Retinoic Acid Treatment Enhances Lipid Oxidation and Inhibits Lipid Biosynthesis Capacities in the Liver of Mice

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Key Words

Vitamin A • Retinoic acid • Body weight • Adiposity • Liver lipid metabolism • Liver fat content

Abstract

Vitamin A, mainly as retinoic acid (RA), is known to affect the development and function of adipose tissues. Treatment with RA reduces body weight and adiposity independent of changes in food intake in mice. Lipid metabolism in liver can have a major impact on whole body adiposity. The aim of this work was to investigate the effects of an *in vivo* treatment with RA on hepatic lipid metabolism in mice. Adult, standard diet-fed mice were treated with different doses of all-trans RA or vehicle (subcutaneous injection) for 4 days before sacrifice. Food intake and body weight changes during treatment were determined, as well as adiposity, liver composition, levels of circulating metabolites and lipoproteins and expression levels of key mRNA species in liver following sacrifice. RA treatment resulted in reduced body weight and adiposity, as expected. In the liver, RA treatment triggered an increase in the mRNA expression levels of peroxisome proliferator-activated receptor alpha, retinoid X receptor alpha, uncoupling protein 2, liver-type carnitine palmitoyltransferase 1,

and carnitine/acylcarnitine carrier, and a reduction in the mRNA expression levels of sterol regulatory element binding protein 1c and fatty acid synthase. Consistent with the changes in gene expression, hepatic triacylglycerol content and circulating VLDL fraction were reduced and levels of circulating ketone bodies increased after RA treatment. These results point to a capacity of active vitamin A forms to shift liver lipid metabolism *in vivo* towards increased catabolism and reduced lipogenesis. These effects might contribute to the reduction of adiposity brought about by RA treatment.

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Introduction

Retinoic acid (RA), the carboxylic acid form of vitamin A, is a nutrient derivative with many remarkable effects on adipocyte biology and whole body adiposity (reviewed in [1, 2]). Previous studies have shown that, in mice, treatment with RA reduces body weight and adipose depot mass independent of changes in food intake [3-9], and improves glucose tolerance and insulin sensitivity linked to effects on adipokine expression [6, 9, 10]. RA-induced body fat loss correlates with activation of brown

adipose tissue (BAT) [3, 4], and with reduced adipogenic/lipogenic capabilities [5] and increased capabilities for oxidative metabolism and thermogenesis in white adipose tissue (WAT) depots [8, 9] and skeletal muscle [9, 11, 12]. Chronic dietary vitamin A supplementation (as retinyl ester) also increases thermogenic potential in BAT and muscle [11, 13, 14], reduces body fat content [13, 14], and partly opposes the development of obesity in dietary [11] and genetic [14] models in mice and rats. A poor vitamin A status, on the other hand, favors an increment of adiposity that in mice correlates with an increased WAT adipogenic potential and a reduced BAT thermogenic potential [4, 5].

RA regulates gene expression in mammals through diverse mechanisms, notably by binding to and activation of two types of retinoid receptors that belong to the nuclear receptor superfamily of ligand-modulated transcription factors: the retinoic acid receptors (RARs), which respond to both all-trans RA (ATRA) and 9-cis RA, and the retinoid X receptors (RXRs), which respond specifically to 9-cis RA, both existing in different isoforms (reviewed in [15]). RAR:RXR heterodimers control the expression of typical retinoid-target genes. In addition, many other nuclear receptors bind to target gene promoters as heterodimers with RXR [16]. The transcriptional activity of some RXR-containing heterodimers (the so-called permissive heterodimers) responds to ligands of either partner and is synergistically activated in the presence of both ligands, providing a mechanism for widespread effects of retinoids on gene expression [16]. In particular, peroxisome proliferator-activated receptors (PPARs) act on target genes as permissive heterodimers with RXR [16, 17]. PPARs are lipid-activated nuclear receptors critically involved in the transcriptional control of lipid metabolism [18].

