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Apo-10'-lycopenoic acid impacts adipose tissue biology *via* the retinoic acid receptors

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ABSTRACT

Apo-10'-lycopenoic acid (apo-10-lycac), a metabolite of lycopene, has been shown to possess potent biological activities, notably via the retinoic acid receptors (RAR). In the current study, its impact on adipose tissue and adipocytes was studied. In microarray experiments, the set of genes regulated by apo-10-lycac treatments was compared to the set of genes regulated by *all-trans* retinoic acid (ATRA), the natural ligand of RAR, in adipocytes. Approximately 27.5% of the genes regulated by apo-10-lycac treatments were also regulated by ATRA, suggesting a common ability in terms of gene expression modulation, possibly *via* RAR transactivation. The physiological impact of apo-10-lycac on adipose tissue biology was evaluated. If it had no effect on adipogenesis in the 3T3-L1 cell model, this metabolite may have a preventative effect against inflammation, by preventing the increase in the inflammatory markers, interleukin 6 and interleukin 1β in various dedicated models. The ability of apo-10-lycac to transactivate the RAR and to modulate the transcription of RAR target gene was brought in vivo in adipose tissue. While apo-10-lycac was not detected in adipose tissue, a metabolite with a molecular weight with 2 Da larger mass was detected, suggesting that a dihydro-apo-10'-lycopenoic acid, may be present in adipose tissue and that this compound could active or may lead to further active RAR-activating apo-10-lycac metabolites. Since apo-10-lycac treatments induce anti-inflammatory effects in adipose tissue but do not inhibit adipogenesis, we propose that apo-10-lycac treatments and its potential active metabolites in WAT may be considered for prevention strategies relevant for obesity-associated pathologies.

1. Introduction

Lycopene is a carotenoid found in multiple vegetables and fruits, such as tomato and tomato-based products, papaya, and watermelon [1]. Its beneficial health effects have been studied extensively, especially in the prevention of prostate cancer where it has been demonstrated to be a potential chemoprotective agent [2,3]. Several studies suggest that lycopene has an impact on adipose tissue metabolism. First, lycopene is the predominant carotenoid in human adipose tissue [4]. Second, it has recently been reported that a higher intake of lycopene was associated with a smaller waist circumference and lower visceral and subcutaneous fat mass [5]. Third, we demonstrated that lycopene inhibited proinflammatory cytokine and chemokine expression in adipose tissue [6]. Finally, it has been firmly established that the concentration of lycopene in adipose tissue is correlated to a reduction in the risk of developing a cardiovascular disease in men [7]. Such beneficial effects of lycopene might be due to its positive effects on adipose tissue. Indeed adipose tissue has a well-established role in the genesis of obesity-associated pathologies, such as insulin resistance and type II diabetes, which are risk factors for cardiovascular diseases.

The metabolism of lycopene remains unclear. After intestinal absorption, which is mediated, at least in part, by SR-B1 [8], lycopene is incorporated into chylomicrons and released into the lymphatic system for transport to the liver [1]. In the plasma, lycopene is transported in LDL and VLDL for distribution to multiple organs. Our group recently demonstrated that CD36 is involved in the uptake of lycopene by adipose tissue and adipocytes [9]. In various organs in ferrets and

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mice, lycopene can be metabolized by β-carotene 9',10'-dioxygenase (BCDO2) [10,11]. This enzyme is responsible for the eccentric cleavage of lycopene (especially the *cis*-lycopene isomers). Indeed, Hu et al. [10] demonstrated that the *cis* isomer of lycopene was metabolized into apo-10'-lycopenal by ferret BCDO2 *in vitro*, and that *all-trans* lycopene supplementation in these animals resulted in the formation of apo-10'-lycopenol in the lung. They also demonstrated that apo-10'-lycopenal can be metabolized into apo-10'-lycopenoic acid (apo-10-lycac) or apo-10'-lycopenol, depending on whether NAD^+ or NADH are present as cofactors. Several other lycopene metabolites were identified in rat liver (apo-8'-lycopenal and apo-12'-lycopenal [12]). Apo-lycopenals were recently detected in human plasma, possibly derived from ingested tomato products or from lycopene *in vivo* metabolism [13].

Among these metabolites, the apo-10-lycac has been shown to be an active metabolite, especially in cancer cells. Indeed, Lian et al. [14] demonstrated that treatment of human bronchial epithelial cells with apo-10-lycac resulted in the nuclear accumulation of Nrf2, which is associated with an induction of phase II detoxifying/antioxidant enzymes [14]. This group previously reported that apo-10-lycac transactivated the retinoic acid receptor β (RARβ) promoter in relatively high concentrations (>3 μM), and induced the expression of RARβ in bronchial and lung cancer cells *in vitro*[15]. These data strongly suggest that apo-10-lycac is highly active in terms of the regulation of gene expression in organs that have an optimal environment for further potential bioactivation to active metabolites.

Interestingly, BCDO2 is highly expressed in adipose tissue [16]. Thus, we hypothesized that the apo-10-lycac and/or other related bioactive derivatives are generated in adipose tissue and have the ability to impact on adipose tissue biology via the transactivation of RAR. In the current study, we evaluated its effects on the transcriptome of adipocytes, as well as on different aspects of adipose tissue biology, including adipogenesis and prevention of inflammation. We also demonstrated that apo-10-lycac treatments induce potent RAR activation *in vivo* and *in vitro*. We conclude that this metabolite affects adipose tissue and adipocyte biology in a manner similar to *all-trans* retinoic acid (ATRA), a well-known natural ligand of RAR.

2. Materials and methods

2.1. Chemicals

Apo-10'-lycopenoic acid (apo-10-lycac) was obtained by organic synthesis as described by Reynaud et al. [17].

2.2. Animal experiments

For *ex vivo* experiments, the care and use of mice were in accordance with French guidelines and approved by the local experimental animals ethics committee. Adult male C57BL/6j mice were housed in a temperature-, humidity- and light-controlled room. Mice were fed a standard chow diet and water *ad libitum*. Mice on a high-fat diet ($n=6$) were fed a diet containing 35% fat for 6 weeks, as described previously [18].

For *in vivo* RAR transactivation experiments, RARE-luc mice (Cgene AS, Olso, Norway) were engineered to express the firefly luciferase gene under the control of RARE (retinoic acid response element) [19]. The animals were housed in standard plastic cages at room temperature (20 ± 2 °C) according to the Hungarian guidelines for the care and use of animals. They had *ad libitum* access to both food and water. Standard laboratory animal diet (*Altromin*, *VRF 1*) was acquired from *Charles River* (Budapest, H). Male *mice* (10–12 *weeks old)* were *used*. In each group, 4–6 animals were analyzed.

For HPLC analysis, six C57BL/6 male *mice* were *used*. Three animals were injected intravenously with lycopene (50 mg/kg body weight), and three animals were treated with vehicle. After 4 h, animals were sacrificed by anesthesia with halothane. Spleens were collected and snap-frozen in liquid nitrogen for HPLC analysis.

