Genetic dissection of retinoid esterification and accumulation in the liver and adipose tissue

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Abstract
Approximately 80–90% of all retinoids in the body are stored as retinyl esters (REs) in the liver. Adipose tissue also contributes significantly to RE storage. The present studies, employing genetic and nutritional interventions, explored factors that are responsible for regulating RE accumulation in the liver and adipose tissue and how these influence levels of retinoic acid (RA) and RA-responsive gene expression. Our data establish that acyl-CoA:retinol acyltransferase (ARAT) activity is not involved in RE synthesis in the liver, even when mice are nutritionally stressed by feeding a 25-fold excess retinol diet or upon ablation of cellular retinol-binding protein type I (CRBP1), which is proposed to limit retinol availability to ARATs. Unlike the liver, where lecithin:retinol acyltransferase (LRAT) is responsible for all RE synthesis, this is not true for adipose tissue where Lrat-deficient mice display significantly elevated RE concentrations. However, when Crbp1 is also absent, RE levels resemble wild-type levels, suggesting a role for Crbp1 in RE accumulation in adipose tissue.

Supplementary key words diacylglycerol acyltransferase 1 • cellular retinol-binding protein type I • 9-cis-retinoic acid or 9-cis-RA • retinol-binding protein or RBP4

Retinoids (vitamin A and its analogs) are important transcriptional regulators that act primarily through three nuclear hormone receptors, retinoic acid receptor (RAR)α, RARβ, and RARγ, to modulate the activities of more than 500 genes (1–3). There is also some, albeit controversial, evidence that retinoic acid (RA) is a physiological ligand contributing importantly to the regulation of peroxisome proliferator-activated receptor-δ (PPARδ)-mediated gene expression (4, 5). The great majority of retinoids present in a healthy well-nourished vertebrate are in the form of retinyl esters (REs) (6–8). REs are also found in the postprandial circulation, where they are present in chylomicrons and chylomicron remnants, and in the fasting circulation, where they are present at relatively low levels in very low density lipoproteins (VLDLs) (6–8). Many tissues have some capacity to synthesize REs from retinol, but REs are most abundant in the liver where approximately 80–90% of the body’s retinoids are found, primarily in hepatic stellate cells (7–10). REs are also relatively abundant in the eyes, lungs, skin, and adipose tissue (7–10). In times of insufficient dietary vitamin A intake, RE stores undergo enzymatic hydrolysis to retinol which is then secreted into the circulation bound to retinol-binding protein (RBP4) (6–10). The accumulation of RE stores within the liver and other tissues relieves the organism from the obligation to acquire this essential micronutrient regularly from its diet; thus providing an evolutionary advantage to the organism.

The literature, based on in vitro studies, indicates that at least two distinct enzymatic activities present in the liver...
and other tissues are able to catalyze RE formation; one involves an acyl-CoA-dependent mechanism and the other an acyl-CoA-independent process (11–15). The acyl-CoA-dependent mechanism is attributed to an acyl-CoA:retinol acyltransferase (ARAT) activity (9–12). The acyl-CoA-independent synthesis of REs was shown early on to involve the transsterification of a fatty acyl group from the sn-1 position of a membrane phosphatidylcholine to retinol, and the enzyme responsible for this transsterification was termed lecithin:retinol acyltransferase (LRAT) (13–15).

Studies of Lrat-deficient (Lrat−/−) mice have established that LRAT is responsible for most, but not all, REs synthesized in the body (16–18). Very few REs can be detected in the liver, eyes, or lungs of Lrat−/− mice fed a control chow diet (17). The sole tissue where substantial REs accumulate in Lrat−/− mice is adipose tissue, where concentrations of REs are elevated by 2- to 3-fold over those measured in matched wild-type (WT) mice (17, 18).

Several groups have reported in vitro studies demonstrating that recombinant diacylglycerol acyltransferase 1 (DGAT1), an enzyme that catalyzes the formation of triglyceride from diglycerides and fatty acyl-CoAs (19–21), also catalyzes the acyl-CoA-dependent esterification of retinol (17, 22, 23). Thus, DGAT1 possesses ARAT activity. Subsequently, through investigations of both CRBPI and CRBPIII (28, 30, 31). Moreover, the literature suggests that ARAT activities may become active logically in catalyzing RE synthesis in mouse skin.