The liver is the main storage site of vitamin A in the body and a highly active metabolic organ that plays a central role in coordinating whole body metabolism. In particular, by functionally interacting with the intestinal tract and adipose tissue the liver plays a key role in various aspects of lipid metabolism (reviewed in [19]). Changes in lipogenesis and/or fat oxidation in liver can underlie the development of non-alcoholic fatty liver and condition the availability of substrates for adipose tissue expansion. Liver lipid metabolism is influenced by a complex system of hormones, hormonally regulated signaling pathways and transcription factors. Among the latter, sterol regulatory element binding protein-1c (SREBP-1c) and PPAR α are key to the transcriptional control of hepatic fatty acid synthesis and oxidation, respectively (reviewed

in [20, 21]). Modulation of the hepatic expression of several genes for proteins involved in lipogenesis by RA and vitamin A status has been reported, both in hepatocyte cell models and in vivo (see [22, 23]). However, studies regarding the effects of vitamin A or its derivatives on liver lipid metabolism have lead to controversial results. For instance, vitamin A deficiency has been reported to associate with both reduced [24] and enhanced [25, 26] hepatic fat catabolism.

The above background prompted us to investigate changes in liver lipid metabolism in response to RA treatment in mice.

Materials and Methods

Animals, diet, ATRA treatment, and tissue collection

Thirteen-week-old NMRI male mice (Charles River, Barcelona, Spain) were used. The animals were kept on wood shavings in plastic cages with 2-3 mice per cage and maintained at 22 °C with a 12:12-hour light-dark cycle (lights on at 08:00 h). Mice fed ad libitum regular laboratory chow (Panlab, Barcelona, Spain; approximately 68% carbohydrate-, 22% protein- and 9%- lipid derived energy; 2.5 IU vitamin A/kcal) received one daily subcutaneous injection of ATRA (Sigma, St. Louis, MO) at a dose of 10 or 100 mg/kg body weight during the 4 days before they were killed (five to six animals per group). Controls were injected the vehicle (100 μ l olive oil). Body weight and energy intake were followed daily during the treatment period. Energy intake was estimated on a per-cage basis from the actual amount of food consumed by the animals and its caloric equivalence. The animals were killed with CO₂ and decapitated at the start of the light cycle. Blood was collected from the neck and serum prepared by centrifugation and frozen at -20 °C. Liver and epididymal white adipose tissue (eWAT) were excised in their integrity, weighted, rapidly frozen in liquid N₂ and stored at -70 °C until analysis. The weight of the eWAT depot expressed as percentage of body weight was previously shown to largely correlate with total body fat content [27] and was used as adiposity index. For serum lipoprotein pattern analysis, an independent experiment was conducted in which fresh serum was collected from control (vehicle-treated) mice and mice treated as described above with an intermediate dose of 50 mg ATRA per kg of body weight per day. Aminotransferase activity in serum, a marker of hepatic damage, was previously shown to be unaffected by the ATRA treatment applied [7]. Indirect evidence of increased circulating levels of ATRA following treatment has been reported previously [6, 9, 10].

All animal experimentation was conducted in accord with accepted standards of humane animal care, and the protocols were approved by our institutional bioethical committee.

Serum measurements

Commercial enzymatic colorimetric kits were used for the determination of serum non-esterified fatty acid (NEFA) pool

