Hepatic retinol secretion and storage are altered by dietary CLA: common and distinct actions of CLA c9,t11 and t10,c12 isomers[S]

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Abstract Conjugated linoleic acid (CLA) is a polyunsaturated fatty acid obtained from ruminant products. Previous studies in rats and pigs showed that a dietary equimolar mixture of c9,t11 and t10,c12 CLA isomers induces changes in serum and tissue levels of retinoids (vitamin A derivatives). However, the mechanism(s) responsible for these actions remain(s) unexplored. Given the numerous crucial biological functions regulated by retinoids, it is key to establish whether the perturbations in retinoid metabolism induced by dietary CLA mediate some of the beneficial effects associated with intake of this fatty acid or, rather, have adverse consequences on health. To address this important biological question, we began to explore the mechanisms through which dietary CLA alters retinoid metabolism. By using enriched preparations of CLA c9,t11 or CLA t10,c12, we uncoupled the effects of these two CLA isomers on retinoid metabolism. Specifically, we show that both isomers induce hepatic retinyl ester accumulation. However, only CLA t10,c12 enhances hepatic retinol secretion, resulting in increased serum levels of retinol and its specific carrier, retinol-binding protein (RBP). Dietary CLA t10,c12 also redistributes retinoids from the hepatic stores toward the adipose tissue and possibly stimulates hepatic retinoid oxidation. Using mice lacking RBP, we also demonstrate that this key protein in retinoid metabolism mediates hepatic retinol secretion and its redistribution toward fat tissue induced by CLA t10,c12 supplementation.—Ortiz, B., L. Wassef, E. Shabrova, L. Cordeddu, S. Banni, and L. Quadro. **Hepatic retinol secretion and storage are altered by dietary CLA: common and distinct actions of CLA c9,t11 and t10,c12 isomers.** ***J. Lipid Res.*** **2009.** 50: **2278–2289.**

Supplementary key words conjugated linoleic acid • retinol • retinyl ester • retinol-binding protein

The maintenance of normal retinoid (vitamin A and its derivatives) homeostasis is required to support many crucial biological functions (1–3). All retinoids in animals are derived from the diet as preformed dietary vitamin A (retinyl esters, retinol, and very small amounts of retinoic acid) from animal products or as β-carotene from vegetables and fruits (4). Within the intestine, ingested vitamin A is packaged into chylomicrons as retinyl ester regardless of its dietary origin (5). In the bloodstream, lipolysis of the chylomicrons generates smaller lipoprotein particles called chylomicron remnants, still retaining retinyl ester (6, 7). Approximately 75% of retinoids within chylomicron remants are cleared by the liver, which is the major site of vitamin A storage and metabolism (8–10). To meet tissue retinoid needs, the liver secretes retinol into the circulation bound to its sole specific transport protein, retinol-binding protein (RBP; also known as RBP4) (11, 12). RBP is a 21 kDa protein with a single binding site for one molecule of all-*trans*-retinol. It is mainly, but not exclusively, synthesized within the hepatocytes. RBP circulates in the blood as a 1:1 molar complex with another serum protein, transthyretin (TTR), preventing retinol-RBP excretion by the kidney (11, 12). In the fasting circulation, retinol-RBP represents approximately 99% of all serum retinoids. Blood levels of retinol-RBP in both humans and animals are maintained very constant except in extreme cases of nutritional intake of vitamin A, protein, calories, and zinc or in response to hormonal factors, stress, and in certain disease states (11, 13, 14).

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Abbreviations: CLA, conjugated linoleic acid; LRAT, lecithin:retinol acyltransferase; RBP, retinol-binding protein; TTR, transthyretin.

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Conjugated linoleic acid (CLA) is a polyunsaturated fatty acid found mainly in ruminant products. CLA is a term given to a group of positional and geometrical isomers of octadecadienoic acid that differ by the placement of conjugated double bonds (15, 16). In experimental animal studies, CLA showed beneficial effects on health. For example, CLA has been reported to protect against the development of cancer and atherosclerosis, stimulate immune functions, normalize impaired glucose tolerance in type-2 diabetes, and induce changes in body mass composition [reviewed in (15, 16)]. Out of the 28 CLA isomers existing in nature, numerous studies have attributed the specific biological activities of CLA intake mainly to the c9,t11 and t10,c12 isomers (15). These two molecules share some common effects but yet show other quite distinct biological activities (15).

Banni et al. (17, 18) noted that the addition of a mixture of CLA isomers (50% CLA c9,t11 and 50% CLA t10,c12) to the diet of female rats for four weeks unexpectedly resulted in increased concentrations of serum retinol and elevated levels of retinol and retinyl ester in liver and mammary tissue. Although the mechanisms of this action remain unexplored, these results raised the intriguing possibility that retinoids may mediate some of the beneficial effects of CLA (17). However, given the multitude of crucial biological functions regulated by vitamin A and the need to maintain retinoid homeostasis for health (1–3), it cannot be ruled out that the perturbations in vitamin A metabolism induced by dietary CLA may possibly have adverse consequences. To address this important biological question, we began to explore the mechanisms through which dietary CLA alters retinoid metabolism.

This novel study was designed to investigate whether CLA c9,t11 and CLA t10,c12 have distinct and/or common effects on vitamin A metabolism, using mice as an experimental animal model. In addition, given the central role played by RBP in maintaining homeostatic levels of retinol in serum and tissues, we took advantage of mice lacking RBP [$RBP^{-/-}$ (19)], to investigate whether this protein is involved in mediating the serum and tissue vitamin A changes induced by dietary CLA.

MATERIALS AND METHODS

CLA isomers

Enriched preparations of CLA isomers c9,t11 and t10,c12 were obtained from Lipid Nutrition (Wormerveer, The Netherlands). The composition of these powder preparations was as follows: CLA c9,t11 contained 73.8% CLA c9,t11, 4.6% CLA t10,c12, 0.4% CLA t,t, and 6.6% saturated fatty acids; CLA t10,c12 contained 71.4% CLA t10,c12, 13.5% CLA c9t,11, 3.5% CLA t,t, and 4.7% saturated fatty acid.

Mouse strains and nutritional manipulation

Since its initial generation (19), the $RBP^{-/-}$ mouse strain was maintained through mating of the heterozygous sibilings from one generation to the next. Our colony of $RBP^{-/-}$ mice has never been backcrossed and the mice have a mixed genetic background (129sv/C57Bl6). Experimental cohorts were generated from breeding pairs of RBP or wild-type littermates that were first generation of offspring from mice that were heterozygous for the RBP gene. Mice were maintained on a regular chow diet manufactured by TestDiet (PicoLab Rodent Diet 20 #5053; Purina Mills, St. Louis, MO) from weaning. This diet provided 25.0, 12.0, and 63.0% calories from protein, fat, and carbohydrates, respectively. The diet also contained 25 IU of vitamin A/g. Starting at 10 weeks of age, $RBP^{-/-}$ and wild-type mice were randomly assigned to one of the following dietary regimens: regular chow diet (control diet), regular diet supplemented with 1% CLA c9,t11, regular diet supplemented with 1% CLA t10,c12, or regular diet supplemented with 1% olive oil. The diets supplemented with CLA or olive oil (also manufactured by TestDiet with the same batch of regular chow diet) contained 99% of control diet + 1% CLA isomer preparation or + 1% olive oil, respectively, thus resulting in the following energy content: 24.3, 14.0, and 61.7% calories from protein, fat, and carbohydrates, respectively. Mice (n = 5 to 6/group) were maintained on the assigned diet for 4 weeks, i.e., until 14 weeks of age. For all the studies, both diet and water were available to the animals on an ad libitum basis. Mice were maintained on a 12:12 light/dark cycle with the period of darkness between 7:00 PM and 7:00 AM. All mice used for these studies were euthanized by CO_2 inhalation between 9:30 and 11:30 AM. All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (20) and were approved by the Rutgers University Institutional Committee on Animal Care.