Numerical manipulations

For nutritional studies, mice were maintained on a chow diet (25 IU of retinol/g of diet) until they reached approximately 3 months of age. At 3 months of age, mice were randomized onto one of two different diets: 1) a retinoid-sufficient (basal) diet providing 22 IU of retinol/g diet (Test Diet, 5755, St. Louis, MO); or 2) a retinoid-excess diet containing 25 times the amount of retinol in the basal diet or 550 IU of retinol/g diet. These purified diets were formulated otherwise according to the AIN-93M formulation (38). Each diet was nutritionally complete for all other nutrients and only differed in their retinol content. Depending on the specific experiment, mice were maintained continuously on one of these diets for up to 12 weeks prior to sacrifice. For these nutritional studies, diet and water were provided to all animals on an ad libitum basis until the time of sacrifice.

HPLC analysis of retinol and REs

Tissue and serum retinol and RE levels were determined by HPLC protocols described previously (24). Briefly, serum, liver, and epididymal adipose tissue were flash-frozen in liquid N2 after dissection and stored immediately at −80°C prior to analysis. Tissues were homogenized in 10 vol of PBS [10 mM sodium phosphate (pH 7.2), 150 mM sodium chloride] using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) set at half-maximal speed for 10 s. An aliquot of serum, the total adipose tissue, or a 200 μl aliquot of the liver homogenate was then treated with an equal volume of absolute ethanol containing a known amount of retinyl acetate as an internal standard. The retinoids present in the homogenates were extracted into hexane. After one backwash against doubly distilled water, the hexane extract was evaporated to dryness under a gentle stream of N2. Immediately upon reaching dryness, the retinoid-containing film was redissolved in 40 μl of benzene for injection onto the HPLC column. The extracted retinoids were separated on a 4.6 × 250 mm Ultrasphere C18 column (Beckmann, Fullerton, CA) preceded by a C18 guard column (Supelco, Bellefonte, PA) using 70% acetonitrile-15% methanol-15% methylene chloride as the running solvent flowing at 1.8 ml/min. Retinol and REs (retinyl palmitate, oleate, linoleate, and stearate) were detected at 325 nm and identified by comparing the retention times and spectral data of experimental compounds with those of authentic standards. Concentrations of retinol and REs in the tissues were quantitated by comparing integrated peak areas of each retinoid against those of known amounts of purified standards. Loss during extraction was accounted for by adjusting for the recovery of internal standard added immediately after homogenization of the samples.

LC/MS/MS analysis of RA

Serum and tissue levels of all-trans-RA were determined by ultra-high-performance liquid chromatography tandem mass spectrometry (LC/MS/MS) using a Waters Xevo TQ MS ACQUITY UPLC system (Waters, Milford, MA). For this analysis, we only employed
LC/MS grade acetonitrile and LC/MS grade water purchased from Thermo Fisher (Pittsburgh, PA). All-trans- and 9-cis-RA were purchased from Sigma-Aldrich. Penta-deuterated all-trans-RA was employed as an internal standard and was purchased from Toronto Research Chemicals (North York, Ontario, Canada). Retinoid concentrations were verified spectrophotometrically using published \( \epsilon \) values (39). Tissue homogenates were extracted using the two-step acid-base extraction described by Kane et al. (40). All-trans-RA was detected and quantified using the multiple reaction monitoring mode employing the following transitions: all-trans-RA, \( m/z \) 301.16→123.00; penta-deuterated all-trans-RA, \( m/z \) 306.15→127.03; and 9-cis-RA, \( m/z \) 301.16→123.00.

**Triglyceride analysis**

Triglyceride concentrations were determined enzymatically using a commercial colorimetric triglyceride kit (Wako), according to the manufacturer’s instructions.