Gene	Primer sequence (forward first, reverse second; 5'→3')	Size (bp) and accession no.
RXR α	TACCCACCACACCCACATT GGTTCCGCTGTCTCTGTGTC	420 NM_011305
PPAR α	CGTTTGTGGCTGGTCAAGTT AGAGAGGACAGATGGGGCTC	117 NM_011144
PPAR δ	GGAACAGCCACAGGAGGA AGGGAGGAAGGGGAGGAA	155 NM_011145
PGC-1 α	AGGAGGGTCATCGTTTGTGG GGAGGCAGAAGAGCCGTC	258 NM_008904
UCP2	CAGTTCTACCAAGGGCTC AGCATGGTCAGGGCACAGTG	469 NM_019354
CPT1L	GCTCGCACATTACAAGGACAT TGGACACCACATAGAGGCAG	268 NM_013495
CAC	GCAGACACAACCAAGTT AAACCAAACCAAGAAGCA	171 NM_020520
OCTN2	ACAGTATCCCGTTGGAGACG ACACCAGGTCCCACTCTGTC	213 NM_011396
ACOX1	CCGCCACCTTCAATCCAG ACTCTTGGGTCTTGGGGTCA	456 NM_015729
FGF21	ACAGATGACGACAGGACAC AGGCTTTGACACCCAGGATT	145 NM_020013
LXR α	GGGCATGATTGAGAAGTTGG GTAGGAAGCCAGGGAGCTGT	206 NM_013839
SREBP1c	AGCCATGGATTGCACATTTG GGTACATCTTTACAGCAGTG	260 NM_011480
FASN	CCAGAGCCCAGACAGAGAAG GACGCCAGTGTTCGTTCC	345 NM_007988
SCD1	GGAATGAACGAGAGAAGGTG CCGAAGAGGCAGGTGTAGAG	227 NM_009127
ACC1	CAACTCAGCAAGACCACAA CTGTTTAGCGTGGGGATGTT	395 NM_133360
ACC2	TTCCAGATGCTAATGGGTTG GCAGGTCCAGTTCTTGTGTT	336 NM_133904
β -actin	ACGGGCATTGTGATGGACTC GTGGTGGTGAAGCTGTAGCC	164 NM_009948

Table 1. Primers used in the PCR.

(Wako Chemicals GmbH, Neuss, Germany) and circulating levels of triacylglycerol (TR0100; Sigma, St. Louis, MO) and 3-hydroxybutyrate (BEN srl, Milan, Italy). For the semi-quantification of serum lipoproteins, five microliters of fresh serum was used to separate lipoprotein fractions using agarose gel electrophoresis in a tris-barbital buffer, followed by staining with a methanol solution of Fat Red 7B (Helena Laboratories, Beaumont, TX), according to the supplier's instructions. The stained bands were quantified using an image processing and analyzing program (GeneTools; Syngene, Frederick, MD).

Tissue composition

A sample of liver was homogenized in PBS (137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer, pH 7.4). The tissue homogenates were filtered through a nylon filter (250 μ m pore size) prior to protein, triacylglycerol and DNA determination.

Protein was measured by the method of Bradford [28]. Tissue triacylglycerol levels were measured using a commercial enzymatic colorimetric kit (TR0100; Sigma, St. Louis, MO). Tissue DNA was determined by a fluorimetric method that uses 3,5-diaminobenzoic acid [29]. A separate sample of tissue was digested in boiling NaOH, precipitated with alcohol, washed and deproteinized with trichloroacetic acid, prior glycogen content measurement by a colorimetric anthrone procedure [30].

Total RNA extraction and semiquantitative RT-PCR analysis

Total RNA was extracted from liver samples using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the supplier's instructions. The yield and purity of total RNA was measured spectrophotometrically. The ratio of absorption at 260 and 280 nm (A_{260}/A_{280}) was between 1.5 and 1.8 for all preparations. Integrity of the RNA extracted was further verified by ethidium bromide staining after electrophoresis in a 1.5% agarose gel. Levels of PPAR α , PPAR β/δ , LXR α , RXR α , peroxisome proliferator-activated receptor γ coactivator 1 α (PGC1 α), uncoupling protein 2 (UCP2), liver-type carnitine palmitoyltransferase 1 (CPT1-L), carnitine/acylcarnitine carrier (CAC), organic cation transporter 2 (OCTN2), acyl-CoA oxidase I (ACOX1), SREBP-1c, fatty acid synthase (FAS), acetyl-CoA carboxylase-1 (ACC1), acetyl-CoA carboxylase-2 (ACC2), fibroblast growth factor 21 (FGF21) and stearoyl-coenzyme A desaturase 1 (SCD1) mRNAs were semi-quantified by RT-PCR assays using the housekeeping gene β -actin as internal control as described previously [12, 31]. Primers sets used are detailed in Table 1.