Body mass compositions analysis

After 4 weeks on the assigned diet, the body mass composition of three mice/group was measured using a LUNAR PIXImus Densitometer (Fitchburg, WI).

HPLC analysis of retinoids

Reverse-phase HPLC analysis was performed to measure serum and tissue retinol and retinyl esters levels (21). Retinoids were separated on a 4.6 × 250 mm Ultrasphere C_{18} column (Beckman, Fullerton, CA) preceded by a C_{18} guard column (Supelco Inc., Bellefonte, PA) using acetonitrile, methanol, and methylene chloride (70:15:15, v/v) as the mobile phase flowing at 1.8 ml/min. A Dionex Ultimate 3000 HPLC system and a computerized data analysis workstation with Chromeleon software were used. Retinol and retinyl esters were identified by comparing retention times and spectral data of experimental compounds with those of authentic standards. The concentrations of retinoids were determined by comparing peak integrated areas for unknowns against those of known amounts of purified standards. Loss during extraction was accounted for by adjusting for the recovery of retinyl acetate, the internal standard added immediately following homogenization of the tissues.

CLA analysis

Total lipids were extracted from serum and tissues using the Folch method (22). Aliquots were mildly saponified as previously described (23) in order to obtain free fatty acids for HPLC analysis. CLA analysis was carried out with a Hewlett-Packard 1100 HPLC system (Hewlett-Packard, Palo Alto, CA) equipped with a diode array detector. A C-18 Inertsil 5 ODS-2 Chrompack column (Chrompack International BV, Middleburg, The Netherlands), 5 µm particle size, 150 × 4.6 mm, was used with a mobile phase of acetonitrile-water-acetic acid (70:30:0.12, v/v) at a flow rate of 1.5 ml/min (24). Conjugated diene unsaturated fatty acids, including arachidonic acid hydroperoxides, were detected at 234 nm. Spectra (195–315 nm) of the eluate were obtained every 1.28 s and were electronically stored. Second-derivative UV spec-

tra of the conjugated diene fatty acids were generated using Phoenix 3D HP Chemstation software (Hewlett-Packard). The spectra were taken to confirm the identification of the HPLC peaks (25). Separation of CLA isomers was achieved by separation of fatty acid methyl esters using a silver ion column (26). Methyl esters were prepared by the addition of 14% boron trifluoride/methanol at room temperature and immediately extracted into a solvent consisting of n-hexane and water (4:3; v/v). After phases separation by centrifugation, the hexane phase was saved and the aqueous phase was further extracted by another round of hexane. The two hexane collections were combined, dried, and redissolved in 500 μl of n-hexane. Separation of CLA isomers was carried out with an Agilent 1100 HPLC system (Agilent, Palo Alto, CA) equipped with a diode array detector. A silver ion chromspher 5 lipid Chrompack column (Chrompack International BV), 5 μm particle size, 250 × 4.6 mm, was used with a mobile phase of n-hexane containing 0.0375% of CH_3CN at a flow rate of 1 ml/min. This technique separates the positional and geometric (*cis* and *trans*) isomers of CLA (26).

Western blot analysis

Serum and tissue content of RBP and TTR, as well as adipose LDL receptor (LDLr) levels, were analyzed by Western blot (27). A rabbit polyclonal anti-rat RBP antiserum (28), a rabbit polyclonal anti-rat TTR serum (29), and a goat polyclonal anti-mouse LDLr (R & D Systems, Inc.) were used for immunodetection. Signals were detected by using a Biorad Chemidoc XRS Molecular Imager System. Albumin, detected upon treating the membranes with either Coomassie or Ponceau S stain, was used as a loading control for serum samples. Albumin, detected by a rabbit polyclonal anti-albumin antibody (Abcam), or actin, detected by a mouse monoclonal anti-actin antibody (Sigma), were used as a loading control for tissue samples. The molecular weight of each detected protein is as follows: RBP, 21 kDa; TTR, 14 kDa; LDLr, 150 kDa; actin, 45 kDa; and albumin, 65 kDa. The quantification of the membranes was completed by densitometry analysis with Quantity One Program (Biorad).

Total RNA extraction and RT real-time PCR analysis

Total RNA was extracted from tissue using RNA Bee (Tel-test Inc. TX) according to the manufacturer's instructions. RNA concentrations were measured by spectrophotometry, followed by DNase I treatment (Roche Diagnostics, IN). Three micrograms of the DNase Treated RNA was reverse transcribed to cDNA using instructions and reagents from Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics). A no-reverse transcriptase control was included. For RT real-time PCR analysis, the Roche Applied Science Lightcycler 480 machine was used, together with the Lightcycler 480 SYBR green I master mix (Roche Diagnostics). A series of experiments were performed to determine relative efficiency and concentrations for each primer of interest. Validation experiments were also performed to confirm that the housekeeping gene, β-actin, was unaffected by the dietary treatments. Primer sequences were as follows: RBP, Fw 5′GGAGAACTTCGACAAGGCTC3′ and Rev 5′CTGCACACACTTCCCAGTTG3′; CYP26A1, Fw 5′GAACCTTATACACGCGCGCAT3′ and 5′CTCTGTTGACGATTGTTTTAGTG3′; LRAT, Fw 5′ATGAAGAACCCAATGCTGGAA3′ and Rev 5′CTAATCCCAAGACAGCCGAAG3′; Cyp2c39, Fw 5′GGAGACAGAGCTGTGGC3′ and Rev 5′TAAAAACAATGCCAAGGCC3′; LPL, Fw 5′ CCCTAAGGACCCCTGAAGAC3′ and Rev 5′ GGCCCGATACAACCAGTCTA3′; β-actin, Fw 5′CGGAGGGAAAGATTCCTCTGGC3′ and Rev 5′AGGGCCGGCACATTGAAGGTCT3′. Final primer reaction conditions were as follows: SYBR Green Master Mix, 200 nM Forward Primer (400 nM for Cyp2c39), 200 nM Reverse Primer (400 nM for Cyp2c39), and PCR grade water. cDNA, no-reverse transcriptase control, and a no template control were amplified using the following conditions: 95°C for 10 min, followed by 40 cycles at 95°C for 10 s, 56°C (59°C for LPL) for 20 s, and 72°C for 30 s. This was followed by a dissociation curve to ensure the absence of any primer dimers. Furthermore, RT real-time PCR products were run on a gel to ensure the gene of interest was amplified. Each sample was run in triplicate and each RT real-time PCR run was repeated twice. Samples were analyzed by relative quantification, where the genes of interest are expressed relative to a chosen calibrator (control) using the ΔΔ CT method. This includes the subtraction of the CT value for the gene of interest from the CT value for the housekeeping gene (β-actin) to obtain ΔCT values. ΔΔCT values were obtained from subtracting ΔCT for each sample from the average ΔCT for the calibrator. The expression of each gene relative to the calibrator was calculated using $2^{(-\Delta\Delta CT)}$.

