All-Trans Retinoic Acid Decreases Murine Adipose Retinol Binding Protein 4 Production

Josep Mercader, Nuria Granados, M. Luisa Bonet and Andreu Palou

Laboratory of Molecular Biology, Nutrition and Biotechnology, Universitat de les Illes Balears, Palma de Mallorca, Spain, CIBER Fisiopatología de la Obesidad y Nutrición (CB06/03), Instituto de Salud Carlos III, Spain

Key Words

Vitamin A • Retinoic acid • Retinaldehyde • Adipokines
• Insulin resistance • Adipocyte

Abstract

Background/Aims: Adipose-derived retinol binding protein 4 (RBP4) might contribute to the development of insulin resistance, and therefore further knowledge of factors regulating it is of interest. Retinoic acid, the acid form of vitamin A, affects the expression of several adipokines related to insulin sensitivity in mice. Here, we sought to investigate its impact on adipose RBP4 production. **Methods:** Changes in RBP4 expression were analyzed in adipose tissues and liver of mice treated *in vivo* with all-trans retinoic acid (ATRA), and in 3T3-L1 adipocytes and adipocytes derived from mouse embryonic fibroblasts exposed to ATRA. **Results:** ATRA treatment in mice increased insulin sensitivity as assessed by the homeostatic model assessment for insulin resistance, and led to a reduction of RBP4 mRNA and protein levels in adipose tissues, a reduction of RBP4 protein but not RBP4 mRNA levels in the liver, and a marked increase in circulating RBP4 protein levels. In adipocyte cell models, ATRA down-regulated RBP4

mRNA levels in a dose-dependent manner: this effect was reproduced by retinaldehyde and retinoid receptors agonists, and correlated with a reduced accumulation of RBP4 protein in the culture medium. **Conclusion:** These results reveal a selective effect of ATRA inhibiting RBP4 expression specifically in adipocytes, and reinforce the concept that vitamin A vitamers may affect insulin sensitivity through effects on adipokine production.

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Introduction

Retinol binding protein 4 (RBP4) is a 21-kDa single polypeptide chain protein best known for being the principal physiological carrier of retinol, the parent vitamin A molecule, in blood. RBP4 helps vertebrates adapt to fluctuations in dietary vitamin A intake by allowing the delivery of retinol from tissue storage sites to target tissues, which express a specific membrane receptor for RBP4 (STRA6) that mediates retinol uptake from vitamin A-loaded RBP4 (holo-RBP4) complexes [1]. Both apo- and holo-RBP4 circulate in blood complexed to plasma transthyretin in a 1:1 molar ratio. Liver and, to a lesser extent, white adipose tissue (WAT) are two main sites of

vitamin A storage and RBP4 synthesis. RBP4 is abundantly expressed in WAT depots [2-4], almost exclusively in the adipocytes and only weakly in the stromal-vascular cells [3], and adipocytes express RBP4 in a differentiation-dependent manner [5].

RBP4 was recently identified by microarray profiling as a gene overexpressed in WAT (but not liver) of adipose-specific GLUT4 knockout mice, which are insulin resistant, and underexpressed in WAT of transgenic mice overexpressing GLUT4 in adipose tissues, which have enhanced insulin sensitivity, suggesting adipocyte-derived RBP4 may act as an insulin resistance factor [6]. In keeping with this role, serum RBP4 levels are elevated in insulin-resistant mouse models; the anti-diabetic drug rosiglitazone reduces serum RBP4 levels and RBP4 mRNA expression in WAT (but not liver) of adipose-specific GLUT4 knockout mice; and genetic and pharmacological interventions that elevate serum RBP4 impair, while those reducing serum RBP4 enhance, insulin sensitivity in mice [6]. In humans, several studies have found a correlation between serum RBP4 levels and the magnitude of insulin resistance in subjects with obesity, impaired glucose tolerance or type 2 diabetes [6-10]. Some authors even reported a negative correlation between serum RBP4 and insulin sensitivity in healthy human subjects [9, 11, 12]. The association between serum RBP4 levels and insulin resistance remains, however, controversial, as several studies failed to find it [13-19], although in some of these reports an association of RBP4 with inflammatory markers and other components of the metabolic syndrome was noticed [17, 18]. Despite these discrepancies, there is evidence of effects of RBP4 counteracting insulin action, as RBP4 has been reported to attenuate insulin signaling in primary human adipocytes [20] and in skeletal muscle of mice [6], to increase hepatic glucose output in mice [6], and to negatively affect insulin secretion by pancreatic beta-cells in humans [16]. In addition, single nucleotide polymorphisms in the RBP4 gene have been found to be associated with susceptibility to type 2 diabetes and insulin resistance in humans [21-23].

