. During hepatic stellate cell activation and RE depletion in culture, PPARβ expression was strongly induced. Exposure to PPARβ agonists resulted in an enhanced proliferation. Recently, it was demonstrated that PPARβ regulates the expression of ACS2 (16) and the reverse cholesterol transporter ATP binding cassette A1 (A17). PPARβ could thus occupy a key position in the transcriptional control of a group of pivotal enzymes that control the channeling of FA into various metabolic pathways. Esterification of retinol might be one of these pathways. VitA deficiency in rats has been shown to potentiate CCl4-induced liver fibrosis (18), whereas chronic hypervitaminosis A results in severe liver fibrosis by a poorly understood mechanism (19). The goal of the present study was to analyze the expression of a number of genes involved in FA and retinol uptake and storage during conversion of hepatic stellate cells into their activated phenotype. The involvement of PPARβ as a possible regulator of vitA esterification and storage was examined.

MATERIALS AND METHODS

Isolation of hepatic stellate cells

Adult male Wistar rats (body weight: 400-500 g) were used in all experiments. Animals were treated according to the guidelines of the Council for International Organizations of Medical Sciences, as required by the Belgian National Fund for Scientific Research. Hepatic stellate cells were isolated from rats by collagenase-pronase digestion, as described previously (20). After isolation, hepatic stellate cells were plated at a density of 1.5 × 10⁴ cells/ml in 250 ml culture flasks (Falcon, Becton Dickinson, Lincoln Park, NJ) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Paisley, Scotland) with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultured hepatic stellate cells were allowed to reach confluency before they were trypsinized and subcultured. Cell growth, purity, and phenotype were evaluated by phase-contrast microscopy and fluorescence microscopy (AXIOVERT100, Zeiss, West Germany).

CCL4 treatment of rats

Adult Wistar rats received repeated (every 72 h) intraperitoneal injections of CCl4-paraffin (50:50, v/v) alone or in combination with subcutaneous injections of retinol dissolved in sunflower oil (20,000 IU/administration) (Sigma) as described previously (21) (n = 5 per group). Control rats received vehicle only (paraffin, paraffin-sunflower oil), whereas a fifth group was injected subcutaneously with retinol-sunflower oil. Rats were sacrificed after 5 weeks, and livers were taken for immunohistochemistry and Northern blotting.

RT-PCR, real time quantitative-PCR, and sequencing

Total RNA was extracted from freshly isolated and cultured hepatic stellate cells using Qiaquick RNA extraction columns (Qiagen, Westburg, The Netherlands). Total RNA was first normalized to the respective hypoxanthine phosphoribosyltransferase (HPRT) Ct-value of the sample and afterwards to the control group. Threshold cycle (Ct) method (22), each sample was normalized to the respective hypoxanthine phosphoribosyltransferase (HPRT) Ct-value of the sample and afterwards to the control group.
terwards to the control (mRNA obtained from day 0 hepatic stellate cells). Fold induction was calculated from these Ct values.

Northern blot analysis

Total RNA was fractionated in a 1% agarose-3% paraformaldehyde gel and transferred onto a nylon membrane (Hybond, Amersham Little Chalfont, England). Hybriizations were carried out using Quickhyb hybridization solution following the manufacturer’s instructions (Stratagene, Little Chalfont, England). Rat-specific probes were produced by RT-PCR and labeled with 32P-deoxyctydine triphosphate (32P-dCTP, 5,000 Ci/ml, ICN Biomedicals, Costa Mesa, CA) using the Rediprime II labeling kit (Amersham). Filters were exposed to Kodak Biomax-MS using intensifying screens at −70°C. Radioactive signals were quantitated using a Bio-Rad G-525 Molecular Imager System (Bio-Rad Laboratories, Nazareth, Belgium).

Immunocytochemistry and fluorescence microscopy

Immunocytochemistry was performed as described previously, using rabbit anti-PPARβ antibodies and different hepatic stellate cell markers: desmin, α-smooth muscle actin (αSMA), and glial fibrillary acidic protein (GFAP) (Sigma, St. Louis, MO) (23). As secondary antibodies, whole IgG affinity-purified anti-rabbit-Cy3 and anti-mouse-Cy2-labeled antibodies (Jackson, Bar Harbor, ME) were used. To detect vitA, coverslips were observed under the fluorescence microscope immediately after formaldehyde (3%) fixation. When excited by 326 nm UV light, vitA emits a natural, blue/green fluorescence that characteristically fades away within 10 s. Digital pictures were taken using a Sony PowerHad camera (Sony, Zeiss) using KS300 Version2 Kontron elektronic software (Zeiss).

Metabolic labeling and immunoprecipitation

Six-day-old hepatic stellate cells were trypsinized and plated in 6-well dishes (100 × 104 cells/well) (Falcon). Cells were allowed to recover 24 h before L165041 (dissolved in DMSO), FA (dissolved in ethanol), or the appropriate vehicles were administered for a total time of 24 h. Hepatic stellate cells were metabolically labeled with 50 μCi/ml [35S]methionine/cysteine (Translabel, 1,000 mCi/mmol, ICN Bio- medicals) in methionine-free DMEM supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml sodium ascorbate (Merck, Darmstadt, Germany), and 64 μg/ml β-aminopropionitrile (Sigma) with or without PPARactivators. Cell layers were collected and immunoprecipitations were carried out as described previously (20).