2.3. Ex vivo culture of adipose tissue explants

Adipose tissue explants of mice were recovered, rinsed in saline buffer and placed in DMEM supplemented with 10% FBS at 37 °C in a 5% CO_2 humidified atmosphere. The medium was changed after 1, 3 and 24 h. Explants of adipose tissues from mice who consumed a high-fat diet or normal chow as a control were treated with apo-10-lycac (2 μM) for 24 h. Adipose tissues of mice fed normal chow were cultured for 24 h with apo-10-lycac, followed by a TNFα stimulation (15 ng/ml; 3 h).

2.4. Bioluminescence imaging and quantification

An Andor IQ imaging system (Belfast, IRL), which consists of an Andor-ixon cooled charged coupled device (CCD) camera, housed in a Unit-one (Birkerod, DK) black box and connected to a computer system, was used for data acquisition and analysis. Groups of 10- to 12-week-old male mice were treated with apo-10-lycac (4 mg/kg body weight), ATRA (50 mg/kg body weight) or DMSO by oral gavage 16 h before screening. Reporter animals received 120 mg/kg D-luciferin (Promega) intra-peritoneally. Mice were euthanized by cervical dislocation 15 min later. Subsequently, organs were rapidly excised and placed under a CCD camera. Organs were kept at −80 °C for later HPLC analysis. Bioluminescence images were taken using a 5-min integration time. The photon signals were quantified by the Andor IQ 1.6. program. Luciferase expression was determined as the integrated intensity/area. Data are presented as the means ± SEM values of 4–6 different animals per treatment group.

2.5. Cell culture

3T3-L1 preadipocytes (ATCC, Manassas, VA, USA) were seeded in dishes with a 3.5-cm diameter at a density of 15×10^4 cells/well. Cells were grown in DMEM supplemented with 10% FBS at 37 °C in a 5% CO_2 humidified atmosphere, as reported previously [20]. To induce differentiation, 2-day post-confluent 3T3-L1 preadipocytes (day 0) were stimulated for 48 h with 0.5 mM isobutylmethylxanthine, 0.25 μmol/L dexamethasone, and 1 μg/ml insulin in DMEM supplemented with 10% FBS. Cells were then maintained in DMEM supplemented with 10% FBS and 1 μg/ml insulin. In order to examine the effect of apo-10-lycac and ATRA on gene expression, 3T3-L1 adipocytes were incubated with 2 μM apo-10-lycac or ATRA for 24 h, as reported previously [21]. To examine the effect of apo-10-lycac on adipocyte differentiation, 2-day post-confluent 3T3-L1 preadipocytes received 2 μM apo-10-lycac or ATRA every 2 days until the end of the experiment at day 9. The data are presented as the mean of three independent experiments, each performed in triplicate. To examine the anti-inflammatory effect of apo-10-lycac and ATRA, 3T3-L1 adipocytes were incubated with molecules (2 μM) for 24 h. These adipocytes were then incubated with TNFα (15 ng/ml) for 3 h. All treatments were performed on day 8. Human preadipocytes were purchased from Promocell (Heidelberg, D) and cultured following the company's instructions. Mature adipocytes (day 14) were incubated with apo-10-lycac (2 μM, 24 h) followed by an incubation with TNFα (15 ng/ml) for 3 h.

2.6. RAR transactivation assay in vitro

The reporter plasmid containing the gene for luciferase under the control of four copies of the Gal4 binding site UAS was transfected in 3T3-L1 cells, together with plasmids CMX-Gal4 or CMX-Gal4-hRARa (generous gift of Dr. Makishima; Nihon University School of Medicine, Tokyo). The transfection was performed using Lipofectamine 2000 (Invitrogen). After overnight incubation with the transfection mixes, the medium was replaced by DMEM supplemented with 10% FBS and

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various concentration of apo-10-lycac or ATRA. The cells were treated for 24 h. The cells were lysed and assayed for luciferase activity using a luciferase assay system (Promega, Madison, WI), which was normalized to β-galactosidase activity as previously described [6]. The transfection experiments were performed in triplicate and repeated three times independently.

2.7. RNA isolation and qPCR

Total cellular RNA was extracted from 3T3-L1 cells and mice epididymal fat pads using TRIzol reagent according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA in 20 μl using random primers and Moloney murine leukemia virus reverse transcriptase. Real Time Quantitative RT-PCR analyses for the genes were performed using the Mx3005P Real-Time PCR System (Stratagene, La Jolla, CA, USA) as described previously [22]. For each condition, expression was quantified in duplicate, and 18S mRNA was used as the endogenous control in the comparative cycle threshold (C_T) method.

2.8. Hybridization arrays and microarray data analysis

RNA quality control was performed on an Agilent 2100 Bioanalyzer (Massy, F) with 6000 Nano Chips according to the manufacturer's instructions and as reported previously [23]. RNA from three independent experiments were pooled per treated group and hybridized to Agilent Whole Human Genome (4x44k; Massy, F). All labeling, hybridization, washing and scanning were performed as described in the manufacturer's protocol. Arrays were scanned with an Agilent Scanner (Massy, F). Data were extracted with Agilent Feature Extraction v9.5.3 and analyzed with Agilent GeneSpring GX v10.0 (Massy, F). Data were determined to be significant based on *P*-value ($P<0.05$) and fold change (1.5 or not). Pathway analyses were performed using Metacore (http://www.genego.com/metacore.php). A scatter plot was generated, and the associated correlation analysis was performed with SPSS 17. Creation of a heatmap was performed with PermutMatrix [24].

2.9. HPLC MS-MS determinations

We used an existing high performance liquid chromatography/mass spectrometry / mass spectrometry (HPLC MS-MS) configuration similar to that already published [25], except a novel and more sensitive mass spectrometer was used (Micromass Quattro Ultima PT, Manchester, UK; donated by Biosystems Int., Evry, F). The separation using HPLC was performed in a manner similar to what has been reported previously [25]. For the detection of apo-10-lycac, we established a specific MS-MS method using APCI (+) setting with 393 to >269 *m/z*, a collision energy of 10 V, a dwell time 0.3 s and a cone voltage of 50 V as parameters for multiple reaction monitoring (MRM) measurements. Detection of lycopene metabolites with a molecular weight of 395 Da was performed using a single ion recording (SIR) setting at 395 *m/z* and a photodiodearray detector (Waters 996, Waters KFT., Budapest, H) placed in series before the MS detector.