Statistical analysis

Normality of the data was determined using the Kolmogorov Smirnov test. Non-normally distributed data were analyzed by a Kruskal-Wallis test followed by a Mann-Whitney test. Normally distributed data were analyzed by *t*-test or ANOVA test with a correction for multiple comparisons using the Fisher's least significant test. Analyses were performed with SPSS statistical software (SPSS 11.0 for Windows Student Version, 2001; SPSS Inc., IL). A p-value <0.05 was used to establish statistical significance.

RESULTS

The effects of different dietary CLA isomers on body weight and body mass composition are independent of RBP

Ten-week-old wild-type and RBP knockout ($RBP^{-/-}$) (19) male mice were maintained for 4 weeks on one of the following four dietary regimens: control diet, control diet supplemented with 1% olive oil, control diet supplemented with 1% CLA c9,t11, or control diet supplemented with 1% CLA t10,c12. We chose the dietary concentration of CLA and the duration of the treatment based on previous reports on rodents (17, 18). As a control for the efficacy of our experimental procedure, we measured serum concentration of CLA c9,t11 and CLA t10,c12 by HPLC analysis (24). In both mouse strains, CLA isomers were only detected when provided with the diet, as expected (**Table 1**).

Dietary intake of CLA has been shown to protect from body weight gain and to reduce body fat mass, depending upon the length of the feeding, the isomer composition of the diet, and the experimental model used. These effects have been attributed mainly to CLA t10,c12 [reviewed in (15, 16)]. On the other hand, increased levels of serum RBP have been shown in obese mouse models and humans, leading to the hypothesis that elevated circulating RBP could be considered a marker of metabolic syndrome (14, 30, 31). To verify whether a link exists between the biological effects of dietary CLA and RBP, body weight of each animal was recorded weekly. In our experiment, mice fed the control diet did not display a significant body weight gain over the four-week feeding period. Indeed, the average body weight gain (% increase from 10 to 14

TABLE 1. Serum levels of CLA isomers in mice fed different diets

Genotype	Diet	CLA Isomers (nmol/ml) c9,t1 1	t10,c12
WT	CTR	nd	nd
	CLA c9,t11	249.3 ± 114.6	nd
	CLA t10,c12	nd	409.4 ± 129.3
RBP$^{-/-}$	CTR	nd	nd
	CLA c9,t1 1	232.7 ± 55.7	nd
	CLA t10,c12	nd	319.5 ± 59.8

Serum levels of CLA isomers were determined by HPLC analysis. Wild-type (WT) and RBP knockout (RBP$^{-/-}$) male mice were maintained on three different dietary regimens for 4 weeks: control diet (CTR), control diet supplemented with 1% CLA c9,t11 (CLA c9,t11), and control diet supplemented with 1% CLA t10,c12 (CLA t10,c12). Serum CLA isomers concentration is expressed as mean ± SD. nd indicates below the limit of detection (< 1 nmol/ml); n = 5 to 6 mice/group.

weeks within the same mouse strain) was 5% and 4% for wild-type and RBP$^{-/-}$ mice, respectively. Although the diets supplemented with fatty acids provided a slightly elevated energy content from fat compared with the control diet (14.0% vs. 12.0%, respectively), only mice fed CLA c9,t11 or olive oil showed a significant increase in body weight over the four weeks feeding treatment (the average body weight gain for the wild-type mice was 17% on the olive oil diet and 12% on the CLA c9,t11 diet and, for the RBP$^{-/-}$ mice, 12% on the olive oil diet and 14% on the CLA c9,t11 diet). In addition, these increases in body weight were also statistically significant when compared with that of the control group within the same strain (**Fig. 1A**). In contrast, lack of body weight gain was observed in mice fed CLA t10,c12, regardless of their genotype (the average body weight gain on this dietary regimen was 6% and 5% for wild-type and RBP$^{-/-}$ mice, respectively), similar to the mice on the control diet (Fig. 1A). Furthermore, food intake among different groups of animals was comparable (data not shown). Our data demonstrate that dietary supplementation with CLA t10,c12 prevents body weight gain otherwise induced by the fatty acid supplementation regardless of the presence or absence of RBP.

Body mass composition analysis was also performed by a LUNAR PIXImus mouse DEXA instrument on three mice from each group at 14 weeks of age (prior to euthanasia). CLA c9,t11 did not affect body mass composition in wild-type nor in RBP$^{-/-}$ mice. In contrast, CLA t10,c12 feeding resulted in a statistically significant reduction of body fat mass and in an increase of lean body mass in both strains (Fig. 1B, C). These results confirm that dietary supplementation with CLA t10,c12 reduces body fat mass regardless of the presence or absence of RBP. Olive oil feeding had the opposite effect on body mass composition compared with that of dietary CLA t10,c12 supplementation although this trend reached statistical significance only in RBP$^{-/-}$ animals (Fig. 1B, C). This observation could be taken to imply that each individual fatty acid has a specific impact on lipid metabolism and, subsequently, on fat deposition (32). Overall, our data demonstrate that RBP does not have a role in mediating the differential effects of CLA c9,t11 and t10,c12 on body weight and body mass composition.