RBP4 regulation by vitamin A has been most studied in the liver, but not in the adipose tissue, and this becomes of special interest in view of the potential role of adipose-derived RBP4 in the modulation of insulin action. It is unclear whether the link between RBP4 and insulin resistance occurs through vitamin A-dependent and/or vitamin A-independent mechanisms [6]. Retinoic acid, the carboxylic acid form of vitamin A, is a well known regulator of gene expression in mammals, mainly through

not solely through binding to and activation of specific retinoid receptors that behave as ligand-modulated transcription factors. Retinoic acid has many remarkable effects on adipocyte biology (reviewed in [24]), including effects on the production of several adipocyte-secreted bioactive proteins (adipokines), such as resistin and leptin [25-27]. The aim of this study was to evaluate RBP4 regulation by retinoic acid in murine adipose tissues and cultured white adipocytes.

Materials and Methods

Animal studies

Twelve-week-old NMRI male mice (CRIFFA, Barcelona, Spain) fed *ad libitum* regular chow (Panlab, Barcelona, Spain) and kept at 22°C with a 12-h light/dark cycle (lights on at 08:00) received one daily subcutaneous injection of all-trans retinoic acid (ATRA, Sigma, St. Louis, MO) at a dose of 10, 50, or 100 mg/kg body weight during the 4 days before they were killed (5-6 animals/group). Controls were injected the vehicle (olive oil). The animals were killed with CO₂ and decapitated at the start of the light cycle. Blood was collected from the neck and serum prepared and frozen at -20°C. Interscapular brown adipose tissue (BAT), inguinal WAT, epididymal WAT, retroperitoneal WAT and liver were excised in their entirety, weighed, frozen in liquid nitrogen and stored at -70°C until analysis. Biometric and blood parameters of animals used in the present study have already been published [28]. Insulin resistance was assessed by the homeostatic model assessment for insulin resistance (HOMA-IR) in additional twelve-week-old NMRI male mice treated as described above with vehicle (n=6) or 50 mg ATRA/kg body weight (n=6) and submitted to a 6-h fast (from 24:00 to 06:00) after the last vehicle/ATRA injection. The HOMA-IR score was calculated from fasting insulin and glucose concentrations using the formula of Matthews et al. [29]: $HOMA-IR = \text{fasting glucose (mmol/L)} \times \text{fasting insulin (mU/L)} / 22.5$. All animal experimentation was conducted according to accepted standards of humane care and use of laboratory animals and the studies were approved by our institutional bioethical committee.

Cell culture and differentiation

3T3-L1 cells (American Type Culture Collection, LGC Deselaers SL, Barcelona, Spain) were grown to confluence in basal medium – Dulbecco's modified Eagle's medium (DMEM; Sigma) with 50 IU/ml penicillin (Sigma), 50 µg/ml streptomycin (Sigma) and 2 mM L-glutamine (Sigma) – supplemented with 10% new born calf serum (Linus, Madrid, Spain). Two days after the cells reached confluence (referred as day 0) they were induced to differentiate in basal medium containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 1 µM dexamethasone (DEX; Sigma), 0.5 mM methylisobutylxanthine (MIX; Sigma) and 1 µg/ml insulin (Sigma) for 2 days, followed by 2 days in basal medium containing 10% FBS and 1 µg/ml insulin. The cells were subsequently refed every other day fresh

basal medium supplemented with 10% FBS (without insulin). Primary mouse embryonic fibroblasts (MEFs) were prepared and cultured essentially as described previously [30]. In brief, MEFs on passage 3-4 were grown to confluence in AmnioMAX™-C100 basal medium (Invitrogen) supplemented with 7.5% AmnioMAX™-C100 supplement (Invitrogen), 7.5% FBS, 50 IU/ml penicillin, 50 µg/ml streptomycin and 2 mM L-glutamine. For differentiation, 2-day postconfluent cells were treated (day 0) with growth medium containing 1 µM DEX, 0.5 mM MIX, 5 µg/ml insulin and 0.5 µM rosiglitazone (BioVision, Mountain View, CA) for 2 days; the cells were subsequently refed every other day fresh medium containing 5 µg/ml insulin and 0.5 µM rosiglitazone, and were deprived of rosiglitazone from day 6 on. In both cell models, adipogenesis was monitored by examination of the cells for lipid accumulation using phase-contrast microscopy. ATRA, all-trans retinaldehyde or the retinoid receptors agonists isopropyl- (E,E)-(R,S)-11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate (methoprene) and p-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB) (all from Sigma) were added together with fresh medium when more than 90% of the cells had acquired the adipose phenotype, at the doses and times indicated in the figure legends; control cells received an equal volume of vehicle (DMSO). Cytotoxicity of ATRA and the retinoid receptors agonists at the working concentrations used over that of DMSO was measured by the lactate dehydrogenase method [31], using a commercial kit (Roche Diagnostics GmbH, Mannheim, Germany).