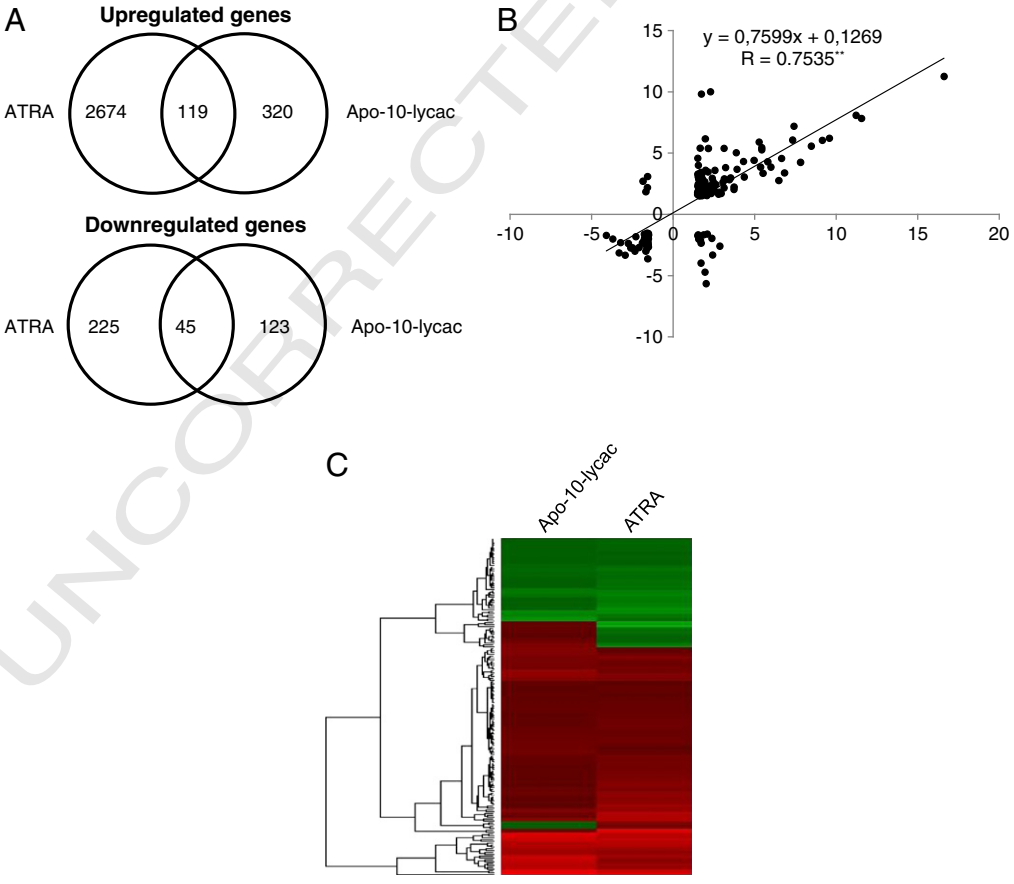

Fig. 1. Comparison of genes regulated by apo-10'-lycopenoic acid and ATRA in 3T3-L1 adipocytes. A. Venn diagrams represent the number of significantly ($P<0.05$) upregulated or downregulated genes in 3T3-L1 adipocytes due to apo-10'-lycopenoic acid treatment in comparison to ATRA treatment and the number of significantly regulated genes in common (overlap). 3T3-L1 adipocytes were incubated with apo-10'-lycopenoic acid (2 μM) or ATRA (2 μM) for 24 h. RNA was extracted and hybridized on a whole mouse genome microarray. Data were computed using Genespring GX10. B. Filtered fold change (fold change of 1.5) of commonly regulated genes after treatment with ATRA (*Y*-axis) and apo-10'-lycopenoic acid (*X*-axis) were plotted. The correlation line was drawn, and the Pearson correlation coefficient and the associated *P*-value were calculated with SPSS 17. C. A heatmap was constructed for these filtered data using PermutMatrix. Apo-10'-lycopenoic acid is abbreviated as apo-10-lycac.

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Table 1
ATRA target genes in adipose tissue and/or adipocytes.

Gene name	Refseq number	Regulation	Fold change	Reference
Leptin	NM_010704	Down	24.75	Felipe [27]
Resistin	NM_022984	Down	1.84	Felipe[27]
CD36	NM_007643	Up	1.54	Han [29]
Adiponectin	NM_009605	Down	1.42	Zhang [26]
CEBPα	NM_007678	Down	3.00	Mercader [30]
RXRα	NM_011305	Down	1.66	Mercader [30]
UCP2	NM_011671	Up	3.33	Mercader [30]
PGC1α	NM_008904	Up	2.54	Mercader [30]
PPARα	NM_011144	Up	3.54	Mercader [30]

2.10. Statistical analysis

Data are expressed as the means ± SEM. Significant differences between control and treated groups were determined by one-way ANOVA, followed by Fisher's PLSD test using Statview software (SAS Institute, Cary, NC, USA). Values of $P<0.05$ were considered significant.

3. Results

3.1. Apo-10'-lycopenoic acid modulates the transcriptome of 3T3-L1 adipocytes in a manner similar to ATRA

To study in detail the impact of apo-10-lycac (structure in Suppl. Fig. 1), in terms of gene expression on adipocytes, we performed microarray experiments. 3T3-L1 adipocytes were incubated with apo-10-lycac (2 μM for 24 h). Since apo-10-lycac effect are supposed to be mediated at least in part by RAR [15], 3T3-L1 were also incubated with ATRA (2 μM for 24 h), a well-known RAR ligand. The impact of these treatments on the transcriptome was evaluated with a fold change filter of 1.5. As seen in Fig. 3, 5093 genes were regulated (2,793 were upregulated and 2300 were downregulated) by ATRA ($P<0.05$) and 607 genes were regulated by apo-10-lycac (439 were upregulated and 168 were downregulated). Remarkably, 164 genes were upregulated or downregulated by both ATRA and apo-10-lycac (119 were upregulated and 45 were downregulated (Fig. 1A)), i.e., 27% of the genes regulated by apo-10-lycac were also regulated by ATRA, which suggests a selective overlap between the two genes lists. In addition, the fold change of genes regulated by ATRA (Y-axis) was plotted against the fold change of genes regulated by apo-10-lycac (X-axis; Fig. 1B). This scatter plot revealed a highly significant ($P<0.01$) linear correlation between the two treatment conditions (Pearson coefficient of correlation 0.7535). The heatmap established using these filtered data convincingly illustrated the strong similarity between the two treatments in terms of their impact on gene expression (Fig. 1C). It is noteworthy that genes previously shown to be modulated by ATRA in adipocytes and/or adipose tissue (Table 1) were found to be regulated [26–31], which validated our microarray experiments. In addition, pathway analysis was performed. For this analysis, we used the list of genes regulated by apo-10-lycac and ATRA treatments. Several pathways were significantly regulated by the two molecules (Table 2). Interestingly, of the pathways identified, the one called "Ligand-dependent transcription of retinoid-target genes" (Metacore nomenclature) was deeply affected: of the 32 genes involved in this pathway, 21 were regulated by the treatments ($P<1.36e-04$). Taken together, these data suggested that apo-10-lycac and ATRA shared similarities in terms of their effects on gene expression. This was in agreement with the hypothesis of a similar action for the same nuclear receptor.