**RNA isolation, reverse transcription, and qualitative real-time PCR**

Total RNA from the liver was isolated using the RNA-Bee (Tel-Test) reagent according to the manufacturer’s instructions. Potential contaminating genomic DNA present in the liver RNA isolates was removed by DNase treatment and chromatography on RNeasy columns (Qiagen). Reverse transcription was performed using random hexamer primers to generate cDNAs according to the supplier’s instructions (Invitrogen). Quantitative polymerase chain reaction (qPCR) was performed for 40 cycles for 15 s at 95°C and 60 s at 60°C using an ABI 7000 sequence detection system (Applied Biosystems). TaqMan probes and primers for *Ppara*, *Pparγ*, *Pparδ*, *Pparβ*, *Chrebp*, *Fas*, *Scd1*, *Acc*, *Cpt1l*, *Dgat1*, *Dgat2*, *Lrat*, *Rarβ* isoform 2 (*Rarβ2*), cytochrome 26A1 (Cyp26A1), cytochrome 26B1 (Cyp26B1), cellular-retinoic acid-binding protein type I (CrbpI), CrbpII, and *18S* transcripts were designed by and obtained from ABI (Applied Biosystems). Quantification of mRNA levels was performed by comparing the \( C_T \) value of each sample to a standard curve generated by serial dilution of the appropriate tissue cDNA. For each of these standard curves, the correlation coefficients were 0.99 or greater. Values are normalized to *18S* rRNA levels.

**Hepatic VLDL production and triglyceride analysis**

To assess the roles or effects of LRAT, Dgat1, and RBP4 in facilitating RE incorporation into nascent VLDLs, mice were fasted for 4 h and then injected with the total lipase inhibitor P-407, at 1 mg/g body weight by ip injection (41, 42). Immediately prior to injection (0 h) and 6 h after injection (a time previously shown to assure a linear rate of triglyceride accumulation in P-407-treated mice (43), serum was obtained and processed for retinoid analysis by HPLC and triglyceride analysis as described above.

**Statistical analyses**

All data were analyzed for statistically significant differences using standard procedures consisting of an unpaired \( t \)-test for comparisons of two groups or an ANOVA followed by post hoc analysis if more than two groups of mice were being compared.

**RESULTS**

The literature has long indicated that an acyl-CoA-dependent enzymatic activity, an ARAT, present in liver homogenates, can catalyze synthesis of REs (9–12). Dgat1, which is expressed in the liver, has been shown to be a physiologically significant ARAT in the intestine and skin (24, 25). It also has been proposed in the literature that ARAT activities can contribute to RE synthesis when retinol is present in excess of normal amounts (27–29). We investigated these possibilities in matched male WT, *Lrat\(^{-/-}\)*, *Dgat1\(^{-/-}\)*, and *Lrat\(^{-/-}\)/Dgat1\(^{-/-}\)* mice fed a diet containing a 25-fold excess of retinol compared with standard dietary levels for 4 weeks. However, we were unable to detect substantial RE concentrations in the livers of *Lrat\(^{-/-}\)* or *Lrat\(^{-/-}\)/Dgat1\(^{-/-}\)* mice (Table 1). This is contrary to what has been reported in the literature by Yamaguchi et al., who proposed, based on cell culture studies, that Dgat1 is the major contributor to the ARAT activity contributing to RE formation in hepatic stellate cells (44), the cellular site for RE storage in the liver (7, 8, 10). These investigators also reported that ablation of Dgat1 expression in cultured cells using antisense oligonucleotides results in increased expression of *Lrat* (44). We were unable to confirm this published finding in our studies of *Dgat1\(^{-/-}\)* mice. *Lrat* mRNA levels assessed by qPCR for matched WT and Dgat1\(^{-/-}\) livers were identical (Fig. 1A). Similarly, *Dgat1* mRNA levels were not different for WT and *Lrat\(^{-/-}\)* livers (Fig. 1B). We also attempted to confirm the published studies of Yamaguchi et al. (44) in vivo, using adenovirus constructs to rescue RE synthesis in *Lrat\(^{-/-}\)* or *Lrat\(^{-/-}\)/Dgat1\(^{-/-}\)* mice. However, adenovirus rescue vectors injected into the circulation of these mice were cleared predominantly by hepatocytes with very little being taken up by hepatic stellate cells, the cellular site of retinoid storage in the liver. Consequently, it was not possible to use this standard approach for rescuing hepatic *Lrat* expression to further validate our findings from nutritional and genetic studies.