Antisense PPARβ mRNA-expressing viral constructs

Full-length PPARβ cDNA was cloned from a pSG5-PPARβ construct and transferred in the antisense orientation into pShuttle-CMV allowing homologous recombination with pAdEasy-1 adenoviral plasmid (24). Empty shuttle vectors pAdTrack and pShuttle-CMV were used for production of respectively GFP-trackable and empty pAdEasy-1 control viruses. Recombinant adenoviral plasmids were generated by homologous recombination in Escherichia coli BJ-5183 cells. High titer viral stocks were produced in 911 cells. Infection of hepatic stellate cells was performed by mild shaking in a limited amount of CDMEM for 2 h.

DNA binding assay

Electrophoretic mobility-shift assays (EMSAs) were performed by radiolabeling (32P]ATP, Amersham) double-stranded oligonucleotides using T4 polynucleotide kinase (Invitrogen). DNA-binding reactions were set up using nuclear extracts derived from isolated hepatic stellate cells and/or PPARβ and RXRα proteins synthesized by in vitro transcription-translation of PSG5-PPARβ and PSG5-RXRα (kind gift of Dr. P. Chambon, Institut de Genetique et de Biologie Moleculaire et Cellulaire, Illkirch, France) using TNT T7 quick-coupled reticulocyte lysate system (Promega). Nuclear extracts (3–5 μg of protein) or reticulocyte lysates (1–2 μl) were incubated with 2 μg of nonspecific competitor DNA (poly[d(lC)] in binding buffer containing 20 mM Hepes, pH 7.5, 60 mM KCl, 5 μg of acetylated BSA, 1 mM DTT, and 5% Ficoll at room temperature for 15 min. For supershift analysis, antibodies were added and the mixture was incubated an additional 60 min. Labeled oligonucleotides (100,000 cpm) were added and the incubation was continued for 20 min. To show the specificity of PPAR-RXR binding to predicted PPREs, an unspecific oligonucleotide (‘5’-caagacacccctgctttcc-3’) derived from the rat cellular retinol binding protein I (CRBP-I) promoter sequence was used. Competition analysis was performed by adding increasing amounts of unlabeled homologous or heterologous oligo to the reaction mixture and an additional incubation of 20 min. The reaction mixtures were electrophoresed on a nondenaturing 4% or 6% polyacrylamide (28:1) Tris-borate-EDTA gel and subjected to autoradiography.

Statistics

All experiments exposing hepatic stellate cells to LCFA, retinol, retinoic acid receptor ligands, and L165041 were done on 7-day-old hepatic stellate cells. For immunoprecipitations of PPARβ, the amounts of immunoprecipitable protein of cell layers were calculated per dish and per 106 cells. The values per 106 cells for cell lysates were expressed relative to the control culture. The number of observations was five per condition. Data are represented as mean ± SD. For Northern hybridizations, data were first normalized to 18S rRNA before ratios of treated-control were calculated (n = 3). Statistical evaluations were performed by calculating confidence intervals. Data are represented as mean ± SD.

RESULTS

PPARβ expression in cultured hepatic stellate cells

Esterification of vitA in hepatic stellate cells requires the uptake and activation of LCFA. Since PPARs regulate the expression of multiple genes involved in FA transport, uptake, and storage, we studied the changes in the expression levels of the different PPAR isotypes (α, β, γ) by RTQ-PCR during hepatic stellate cell culture (n = 3). In parallel, the mRNA expression level of ACS2, a gene transcriptionally regulated by PPARs (16), was analyzed. RTQ-PCR was performed on mRNA obtained from isolated hepatic stellate cells cultured up to 7 days. As shown in Fig. 1A, a strong 12-fold increase of PPARβ mRNAs was observed over time, whereas PPARα mRNA levels remained unchanged. As described previously, PPARγ mRNA expression was low and further reduced over time (25–27). The relative expression levels of the α, β, and γ isotypes in total liver tissue and in 3-day-old hepatic stellate cells were shown by Northern hybridization analysis (insert of Fig. 1A). A clear signal was found for PPARα expression in total liver tissue, reflecting its expression in hepatocytes. PPARγ has been shown to be expressed mainly by Kupffer cells (28). In hepatic stellate cells, a strong signal was found for PPARβ using only 5 μg total RNA, whereas PPARα and γ mRNAs remained undetectable in 20 μg total RNA. In keeping with this observation, RTQ-PCR analysis revealed that the mRNA for ACS2 was induced in parallel with the PPARβ transcripts.
Expression of genes involved in FA uptake and activation, and in retinol esterification and storage

Little is known about the expression of genes related to FA uptake and binding and genes involved in vitA binding, esterification, and metabolism under hepatic stellate cell culture conditions. By Northern blot hybridization we examined the expression of FAT/CD36, which plays an essential role in FA uptake (29), and FABPs involved in the solubilization of FA, retinol, and RA. We further examined which ACS subtype (ACS1, 2, or 3) was expressed, and whether LRAT, which plays an important role in the esterification of vitA, was expressed.

Northern blot hybridizations were performed on total RNA obtained from cultured hepatic stellate cells at different time points. The expression of PPARα, PPARβ, PPARγ, FAT/CD36, ACS1, ACBP, B-FABP, L-FABP, CRBP-I, LRAT, RBP, and CRABP-I mRNAs was analyzed. The expression of PPARα, PPARβ, and PPARγ was evaluated on days 3, 7, 14, 21, and 28 in hepatic stellate cell cultures.