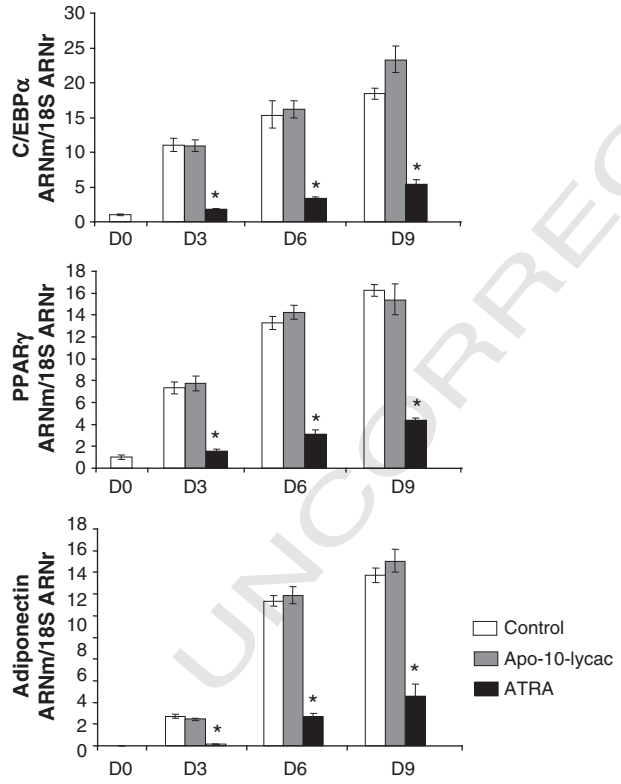

Fig. 2. Apo-10'-lycopenoic acid has no influence on 3T3-L1 adipocyte differentiation. At 2-days post-confluence, 3T3-L1 preadipocytes (day 0) were stimulated to induce differentiation with 0.5 mM isobutylmethylxanthine, 0.25 μM dexamethasone, and 1 μg/ml insulin, for 48 h. To examine the effect of apo-10'-lycopenoic acid on adipocyte differentiation, 2-days post-confluent 3T3-L1 preadipocytes received apo-10'-lycopenoic acid (2 μM) every 2 days or ATRA (2 μM) as a positive control until the end of the experiment at day 9. Total RNA was extracted and reverse transcribed with MMLV. Real time PCR was performed using specific primers. 18S rRNA was used as the endogenous control. Data are presented as means ± SEM; * represents a significant difference treated ATRA condition and control; $P<0.05$. Apo-10'-lycopenoic acid is abbreviated as apo-10-lycac.

3.2. Apo-10'-lycopenoic acid does not modulate adipogenesis in 3T3-L1 cells

Because adipogenesis, a major event in adipocyte biology, is inhibited by ATRA [32], we evaluated whether apo-10-lycac was also able to inhibit adipogenesis in a similar manner. 3T3-L1 cells were incubated in the presence of apo-10-lycac (2 μM) or ATRA (2 μM) during the differentiation process and the expression of PPARγ, C/EBPα and adiponectin were evaluated as reporters for the extent of adipogenesis. As expected, we observed an increase in the level PPARγ mRNA at different points in the differentiation process (×7.3, ×13.3 and ×16.2 at days 3, 6 and 9, respectively; Fig. 2). The same pattern of results was observed for the two other genes, C/EBPα and adiponectin, demonstrating normal differentiation of adipocytes. In contrast to ATRA, which strongly inhibited the PPARγ, C/EBPα and adiponectin mRNA expression levels, apo-10-lycac did not affect the expression of these mRNAs, which strongly suggests that apo-10-lycac did not have an impact on adipogenesis in 3T3-L1 cells.

3.3. Apo-10'-lycopenoic acid reduces the production of proinflammatory markers by adipose tissue and adipocytes

Low-grade inflammation of adipose tissue is a well-established contributor to the development of obesity-associated pathologies. It has been demonstrated that ATRA modulates inflammation via RAR and NF-κB [33]. Therefore, we tested whether this property was shared with apo-10-lycac. For this purpose, 3T3-L1 cells were incubated with apo-10-lycac (2 μM) or ATRA (2 μM) for 24 h, followed by incubation with TNFα (15 ng/ml) for 3 h. As expected, we observed an increase of IL-6 (×9.7) and IL-1β (×3.7) mRNA levels after TNFα incubation. This induction was partially reversed (IL-6 (−20%), IL-1β (−40%) and IL-6 (−20%), IL-1β (−60%)) (Fig. 3A) when the cells were pretreated

Table 2
Pathways regulated by both apo-10'-lycopenoic acid and ATRA.

Map	Map Folders	*P*-value
Cytoskeleton remodeling_Role of PKA in cytoskeleton reorganization	Protein function/kinases regulatory processes/ cytoskeleton remodeling	5,77E-09
Cytoskeleton remodeling_Cytoskeleton remodeling	Congenital, hereditary, and neonatal diseases and abnormalities regulatory processes/cytoskeleton remodeling	1,76E-07
Development_Role of IL-8 in angiogenesis	Protein function/G-proteins/GPCR regulatory processes/ development/angiogenesis	6,84E-07
Cell adhesion_Integrin-mediated cell adhesion and migration	Regulatory processes/cell adhesion	3,04E-06
Transcription_Sin3 and NuRD in transcription regulation	Regulatory processes/transcription	6,81E-06
Cell adhesion_Role of tetraspanins in the integrin-mediated cell adhesion	Regulatory processes/cell adhesion	1,45E-05
Cytoskeleton remodeling_Integrin outside-in signaling	Regulatory processes/cell adhesion regulatory processes/ cytoskeleton remodeling	2,28E-05
Transport_Clathrin-coated vesicle cycle	Disease maps/lung diseases/cystic fibrosis regulatory processes/Transport	2,32E-05
Cell adhesion_Integrin inside-out signaling	Regulatory processes/cell adhesion	2,45E-05
Cell adhesion_Endothelial cell contacts by non-junctional mechanisms	Regulatory processes/cell adhesion	2,66E-05
Cytoskeleton remodeling_Fibronectin-binding integrins in cell motility	Regulatory processes/cell adhesion regulatory processes/ cytoskeleton remodeling	3,08E-05
Cell adhesion_Chemokines and adhesion	Congenital, hereditary, and neonatal diseases and abnormalities protein function/cyto/chemokines Regulatory processes/Cell adhesion	4,86E-05
Cytoskeleton remodeling_Regulation of actin cytoskeleton by Rho GTPases	Protein function/G-proteins/RAS-group Regulatory processes/ cytoskeleton remodeling	6,19E-05
Regulation of CFTR activity (norm and CF)	Congenital, hereditary, and neonatal diseases and abnormalities disease maps/lung diseases/cystic fibrosis	1,04E-04
Transcription_Ligand-Dependent Transcription of Retinoid-Target genes	Protein function/transcription factors	1,37E-04
Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodeling	Congenital, hereditary, and neonatal diseases and abnormalities regulatory processes/cytoskeleton remodeling	2,05E-04
Cell adhesion_Histamine H1 receptor signaling in the interruption of cell barrier integrity	Protein function/G-proteins/GPCR Regulatory processes/Cell adhesion	2,22E-04
Apoptosis and survival_BAD phosphorylation	Regulatory processes/Apoptosis and survival	2,41E-04
Neurophysiological process_Receptor-mediated axon growth repulsion	Congenital, hereditary, and neonatal diseases and abnormalities regulatory processes/cell adhesion regulatory processes/cytoskeleton remodeling regulatory processes/development/neurogenesis regulatory processes/neurophysiological process	2,81E-04

with apo-10-lycac and ATRA respectively. These effects were confirmed in two additional models in the case of apo-10-lycac. Inguinal adipose tissue explants of mice fed a high-fat diet were incubated *ex vivo* with apo-10-lycac (2 μM) for 24 h. We observed an important decrease of IL-6 (−58%) and IL-1β (−82%) mRNA levels in explants that were incubated in the presence of apo-10-lycac compared to the control (Fig. 3A). We also used human primo cultures. Cells were incubated with apo-10-lycac (2 μM) for 24 h, followed by incubation with TNFα (15 ng/ml) for 3 h. As expected, we observed an increase of IL-6 (×16.7) and IL-1β (×17.2) mRNA levels after TNFα incubation. This induction was partially reversed (IL-6 (−20%) and IL-1β (−30%)) (Fig. 3B) when the cells were pretreated with apo-10-lycac treatment. Taken together, these data strongly suggest that apo-10-lycac and ATRA exert anti-inflammatory effects in adipose tissue and adipocytes.