The literature indicates that Dgat1 contributes to triglyceride-rich lipoprotein (VLDL) secretion from hepatocytes (45, 46). Because REs are present in VLDLs, we asked whether Dgat1 might act to facilitate RE incorporation into VLDLs. Figure 2 provides evidence that LRAT is responsible for the synthesis of most REs that are incorporated into VLDLs and secreted from the liver. When RE concentrations were normalized for VLDL triglyceride levels, these concentrations were not different for WT or Dgat1\(^{-/-}\) mice. Very little RE was detected in VLDLs obtained from *Lrat\(^{-/-}\)* mice. Thus, LRAT-catalyzed RE expression appears to be primarily responsible for most of the triglyceride-rich lipoprotein (VLDL) secretion from hepatocytes (45, 46).

**Table 1**. Hepatic RE concentrations for 3-month-old male WT, *Lrat\(^{-/-}\)*, *Lrat\(^{-/-}\)/Dgat1\(^{-/-}\)*, *CrbpI\(^{-/-}\)*, and *Lrat\(^{-/-}\)/CrbpI\(^{-/-}\)* mixed C57Bl/6J/129sv genetic background mice

| Strain                  | n | Hepatic RE (n mole/g tissue) |
|------------------------|---|-----------------------------|
| WT                     | 5 | 4272.0 ± 828.0              |
| *Lrat\(^{-/-}\)*       | 4 | 0.1 ± 0.1 \(^{a,b}\)       |
| *Lrat\(^{-/-}\)/Dgat1\(^{-/-}\)* | 4 | 0.1 ± 0.1 \(^{a,b}\)       |
| *CrbpI\(^{-/-}\)*      | 5 | 679.5 ± 205.8 \(^{c}\)     |
| *Lrat\(^{-/-}\)/CrbpI\(^{-/-}\)* | 5 | 5.0 ± 3.1 \(^{c}\)       |

Mice were maintained for 4 weeks on a diet providing 25 times more retinol than a standard vitamin A-sufficient basal diet. Prior to being placed on the excess-retinol diet, all mice were maintained on a weaning on a standard vitamin A-sufficient chow diet. All values are given as mean ± SD.

\(^{a}\)P < 0.01 different from WT mice.

\(^{b}\)P < 0.05 different from *CrbpI\(^{-/-}\)* mice.

\(^{c}\)P < 0.05 different from *Lrat\(^{-/-}\)* mice.
REs that are incorporated into VLDLs. Interestingly, mice totally lacking expression of \textit{Rbp4}, and hence unable to mobilize hepatic retinol (36), are able to mobilize REs from the liver bound to VLDL at levels that are identical to those of WT mice (Fig. 2).

Cellular retinol-binding proteins, like CRBPI, which is highly expressed in the liver, have been proposed to sequester retinol and prevent it from being acted upon by ARAT activities (27–29). To address whether this might account for our inability to demonstrate the existence of a hepatic \textit{ARAT} in vivo, we conventionally bred \textit{Lrat}\textsuperscript{−/−} mice to generate mice deficient in both genes, \textit{CrbpI}\textsuperscript{−/−} and \textit{Dgat1}\textsuperscript{−/−} mice. Moreover, expression levels for a number of RA-inducible genes are likely elevated in the livers of these mutant mice.