Statistical analysis

Data are expressed as means $\pm$ SEM. Multiple comparisons were assessed by ANOVA of repeated measures. Single comparisons between groups were assessed by Student's *t*-test or one-way ANOVA followed by least-significance difference (LSD) post-hoc comparison. Results were considered as statistically significant when $P < 0.05$. The analyses were performed with SPSS 14.0 for windows (SPSS, Chicago, IL).

Results

Effects of ATRA treatment on biometric, metabolic and liver parameters in mice

ATRA treatment led to a significant reduction in body weight (Fig. 1A) and body fat (Fig. 1C) without significantly affecting energy intake (Fig. 1B), in accordance with previous reports [3-8]. ATRA-induced body weight reduction was dose- and time-dependent ($P < 0.05$ for the interactive effect between ATRA dose and time of treatment in ANOVA of repeated measures). Dose-dependent differences became significant at the end of treatment ($P < 0.005$ in ANOVA one-way), when animals treated with 10 and 100 mg ATRA/kg body weight/day

Fig. 1. Effect of ATRA treatment on body weight (A), food intake (B) and adiposity index (C) of mice. Thirteen-week-old NMRI male mice fed with standard chow received (indicated by ↓) a daily subcutaneous injection of vehicle (olive oil) or ATRA at a dose of 10 or 100 mg/kg body weight during the 4 days before they were killed (at 5th day). Data represent the means ± SEM of at least 5 animals per group. Energy intake represents the means ± SEM of 2 cages (2-3 animals each) per group, and was estimated from the actual amount of food consumed by the animals and its caloric equivalence. The weight of the epididymal fat depot expressed as percentage of body weight was previously shown to largely correlate with total body fat content [27]. Significant differences ($P < 0.05$) were tested by ANOVA-one way and LSD post hoc comparison. Values not sharing a common letter were statistically different: a and b refer to differences between ATRA doses following 4 days of treatment; z and y refer to differences between days of treatment with 100 mg ATRA/kg/day.

exhibited a 6 and 14% body weight reduction as compared with vehicle-treated control animals, respectively. Time-dependent differences became significant in the

Fig. 2. Effect of ATRA treatment on serum parameters (A) and on lipoprotein profile (B) of mice. Thirteen-week-old NMRI male mice fed with standard chow received a daily subcutaneous injection of vehicle (olive oil) or the indicated ATRA doses during the 4 days before they were killed. Serum lipoprotein analysis was performed by electrophoresis as described in the Material and Methods section, using 5 µL of fresh serum. Data are the means ± SEM of at least 5 animals per group. Significant differences ($P < 0.05$) were tested by Student's t test. NEFA, non-esterified fatty acids.

animals treated with 100 mg ATRA/kg body weight/day, in which body weight reductions of 1, 3, 9 and 14% were observed after 1, 2, 3 and 4 days of treatment compared with day 0, respectively ($P < 0.05$ in ANOVA one-way) (Fig. 1A).

Circulating 3-hydroxybutyrate levels were moderately increased (by 30%, $P < 0.05$) in mice treated with the high ATRA dose (100 mg per kg of body weight), suggesting increased hepatic fatty acid catabolism (Fig. 2A). Trends to reduced circulating levels of triacylglycerol and NEFA in the ATRA-treated animals did not reach statistical significance ($P = 0.162$ and $P = 0.135$, respectively) (Fig. 2A). No changes were observed in serum glucose and insulin levels (data not shown), in accordance with previous reports [8, 10].