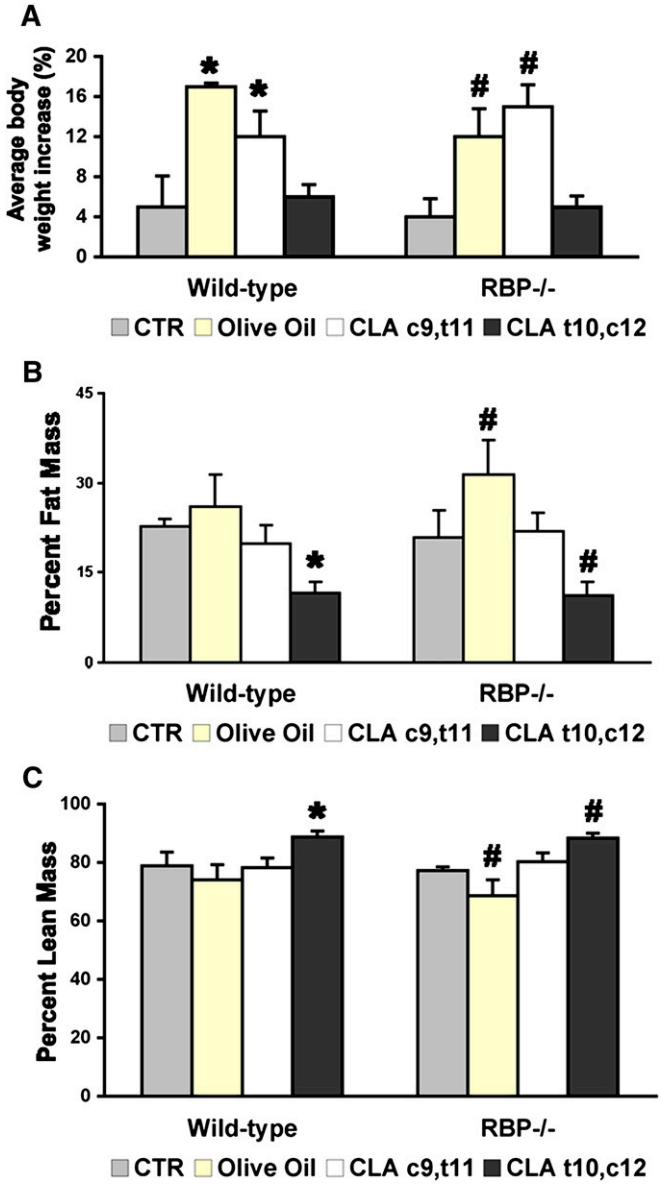

Fig. 1. Effects of dietary CLA on body weight and composition in wild-type and RBP$^{-/-}$ mice. A: Percent change in body weight (10 weeks vs. 14 weeks); n = 4 to 6 mice/group. B: Percent body fat mass. C: Percent body lean mass. Body composition was measured in three mice per group by a Lunar PIXImus Densitometer Dexa analysis at 14 weeks of age, prior to euthanasia. Statistical analysis was performed by ANOVA test. * indicates statistically significant differences between treatment and control groups within the wild-type strain ($P < 0.05$). # indicates statistically significant differences between treatment and control groups within the RBP$^{-/-}$ strain ($P < 0.05$). Results are expressed as mean ± SE in panel A and as mean ± SD in panels B and C.

Differential effects of CLA isomers on circulating levels of retinol and RBP

To quantify the effect of CLA c9,t11 and t10,c12 on serum retinol levels in mice, we used reverse-phase HPLC analysis (21). As shown in **Table 2**, neither CLA c9,t11 nor olive oil feeding induced a statistically significant change in circulating retinol concentrations in wild-type animals. In contrast, serum retinol levels increased approximately 2-fold when CLA t10,c12 was administered with the diet to

TABLE 2. Effect of different dietary CLA isomers on serum retinol levels

Genotype	Diet			
	CTR	Olive Oil	CLA c9,t1 1	CLA t10,c12
WT	33.0 ± 9.3	39.7 ± 13.3	40.5 ± 8.5	71.7 ± 9.0*
RBP$^{-/-}$	4.0 ± 1.2*	3.9 ± 1.4*	4.0 ± 0.4*	2.8 ± 0.6*

Serum retinol levels (μg/dl) were determined by reverse phase HPLC. Wild-type (WT) and RBP knockout (RBP$^{-/-}$) male mice were maintained on four different dietary regimens for 4 weeks: control diet (CTR), control diet supplemented with 1% olive oil, control diet supplemented with 1% CLA c9,t11 (CLA c9,t11), and control diet supplemented with 1% CLA t10,c12 (CLA t10,c12). Serum retinol concentration is expressed as mean ± SD. Statistical analysis by ANOVA test. *$P < 0.05$ versus wild-type group on control diet; n = 5 to 6 mice/group.

this strain (Table 2). Due to the lack of RBP, RBP $^{-/-}$ mice showed reduced serum retinol levels compared with wild-type mice on the control diet (19) (Table 2). However, unlike wild-type mice, serum retinol levels in RBP$^{-/-}$ animals were not affected by any of the diets (Table 2).

Because no differences were found in circulating retinol levels between mice fed the control or the olive oil diet regardless of the genotype of the animals, further serum analyses were restricted to mice fed the control diet in comparison to those fed the CLA isomers. Retinol circulates in the bloodstream in a ternary complex with RBP and TTR (11). Therefore, we analyzed the serum concentrations of these two proteins in wild-type and RBP$^{-/-}$ mice by Western blot analysis. As shown in **Fig. 2A** and 2B, dietary CLA t10,c12 resulted in a 2.5-fold elevation of circulating RBP levels in wild-type mice. In contrast, serum RBP levels did not change in wild-type mice fed CLA c9,t11. As previously reported (19), RBP was not present in the serum of RBP$^{-/-}$ mice. In both wild-type and RBP$^{-/-}$mice, circulating levels of TTR were not affected by any of the CLA diets (Fig. 2A, C, D).

Our data demonstrate a differential action of the two CLA isomers on circulating levels of retinol and RBP. Specifically, only the diet supplemented with CLA t10,c12 increases the concentration of serum retinol and its specific transport protein. The absence of RBP abolishes the effect of this isomer on circulating retinol levels. Neither of the two CLA isomers influences serum TTR regardless of the presence or absence of RBP.

Effects of CLA isomers on retinoid homeostasis in liver and adipose tissue

The complex retinol-RBP is secreted from the liver to meet the vitamin A requirements of the periphery of the body (11, 12). Therefore, we set out to investigate whether CLA c9,t11 and t10,c12 isomers affect hepatic secretion of retinol and, more broadly, retinoid homeostasis in this tissue. As both RBP and TTR are expressed in the liver (11), we first measured the hepatic expression levels of these proteins in wild-type mice by Western blot analysis. CLA t10,c12 feeding resulted in a 2-fold increase in RBP levels,