We were able to confirm this finding and able to extend it to \textit{CrbpI}\textsuperscript{−/−} and \textit{Lrat}\textsuperscript{−/−}/\textit{CrbpI}\textsuperscript{−/−} mice, which also showed elevated levels of \textit{Cyp26A1} mRNA (Fig. 4A). In addition to elevated expression of \textit{Cyp26A1}, we observed statistically significant elevations in hepatic expression of another RA-inducible transcript, \textit{Rasβ2}, for \textit{Lrat}\textsuperscript{−/−} and \textit{Lrat}\textsuperscript{−/−}/\textit{CrbpI}\textsuperscript{−/−} mice (Fig. 4B). However, we did not detect differences in hepatic mRNA expression levels of \textit{CrbpI} or \textit{CrbpII}. Thus, expression levels for a number of RA-inducible genes are likely elevated in the livers of these mutant mice. It is generally assumed that elevated expression levels of \textit{Cyp26A1} and \textit{Rasβ2} reflect elevated cellular all-trans-RA
concentrations but, as far as we are aware, this has not been directly established. Consequently, we assessed serum and hepatic all-trans-RA concentrations for Lrat\(^{-/-}\) and matched WT mice using very sensitive LC/MS/MS methodologies (Fig. 4C–E). Our LC/MS/MS methods allowed for a very clean separation of all-trans-RA in tissue extracts. We did not encounter any problems that might be associated with matrix effects for either the LC separations (Fig. 4D) or the fragmentation as assessed from the daughter ion spectrum of the endogenous all-trans-RA (Fig. 4E). Surprisingly, and contrary to what has been inferred based on gene expression data, serum and hepatic steady-state concentrations of all-trans-RA were not elevated for Lrat\(^{-/-}\) compared with WT mice (Fig. 4C). These levels were actually significantly reduced in the serum and livers of the mutant mice. This was also the case for hepatic all-trans-RA levels for Cebp\(^{+/+}\) and Lrat\(^{-/-}\)/Cebp\(^{+/+}\) mice as well (data not shown). We take this to indicate that elevated expression of CYP26A1 results in increased catabolism and lower hepatic all-trans-RA concentrations.

We were also interested in measuring 9-cis-RA concentrations in addition to all-trans-RA in Lrat-deficient adipose tissue. However, 9-cis-RA was not present in the livers at a level that we felt we could accurately measure. This can be seen in the LC/MS/MS profile provided as Fig. 4D. The peak for all-trans-RA is very substantial for this liver extract, and for all other liver extracts we analyzed. There is a small peak with a retention time of approximately 8.15 min, which is the retention time at which authentic 9-cis-RA elutes. Given the size of this peak, it is possible that the small amount of 9-cis-RA present may have been formed as an artifact during extraction and processing, because it is well known that all-trans-RA can undergo some isomerization to its cis-isomers.

To understand whether DGAT1 is responsible for the REs present in Lrat-deficient adipose tissue, we measured total retinol levels (retinol + REs) for epididymal fat pads obtained from mice lacking both Lrat\(^{-/-}\) and Dgat1\(^{-/-}\), Lrat\(^{-/-}\)/Dgat1\(^{-/-}\) mice. These levels were not statistically different for Lrat\(^{-/-}\) or Lrat\(^{-/-}\)/Dgat1\(^{-/-}\) mice (Fig. 5A). Because Cebp is expressed in adipose tissue, in a separate study we asked whether the absence of Cebp affects adipose retinol levels as it does in the liver. Indeed, adipose tissue total retinol levels, which are elevated by approximately 3-fold for Lrat\(^{-/-}\) compared with WT mice, were diminished in adipose tissue from matched Lrat\(^{-/-}\)/Cebp\(^{-/-}\) mice to levels identical to WT mice (Fig. 5B). We also undertook studies to identify whether there might be differences in expression of known RA-responsive genes in adipose tissue obtained from these mice. However, unlike the liver, we did not detect statistically significant differences in mRNA expression levels for Rar\(\beta\)2, Cyp26A1, or Cyp26B1 for the different mouse lines (data not shown). We also did not observe differences in Rbp4, Crabp1, or Crabp2 mRNA levels between the different lines.

While studying the Lrat\(^{-/-}\)/Cebp\(^{-/-}\) mice, we observed visually that these mice seemed to accumulate more hepatic fat than WT mice. We assessed this possibility in age- and diet-matched male WT, Lrat\(^{-/-}\), Cebp\(^{+/+}\), and Lrat\(^{-/-}\)/Cebp\(^{-/-}\) mice. Both Cebp\(^{+/+}\) and Lrat\(^{-/-}\)/Cebp\(^{-/-}\) mice showed a statistically significant elevation in fasting triglyceride levels compared with WT mice (Fig. 6A). Although Lrat\(^{-/-}\) mice tended to have higher hepatic fasting triglyceride concentrations than WT mice, statistical significance was not reached. To gain insight into the molecular basis for the elevated fasting triglyceride levels observed for Cebp\(^{-/-}\) and Lrat\(^{-/-}\)/Cebp\(^{-/-}\) mice, we investigated expression of a number of key regulators of hepatic fat metabolism, Ppara, Pparg, and Pparg. As seen in Fig. 6B, Pparg gene expression was significantly down-regulated in the livers from Lrat\(^{-/-}\), Cebp\(^{-/-}\), and Lrat\(^{-/-}\)/Cebp\(^{-/-}\) mice. No significant differences in hepatic expression of either Ppara or Pparg were observed for any of the mutants including the carbohydrate response element-binding protein (Chrebp), a regulator of glucose and lipid metabolism (data not shown). The body weights of age-, gender-, and diet-matched male WT,
nificantly different nor were the expression levels of (data not shown). We also examined possible changes in