In contrast with the observed reduction of WAT mass, fresh liver weight and liver weight as percentage of body weight were not significantly affected by ATRA treatment at any of the doses tested (Table 2). However, the treatment brought about a dose-dependent reduction of liver triacylglycerol content, which reached a signifi-

	ATRA -treatment			ANOVA (<i>P</i> value)
	Vehicle	10 mg/kg	100 mg/kg	
Liver mass (g)	2.07 ± 0.04	1.85 ± 0.07	1.77 ± 0.13	
Liver mass (g/100 g body weight)	4.99 ± 0.17	4.72 ± 0.08	4.95 ± 0.35	
Protein (mg/g wet tissue)	114 ± 5	118 ± 5	112 ± 5	
DNA (mg/g wet tissue)	4.23 ± 0.42	4.92 ± 0.47	5.74 ± 0.46	
Glycogen (mg/g wet tissue)	22.7 ± 5.9 ^a	11.6 ± 2.6 ^b	4.6 ± 2.2 ^b	<i>P</i> < 0.05
Triacylglycerol (mg/g wet tissue)	34.4 ± 6.9 ^a	25.7 ± 1.6 ^a	11.3 ± 3.2 ^b	<i>P</i> < 0.05

Table 2. Effect of ATRA treatment on liver mass and liver composition of mice. Thirteen-week-old NMRI male received a daily subcutaneous injection of ATRA at a dose of 10 or 100 mg/kg body weight during the 4 days before they were killed. Control animals received the vehicle (olive oil). Data are the means ± SEM of at least 5 animals per group. Significant differences were tested by ANOVA-one way and LSD post hoc comparison. Values in the same row not sharing a common letter were statistically different: a and b refer to differences between ATRA doses following 4 days of treatment.

Fig. 3. Effect of ATRA treatment on the expression of genes related to fatty acid oxidation in liver of mice. Thirteen-week-old NMRI male mice fed with standard chow received a daily subcutaneous injection of vehicle (olive oil) or ATRA at a dose of 10 or 100 mg/kg body weight during the 4 days before they were killed. The expression levels of the indicated mRNAs were semiquantified by RT-PCR and normalized to the expression of the housekeeping gene β -actin. Data are the means ± SEM of at least 5 animals per group and are expressed relative to the mean value of the control, vehicle-injected, group, which was set to 100. Significant differences were tested by one-way ANOVA and LSD post hoc comparison. For each gene, values not sharing a common letter were statistically different: a, b and c refer to differences between ATRA doses following 4 days of treatment.

cant ($P < 0.05$) 3-fold reduction in the group treated with the high ATRA dose (Table 2). Liver glycogen content was also significantly reduced in the ATRA-treated mice, whereas protein and DNA content per gram of fresh liver remained unchanged (Table 2).

Effects of ATRA treatment on serum lipoprotein pattern in mice

Serum lipoprotein profiles in control (vehicle-treated) and 50 mg ATRA/kg body weight-treated mice were qualitatively analyzed on agarose gels stained with Fat Red 7B, followed by quantification of the stained bands.

ATRA treatment brought about a reduction in pre-beta VLDL levels accompanied by an increase in beta LDL levels ($P < 0.05$ for both parameters, Student's *t*-test) (Fig. 2B). No changes were observed in serum alpha HDL lipoprotein levels.

Effects of ATRA treatment on hepatic expression of genes related to fatty acid oxidation and lipogenesis in mice

Fig. 3 summarizes changes elicited by ATRA treatment in the hepatic mRNA expression levels of genes encoding transcription-related factors, metabolic enzymes,

Fig. 4. Effect of ATRA treatment on the expression of genes related to fatty acid biosynthesis in liver of mice. Thirteen-week-old NMRI male mice fed with standard chow received a daily subcutaneous injection of vehicle (olive oil) or ATRA at a dose of 10 or 100 mg/kg body weight during the 4 days before they were killed. The expression levels of the indicated mRNAs were semiquantified by RT-PCR and normalized to the expression of the housekeeping gene β -actin. Data are the means $\pm$ SEM of at least 5 animals per group and are expressed relative to the mean value of the control, vehicle-injected, group, which was set to 100. Significant differences were tested by one-way ANOVA and LSD post hoc comparison. For each gene, values not sharing a common letter were statistically different: a and b refer to differences between ATRA doses following 4 days of treatment.