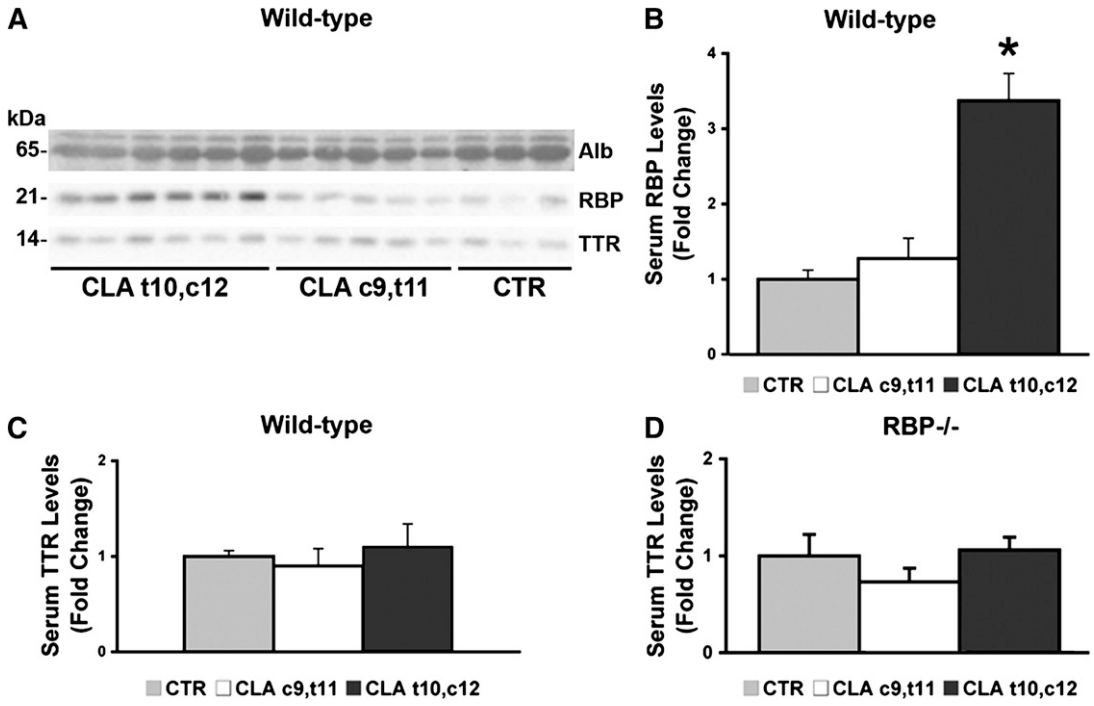

Fig. 2. Serum RBP and TTR protein levels upon dietary CLA intake. A: A representative Western blot analysis of serum RBP and TTR levels in wild-type mice fed the control diet or the CLA isomers, as indicated at the bottom of the panels. The molecular weight of each detected protein is indicated on the left side. Quantification of (B) serum RBP levels in wild-type mice, (C) serum TTR levels in wild-type mice, and (D) serum TTR levels in RBP knockout mice (RBP$^{-/-}$). Quantification of Western blots was performed by densitometry of the bands detected upon Western analysis. Albumin (Alb) was used as a loading control, as described in Materials and Methods. A reference sample was loaded on each gel to allow for normalization between gels. Results (expressed as mean ± SD) are shown as a fold change in respect to wild-type mice on the control diet. Statistical analysis was performed by ANOVA. * indicates statistically significant differences between treatment and control group ($P < 0.05$). Five to six mice per group were analyzed at 14 weeks of age.

whereas these did not change when the mice were fed CLA c9,t11 (**Fig. 3A**). In contrast to the protein, mRNA levels of hepatic RBP measured by RT real-time PCR analysis were not affected by the CLA dietary supplementation (data not shown). No statistically significant differences were observed in hepatic levels of TTR regardless of the CLA isomer (Fig. 3B). These data are consistent with the hypothesis that CLA t10,c12 stimulates secretion of the complex retinol-RBP from the liver through mechanisms that do not involve transcriptional events.

We next measured liver retinol and retinyl ester concentrations in wild-type mice by reverse-phase HPLC analysis (21). As shown in Fig. 3C and D, CLA c9,t11 feeding did not affect retinol concentrations but significantly increased retinyl ester levels. In contrast, when mice were fed the CLA t10,c12 diet, hepatic retinyl ester levels remained steady and retinol declined (Fig. 3C, D). No differences were observed in liver retinol and retinyl ester levels between mice fed the control or the olive oil diet (data not shown). To further investigate the correlation between intake of CLA and hepatic levels of vitamin A, we performed RT real-time PCR analysis to measure hepatic mRNA levels of lecithin:retinol acyltransferase (LRAT), the main enzyme that esterifies retinol into retinyl ester, the storage form of retinoids (33–35). In addition to being highly expressed in liver, the *LRAT* gene is regulated by retinoic acid, the active retinoid form, and its expression correlates with the tissue storage activity (21, 36–38). As shown in Fig. 3E, LRAT mRNA expression levels were upregulated in livers of wild-type mice fed either CLA diet. We interpret these data as an indication that dietary supplementation of either CLA isomer stimulates accumulation of retinyl ester in the liver. However, only with CLA t10,c12 intake is this followed by secretion of retinol via RBP in the bloodstream.

Dietary CLA has been shown to induce hepatic mRNA and protein accumulation of several lipid-metabolizing enzymes, including cytochrome P450 4A1 [Cyp4A1 (39, 40)]. Members of the cytochrome P450 family of enzymes, such as Cyp26A1 and Cyp2c39, are also known to oxidize retinoic acid into more polar inactive metabolites and thus, contribute to maintaining retinoid homeostasis in tissues (21, 38, 41, 42). In particular, Cyp26A1 is regulated by retinoic acid action and its expression correlates with the tissue levels of this vitamin A metabolite (42, 43). Hence, Cyp26A1 is considered a molecular marker to monitor the retinoid status of a tissue (38). To gain insights on whether CLA feeding may also alter retinoid catabolism in liver, we measured hepatic mRNA levels of Cyp26A1 and Cyp2c39 by RT real-time PCR analysis. We showed that the mRNA levels of these two enzymes were significantly elevated only in wild-type mice fed CLA t10,c12 (Fig. 3F, G).

Taken together, our data indicate a specific effect of CLA t10,c12 intake on hepatic retinol secretion and suggest that hepatic retinoid oxidation may also be influenced by this isomer of CLA.

Finally, we examined whether the excess retinol secreted from the liver bound to RBP upon dietary intake of CLA t10,c12 alters vitamin A metabolism in the target tissues. Among these, adipose tissue has been proposed to contribute significantly to the regulation of whole body retinoid homeostasis (44–46). Therefore, we measured retinol and retinyl ester levels in fat tissue of wild-type mice (21). Adipose retinol concentrations did not change with either of the two isomers (Fig. 3H). In contrast, only CLA t10,c12 induced a statistically significant increase in retinyl ester levels of this tissue (Fig. 3I). It is known that adipose tissue expresses RBP (11) although the function of the protein in this extrahepatic tissue remains unclear (47). As shown in Fig. 3J, the levels of RBP in adipose tissue were not affected when wild-type mice were fed either CLA isomer. TTR was not detected in adipose, as previously reported (48). In addition, mRNA expression levels of LPL and LDLr protein levels were both downregulated in the adipose tissues of mice fed CLA t10,c12 (supplementary Fig. I). Because both LPL and LDLr mediate tissue uptake of chylomicrons (49, 50), these results rule out the possibility that at least some of the increase in adipose retinyl esters is due to an increase in uptake of retinol from the chylomicrons. Overall, our findings support the hypothesis that when wild-type mice are fed the diet supplemented with CLA t10,c12, the retinol secreted from the liver bound to RBP is redistributed toward the periphery of the body, as seen by retinyl ester accumulation in adipose tissue.

Hepatic but not fat retinyl ester accumulation induced by CLA feeding is independent of RBP

To verify whether RBP would also mediate hepatic retinyl ester accumulation upon CLA feeding and to further confirm the central role played by this protein in redistributing retinol from the liver toward the periphery of the body upon CLA t10,c12 intake, we analyzed the effects of dietary CLA supplementation in liver and adipose tissues of the $RBP^{-/-}$ mice. We first measured liver mRNA levels of LRAT as a marker for the retinyl ester storage activity of this tissue (33–35). The fact that both CLA isomers induced a modest but statistically significant increase in hepatic LRAT expression levels (**Fig. 4A**) suggests that accumulation of retinyl ester in the liver could still be stimulated by dietary CLA in the absence of RBP. However, retinol and retinyl ester levels remained steady in the liver of $RBP^{-/-}$ mice fed CLA c9,t11, whereas CLA t10,c12 induced a statistically significant reduction in the concentration of both hepatic retinol and retinyl ester (Fig. 4B, C). Notably, we had previously reported increased Cyp2c39 mRNA levels in the liver of $RBP^{-/-}$ mice on a regular chow diet (51) and hence, proposed that elevation of Cyp2c39 expression in liver of this strain could account for turnover of hepatic retinoid reserves (51). Therefore, we wondered whether an upregulation of the mRNA levels of this gene would occur upon CLA feeding in $RBP^{-/-}$ mice, providing a potential explanation for the apparent lack of retinyl ester accumulation in the liver of this strain fed with either CLA isomer. To prove this hypothesis, we analyzed hepatic mRNA levels of both Cyp2c39 and Cyp26A1 by RT real-time PCR analysis. First, Cyp26A1 and Cyp2c39 mRNA in $RBP^{-/-}$ animals showed more robust basal ex-