Fig. 1. Expression of PPAR-subtypes under hepatic stellate cell culture conditions. A: RTQ-PCR analysis for the expression of PPARα, β, γ, and ACS2 in hepatic stellate cell cultures. Total RNA was obtained from isolated hepatic stellate cells on days 0, 1, 3, and 7 in culture. Amplification signals were expressed relative to the signal derived from freshly isolated hepatic stellate cells. The insert shows a representative Northern blot analysis result, demonstrating the relative expression levels of PPARα, β, and γ in total liver tissue and in hepatic stellate cells cultured for 3 days. Hybridizations were carried out with rat-specific probes for PPARα, β, and γ using 20 μg (rPPARα and γ) or 5 μg (rPPARβ) of total RNA per lane. B: Representative Northern blot analysis, showing the expression of PPARβ and CD36, ACS1, ACBP, B-FABP, L-FABP, CRBP-I, LRAT, RBP, and CRABP-I mRNAs during hepatic stellate cell culturing. Gene expression was evaluated on days 3, 7, 14, 21, and 28 in hepatic stellate cell cultures.
different moments during their transdifferentiation process (day 0–28, n = 4). As shown by Fig. 1B, PPARβ mRNA expression was transiently induced during hepatic stellate cell culture transition, reaching maximal expression around day 7. As already demonstrated by RTQ-PCR for the ACS2 transcript (vide supra), CD36 mRNA, a PPAR-dependent gene, was induced, closely following the transient rise of PPARβ. Both ACS1 and ACS2 mRNAs were expressed by freshly isolated hepatic stellate cells, but in contrast to ACS2, ACS1 mRNA expression became undetectable when hepatic stellate cells were cultured. The mRNA levels of both brain- and liver-FAs binding proteins (B-FABP and L-FABP) were significantly reduced between days 0 and 3, and remained low throughout culture (days 3 to 28), whereas Acyl-CoA binding protein (ACBP) mRNA was induced between days 0 and 7. Unexpectedly, the mRNA levels of CRBP-I and LRAT, both involved in the esterification of retinol, were induced between days 0 and 3; increased expression was sustained until day 21. CRABP-I mRNA levels were gradually reduced between days 3 and 7 of culture, whereas retinol binding protein (RBP) mRNA expression could only be demonstrated in freshly isolated cells. At all time-points, we were unable to demonstrate significant expression of mRNA for ACS3, CRBP-II, and CRABP-II by Northern blotting. These observations suggest that the induced expression of CD36, ACS2, CRBP-I, and LRAT might reflect a role of these proteins in the physiological alterations related to vitA storage.

**Effect of L165041 on the expression of CD36, FABPs, CRBP, and LRAT**

To reveal whether activation of PPARβ might influence the expression of CD36, B-FABP, CRBP-I, and LRAT, activated hepatic stellate cells were exposed for 24 h to synthetic PPARβ agonist (L165041) (30). Figure 2A shows a representative autoradiograph of the hybridization experiments. Hybridization signals were expressed relative to the control condition (n = 4). The mRNA levels of CD36 and B-FABP were significantly induced after exposure to 1 μM L165041 (+32.4 ± 9.7% and +49.5 ± 7.1%, respectively, P < 0.05). CRBP-I mRNA levels were strongly induced by +60.6 ± 4.1% (P < 0.05). Since the mRNA levels for L-FABP were low at all moments and since they were not influenced by the expression of antisense PPARβ mRNA, we did not further study the effect of L165041 on this FA binding protein. Unexpectedly, the mRNA levels of LRAT were strongly induced by exposure to the PPARγ-specific agonist L165041 (+190.3 ± 92.1%; P < 0.05). These observations indicate that PPARβ might contribute to regulating the expression of both CRBP-I and LRAT during hepatic stellate cell activation under culture conditions.

**Effect of retinol and RAR/RXR-selective agonists on the expression of CRBP-I and LRAT**

The expression of CRBP-I and LRAT in cells and tissues has been demonstrated to depend upon the retinoid status. To further investigate the contribution of PPARβ signaling to inducing the expression of CRBP-I and LRAT, we tested the effect of exposure of 7-day-old hepatic stellate cells to retinol, to retinoic acid receptor (RAR) (AGN191183), and to an RXR-specific agonist (AGN194204) in comparison with the effect of a specific RAR antagonist (AGN193109) (Fig. 2B, C). In line with the presence of an RAR-responsive element (RARE) in the promoter of the CRBP-I gene (31), the mRNA expression of CRBP-I was significantly induced by 1 μM AGN191183 (+57.2 ± 47.0%, P < 0.05) and 5 μM retinol (+23.6 ± 7.3%, P < 0.05) and was reduced by incubation with an RAR antagonist AGN193109 (~30.9 ± 15.8% P < 0.025). Surprisingly, exposure to 25 μM retinol resulted in a significantly re-