Real-time PCR

Total RNA was extracted from cultured cells and tissues using Trizol reagent (Invitrogen) according to the supplier's instructions. For cDNA synthesis, 1 µg total RNA was denatured at 65°C for 10 min prior to being reverse transcribed using murine leukemia virus reverse transcriptase (Perkin-Elmer, Madrid, Spain) in the presence of 50 pmol of random primers, in a Perkin-Elmer 2400 Thermal Cycler. After diluting the cDNA samples, real time PCR analyses were conducted using the LightCycler System with SYBR Green I sequence nonspecific detection (Roche Diagnostic GmbH, Mannheim, Germany) and LRP10 [32] and β-actin as reference genes. Sense and antisense primers used were designed with specific primer analysis software Primer3 (Whitehead Institute for Biomedical Research, Cambridge, MA), and their specificity analyzed by the ENTREZ and BLAST databases utilities (National Center for Biotechnology Information, Bethesda, MD). The primers used were: for RBP4, 5'-ACT GGG GTG TAG CCT CCT TT-3' and 5'-GGT GTC GTA GTC CGT GTC G-3'; for LRP10, 5'-TCC CCT TTC TTC TCC TCC TC-3' and 5'-TTACCG TCT GTT CCT TGC TG-3'; and for β-actin, 5'-TAC AGC TTC ACC ACC AGC-3' and 5'-TCT CCA GGG AGG AAG AGG AT-3'.

Immunoblotting analysis

Adipose tissue and liver samples were homogenized in phosphate-buffered saline supplemented with protease inhibitors using a Teflon/glass homogenizer. After centrifugation at 500 g for 10 min at 4°C, the supernatant was used for total protein quantification with the BCA protein assay

Adipose depot	RBP4 mRNA levels (% of liver level)
epididymal WAT	6.20 ± 0.77
retroperitoneal WAT	23.25 ± 7.56
inguinal WAT	6.31 ± 2.10
interscapular BAT	10.27 ± 1.17

Table 1. RBP4 mRNA levels in adipose depots of NMRI mice: Relative RBP4 mRNA levels were determined by real time PCR in the indicated adipose depots and liver of twelve-week-old NMRI male mice (n=6) using β-actin as reference gene. Results are expressed as a percentage of the relative RBP4 mRNA levels in liver, which was set as 100%.

kit (Pierce, Rockford, IL, USA). Protein in supernatants (20-60 µg) and in 3 µl of serum was solubilized and boiled in Laemmli sample buffer containing 20% 2-mercaptoethanol, fractionated in 13% SDS-PAGE gels, and electrotransferred onto PVDF membranes (Bio-Rad, Madrid, Spain). Black amide B10 staining provided visual evidence for correct loading and blotting of proteins. After blocking, membranes were incubated with polyclonal anti-mouse RBP4 antibody raised in rabbits (Axxora, LLC, San Diego, CA). Horseradish peroxidase-conjugated anti-rabbit IgG antibody was used as secondary antibody (from Amersham Biosciences, UK). The immunocomplexes were revealed using an enhanced chemiluminescence detection system (Amersham Biosciences, Barcelona, Spain). Membranes were exposed to Hyperfilm ECL (Amersham Biosciences), and bands in films were analyzed by scanner photodensitometry and quantified using the BioImage program (Millipore, Bedford, MA). Serum RBP4 was also assessed by immunoblotting after fractionation of whole serum by nondenaturing PAGE (7% acrylamide), which has been reported to allow the separation of retinol bound-, retinoic acid bound- and apo-human RBP4 complexes [33-35]. Delipidized serum from control mice was prepared as previously described to serve as a marker for the apo-RBP4:transthyretin complex in nondenaturing PAGE [35].

Statistical analysis

Data are presented as mean ± SEM. Statistical significance of effects was assessed by Student's *t*-test or one-way ANOVA followed by least-significance difference (LSD) post-hoc comparison. Results were considered statistically significant when *P* < 0.05.