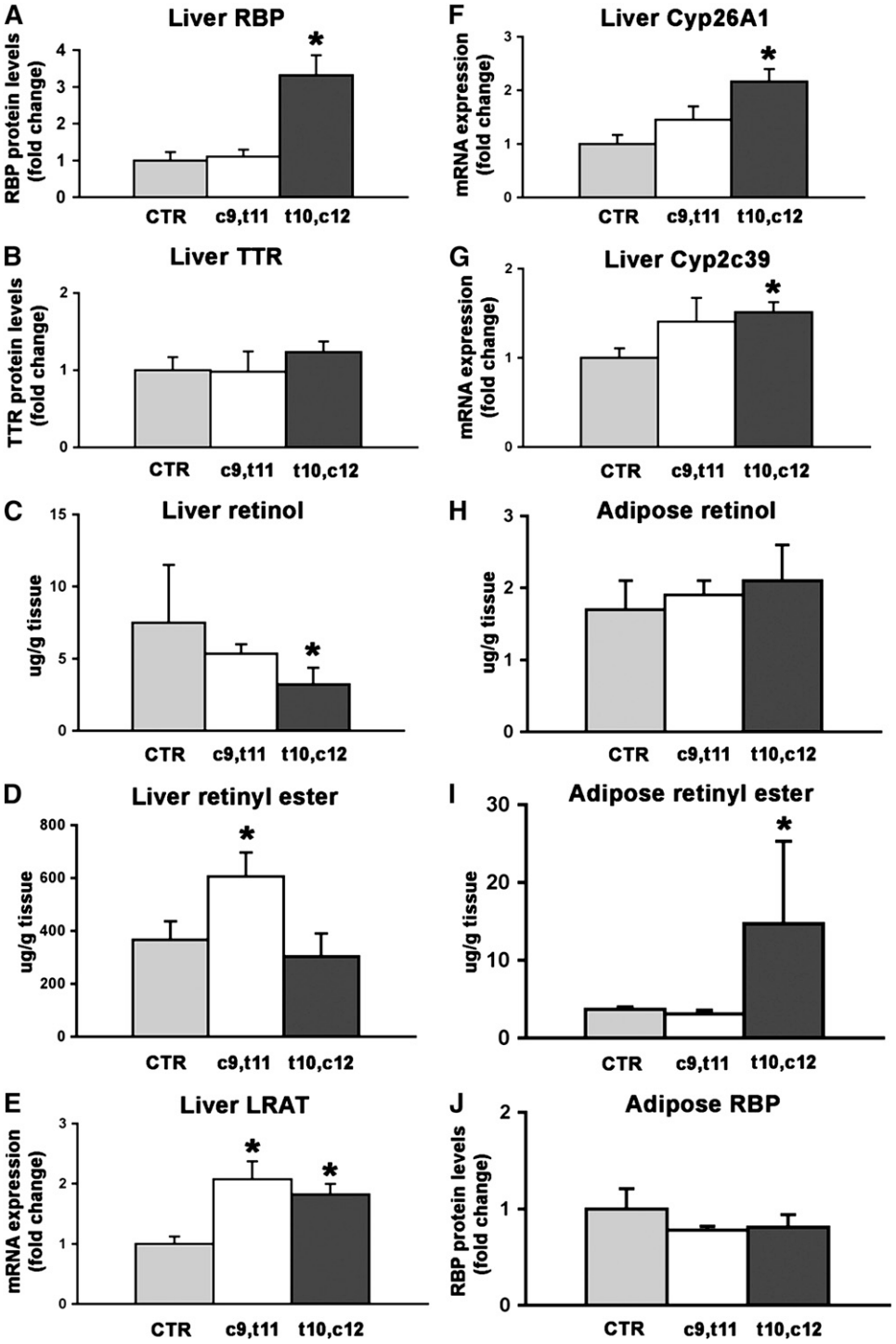

Fig. 3. Effects of dietary CLA on tissue retinoid metabolism in wild-type mice. A: RBP and (B) TTR protein levels in the liver of wild-type mice. Analysis was performed by Western blot followed by densitometry. Albumin was used as a loading control. Results (expressed as mean ± SD) are shown as fold change from the reference (wild-type mice on the control diet). C: Retinol and (D) retinyl ester levels in the liver of wild-type mice. Retinoid concentrations were measured by reverse-phase HPLC and expressed as μg/g of tissue (mean ± SD). Hepatic mRNA levels of (E) LRAT, (F) Cyp26A1, and (G) Cyp2C39 in wild-type mice. Measurements were performed by RT real-time PCR analysis. Values are expressed as mean ± SE using the $2^{-\Delta\Delta CT}$. H: Retinol and (I) retinyl ester levels in the adipose tissue of wild-type mice. Retinoid concentrations were measured by reverse-phase HPLC and expressed as μg/g of tissue. J: RBP protein levels in adipose tissue of wild-type mice. Analysis was performed by Western blot followed by densitometry. Actin was used as a loading control. Results (expressed as mean ± SD) are shown as a fold change from the reference (wild-type mice on the control diet). Statistical analysis for all parameters was performed by ANOVA; n = 4 to 6 mice per group, except in (H) and (I) where n = 3. *$P < 0.05$ versus the wild-type on the control diet.