3.4. Apo-10'-lycopenoic acid transactivates RAR in vivo, in vitro and modulates the transcription of RAR target genes in mouse adipose tissue

We took advantage of the RARE-luciferase mouse model [19], in order to examine the ability of apo-10-lycac to transactivate RAR *in vivo*, in adipose tissue. As expected, we observed a strong induction of the luciferase reporter in white adipose tissue (WAT; Fig. 4A). This ability of apo-10-lycac to transactivate RAR was confirmed in vitro in 3T3-L1 cells (Fig. 4B). The maximum transactivation (about 25%) of RAR for apo-10-lycac was obtained for the 2 μM concentration, whereas ATRA significantly induced RAR transactivation for lower concentration (1 μM).

In addition, we evaluated the induction of CYP26A1 and RARβ (Fig. 4C), two well-known RAR target genes in adipose tissue [34]. Upon ATRA and apo-10-lycac treatment, these genes were strongly induced (82 and 10 fold induction respectively for CYP26A1; 1.8 and 2.3 fold induction respectively for RARβ). Interestingly, apo-10-lycac significantly induced ($P<0.05$) the reporter gene expression in the lung and intestine, while transactivation was not observed in other tested organs (Suppl. Fig. 2). Therefore, apo-10-lycac seems to be an activator of RAR in specific organs, suggesting that an organ specific environment is needed for apo-10'-lycopenoic acid transactivation of RAR-signaling.

To confirm the regulation of several other ATRA target genes by apo-10-lycac in adipose tissue, we evaluated the mRNA level of a panel of genes, chosen from Table 1 that were regulated in the same manner by both ATRA and apo-10-lycac in microarray experiments. Therefore, validation was conducted for PPARα, RXRα, leptin and CEBPα. As shown in Fig. 5, we observed a downregulation of leptin, CEBPα and RXRα and an upregulation for PPARα, confirming thus *in vivo* the microarray data. Together, these data supported the effect of apo-10-lycac on the expression of known ATRA target genes *in vivo*.

3.5. Apo-10'-lycopenoic acid is not present in human adipocytes and mouse adipose tissue

To detect apo-10-lycac in tissues or cells, we used an HPLC MS-MS approach. With a protocol analysis specifically primed for apo-10-lycac using MRM conditions with 393→269 *m/z*, a small peak was detected only in the spleen of mice that were treated intravenously with lycopene (Fig. 6A). Based on these weak criteria we just can speculate that apo-10-lycac is an endogenous metabolite of lycopene in mice. In addition, potential *cis*-isomers of apo-10-lycac are indicated by the box in scattered lines in Fig. 6A. In the white adipose tissue (WAT) of mice orally treated with vehicle control, lycopene or apo-

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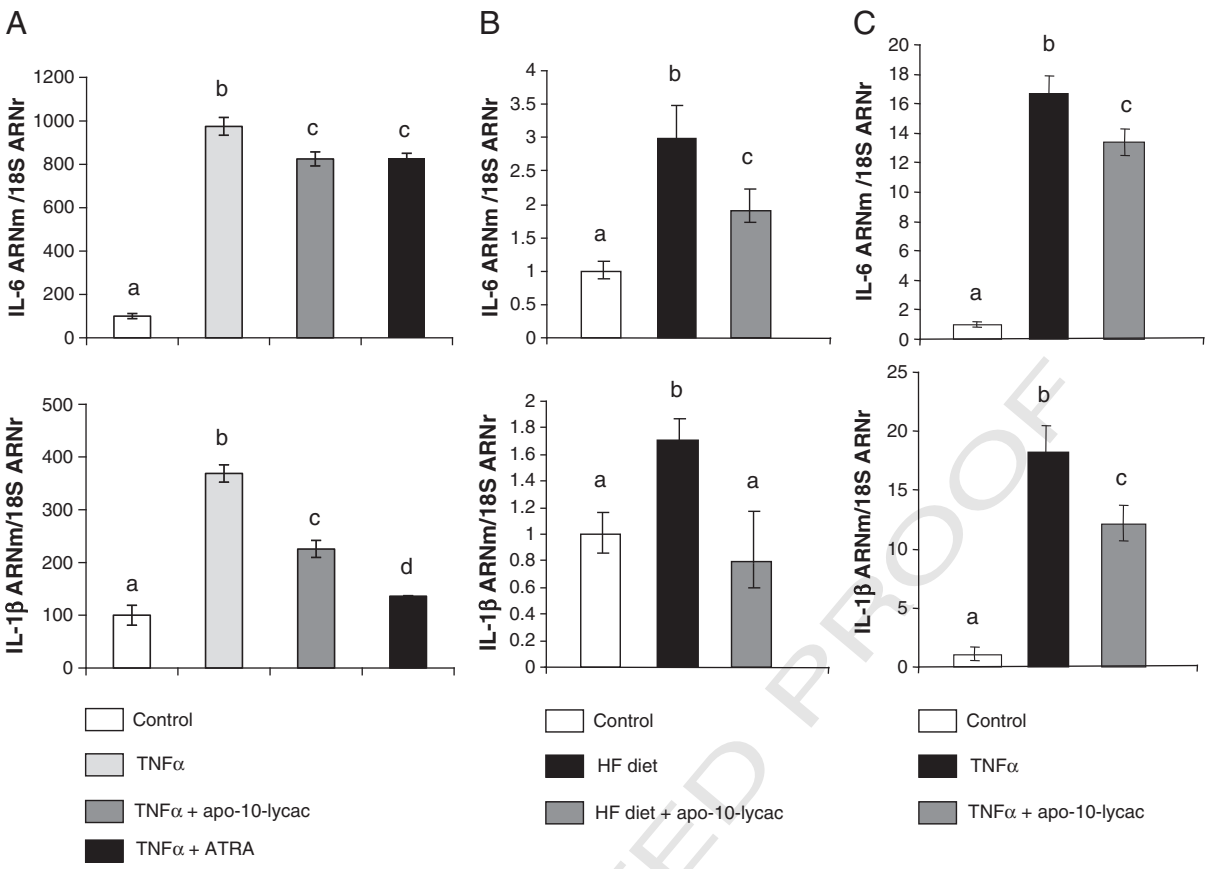

Fig. 3. Apo-10'-lycopenoic acid decreases inflammation in 3T3-L1 cells, in adipose tissue explants of mice subjected to HFD in *ex vivo* culture and in primo culture of human mature adipocytes *in vitro*. A. 3T3-L1 adipocytes were incubated with apo-10'-lycopenoic acid (2 μM) or ATRA (2 μM) for 24 h and then incubated with TNFα (15 ng/ml) for 3 h. B. Adipose tissue of mice force fed with a high-fat diet for 6 weeks was recovered and incubated with apo-10'-lycopenoic acid (2 μM) for 24 h C. Human adipocytes were incubated with apo-10'-lycopenoic acid (2 μM) for 24 h and then incubated with TNFα (15 ng/ml) for 3 h. RNAs were extracted and reverse transcribed with MMLV. Real time RT-PCR was performed using specific primers. 18S rRNA was used as the endogenous control. Data are presented as means ± SEM; Bars not sharing the same letter were significantly different, $P<0.05$. Apo-10'-lycopenoic acid is abbreviated as apo-10-lycac.