Results

RBP4 mRNA expression pattern in mouse fat depots

The anatomic pattern of RBP4 mRNA expression in the different fat depots has been reported in the rat [3] but not, to our knowledge, in the mouse. We compared

	Control	ATRA treatment			ANOVA
		10 mg/kg	50 mg/kg	100 mg/kg	
epididymal WAT	100 ± 15 ^a	78 ± 12 ^{ab}	47 ± 8 ^{bc}	69 ± 7 ^{ac}	$P<0.05$
inguinal WAT	100 ± 20 ^a	24 ± 7 ^b	20 ± 5 ^b	7 ± 3 ^b	$P<0.001$
retroperitoneal WAT	100 ± 13 ^a	60 ± 17 ^b	62 ± 5 ^b	58 ± 16 ^b	$P<0.05$
interscapular BAT	100 ± 11	100 ± 15	86 ± 17	54 ± 7	$P=0.056$
liver	100 ± 6	74 ± 5	87 ± 7	93 ± 24	

Table 2. Effect of ATRA treatment on RBP4 mRNA levels in adipose depots and liver of NMRI mice: RBP4 mRNA levels were determined in the indicated tissues of twelve-week-old NMRI male mice treated with vehicle (olive oil) or ATRA (one daily subcutaneous injection, at the indicated dose, during the 4 days before the animals were killed). Data are the mean ± SEM of 8-12 animals per group distributed in two independent experiments and are expressed relative to the mean value of the control group, which was set as 100%. Relative levels of RBP4 mRNA were analyzed by real time PCR using β -actin as the reference gene. Values within a row not sharing a common letter are statistically different by LSD post-hoc comparison ($P < 0.05$).

the expression levels of RBP4 mRNA in visceral WAT depots (retroperitoneal and epididymal), subcutaneous (inguinal) WAT and interscapular BAT of NMRI mice relative to the liver levels by real time PCR (Table 1). Levels of RBP4 mRNA in the retroperitoneal, epididymal, inguinal and interscapular mouse adipose depots averaged, respectively, 23.3%, 6.2%, 6.3% and 10.3% the levels in mouse liver. This expression pattern is similar to the rat pattern except for the epididymal depot, for which RBP4 expression was proportionally higher in the rat [3]. In humans, a higher RBP4 gene expression in visceral *versus* subcutaneous fat and correlation of RBP4 serum levels with intra-abdominal fat mass have been reported [36, 37].

ATRA treatment in NMRI mice led to reduced RBP4 mRNA and protein levels in fat depots and triggered an increase in serum RBP4 protein levels.

We first examined RBP4 regulation by ATRA in a model of acute ATRA treatment *in vivo* that has previously been shown to improve glucose tolerance and insulin sensitivity, to decrease body weight and adiposity independent of changes in food intake, and to suppress adipose expression and circulating levels of resistin and leptin in the treated mice [25, 27, 28, 38-40]. This treatment also increases thermogenic capacity in BAT [38, 39] and thermogenic and fat oxidation capacity in skeletal muscle [41], and favors the acquisition of BAT-like properties in WAT depots [28], without increasing aminotransferase activity in serum [27] – a marker of hepatic damage, which can arise from vitamin A excess.

Further confirming an effect of this treatment in increasing insulin sensitivity, we report here that the HOMA-IR score was lower in mice treated with 50 mg ATRA/kg body weight than in vehicle-treated mice (ATRA-treated group, 1.69 ± 0.35 ; vehicle-treated group, 4.22 ± 0.91 ; $P < 0.05$, Student's *t* test).

ATRA treatment triggered a significant down-regulation of RBP4 mRNA levels in all WAT depots examined, but not in the liver (Table 2). Down-regulation was especially marked in the inguinal WAT depot, where RBP4 mRNA levels were already reduced by 76% at the lowest ATRA dose tested (10 mg per kg body weight) and almost completely suppressed at the highest (100 mg ATRA per kg body weight). In the retroperitoneal WAT depot, RBP4 mRNA levels were reduced by 40% following treatment with 10 mg ATRA per kg body weight, and treatment with higher doses of ATRA resulted in similar reductions. In the epididymal WAT depot, down-regulation was maximal (53% reduction) in the animals treated with 50 mg ATRA per kg of body weight. A down-regulatory effect of ATRA treatment on RBP4 mRNA expression in the interscapular BAT depot did not reach statistical significance ($P=0.056$) but became evident (46% decrease) at the highest ATRA dose used.