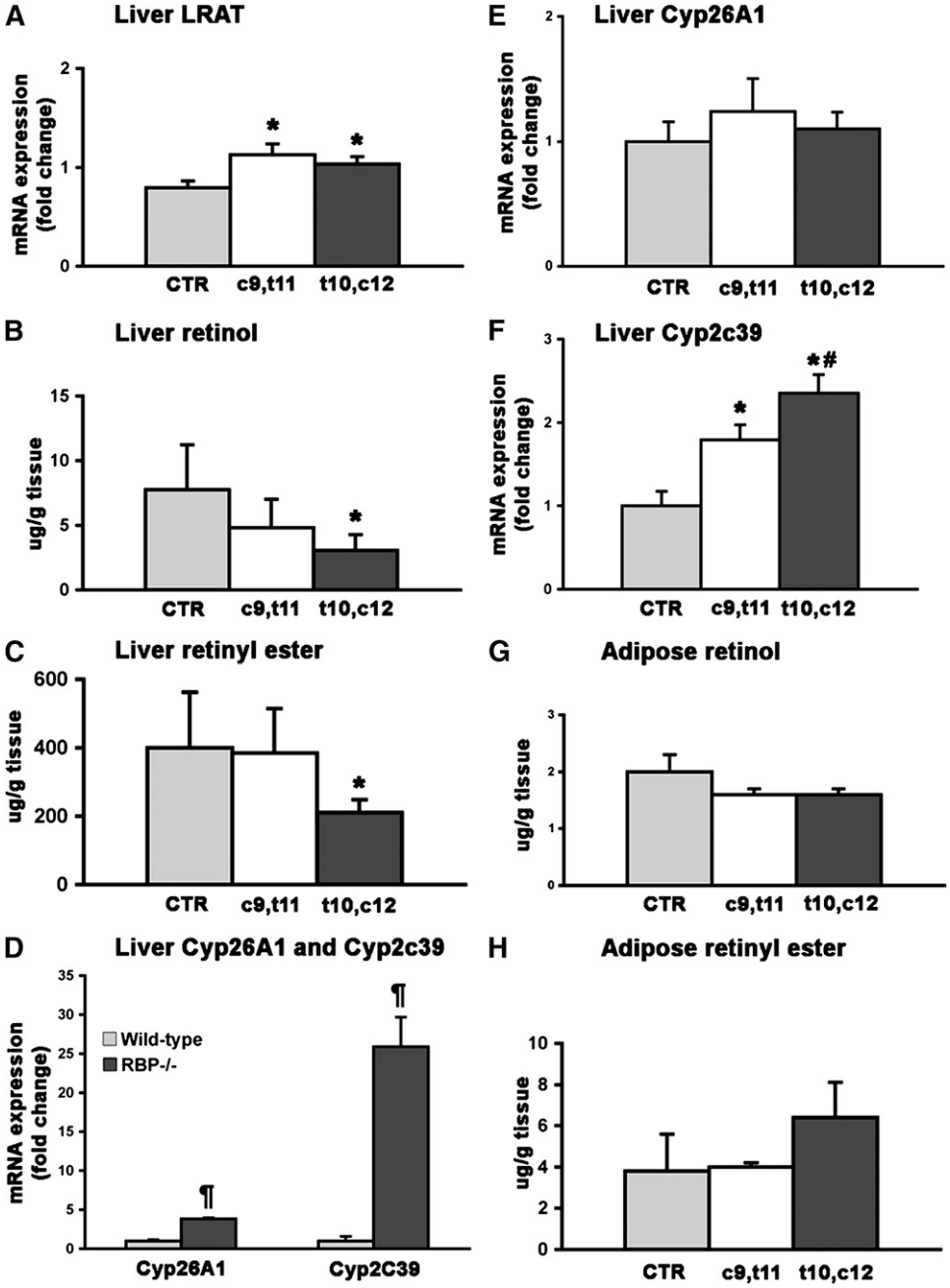

Fig. 4. Effects of dietary CLA on retinoid metabolism in RBP knockout mice ($RBP^{-/-}$). A: Hepatic mRNA levels of LRAT measured by RT real-time PCR analysis. Values are expressed as mean ± SE using the $2^{-\Delta\Delta CT}$. B: Retinol and (C) retinyl ester levels in the liver of $RBP^{-/-}$ mice. Retinoid concentrations were measured by reverse-phase HPLC and expressed as μg/g of tissue (mean ± SD). D: Hepatic mRNA levels of Cyp26A1 and Cyp2c39 in wild-type and $RBP^{-/-}$ maintained on the control diet. Measurements were performed by RT real-time PCR analysis. Values are expressed as mean ± SE using the $2^{-\Delta\Delta CT}$. E: Cyp26A1 and (F) Cyp2c39 hepatic mRNA levels in $RBP^{-/-}$ mice. Measurements were performed by RT real-time PCR analysis. Values are expressed as mean ± SE using the $2^{-\Delta\Delta CT}$. G: Retinol and (H) retinyl ester in adipose tissue of $RBP^{-/-}$ mice. Retinoid concentrations were measured by reverse-phase HPLC and expressed as μg/g of tissue (mean ± SD). Statistical analysis for all parameters was performed by ANOVA. * $P < 0.05$ versus $RBP^{-/-}$ mice on the control diet; ¶ $P < 0.05$ versus wild-type on control diet; and # $P < 0.05$ versus $RBP^{-/-}$ mice on the CLA c9,t11 diet; n = 4 to 6 mice per group, except in (G) and (H) where n = 3.

pression levels when compared with wild-type mice (Fig. 4D). Second, whereas Cyp26A1 levels remained unchanged with either CLA isomer (Fig. 4E), hepatic mRNA Cyp2c39 levels were significantly upregulated in $RBP^{-/-}$ mice fed CLA c9,t11 or CLA t10,c12 (Fig. 4F). Interestingly, the levels of Cyp2c39 in mice fed CLA t10,c12 were also significantly increased compared with those of mice supplemented with CLA c9,t11 (Fig. 4F). Overall, these data suggest that an elevated turnover of the hepatic retinoid reserves could mask the accumulation of retinyl ester

taking place in the liver of RBP$^{-/-}$ mice upon CLA feeding. They also suggest that lack of RBP does not abolish the potential effects of CLA t10,c12 on hepatic retinoid oxidation.

In the absence of RBP, no statistically significant changes were observed in adipose tissue retinol and retinyl ester levels regardless of the type of CLA isomer supplemented with the diet (Fig. 4G, H). These data confirm our hypothesis that the redistribution of retinol from the liver toward the periphery of the body upon CLA t10,c12 intake is mainly mediated by RBP.

DISCUSSION

In this report, we set out to investigate the mechanisms by which two CLA isomers (CLA c9,t11 and CLA t10,c12) induce changes in serum and tissue retinoid levels in mice. By feeding the animals with diets supplemented with enriched preparations of CLA c9,t11 or CLA t10,c12, we were able to uncouple the specific effects of these two distinct isomers on vitamin A metabolism.

Our data demonstrate that dietary CLA increases serum retinol levels in mice. Specifically, our studies are the first to identify CLA t10,c12 as the isomer responsible for this action (Table 2). Moreover, we showed that circulating RBP is also elevated only in CLA t10,c12-fed wild-type animals (Fig. 2). Retinol-RBP circulates bound to another serum protein, namely TTR, forming a complex that is critical for maintaining retinol-RBP concentration (52, 53). Serum TTR levels remained constant under this dietary regimen (CLA t10,c12; Fig. 2). However, as plasma TTR concentrations are normally 2 to 3 molar higher than those of RBP (53), the excess retinol in the bloodstream of these mice is still likely to circulate in an RBP-TTR complex.

The major site of synthesis and secretion of RBP is the hepatocyte, from where this protein mobilizes the retinoid stores (11, 12). Therefore, we hypothesized that the excess retinol in the circulation of wild-type mice fed CLA t10,c12 is secreted from the liver bound to RBP; i.e., this isomer stimulates hepatic secretion of the complex retinol-RBP. In this case, in the absence of changes in hepatic RBP mRNA levels (data not shown), we would have likely expected that hepatic RBP levels would be lower and not higher as we reported (Fig. 3A). However, the molecular mechanisms controlling retinol-RBP secretion from the liver have not been fully elucidated. Hence, our data are consistent with the hypothesis that the regulation of RBP secretion may not (or not only) take place at the transcriptional level and that other events (translational or post-translational, for example) could likely play a role in this process.

The increase in LRAT mRNA levels in the liver of mice fed CLA t10,c12 also suggests that retinyl ester accumulation occurs with this isomer (Fig. 3E). However, no change in the hepatic retinyl ester concentration was observed under this dietary regimen (CLA t10,c12; Fig. 3D). This discrepancy in our data is only apparent because retinol and retinyl ester measurements reflect a steady state vitamin A status of the tissue and do not exclude a rapid turnover of retinyl ester accumulated in the liver through secretion in the bloodstream of retinol bound to RBP.