10-lycac, no peak that co-eluted with apo-10-lycac was observed. In addition, no compound with a co-elution of apo-10-lycac was found in human cultured adipocytes with/without lycopene treatment (Fig. 6B) which indicates a quick and completely metabolism of apo-10-lycac in WAT. However, with the mass spectrometer in a single ion recording (SIR) configuration set at 395 *m*/z, which represents apo-10-lycac plus 2 Da, we detected a peak that has a retention time that is slightly longer than apo-10-lycac. This peak strongly increased in mouse WAT after lycopene and apo-10-lycac treatment in comparison with vehicle-treated animals (Fig. 6C). This suggests that a dihydro-apo-10'-lycopenoic acid was present within adipose tissue and could be an endogenous derivative, as well as a nutritionally relevant lycopene metabolite. The exact chemical structure of this dihydro-apo-10'-lycopenoic acid compounds still remained elusive because of nine different positions of hydrogenation and isomerisation of various conjugated double bounds. Exact chemical identification using MS-MS examination was impossible yet because of low endogenous concentrations of this dihydro-apo-10'-lycopenoic acid. One additional information that we obtained is the UV spectra of this dihydro-apo-10'-lycopenoic acid with an UVmax of 398 nm (Fig. 6D), this UVmax indicated that we may have six conjugated double bounds and an additional conjugated COOH-group [17]. As a reference we had apo-14'-lycopenoic acid with six conjugated double bounds and an additional conjugated COOH-group with an UVmax of 399 nm [17]. Thereby we postulated that 7,8-dihydro-apo-10'-lycopenoic acid might be the novel endogenous and nutritional relevant lycopene/apo-10'-lycopenoic acid metabolite (Fig. 6E). Further investigation using an organic-synthetic approach is needed to conclusively determine the exact chemical structure of this potential metabolite of lycopene.

4. Discussion

In the present study, we report for the first time the effects of apo-10'-lycopenoic acid (apo-10-lycac), a putative human and mouse metabolite of lycopene, on adipose tissue/adipocytes. These data strengthen the role of lycopene and/or metabolites on adipose tissue and adipocytes [1,5,6].

Firstly, we determined the impact of apo-10-lycac on gene expression in adipose tissue, we performed microarrays experiments. The effect of apo-10-lycac was compared to ATRA, a classical RAR ligand. We used adipocytes as a model because they make up the main cellular population of adipose tissue. Apo-10-lycac and ATRA were found to induce highly similar gene expression profiles. Several classical ATRA target genes in adipose tissue/adipocytes were found to be regulated under our conditions, validating our microarray results. Furthermore, several target genes of apo-10-lycac treatments *in vitro* were shown to be regulated in a mouse model, which reinforces the physiological relevance of the results obtained *in vitro*.

It is noteworthy that genes are more differentially affected by ATRA than by apo-10-lycac, in terms of fold change in expression, as reflected by the slope of the correlation line (Fig. 1B). Such a discrepancy might be due to the lower efficiency of apo-10-lycac to transactivate RAR as compared to ATRA (Fig. 4B) as well as effects of ATRA on gene expression *via* non-genomic mechanisms that may not be

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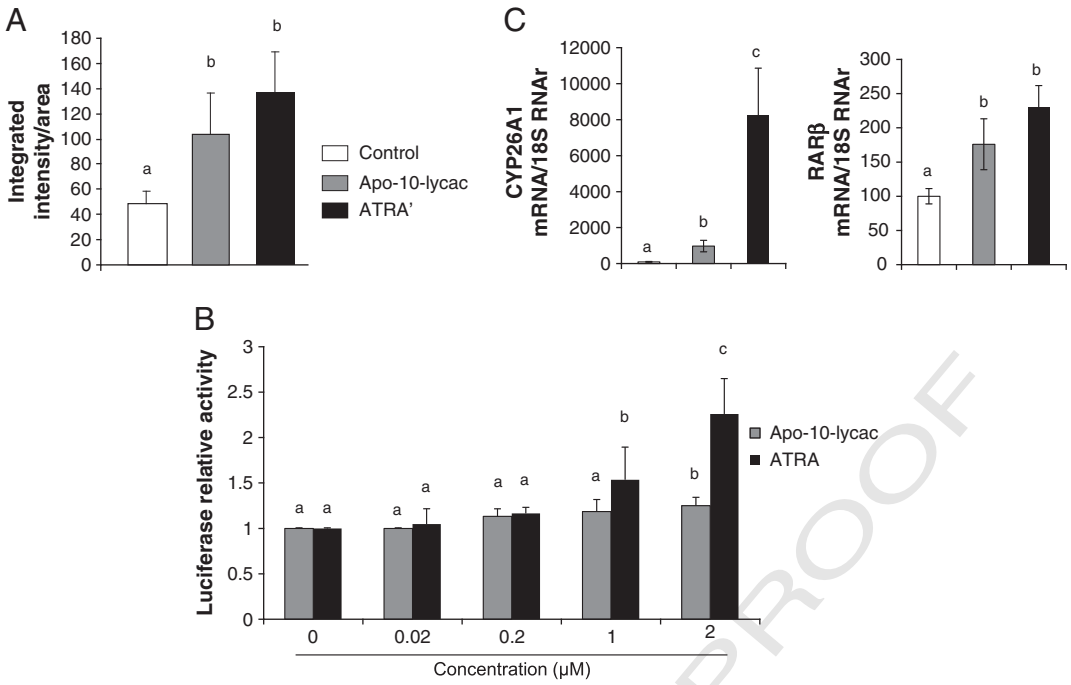

Fig. 4. Apo-10'-lycopenoic acid transactivates RAR in vivo, in vitro and induces CYP26A1 and RARβ in adipose tissue of mice *in vivo*. A. Ten- to twelve-week-old RARE-luc mice, engineered to express firefly luciferase gene under the control of RARE, were treated with apo-10'-lycopenoic acid (4 mg/kg body weight; apo-10'-lycac), ATRA (50 mg/kg body weight) or DMSO by oral gavage 16 h before screening. Fifteen minutes after luciferin injection, white adipose tissue (WAT) and lungs were rapidly excised and placed in a tight light chamber for bioluminescence screening. Luciferase expression is expressed as integrated intensity/area. B. Mice were force fed apo-10'-lycopenoic acid (4 mg/kg body weight). Epididymal adipose tissue was recovered 16 h later. Total RNA was extracted and reverse transcribed using MMLV. Real time RT-PCR was performed using specific primers. 18S rRNA was used as the endogenous control. Means ± SEM are shown; values for 4–6 different animals per treatment group are shown. C. 3T3-L1 cells were transiently transfected with plasmids coding for Gal4-RAR and TK-MH100x4-Luc. Cells were treated for 24 h with various concentrations of apo-10-lycac or ATRA. The β-galactosidase and luciferase dosages were performed as described in Materials and Method section. Bars not sharing the same letter were significantly different, $P<0.05$.; Apo-10'-lycopenoic acid is abbreviated as apo-10-lycac.