signals and transport proteins for which there is evidence of their involvement in lipid catabolism. PPAR α and PPAR β/δ are key to the transcriptional control of fatty acid oxidation, acting mainly in liver and skeletal muscle, respectively [18]. Hepatic PPAR α mRNA levels were modestly but significantly increased in the group treated with the high ATRA dose (100 mg per kg of body weight), in which hepatic PPAR β/δ mRNA levels were, on the contrary, down-regulated. Hepatic RXR α mRNA levels were robustly increased in a dose-dependent manner after ATRA treatment. RXRs are obligate heterodimer partners for PPARs to efficiently act on target gene promoters [16], and RXR α is the most abundant RXR isoform in the adult liver [32]. Hepatic mRNA expression levels of UCP2 (which may play a role favoring fatty acid oxidation [33]), CPT1-L (the rate-limiting enzyme in long-chain fatty acyl-CoA uptake and oxidation in mitochondria) and CAC (which transports acylcarnitine esters into the mitochondria in exchange of free carnitine) were increased after ATRA treatment. No noticeable effect of

treatment was observed on the hepatic mRNA levels of: PGC1 α (a transcriptional cofactor for genes related to mitochondria biogenesis and function), ACOX1 (a rate-limiting enzyme of peroxisomal fat oxidation), OCTN2 (which acts as a transporter of carnitine into the cell), and FGF21 (a liver-born secreted signal that has been related to the control of liver ketogenesis and WAT lipolysis).

We also analyzed the mRNA levels in liver of several genes related to lipogenesis (Fig. 4). Expression levels of SREBP-1c and its target gene FAS, which is directly involved in the *de novo* fatty acid synthesis through its catalytic activity, were both dramatically reduced after ATRA administration, already at the low dose used. No effects of treatment were observed on the mRNA levels of LXR α , which as a heterodimer with RXR is required for the active transcription of the SREBP-1c gene (see [34]), and SCD1, a desaturase enzyme which might be required for efficient hepatic triacylglycerol synthesis [35]. We also measured the hepatic mRNA expression levels of ACC1 and ACC2, whose activities have been linked to the generation of malonyl-CoA for *de novo* fatty acid synthesis and the control of mitochondrial fatty acid oxidation through allosteric inhibition of CPT1, respectively [36]. Expression of ACC1 was increased in mice treated with the high ATRA dose, perhaps reflecting a compensatory mechanism to counteract liver lipid depletion observed at this dosage, while expression of ACC2 was unaffected by ATRA treatment.

Discussion

Findings in this work strongly suggest that ATRA treatment in intact mice favors repartitioning of fatty acids in the liver away from triacylglycerol storage and towards oxidation. At the molecular level, this effect appears to be mediated by (i) an increased hepatic expression of genes encoding regulatory and metabolic proteins that favor fatty acid oxidation (PPAR α , RXR α , CPT1-L, CAC, UCP2), and (ii) a decreased hepatic expression of genes encoding proteins involved in lipogenesis (SREBP-1c, FAS). Consistent with this pattern of gene expression changes, ATRA treatment led to a reduction in liver triacylglycerol content and circulating VLDL fraction, and to increased levels of circulating 3-hydroxybutyrate.

Subcutaneous administration of ATRA at the same or comparable dosages as those used in this work has been used in previous studies dealing with ATRA effects on energy balance and adiposity. These studies have

shown that ATRA treatment leads to loss of body weight and fat mass without reducing energy intake [3-9], effects that have been traced mainly to enhanced energy utilization in adipose tissues and skeletal muscle [3-5, 8, 9, 11, 12]. Results in this work strongly suggest that changes in liver lipid metabolism might also contribute to the slimming effect of ATRA treatment. Moreover, considering the close relationship that exists between hepatic fat content and insulin resistance [37], reduction in hepatic fat content might be one specific factor contributing to the previously reported increase in systemic insulin sensitivity following ATRA treatment in mice [6, 9, 10].