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**Fig. 2.** Effect of PPAR activators on the expression of potential downstream genes. A: Representative Northern blots (10 μg total RNA) showing the effect of exposure of activated hepatic stellate cells (day 7) to L165041 (1 μM, 24 h) on the mRNA levels of CD36, B-FABP, CRBP-I, and LRAT. B: Northern blot analysis for CRBP-I. Seven-day-old hepatic stellate cells were exposed (24 h) to retinol (5 μM and 25 μM), a retinoic acid receptor (RAR)-selective agonist (1 μM AGN191183), a retinoid X receptor (RXR)-specific agonist (1 μM, AGN194204), a specific RAR antagonist (1 μM, AGN193109), and the fatty acids (FAs) arachidonic acid (100 μM), linoleic acid (100 μM), palmitate (25 μM), and 2-bromopalmitate (25 μM) on the expression of CRBP-I. C: Northern blot analysis for LRAT. Abbreviations used: Al, AGN191183; AII, AGN 193109; AIII, AGN194204; 5, 5 μM retinol; 25, 25 μM retinol; 2bP, 2-bromopalmitate; PA, palmitate, LA linoleic acid; AA, arachidonic acid. Signals were normalized to 18S and expressed relative to the control condition (n = 3). Data are expressed as mean ± SD. * Significant at P < 0.05 level, ** P < 0.025, *** P < 0.01.
duced expression of CRBP-I ($\text{mean} \pm \text{SD} = 50.4 \pm 26.1\%$, $P < 0.01$). The expression of CRBP-I mRNA was reduced by the RXR agonist AGN194204 ($\text{mean} \pm \text{SD} = 23.0 \pm 16.5\%$, $P < 0.025$). Since storage of vitA depends on esterification with FAs, which are potential precursors for PPAR agonists (32), we evaluated the effect of administration of arachidonic acid, linoleic acid, palmitate, and the nonmetabolizable 2-bromopalmitate. As shown for L165041, administration of 2-bromopalmitate, known to be a potent PPAR$\gamma$ agonist (33), enhanced CRBP-I levels ($\text{mean} \pm \text{SD} = 41.1 \pm 13.0\%$, $P < 0.01$) to the same extent as AGN194204, whereas no significant effect was observed for arachidonic acid ($99.9 \pm 17.5\%$), palmitate ($102.7 \pm 5.3\%$), and linoleate ($88.6 \pm 16.8\%$).

In contrast, the expression of LRAT mRNA was significantly induced after exposure to 5 $\mu$M retinol ($\text{mean} \pm \text{SD} = 33.4 \pm 7.2\%$, $P < 0.025$), AGN191183 ($\text{mean} \pm \text{SD} = 30.2 \pm 15.9\%$), 2-bromopalmitate ($\text{mean} \pm \text{SD} = 41.4 \pm 13.1\%$, $P < 0.025$) and arachidonic acid ($\text{mean} \pm \text{SD} = 43.2 \pm 24.9\%$, $P < 0.05$), palmitate ($\text{mean} \pm \text{SD} = 39.1 \pm 12.3\%$, $P < 0.025$), or linoleic acid ($\text{mean} \pm \text{SD} = 40.3 \pm 20.5\%$, $P < 0.05$) (Fig. 2C). The administration of 25 $\mu$M vitA resulted in a significantly reduced expression of LRAT ($\text{mean} \pm \text{SD} = 49.8 \pm 21.6\%$, $P < 0.01$). Northern blot analysis did not show an effect of the RAR antagonist or RXR specific agonist on the mRNA expression of LRAT ($90.1 \pm 4.2\%$ and $89.5 \pm 10.5\%$, respectively). Thus, the administration of PPAR$\gamma$ and RAR ligands induce the expression of CRBP-I to the same extent, whereas the PPAR$\delta$ ligand L165041 (vide supra) was more effective than retinoids in enhancing LRAT mRNA levels.

**Involvement of PPAR$\gamma$ in the expression of CD36, FABPs, CRBP, and LRAT**

To investigate whether PPAR$\gamma$ signaling might play a role in the gene expression of CD36, FABPs, CRBP, and LRAT in hepatic stellate cells, and since no specific PPAR$\gamma$ antagonists are described, antisense PPAR$\gamma$ mRNA-expressing adenoviruses were used. Previously, we demonstrated that infection of activated hepatic stellate cells (day 7) with these antisense viruses significantly reduced de novo PPAR$\gamma$-protein synthesis (15). In the present study, total mRNA of 7-day-old hepatic stellate cells was extracted 24 h after infection with anti-PPAR$\gamma$ adenovirus (MOI 25) and analyzed for the expression of CD36, B-FABP, L-FABP, CRBP-I, and LRAT mRNAs. Uninfected cells and hepatic stellate cells infected with control adenoviruses (empty adenovirus, GFP-expressing adenovirus) were used as controls. Infection with GFP-expressing viruses was used as internal control to show the percentage of infected hepatic stellate cells. Using MOI 25, more than 95% of the infected hepatic stellate cells expressed GFP (not shown). As shown by Fig. 3, infection with antisense PPAR$\gamma$ mRNA-expressing viruses significantly reduced the mRNA levels of CD36 ($\text{mean} \pm \text{SD} = 82.8 \pm 0.7\%$), B-FABP ($\text{mean} \pm \text{SD} = 56.5 \pm 5.3\%$), and CRBP-I ($\text{mean} \pm \text{SD} = 66.4 \pm 10.0\%$) ($n = 3$, $P < 0.01$) compared with uninfected hepatic stellate cells, whereas no effect could be found on the mRNA levels of L-FABP ($\text{mean} \pm \text{SD} = 115.43 \pm 11.2\%$). Surprisingly, and in contrast to the other genes under investigation, the mRNA levels of LRAT were significantly induced ($\text{mean} \pm \text{SD} = 67.6 \pm 19.1\%$) in hepatic stellate cells infected with empty control viruses ($n = 3$, $P < 0.01$). This observation, which was beyond the scope of this study and therefore not further studied, might indicate that the expression of this gene responds to stress situations such as infection. Compared with uninfected control hepatic stellate cells, however, LRAT mRNA levels were significantly reduced ($\text{mean} \pm \text{SD} = 40.3 \pm 12.5\%$) upon infection with the anti-PPAR$\gamma$ virus in all experiments performed ($n = 3$, $P < 0.01$). Our results obtained with the anti-PPAR$\gamma$ virus suggest that the observed changes in mRNA expression levels could relate to reduced binding of PPAR$\gamma$-RXR dimers to the respective promoter regions.