shared with apo-10-lycac. Indeed, ATRA has been reported to impact several signaling pathways, including protein kinases, p38MAPK and Erk1/2 [35–37]. Currently, similar effects have not been reported for apo-10-lycac. Conversely, apo-10-lycac-treatment regulated genes were not regulated by ATRA. This might be due to the ability of apo-10-lycac-treatment to activate the transcription factor Nrf2 [14]. Currently, the effect of ATRA on the activation of Nrf2 remains unclear because the literature reports both activation [38] and inhibition [39].

Pathway analysis revealed a common pattern of effects, especially a common impact on the retinoid target gene transcription. This implies that both apo-10-lycac and ATRA directly or indirectly regulate several actors (mainly cofactors) involved in the transcription of

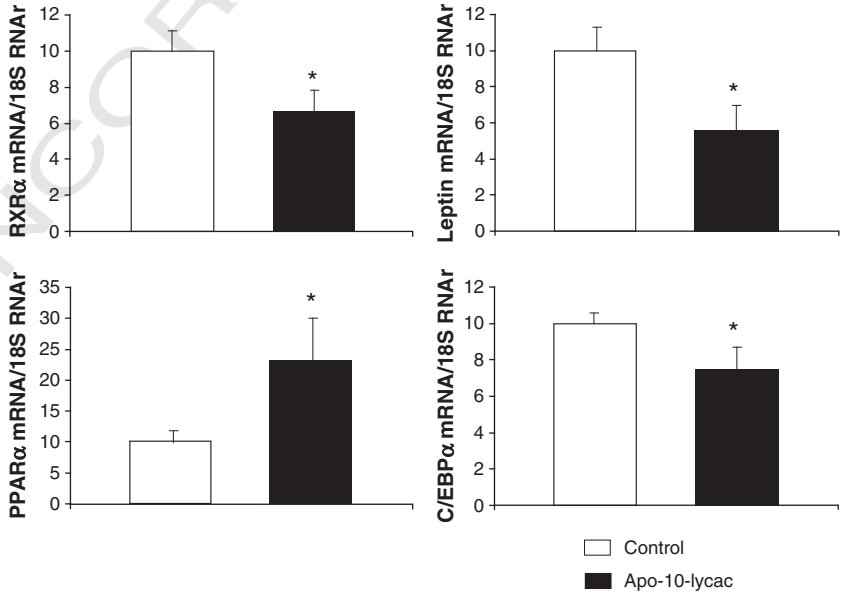

Fig. 5. Expression of leptin, RXRα, PPARα and C/EBPα in the adipose tissue of mice in response to force feeding with apo-10'-lycopenoic acid. Mice were force fed apo-10'-lycopenoic acid (4 mg/kg body weight). Epididymal adipose tissue was recovered 16 h later. Total RNA was extracted and reverse transcribed using MMLV. Real time RT-PCR was performed using specific primers. 18S rRNA was used as the endogenous control. Data are presented as means ± SEM; *$P<0.05$. Apo-10'-lycopenoic acid is abbreviated as apo-10-lycac.