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Statistical significance: a, b,
elts. A: Total retinol levels are signifi cantly elevated for 3-month-old male chow-fed

compared with WT, chow-fed WT (n = 5), and penta-deuterated all-

E: Fragmentation spectra for authentic all-

trans-RA standard (upper spectrum) and for the endogenous all-

trans-RA (at-RA, retention time 8.29 min) but observed no significant differences (data not shown). As shown in

Fig. 6C, we observed a marked downregulation in expression of the key regulatory enzyme Pdk4, which is a known target gene for Ppar6 transcriptional regulation (47).

DISCUSSION

ARAT activities are not involved in RE synthesis in the liver

The literature indicates that ARATs are involved in the synthesis of hepatic REs (9–12, 28, 29). We have reported that DGAT1 can act as a physiologically significant ARAT in the mouse intestine (24) and Shih et al. (25) established that DGAT1 acts physiologically as an ARAT in mouse skin. It is well established that DGAT1 acts to facilitate triglyceride storage/metabolism and lipid droplet formation in the liver (19–21). Because DGAT1 is highly expressed in the liver, this raises a question as to whether DGAT1 might also act as an ARAT in the liver. Moreover, DGAT1 is expressed both in hepatocytes and in hepatic stellate cells (44), the cellular site in the liver where REs are stored and where LRAT is primarily expressed (48). Even though our earlier studies of Lrat−/− mice established that these mutant mice have very low levels of hepatic REs (<0.1% of matched WT levels) suggesting that LRAT is responsible for the preponderance of hepatic RE synthesis when mice are maintained on a standard chow diet (17), the literature suggests a role for an ARAT in hepatic RE formation. This extensive literature maintains that tissue ARAT activities may only become active when high levels of retinol are available and/or when the capacities of CRBPs like CRBPI and CRBPII to bind retinol and channel it to LRAT have been exceeded (27–29, 49). Indeed, our earlier work, which established DGAT1 as a physiologically relevant ARAT in the intestine, also established that one of the actions of CRBPII in the intestine was to channel retinol to LRAT for esterifi cation (23). To directly address these possibilities, we employed a nutritional approach, feeding a 25-fold excess retinol diet for 4 weeks, coupled with a genetic approach, in an attempt to demonstrate LRAT-independent RE formation. Our data do not support the idea that an acyl-CoA-dependent ARAT enzyme(s) contributes to hepatic RE formation in vivo. Our data are consistent with

Fig. 4. A: Cyp26A1 mRNA levels are signifi cantly elevated in the livers of 3-month-old male chow-fed

CrbpI−/− (n = 5), Lrat−/− (n = 5), and Lrat−/−/CrbpI−/− (L/C−/−) (n = 7) mice compared with age- and gender-matched WT (n = 6) mice. mRNA levels were determined in triplicate for each liver by qPCR. Expression levels are normalized for hepatic expression of 18S rRNA. Statistical signifi cance: a, P < 0.01 compared with WT mice. B: Rarb2 mRNA levels are signifi cantly elevated in the same livers from Lrat−/− and Lrat−/−/CrbpI−/− (L/C−/−) mice compared with WT mice. mRNA levels were determined in triplicate for each liver by qPCR. Expression levels are normalized for hepatic expression of 18S rRNA. Statistical signifi cance: a, P < 0.05 compared with WT mice. C: Serum and liver all-trans-RA concentrations are signifi cantly lower for Lrat−/− (n = 9) compared with WT (n = 9) mice. Statistical signifi cance: a, P < 0.01 compared with WT mice. D: A representative LC/MS/MS profi le for RA for an extract obtained for a 3-month-old male Lrat−/− liver showing the multiple reaction monitoring peaks due to all-trans-RA (at-RA, retention time 8.29 min) and penta-deuterated all-trans-RA (at-RA-d5, retention time 8.22 min) employed as the internal standard. E: Fragmentation spectra for authentic all-trans-RA standard (upper spectrum) and for the endogenous all-

trans-RA detected in an Lrat−/− liver extract (lower spectrum).