**Promoter analysis of CRBP-I**

The effects of L165041 and antisense-PPAR$\gamma$ mRNA suggested that the observed changes in the CRBP-I mRNA level could relate to a reduced binding of PPAR$\gamma$-RXR dimers to its promoter region. Analysis of the sequence of the mouse and rat CRBP-I promoter using Genomatix MatInspector calculated the presence of two imperfect DR-1 elements that were potential PPREs (defined PPRE1 and PPRE2) (Acc.nr. S45496 and X60367) (Fig. 4A). In contrast to the previously described RARE (31), these predicted rodent PPREs were not conserved in the human CRBP-I promoter. The presence of an hPPRE, which was not conserved in rodents, was predicted at a position close to the transcription start site. The ability of the potential rodent PPREs and the DR2-like RARE to bind PPAR-RXR het-
Fig. 4. Potential PPAR-responsive elements (PPREs) in the CRBP-I promoter. A: Alignment of the rat, mouse and human CRBP-I promoter showing the presence of predicted rodent PPREs (PPRE1, PPRE2) and RAR-responsive element (RARE). The numbers refer to the position relative to transcription start site. PPRE1 and PPRE2 are present in both the rat and mouse promoters, whereas the RARE is conserved between humans and rodents. B: Electrophoretic mobility-shift assay using oligonucleotides corresponding to the consensus PPRE oligonucleotide of the L-FABP promoter (C) and rPPRE1 (1), rPPRE2 (2), and rRARE (A), and in vitro-translated PPAR-RXR proteins and nuclear hepatic stellate cell extracts. C: Competition analysis. Reticulocyte lysates were incubated with labeled consensus oligonucleotides for 20 min, and competition analysis was performed by adding increasing amounts of unlabeled homologous (C) or heterologous (PPRE1-2, RARE, ASP) oligo to the reaction mixture with an additional incubation of 20 min.
effects of LCFA, and PPARα and RXR, and nuclear hepatic stellate cell extracts. There was no significant binding of in vitro-translated PPARs (PPARα and β), RXR, and nuclear hepatic stellate cell extracts. There was no significant binding of in vitro-translated PPARs in the absence of RXR. In contrast, in the presence of RXR there was binding of both PPARβ and PPARα to these oligonucleotides, as shown by the appearance of a slower mobility protein-DNA complex (Fig. 4B, results for PPARα not shown). To investigate whether binding of PPARβ-RXR to these sequence elements was specific, competition analysis was performed using the previously described consensus PPRE of the L-FABP promoter (denoted C) (34). Reticulocyte lysates were incubated with labeled consensus oligonucleotide for 20 min, and competition analysis was performed by adding increasing amounts of unlabeled homologous (C) or heterologous (PPRE1-2, RARE, ASP) oligo to the reaction mixture with an additional incubation of 20 min. Within this time period, RARE and both PPRE1 and PPRE2 were able to displace binding of the PPARβ-RXR heterodimer from the consensus sequence (Fig. 4C). Cold DR2 RARE oligonucleotide competed more efficiently than cold DR1 PPRE1 and 2 for binding of the PPAR-RXR dimer. As shown in Fig. 4B, the predicted PPREs and RARE also bound nuclear proteins extracted from 7-day-old hepatic stellate cells. The pattern of the retarded bands (two or more closely comigrating bands) appeared to be more complex than those generated by in vitro-translated proteins. This complexity could be the consequence of the presence in the nuclear extracts of other proteins, i.e., various RARs, RXRs, HNF-4, and/or COUPs, able to bind to these direct repeat elements. This was confirmed by competition and supershift analysis (Fig. 5). Nuclear hepatic stellate cell extracts were incubated with RARE or consensus PPRE. Addition of excess unlabeled homologous oligonucleotide abolished complex formation, whereas only one of both DNA-protein complexes was selectively displaced after administration of heterologous oligo. Addition of antibodies directed against RARβ, PPARβ, and RXRs selectively disrupted complex formation of only one of the two retarded bands. PPARβ-RXR heterodimers might thus compete with RAR-RXR dimers for binding to regulatory regions in the CRBP-I promoter.

Effects of specific PPARβ, RAR, and RXR ligands on hepatic stellate cell phenotype

Under normal culture conditions, hepatic stellate cells are described to gradually loose their vitA reserves (see also Fig. 6A). Several authors have shown that administration of retinol and/or retinoic acids (RAs) could induce de novo lipid droplet formation in activated hepatic stellate cells (6, 35). These changes were accompanied by decreased proliferation and delay of activation (36–38). PPARβ has been shown to play a central role in FA-controlled differentiation of preadipocytes (39). To evaluate the effect of PPARβ activation on the vitA-storing hepatic stellate cells phenotype, we studied the effect of retinol-, RAR-, and RXR-specific agonists (AGN191183 and AGN194204), different LCFA, and PPARβ agonists (2-bromopalmitate, L165041) on the phenotype of 7-day-old hepatic stellate cells. At this time-point, control cultures contained few lipid droplets, which are sustained up to day 28. Activated, 7-day-old cells were exposed for 24 h to the different compounds. Lipid droplets were visualized by phase contrast microscopy. PPARβ expression was shown by immunocytochemistry. As shown by vitA autofluorescence (excitation light of 326 nm) and by phase contrast microscopy (Fig. 6C and insert), exposure to (10 μM to 25 μM) retinol resulted in the formation of new cytoplasmatic lipid droplets, whereas lower concentrations (1 μM to 5 μM) failed to induce lipid droplets (results not shown). No phenotypical changes could be observed after exposure to different concentrations (10 μM and 1 μM) of the RAR- and RXR-selective agonists AGN191183 and AGN194204 and after exposure to 2 bromopalmitate (25 μM, not shown) and L165041 (1 μM, Fig. 6D), whereas the formation of numerous small lipid droplets surrounding the nucleus was induced after incubation with LCFA (Fig. 6E–G). The morphology of these newly formed droplets resembled the physical appearance and distribution of the vitA-rich droplets present in quiescent cells (Fig. 6A). By applying autofluorescence, the presence of retinol in these droplets was demonstrated. No further increase in the number, size, and distribution of these droplets was found after administration of retinol in combination with FA and or L165041. Biochemical analysis should be performed to quantify and evaluate the composition of the lipid droplets after this combined treatment. From our observations, we hypothesize that activated hepatic stellate cells remain capable of synthesizing and storing vitA esters and depend mainly on the administration of LCFA to the culture media. The vitA-storing hepatic stellate cell phenotype, however, could not be restored by the administration of synthetic PPAR, RAR, and RXR agonists.