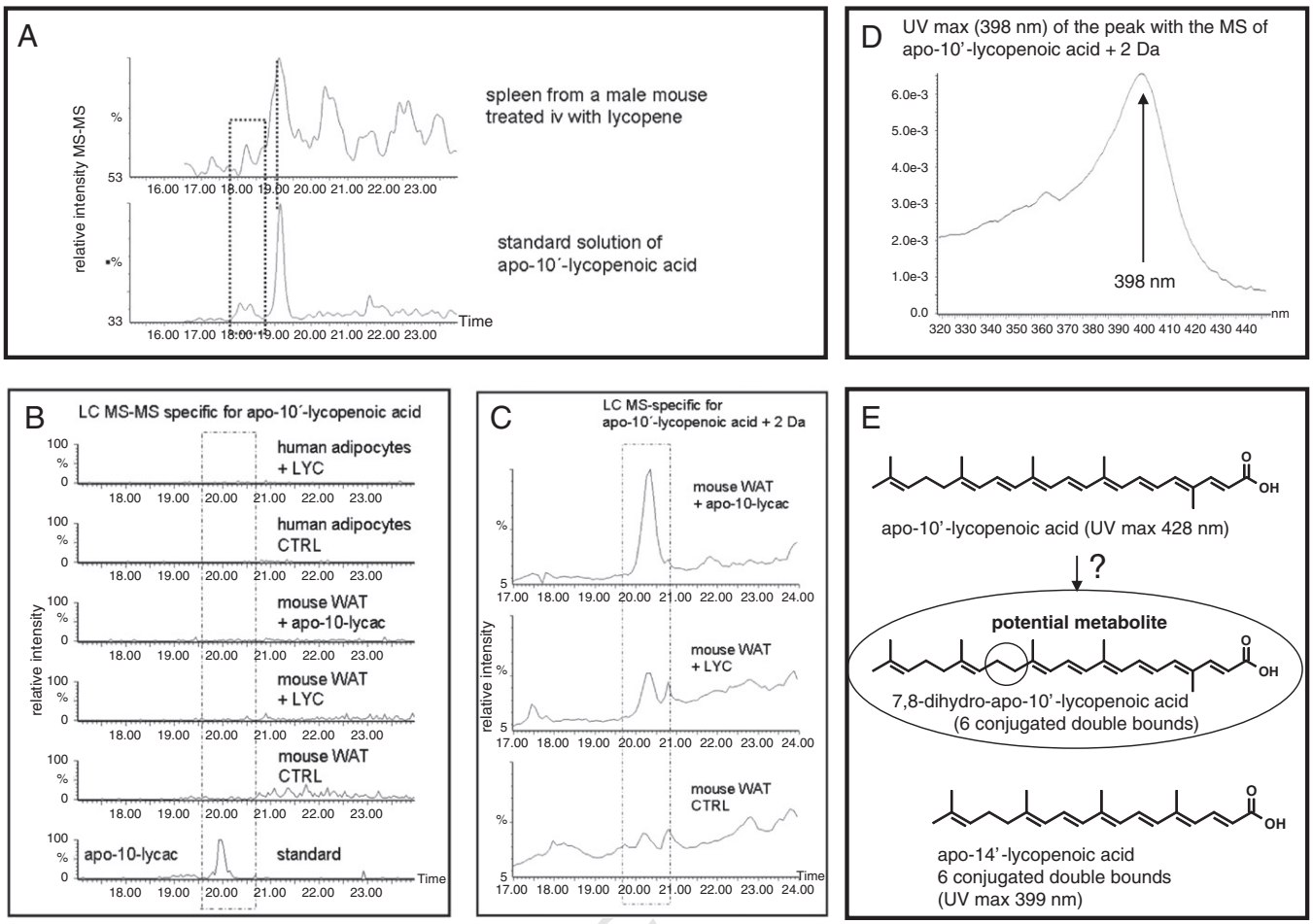

Fig. 6. Apo-10'-lycopenoic acid is not present in adipose tissue from mice treated with lycopene or apo-10'-lycopenoic acid as well as in human adipose tissue treated with lycopene, but a related compound dihydro-apo-10'-lycopenoic acid may be present. A. Male mice were treated intravenously with an aqueous lycopene-beadlet solution (50 mg/kg body weight). In vehicle treated animals, no peak was present and the data are not shown here. Using HPLC MS-MS with apo-10'-lycopenoic acid primed MRM settings we observed a peak in the spleen, which co-eluted with apo-10'-lycopenoic acid (marked by the scattered line), and small peaks, which co-eluted before and may correspond to potential geometric isomers of apo-10'-lycopenoic acid (marked by the scattered line box). In other tissues of lycopene-treated animals, such as serum, WAT, testis and liver, no co-eluting peak was observed. B. RARE-reporter mice with lycopene or apo-10'-lycopenoic acid treatment with up-regulated RARE activity and human adipocytes treated with/without lycopene were analyzed by HPLC MS-MS with apo-10'-lycopenoic acid primed MRM settings. C. RARE reporter mice with lycopene or apo-10'-lycopenoic acid treatment induced up-regulation of RARE activity were analyzed by HPLC MS-MS with SIR settings for 395 *m/z*. D. UV spectra of the peak with the MS of apo-10'-lycopenoic acid + 2 Da (see Fig. 6C). E. 7,8-dihydro-apo-10'-lycopenoic acid as a proposed chemical structure as an apo-10'-lycopenoic acid/lycopene-metabolite including a comparable structure of apo-14'-lycopenoic acid, a derivative with 6 conjugated double bounds. Apo-10'-lycopenoic acid is abbreviated as apo-10-lycac, lycopene as LYC, and control as CTRL.

RAR target gene. In addition, we have demonstrated that apo-10-lycac transactivates RAR in adipose tissue *in vivo* and *in vitro* in adipocytes. Such a transactivational effect was suspected from *in vitro* data in lung [15]; however, we report here, for the first time an *in vivo* effect as well as an effect *in vitro* in adipocytes. Interestingly, both molecules could have a strong impact on the transcription of retinoid target genes, not only *via* activation of RAR, but also *via* the induction of the transcription of several genes involved in this pathway.

Due to the large shape of apo-10-lycopenoic acid we do not expect a direct interaction with the RARs and postulate further smaller downstream metabolites which may directly bind and directly initiated RAR-mediated signaling. Unfortunately these ligands are expected to be present in ultra-low endogenous concentrations comparable like retinoic acids [40] even after apo-10'-lycopenoic acid or lycopene treatments. Studies about the existence, endogenous presence and structure of direct RAR-interacting lycopene-metabolites are currently under investigation in our laboratories.

Based on these effects of apo-10-lycac on adipose tissue/adipocyte gene transcription *via* RAR, we sought to evaluate the impact of this molecule on two physiological processes that occur in adipose tissue and are directly related to physiopathological disorders. Therefore, we studied the effect of apo-10-lycac on adipogenesis and adipose tissue/adipocyte inflammation because ATRA has displayed some effects on these processes, notably through RAR [31–33]. Adipogenesis is related to the process of hyperplasia within adipose tissue. Unlike ATRA, which is a well-known inhibitor of adipogenesis at high concentration [32], apo-10-lycac treatments had no effect on adipogenesis under these conditions. This lack of effect of apo-10-lycac at this concentration (2 μM) could be due to its weaker activity (as shown in microarrays experiments and in transactivation experiments; Figs. 1B and 4B) as compared to ATRA. This point is particularly important to underline, since at low concentration ATRA display inverse effect on adipogenesis. Thus apo-10-lycac could present similar effects of a low concentration of ATRA. If we consider that adipogenesis, and particularly adipogenesis in subcutaneous adipose tissue, is a beneficial process that might participate to limit the prevalence of obesity-related pathologies [41], the lack of negative effects of apo-10-lycac treatment on adipogenesis may be considered beneficial, however the origin of this discrepancy between apo-10-lycac and ATRA on adipogenesis would require further investigations.

In addition, we demonstrated that apo-10-lycac was able to modulate inflammation in adipose tissue and adipocytes. This process is also deeply involved in the genesis of obesity-related pathologies, such as insulin resistance, where inflammatory markers are known to

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have a strong negative impact [42–44]. Therefore, by showing that apo-10-lycac could reduce the expression of some proinflammatory markers, we can hypothesize that this molecule has a beneficial effect on inflammation-related pathologies, such as insulin resistance, like lycopene [6]. Such an effect is probably due to the reduction of NF-κB activity that is mediated by the retinoic acid receptor, as previously reported [6,33]. These data support a beneficial role of apo-10-lycac in the prevention of obesity-related pathologies; however, additional experiments are required to demonstrate this *in vivo*.

We finally wanted to determine in this study whether apo-10-lycac or related derivatives are present in tissues. The presence of apo-10-lycac was partially confirmed in mouse spleen after intravenous injection, this co-elution is just a weak criteria but full identification using MS-MS techniques is not possible due to very low concentration of this derivative in the spleen. However, apo-10-lycac was not present in adipose tissue or human adipocytes following a regimen or incubation with lycopene or apo-10-lycac. Interestingly, we found a derivative with a mass of apo-10-lycac plus 2 Da using a highly sensitive HPLC MS-MS analysis. This finding suggests that one of the eight conjugated double bonds is hydrogenated; supporting the hypothesis that apo-10-lycac is quickly and completely metabolized in adipose tissue and adipocytes. Using an UV detector we additionally found out that six double bounds plus an additional conjugated COOH-group may be present and lets us propose that this compound is 7,8-diyhdro-apo-10'-lycopenoic acid. Such a compound could be produced by Retsat which is able to metabolize retinoic acid to dihydroretinoic acid [45], but so far, we cannot conclusively determine that this metabolite is *in fine* the active derivative of apo-10-lycac. Due to the complex structure and potential geometric isomers of this potential novel derivative, this peak could not be conclusively identified in this study. Studies to completely identify this novel lycopene metabolite, including its biological activity, are ongoing.

To conclude, in the present study, we show that In adipocytes, apo-10-lycac treatment exhibits important similarities with ATRA in terms of gene regulation. Consistently we reported that apo-10-lycac treatments can initiate transactivation of RAR in a transgenic reporter animal model, and induce the expression of several RAR target genes. Moreover, it possesses anti-inflammatory properties in adipose tissue and adipocyte models, but it does not have an effect on adipogenesis. While this compound was not found in adipose tissue/adipocytes, we identified a dihydro-metabolite of apo-10-lycac with a proposed structure of 7,8-dihydro-apo-10'-lycopenoic acid that will require further study. Thus, apo-10-lycac treatment and its further metabolites may be considered as potentially relevant compound in the context of obesity/diabetes prevention.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbalip.2011.09.002.

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