The systemic conditions triggered by ATRA treatment may favor the transcriptional activity of permissive PPAR:RXR heterodimers in liver by ensuring the provision of both fatty acid agonist of the PPAR moiety, through enhanced lipid mobilization in WAT, and 9-*cis* RA, which can be produced from ATRA through isomerization [38]. In fact, many of the gene expression changes that we found might be explained by activation of PPAR α , the main hepatic PPAR isoform and a central regulator of liver lipid metabolism [21]. CPT1-L is a direct target gene of PPAR α in liver [21]. PPAR α agonists induce UCP2 gene transcription in mouse liver through an indirect mechanism [39], and positive effects of retinoids on UCP2 gene transcription have been attributed to their capacity to activate PPAR:RXR, rather than RAR:RXR [40]. The Slc25a20 gene, which encodes CAC, is a PPAR α target gene whose expression in cultured hepatocytes is induced by both PPAR α agonists and 9-*cis* RA [41]. Activation of PPAR α may also be involved in the down-regulation of SREBP-1c and FAS mRNA levels in the liver of ATRA-treated mice, as evidence from cell studies indicates that PPAR α activation can suppress lipogenic gene expression in hepatocytes through repression of the LXR-SREBP-1c pathway [34, 42]. In addition, opposite circadian rhythms of expression of FAS and PPAR α mRNA have been reported in the liver of normal mice [43]. However, the involvement of PPAR α in liver lipogenic gene expression is complex, as PPAR α was shown to be required for the normal response of lipogenic genes to circadian rhythms and re-feeding [43, 44], and there is evidence that pharmacological PPAR α activators can induce both fatty acid oxidation genes and lipogenic genes in liver of rodents [45, 46]. Interestingly, some known target genes of PPAR α in the liver - such as the FGF21 encoding gene [47, 48], the OCTN2 encoding gene [49] and the ACOX encoding gene [21] - were not induced following ATRA treatment

in our system, which suggests that permissivity of the PPAR α :RXR heterodimer might be gene-specific. Gene-specific permissivity has been reported for some RXR-containing nuclear receptor heterodimers, such as the LXR:RXR [50].

ATRA treatment brought about a robust increase in hepatic RXR α mRNA levels, which may translate into increased possibilities of formation of RXR-containing heterodimers. Increased RXR α expression has also been reported in skeletal muscle of ATRA-treated mice [12], while in other tissues, such as BAT, reduced RXR α expression following ATRA treatment was reported [51, 52]. Overall, changes elicited by ATRA treatment on the hepatic mRNA levels of PPAR β/δ (down-regulation), LXR α (no changes), PPAR α (modest increase) and RXR α (robust increase) found in this work might be consistent with favored PPAR α :RXR α heterodimer formation, which would add to treatment effects favoring PPAR α :RXR α heterodimer activation.

Metabolic outcomes observed in this work are also consistent with increased PPAR α :RXR-mediated responses in the liver of ATRA-treated mice. Similar to ATRA-treatment, activation of PPAR α :RXR by synthetic PPAR α agonists reduces hepatic steatosis, decreases the production of triacylglycerol-rich VLDL, and decreases plasma triacylglycerol levels [53]. Similar effects have been reported following treatment of rodents with RXR-specific agonists [17, 54] - although there are results conflicting [50, 55] - and with n-3 polyunsaturated fatty acids, which activate hepatic PPAR α and inhibit SREBP-1c expression and maturation [56]. The reduction of hepatic glycogen content after ATRA treatment in mice that we found may also represent a PPAR α :RXR-mediated response, as it has been described in rats and pigs treated with a PPAR α -selective agonist (clofibrate) [57, 58]. Of note, both in clofibrate-treated rats and in ATRA-treated mice reduction of hepatic glycogen stores takes place despite improved glucose tolerance in the treated animals (this work and [6, 9, 57]). The concurrence of these two seemingly contradictory effects in both experimental paradigms reinforces our conclusion that ATRA treatment enhances PPAR α -mediated responses in liver of mice.