Effects of LCFA and a specific PPARβ ligand on PPARβ expression

To reveal whether the phenotypical effects observed after exposure of activated hepatic stellate cells to LCFA and L165041 might reflect changes in PPARα, β, and γ expression levels, immunoprecipitations were performed. TriPLICATE cultures of 7-day-old hepatic stellate cells were exposed to different compounds for 24 h and metabolically labeled with trans [35S]methionine/cysteine.

Figure 7A shows a representative immunoprecipitation result, using 10 μM and 1 μM L165041, linoleic acid, arachidonic acid, and palmitate. Control cells were treated with the same concentration of vehicle (DMSO and ethanol, respectively). Immunoprecipitations were performed using a rabbit anti-mouse PPARβ antibody. The de novo protein synthesis of PPARβ was significantly induced after exposure to arachidonic acid (+98 ± 25%), linoleic acid (+77 ± 29%), and palmitate (+89 ± 10%) (n = 5, P < 0.05) (Fig. 7B). No changes, however, were found after exposure to the PPARβ-specific ligand L165041. Northern blot analysis showed that the expression of PPARα and γ was not induced by one of these conditions. Administration of specific ligands for these receptors (clofibrate and ciglitizone, respectively) failed to induce PPARα and/or γ expression.
Lipid droplet formation in antisense-PPARβ-infected cells

Exposure to different PPARβ agonists resulted in changed expression levels of CD36, B-FABP, CRBP-I, and LRAT, but failed to induce the formation of new lipid-vitA droplets in activated hepatic stellate cells. To investigate whether PPARβ might play a direct role in lipid droplet formation, activated 7-day-old hepatic stellate cells were infected with empty control viruses (Fig. 8A) or antisense PPARβ RNA viruses. Twenty-four hours after infection, cells were incubated with a single dose of arachidonic acid, linoleic acid, or L165041 for 48 h. As shown in Fig. 8B, control cells infected with the antisense PPARβ virus obtained an elongated shape, showed a reduced number of processes, and were devoid of lipid droplets. These changes were reversed after exposure to LCFA, and new lipid droplets were formed (Fig. 8D–F). Addition of L165041 failed to restore the lipid-storing phenotype of antisense PPARβ-mRNA infected hepatic stellate cells.

Expression of PPARs during high- or low-vitA fibrogenesis

CCL4-induced liver fibrogenesis has been shown to be associated with reduced levels of retinol, whereas the RE levels remain unaffected (40). To investigate whether in vivo activated hepatic stellate cells remain capable of storing retinol, liver fibrosis was induced in rats by CCL4 treatment. One group of rats (n = 5) was administered CCL4, whereas a second group received a combination of CCL4 and retinol. Control rats were injected with vehicle (paraffin, paraffin-sunflower oil) or retinol. Fibrogenesis was more severe in rats exposed to retinol and CCL4 compared with rats treated with CCL4 only (Fig. 9A, B). In control livers, vitA autofluorescence was weak and quickly faded away. After addition of retinol, vitA autofluorescent spots were detected mainly perportal. CCL4 exposure caused a serious increase in bright autofluorescent sites both in rats exposed to CCL4 (Fig. 9C–F) and in rats receiving the combination of retinol and CCL4 (Fig. 9G, H). VitA autofluorescence was mainly found in periportal areas and in cells at the edges of the developing fibrotic septa. VitA-storing cells were identified as hepatic stellate cells by immunocytochemistry for PPARβ, desmin, GFAP, and α-smooth muscle actin. In comparison with rats treated with CCL4, more vitA autofluorescence was found in the septal fibrotic areas in liver tissue from vitA/CCL4-treated animals. A close association was found between vitA autofluorescence and signals derived for PPARβ. These results demonstrate hepatic stellate cells activated in vivo by CCL4 injury remain capable of storing vitA, and that vitA storage during fibrogenesis can be induced by simultaneous treatment with vitA. By Northern blot analysis, we showed that the expression of PPARα, β, and CRBP-I mRNA was induced in CCL4-treated animals and became further enhanced by the coadministration of retinol (Fig. 10). No changes were observed in the control animals exposed to vehicle or retinol. The expression of PPARγ, however, which was poorly detectable in normal liver tissue, was significantly reduced in CCL4-induced fibrotic livers. In contrast, its expression was elevated in animals administered sunflower oil and retinol dissolved in sunflower oil.