Moreover, our data have also shown, for the first time, that CLA t10,c12 feeding upregulates the mRNA levels of Cyp26A1 and Cyp2c39 in the liver of wild-type animals (Fig. 3F, G). These two enzymes are known to oxidize retinoic acid into more polar inactive metabolites (41, 43), thus contributing to maintaining retinoid homeostasis in tissues. In addition, Cyp26A1 mRNA levels are upregulated by retinoic acid (and so are those of LRAT) and hence are considered an indirect means to evaluate the retinoic acid status of a tissue (38, 43). Thus, increased hepatic mRNA Cyp26A1 levels may suggest that tissue retinoic acid levels could be elevated. Based on these observations, we cannot exclude that CLA t10,c12 also stimulates hepatic retinoid oxidation, which will further contribute to the turnover of the liver retinoid reserve under CLA t10,c12 feeding. Additional experiments are needed to unequivocally confirm this hypothesis.

In the case of wild-type mice fed the CLA c9,t11 diet, the increased concentration of hepatic retinyl ester (Fig. 3C, D) and the upregulation of the mRNA levels of LRAT in the liver (Fig. 3E) clearly demonstrate that the intake of this isomer induces hepatic retinyl ester accumulation. However, the lack of change in serum retinol and RBP levels (Table 2 and Fig. 2) and hepatic RBP protein (Fig. 3A) in mice fed CLA c9,t11 indicate that vitamin A accumulation in the liver stores is not followed by secretion of the retinol-RBP complex under this dietary regimen. Interestingly, hepatic mRNA levels of Cyp26A1 and Cyp2c39 are also unaffected by this dietary regimen in wild-type mice (Fig. 3F, G), confirming that hepatic retinoid oxidation may be stimulated only by CLA t10,c12 feeding.

The reasons for these differential effects of the two CLA isomers on hepatic vitamin A metabolism are not presently clear. It is known that when mice are supplemented with the CLA t10,c12 isomer, the liver becomes steatotic and increases in mass up to four times [reviewed in (54)]. In agreement with this notion, we observed elevated levels of triglycerides exclusively in the livers of animals fed CLA t10,c12 [CTR: 12.4 ± 4.2; CLA c9,t11: 8.2 ± 2.5; CLA t10,c12: 94.0 ± 24.7. Values (μmol/g of liver) are expressed as mean ± SD; n = 4 to 6 samples/group]. We found it intriguing that hepatic steatosis and increased hepatic retinol secretion (and possibly oxidation of retinoids) only take place upon CLA t10,c12 supplementation. We speculate that the stimulation of hepatic retinol secretion (and possibly oxidation of retinoids) induced by intake of this isomer may be part of a compensatory mechanism of the tissue to counter the excessive accumulation of lipids, including retinoids. Notably, serum RBP levels have been shown to be elevated in patients with nonalcoholic fatty liver disease (55).

The origin of the excess vitamin A that accumulates in the liver of mice fed CLA remains unclear. The presence of dietary fat in the intestine can stimulate retinyl ester digestion by enhancing the secretion of pancreatic enzymes and bile salts and providing products of lipid digestion, which serve as components of micelles. In addition,

fat ingestion promotes vitamin A absorption by providing the lipid components for intestinal chylomicron assembly [reviewed in (56)]. Because CLA is a naturally occurring polyunsaturated fatty acid, we hypothesize that dietary CLA may increase the intestinal absorption of vitamin A, thus resulting in its accumulation in the storage tissues. Nevertheless, as we did not observe any effects of olive oil supplementation on serum or tissue levels of retinol and retinyl ester regardless of the genotype of the mice (Table 2 and data not shown), we speculate that each individual fatty acid may have a specific impact on intestinal vitamin A absorption. Further experiments are needed to unequivocally confirm these hypotheses.

To rule out that the excess retinol in the circulation bound to RBP upon CLA t10,c12 feeding is the result of vitamin A mobilization from the periphery of the body, we analyzed the retinoid status of the adipose tissue, another important site of retinoid storage that expresses RBP (11, 44–46). In wild-type mice, adipose RBP protein levels remained unaffected upon CLA feeding with either isomer (Fig. 3J). In addition, only CLA t10,c12 supplementation resulted in elevated levels of retinyl ester in fat tissue with no changes in retinol concentration (Fig. 3I, H). We find it unlikely that the increase in adipose retinyl esters is due to an increase in uptake of retinol from the chylomicrons. Indeed, when the complex retinol-RBP is absent (i.e., in the $RBP^{-/-}$ animals), the dietary supplementation with CLA does not affect retinyl ester levels of the adipose tissue (Fig. 4H). This would not be the case if at least some of the elevation in fat retinyl esters observed upon CLA t10,c12 feeding in wild-type mice would be due to an increased uptake of retinol from the chylomicrons. In addition, CLA t10,c12 feeding downregulates LPL (mRNA levels) and LDLr (protein levels) (supplementary Fig. I), two key mediators of the tissue uptake of chylomicrons (49, 50). We take these results as an indication that the CLA-stimulated secretion of retinol-RBP does not take place from fat and that the liver redistributes the excess retinol bound to RBP toward the fat tissue, where it is stored as retinyl ester.

In contrast to experiments performed with pigs (57), liver RBP mRNA levels did not change in our study regardless of the dietary supplementation (data not shown). Also, hepatic retinol levels did not increase upon CLA feeding as reported in experiments performed with rats (17, 18). Possibly, these are species-specific differences in the response of the tissues to the CLA supplementation. Alternatively, different techniques (semiquantitative PCR analysis (57) vs. real-time PCR analysis to measure RBP mRNA levels) or the use of enriched preparations of isomers versus preparations containing the same amounts of each isomer (17, 18, 57) could account for these discrepancies.

The availability in our laboratory of a mouse strain lacking RBP (19) enabled us to confirm the central role played by this protein in mediating the perturbations of vitamin A metabolism induced by CLA feeding. Mice lacking RBP fail to exhibit increased serum retinol levels when fed CLA t10,c12 (Table 2). These data, together with the observation that adipose retinyl ester levels in $RBP^{-/-}$ mice remain steady under this dietary regimen (Fig. 4G, H), demonstrate that hepatic secretion of retinol-RBP and its redistribution toward fat tissue induced by CLA t10,c12 are impaired by the absence of the specific transport protein for retinol. Lack of RBP does not affect the ability to build hepatic retinyl ester stores (51). However, the liver of the $RBP^{-/-}$ mice does, to a significant extent, turn over its retinoid stores. It does so without secreting retinol bound to RBP but rather increasing the catabolic metabolism of retinoid in this tissue, as suggested by the upregulation of hepatic Cyp2c39 mRNA levels [(51) and Fig. 4D]. It seems reasonable to propose that the basal high levels of Cyp2c39 mRNA levels [(51) and Fig. 4D] and the even more robust upregulation of this gene seen upon CLA feeding (Fig. 4F) could stimulate a rapid turnover of the hepatic retinoid reserve that would then account for the failure to accumulate retinyl ester in the liver of the $RBP^{-/-}$ mice supplemented with CLA isomers (Fig. 4C) despite the upregulation of the LRAT mRNA levels (Fig. 4A).

In conclusion, our study shows a dramatic effect of dietary CLA on murine retinoid metabolism and allows, for the first time, the identification of both distinct and common actions of two biologically active isomers of CLA, CLA c9,t11 and t10,c12, in influencing hepatic retinol storage, secretion, and delivery to the periphery of the body. Our study provides evidence that RBP mediates hepatic retinol secretion and its redistribution toward fat tissue induced by CLA t10,c12 supplementation.

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