Fig. 5. Epididymal adipose tissue total retinol (retinol + REs) levels. A: Total retinol levels are signifi cantly elevated for 3-month-old male chow-fed Lrat−/− (n = 12) and Lrat−/−/Dgat1−/− (L/D−/−) (n = 13) mice compared with WT (n = 8) or Dgat1−/− (n = 4) mice. All values are given as means ± SD. Statistical signifi cance: a, P < 0.01 compared with WT mice or Dgat1−/− mice. B: Total retinol levels are signifi cantly lower in Lrat−/−/CrbpI−/− (L/C−/−) mice compared with WT, CrbpI−/−, or Lrat−/− mice. Epididymal adipose tissue retinol and RE levels were assessed for 3-month-old male chow-fed WT (n = 5), CrbpI−/− (n = 10), Lrat−/− (n = 8), and Lrat−/−/CrbpI−/− (n = 22) mice. All values are given as means ± SD. Statistical signifi cance: a, P < 0.01 compared with WT mice or CrbpI−/− mice; b, P < 0.01 compared with Lrat−/− mice.

Lrat−/−, CrbpI−/−, and Lrat−/−/CrbpI−/− mice were not signifi cantly different nor were the expression levels of Pparγ in adipose tissue obtained from these different genotypes (data not shown). We also examined possible changes in expression for genes involved in hepatic lipogenesis (Fas, Sedl, and Ace) and fatty acid oxidation (Cpt1) but observed no signifi cant differences (data not shown). As shown in


the conclusion that LRAT is solely responsible for hepatic RE synthesis. This includes both RE storage in hepatic stellate cells and RE incorporation into nascent VLDLs in hepatocytes. Although DGAT1 is a physiologically relevant ARAT in the intestine and skin (24, 25), we were unable to obtain any evidence that it has this role in the liver. Moreover, contrary to what has been proposed by Yamaguchi et al. (44) from cell culture studies, we observed no relationship between Lrat and Dgat1 gene expression in the liver that suggests coordinated gene regulation as proposed by these authors.

CRBPI acts to prevent catabolism and loss of hepatic retinol

It has been proposed that CRBPI prevents retinol from being converted to REs by ARAT activities or exposure to nonspecific enzymes that may catalyze retinol oxidation (27–29, 34, 49, 50). Our data do not support the notion that CRBPI acts to prevent hepatic or adipose ARAT activities, like DGAT1, from catalyzing RE synthesis. Rather, our data are convincing that CRBPI prevents elimination or loss of retinol from the liver, and from adipose tissue as well (see Fig. 3). The absence of CRBPI from Lrat−/− mice (in Lrat−/−/CrbpI−/− mice), which possess no REs and hence hepatic retinol levels and metabolism can be very cleanly assessed, results in an 8- to 20-fold reduction in the level of hepatic retinol. Molotkov et al. (50) have proposed that hepatic CRBPI limits nonspecific oxidation of retinol by alcohol dehydrogenase 1 and proposed that this increases the ability of hepatic “esterifying enzymes” to produce REs for storage. Because retinol cannot be esterified in the livers of Lrat−/−/CrbpI−/− mice, our data establishes directly that hepatic CRBPI prevents loss of retinol from the liver. Interestingly, although the simple absence of CRBPI from adipose tissue does not affect the total retinol (retinol + REs) level found in adipose tissue (Fig. 5B), the absence of CRBPI from Lrat−/− mice results in a significant reduction of adipose total retinol. Total retinol levels present in Lrat−/− adipose tissue are approximately 2- or 3-fold elevated over those of age-, gender-, and diet-matched WT mice (17) (Fig. 5B). The absence of CRBPI from Lrat-deficient adipose tissue results in adipose tissue total retinol levels which are similar to those of matched WT mice. There are two possible bases for this observation. It is possible, that like in the liver, CRBPI prevents oxidation and loss of adipose retinol. However, because adipose total retinol levels are similar for WT and Cyp26A1−/− mice, our data establishes directly that hepatic CRBPI prevents oxidation and loss of adipose retinol. However, because adipose total retinol levels are similar for WT and Cyp26A1−/− mice, we believe that this is unlikely. Alternatively, because the molecular identity of the enzyme(s) responsible for RE formation in Lrat−/−/Dgat1−/− adipose tissue is not known, possibly there is a previously unsuspected CRBPI-dependent retinol esterifying activity present in adipose tissue. This possibility needs to be explored in future research.