DISCUSSION

PPARβ has recently been associated with various biological functions, including regulation of the cholesterol and lipid metabolism, oligodendrocyte differentiation, embryo implantation, and development of colorectal cancer (17, 41–44). Moreover, PPARβ has been shown to play a critical role in the response of keratinocytes to inflamma-
tion by controlling the balance between cell death and proliferation (45). Enhanced PPARβ expression during hepatic stellate cell transdifferentiation was therefore suggestive of a regulatory role of the receptor in their activation process. Alternatively, it might also reflect a response of the activated hepatic stellate cells to their altered vitA-lipid status. The objective of the current study was to evaluate the expression of a number of genes directly or indirectly involved in the mechanisms behind retinol esterification and storage using the hepatic stellate cell as an

Fig. 6. Vitamin A (vitA) storage in activated hepatic stellate cells. Phase contrast microscopy (A) showing the disappearance of the vitA-rich lipid droplets during activation of hepatic stellate cells under culture conditions (day 1 to day 7). Fluorescence microscopy (326 nm) and phase contrast microscopy (inserts) showing the presence of vitA-rich droplets in 7-day-old control hepatic stellate cells (B) and in cells treated with retinol (25 μM) (C), L165041 (1 μM) (D), arachidonic acid (100 μM) (E), linoleic acid (100 μM) (F), or palmitate (10 μM) (G) (100 μm bar scale). Immunocytochemistry was performed to show PPARβ expression.
experimental model expressing high levels of PPARβ, whereas PPARα and PPARγ remain barely detectable.

PPARβ has been shown to regulate the expression of membrane-bound FAT/CD36 (46) and ACS2 (16), thereby facilitating both uptake and metabolism of lipids by converting FA into acyl-CoA derivatives. During hepatic stellate cell activation, we observed a strong correlation between the mRNA expression profiles of CD36, ACS2, and PPARβ. Moreover, the transcription of the CD36 gene was significantly inhibited after infection with antisense PPARβ mRNA-expressing viruses. It is not known whether the promoter of the ACS2 gene contains a PPRE, but functional PPREs were identified in the promoter of the CD36 (47) and ACS1 genes (48). Our results are in accordance with the observations of Basu-Modak et al. (16), showing that PPARβ expression in reaggregated rat brain cell cultures overlaps with ACS2, but not with ACS1 and ACS3.

Uptake and utilization of FA is influenced by a family of FABPs, of which several members have been shown to contain PPREs in their promoter regions. Some FABPs display ligand selectivities that closely resemble those of nuclear receptors such as PPARs and RARs and are shown to function as a gateway to deliver lipid soluble ligands to these receptors (49–51). During hepatic stellate cell culture, the mRNA levels of B-FABP and L-FABP were reduced and antisense PPARβ mRNA expression had no effect on L-FABP mRNA levels. L-FABP has been shown to interact with

Fig. 7. Effect of L165041 and FA on PPARβ expression. Hepatic stellate cells (day 7) were exposed to L165041 and different FAs. For immunoprecipitation analysis, cells were metabolically labeled the last 24 h with [35S]methionine translabel. A: Representative immunoprecipitation result showing the effect of exposure of activated hepatic stellate cells (day 7) to L165041 (10 μM and 1 μM) and different FAs (C, vehicle; LA: 100 μM linoleic acid; AA, 100 μM arachidonic acid; PA, 25 μM palmitate) on the expression of PPARβ mRNA. Cell lysates were immunoprecipitated with primary antibodies against PPARβ. Immunoprecipitates were subjected to 5% SDS-PAGE under reducing conditions. B: Quantitative analysis of immunoprecipitated bands. The amounts of immunoprecipitable protein were calculated per 10^6 cells. The ratios of treated versus control were calculated. Data are expressed as mean ± SD. The number of observations (n) was five. * Significant at P < 0.05 level.

Fig. 8. Phase-contrast microscopy evaluating the phenotypical effects of viral antisense PPARβ mRNA expression. Twenty-four hours after infection with empty control virus (A, C, E, G) or antisense PPARβ viruses (B, D, F, H), cells were exposed to different FAs [Arachidonic acid (100 μM) (C, D), linoleic acid (100 μM) (E, F)] and L165041 for an additional 24 h.
PPARα and γ, but not with PPARβ (50). In contrast, the expression of CRBP-I, another FABP-family member, was induced during hepatic stellate cell activation (52), thereby following the rise of PPARβ. Antisense PPARβ viruses reduced CRBP-I transcript levels significantly. Analysis of the rodent CRBP-I promoter predicted two potential PPREs, which were shown to bind PPAR-RXR dimers in electrophoretic mobility assays. Competition analysis showed that PPARβ-RXR binding to a consensus DR1-like PPRE was actively competed for binding to the DR2-like
serves under culture conditions, however, we observed a decrease in LRAT expression. During the depletion of the hepatic stellate cells, vitA remnant retinol available for delivery to peripheral tissues (70) is conserved, and functional curtailment of LRAT is believed to maintain vitA homeostasis in transdifferentiating hepatic stellate cells.

In liver, probably due to impaired delivery of retinol to LA (64). LRAT, the expression of which depends on the retinoid status (65, 66) and responds to RAR-selective retinoids (67), requires RBP-bound vitA as a substrate for optimal activity (68, 69). In the liver of vitA-deficient animals, LRAT expression was virtually absent (65). This functional curtailment of LRAT is believed to maintain retinol available for delivery to peripheral tissues (70). During the depletion of the hepatic stellate cells, vitA reserves under culture conditions, however, we observed a strong induction of LRAT mRNA levels and exposure of activated hepatic stellate cells to L165041 upregulated LRAT mRNA levels more effectively than retinoids. PPARβ might thus not only regulate CRBP-I transcription, but could also be involved in the regulation of LRAT. This is also supported by our observations showing a significant reduction of LRAT after viral antisense PPARβ mRNA expression. The sequence of the LRAT promoter and the presence of nuclear receptor-responsive elements remain to be determined.