Evidence has been provided that ATRA can serve as a ligand for PPAR β/δ [59], and that anti-obesity effects of ATRA in mice are due in part to PPAR β/δ activation in skeletal muscle and WAT [9]. This opens the possibility that direct activation of PPAR β/δ in liver explains the induction of fatty acid oxidation-related genes that we observed. However, transcriptional profiling has recently

revealed that PPAR β/δ does not regulate fatty acid oxidation potential in the liver [60]. Also against a major role of PPAR β/δ in mediating liver changes observed in this work could be the fact that hepatic PPAR β/δ mRNA levels themselves were reduced following ATRA treatment.

Studies regarding vitamin A effects on liver lipid metabolism have produced controversial results. Our findings are in keeping with previous reports showing an increased rate of hepatic fatty acid utilization and oxidation in rats acutely fed high vitamin A (retinol) doses [61]; decreased expression of fatty acid oxidation-related genes and increased triacylglycerol accumulation in liver of mice with vitamin A deficiency [24]; and the induction of the expression of fatty acid oxidation-related genes in the liver following acute oral ATRA treatment in vitamin A-deficient mice [24]. Also in keeping with our findings, genetic disruption of RA signaling led to impaired hepatic mitochondrial beta-oxidation of fatty acids and/or liver steatosis in mice [62, 63], effects that were partly reversed by oral ATRA administration [62]. In addition, treatment with ATRA increased the rate of oxidation of an exogenously administered fatty acid in human HepG2 hepatoma cells (Amengual J, Ribot J and Palou A, submitted manuscript). However, others have reported a shift towards increased fat catabolism in liver of rats with vitamin A deficiency [25, 26]. Moreover, high doses of retinoids can induce hypertriglyceridemia in rats and humans, an effect that appears to involve both increased hepatic production of VLDL and suppression of lipoprotein lipase activity in peripheral tissues (see [55]). Of note, retinoid-induced hypertriglyceridemia has been attributed to RAR activation [64], as well as to the ability of retinoids to activate the LXR:RXR heterodimer on the promoter of lipogenic genes [50]. In keeping, RA was shown to induce SREBP-1c and FAS expression in HepG2 cells likely by enhancing the activity of LXR:RXR heterodimers [22], and retinoids were shown to induce SCD1 expression likely by activating RAR [23]. In contrast, we report here a strong down-regulation of SREBP-1c and FAS expression and no changes in SCD1 expression in the liver of ATRA-treated mice. It would appear that, *in vivo*, the net effect of retinoids on liver lipid metabolism depends on the balance between PPAR:RXR, RAR:RXR and LXR:RXR activities, which can be affected in its turn both by the

chemical nature of the retinoid under investigation and by systemic effects of treatment, absent in cell culture settings and possibly variable *in vivo* depending on treatment conditions. In our experimental *in vivo* setting, acute ATRA treatment effectively mobilizes WAT triacylglycerol stores [3-8] and, under these conditions, PPAR α :RXR-mediated responses could conceivably prevail and even block RAR:RXR- and LXR:RXR-mediated responses in the liver, resulting in increased fat catabolism and reduced lipogenesis as main metabolic outcomes.

While our results suggest that reduction in circulating VLDL after ATRA treatment is due, at least in part, to reduced hepatic VLDL production, the simultaneous though less marked increase in circulating LDL is suggestive of increased VLDL catabolism as well in ATRA-treated mice. In particular, it is conceivable that changes favoring fatty acid uptake and utilization by peripheral tissues such as BAT and skeletal muscle known to be triggered by ATRA treatment [3, 4, 8, 11, 12] are fueling increased VLDL catabolism in ATRA-treated mice.

In summary, results in this work point to a capacity of active vitamin A forms to shift liver lipid metabolism *in vivo* towards increased catabolism and reduced lipogenesis, which might contribute to reported effects of ATRA in reducing whole body adiposity. The results suggest that vitamin A vitamers might be useful as protective agents against the development of liver steatosis in situations of increased free fatty acid inflow into the liver, which include high fat diet feeding and visceral obesity, but also rapid weight loss.

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