Elevated hepatic mRNA levels for known RA-responsive genes should not be taken to indicate that hepatic steady-state RA concentrations are elevated

Liu and Gudas (18) have demonstrated that Cyp26A1 mRNA expression is elevated in the livers of Lrat−/− mice. Earlier studies showed Cyp26A1 mRNA expression is induced either by acute loading with RA or long-term exposure to dietary retinoids, whereas expression was downregulated upon administration of a retinoid-deficient diet (51, 52). We have confirmed the published observation of Liu and Gudas (18) that Cyp26A1 expression is elevated in the livers of Chow-fed Lrat−/− mice and have established further that expression of the retinoid-responsive transcription factor RARβ2 is also elevated in the livers of Chow-fed

Fig. 6. A: Fasting triglyceride levels are significantly elevated in the livers of 3-month-old male chow-fed CrbpI−/− and Lrat−/−/CrbpI−/− (L/C−/−) mice compared with matched WT mice. Groups of WT, Coby−/−, Lrat−/−, and Lrat−/−/CrbpI−/− mice (n = 6 per strain) were fasted in the morning for 4 h after diet was removed from their housing prior to sacrifice. Statistical significance: a, P<0.05 compared with WT mice. Livers (n = 6 per strain) were taken for RNA isolation and assessment of Pparδ (B) and Pdk4 (C) mRNA levels by qPCR. All values are given as means ± SD. Statistical significance: a, P<0.01 compared with WT mice.
increased RA-responsive gene expression is associated with elevated triglyceride levels

While undertaking our investigations aimed at understanding hepatic retinoid storage, we observed that the fasting livers of CrbpIΔΔ and LratΔΔ mice accumulate significantly more triglyceride than matched chow-fed WT mice (Fig. 6). Livers from these strains also show elevated expression of RA-responsive gene expression. The literature suggests linkages between retinoid storage, metabolism, and actions and the development of fatty liver. Included in this literature are studies reporting ablation of hepatic retinoid receptor signaling resulting in hepatic steatosis (55), ablation of carotenoid-15,15′-oxygenase (which abolishes retinoid production from β-carotene) (56), and knockout of CrbpII (57), nutritional studies carried out in mice or rats (58–61), studies of retinoid effects on hepatic endocannabinoid signaling (62), and human observational studies (63, 64). However, the specific mechanisms underlying these observations are not well-established. Given the focus of our research, we undertook only a limited survey to identify possible general molecular pathways that might be responsible for the observation. To this end, we examined by qPCR expression levels of a number of key regulators of hepatic lipid metabolism. We did not detect significant differences between matched mutant and WT mice in hepatic expression of regulatory genes commonly associated with hepatic steatosis, specifically Ppara and Ppary. Strikingly though, Pparα mRNA levels were downregulated by more than 75% and levels of the Pparα target gene Pitx4 (47) were similarly downregulated in the livers of CrbpΔΔ/−, LratΔΔ/−, and LratΔΔ/−/CrbpΔΔ/− mice. Although it is generally believed that PPARα exerts its effects on lipid metabolism primarily through actions in skeletal muscle (65), there is evidence that PPARα also controls hepatic energy substrate homeostasis through coordinated regulation of glucose and fatty acid metabolism (66). Interestingly, all-trans-RA has been proposed to be a PPARα agonist (4, 5). We believe that the observations of elevated hepatic triglyceride accumulation in CrbpΔΔ/− and LratΔΔ/−/CrbpΔΔ/− mice and elevated RA-responsive gene expression in these livers are directly related. However, further investigations will be needed before this possibility can be conclusively established.

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