The induction of CRBP-I and LRAT mRNAs during hepatic stellate cell activation both involved in retinol esterification, and is in contrast with the simultaneous depletion of their RE reserves. Our observations might reflect a compensatory mechanism during which the cultured cells try to rescue their vitA stores. This interpretation is supported by several observations: 1) small lipid droplets remain present in the cytoplasm of hepatic stellate cells during prolonged culture (up to 28 days); 2) by the rapid formation of RE-rich lipid droplets after LCFA or retinol administration; and 3) by the reduced expression of CRBP-I and LRAT when higher concentrations of retinol were administered. De novo VitA storage did not require additional treatment with retinol to the cultures. Apparently, the amount of vitA bound to RBP and/or albumin present in the 10% fetal bovine serum-enriched media was sufficient for the formation of new vitA-rich droplets after the exposure to LCFA only. Enhanced RE storage in hepatic stellate cell cultures after addition of FA has been reported previously (6, 71, 72). Cultured stellate cells preferentially take up and esterify RBP-bound retinol over free retinol (6). Thus, in contradiction to what is generally believed, cultured hepatic stellate cells seem not to lose the capacity to take up and esterify retinol, as reflected by the induced expression of CRBP-I and LRAT and by the presence of autofluorescent vitA droplets. The capacity to sustain their cytoplasmic RE reserves during prolonged culture, however, seems to become inaccurate. The above observations suggest that retinoid depletion results from an inability of the activated cells to convey FAs rather than their retinol-esterification pathway becoming impaired. This might reflect changes in the expression levels of proteins that take-up and/or channel FA and retinoids, such as the decreased expression of PPARγ, FABPs, ACS-I, RARs, and CRABP-I during hepatic stellate cell culture. Expression of antisense PPARβ mRNA resulted in a phenotype devoid of lipid droplets, but could not prevent the formation of new lipid droplets after addition of LCFA. In this context, however, it is also appropriate to emphasize that our study describes phenotypical changes evaluated by phase-contrast and fluorescence microscopy, and that LCFA was added to the media dissolved in ethanol. FAs added in such a manner may, next to their presumed interaction with CD36 and FABPs, be taken up in an unphysiological way.

Although the pathways behind vitA-depletion during hepatic stellate cell activation are not yet elucidated, ester hydrolysis occurs intracellularly, releasing retinol and FA (6, 73). Previous studies have reported that hepatic stel-
late cells contained trace amounts of RBP mRNA and/or protein (74). In this study, we failed to demonstrate RBP mRNA expression in cultured hepatic stellate cells, whereas a relatively strong signal was found in freshly isolated cells. If RBP mRNA is present in stellate cells and its expression is immediately lost upon culture, this might link to the altered metabolism and/or mobilization of the retinoid stores. In contrast, it was argued previously that the detection of RBP protein and mRNA in stellate cell isolates might reflect contaminating hepatocyte-derived debris (73, 75).

Free retinol could also be converted into RA (76). This alternative, however, seems unlikely since hepatic stellate cells in vitro rapidly lose their capacity to respond to retinoids, which is reflected by decreased retinoid acid and RA levels (52, 53). Here, we show that CRABP-I mRNA levels, after a transient rise between days 0 and 3, were reduced at later time-points. Functional assays have shown that the liver contains several distinct RE hydrolases (REHs) (77). In hepatic stellate cells, however, these enzymes have not been identified (73, 78) and molecular probes remain poorly available. Their enzymatic activities that are independent of the retinoid status (78) are 10- to 30-fold faster than esterification by LRAT and/or ARAT (64, 76). VitA depletion in activated hepatic stellate cells might thus be driven by REH activity. Further studies combining both descriptive and biochemical analysis are required to elucidate at what level hepatic stellate cells fail to sustain their vitA reserves and what the precise role of PPARβ is.

Although there is strong evidence that retinoids regulate hepatic stellate cell proliferation and collagen synthesis in culture, in vivo studies on the relationship between vitA and liver fibrosis are conflicting (79). The present in vivo microscopic study confirms the results of previous studies on vitA potentiation of CCl4-induced liver injury (40, 79, 80). Fibrotic liver disorders are commonly associated with reduced serum and liver vitA levels. It remains, however, poorly documented whether the development of vitA deficiency reflects an actual depletion of the RE reserves of the activated myofibroblast-like hepatic stellate cells (81, 82). Recently, it was shown that in animals fed a vitA-depleted diet, CCl4 exposure induced a significant increase of the total liver RE levels compared with non-CCl4-treated controls (40). In agreement with our observations showing colocalisation of vitA autofluorescence with PPARβ, desmin-, GFAP-, and αSMA-positive hepatic stellate cells, the authors showed an increase of bright vitA autofluorescent sites after exposure to CCl4 in rats fed both a high- or low-vitA diet. Uchig et al. (83) have recently shown that activated hepatic stellate cells maintain high CRBP-I protein levels in culture and during CCl4-induced liver fibrosis. In our study, chronic CCl4 injury resulted in elevated levels of PPARβ and CRBP-I. Enhanced CRBP-I expression during wound healing phenomena in endothelia, skin, and liver has been indicated as a marker for activation and differentiation of specialized myofibroblastic cells (84, 85). Interestingly, skin wound closure is delayed in PPARβ± animals as compared with wild-type animals (86).

In conclusion, the changed expression of proteins involved in FA and retinol uptake and storage during hepatic stellate cell activation was evaluated. PPARβ signalling might affect the expression of CD36, involved in FA uptake and activation, and CRBP-I and LRAT, involved in retinoid binding and esterification. Our results provide a basis to further study the mechanisms behind vitA-storage and depletion in activated hepatic stellate cells.

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