Immunoblotting following SDS-PAGE was used to estimate total RBP4 protein levels in tissues and serum. In keeping with the down-regulation seen at the mRNA level, RBP4 protein levels were significantly reduced in the epididymal WAT depot (Figure 1a) and had a tendency (that just failed to reach statistical significance, $P=0.056$) to be reduced in the inguinal WAT depot of ATRA-treated mice as compared with vehicle-treated controls (Figure

Fig. 1. Effect of ATRA treatment on tissue and serum RBP4 protein levels in NMRI mice. RBP4 protein levels were analyzed by immunoblotting after SDS-PAGE in epididymal WAT (a), inguinal WAT (b), liver (c), and serum (d, right) of twelve-week-old NMRI male mice treated with vehicle (control group) or 50 mg ATRA/kg body weight (one daily subcutaneous injection during the 4 days before the animals were killed). The blot to the left of panel d shows immunodetection of serum RBP4 complexes after nondenaturing PAGE (7% acrylamide) in control mice and ATRA-treated mice; delipidized serum (D) was run to serve as a marker for the apo-RBP4:transthyretin complex. Data in the bar diagrams are the mean $\pm$ SEM of 7-10 animals per group distributed in two independent experiments, and are expressed relative to the mean value of the control group, which was set as 100%; at the bottom of each bar diagram representative Western blots are shown (60 μ g of total WAT protein, 20 μ g total liver protein and 3 μ l of serum per lane). Student's *t* test significance: *, $P < 0.05$; **, $P < 0.01$.

1 a and b, respectively). RBP4 protein content was also significantly decreased after ATRA treatment in the liver (Figure 1c), despite no treatment effect on liver RBP4 mRNA levels (Table 2). Serum total RBP4 levels were approx. 6-fold higher in the ATRA-treated mice than in the vehicle-treated mice (Figure 1d left). We also performed immunodetection of RBP4 following nondenaturing PAGE of whole serum. Results of this analysis indicated the presence in the serum of ATRA-treated mice of RBP4 complexes of higher mobility than those present in the serum of control mice (Figure 1 d, right). Delipidized mouse serum, which was used to locate the apo-RBP4:transthyretin complex, gave a band with mobility in between that of the RBP4 complexes present in serum of control and ATRA-treated mice. In the light of previous literature [33-35], this pattern of electrophoretic mobilities is consistent with serum complexes consisting mainly of retinol-RBP4:transthyretin

in the control mice, and of retinoic acid-RBP4:transthyretin in the ATRA-treated mice.

ATRA reduced RBP4 mRNA expression and the amount of secreted RBP4 in cultured white adipocytes.

We next examined RBP4 regulation by ATRA in adipocyte cell model systems. Exposure of mature 3T3-L1 adipocytes to 1 μ M ATRA reduced RBP4 mRNA expression levels by approx. 50% after 36 h and 48 h treatment (Figure 2a). ATRA-induced down-regulation of RBP4 gene expression in 3T3-L1 adipocytes was dose-dependent, reaching a plateau at 1 μ M (Figure 2b). Exposure to 1 μ M rosiglitazone for 48 h also resulted in a significant ($P < 0.05$), 40% decrease of RBP4 mRNA levels in 3T3-L1 adipocytes, in keeping with previous results *in vivo* [6].

Since RBP4 was expressed at very low levels in

Fig. 2. ATRA down-regulates RBP4 mRNA levels in 3T3-L1 mature adipocytes. Differentiated 3T3-L1 adipocytes (on day 8-10) were treated with vehicle (DMSO) and 1 μ M ATRA for the indicated time-intervals (a) or the indicated ATRA concentrations for 48 h (b) before harvesting and total RNA extraction. RBP4 mRNA levels were analyzed by real time PCR and normalized to the expression of β -actin. Data are expressed relative to the mean value of the vehicle-treated cells, which was set as 100%, and are the mean $\pm$ SEM of $n=5$ -12 plates (a) or $n=7$ -10 plates (b) per treatment condition, distributed in at least two independent experiments. *, $P < 0.05$, Student's t test; **, $P < 0.01$, Student's t test; ATRA, effect of ATRA treatment, one-way-ANOVA. In panel b, bar values not sharing a common letter are statistically different by LSD post-hoc comparison ($P < 0.05$).

3T3-L1 adipocytes, making reproducibility difficult, we sought to contrast the above results in an alternate adipocyte cell model system, namely primary MEFs-derived adipocytes, which we found express RBP4 mRNA at much higher levels (approx. 100-fold higher). Exposure of primary MEFs-derived adipocytes for 48 h to 0.1 μ M, 1 μ M and 10 μ M ATRA reduced RBP4 mRNA levels by 46%, 56% and 69%, respectively (Figure 3a). Exposure to ATRA also reduced the amount of RBP4 protein accumulated in the culture medium (Figure 3b), indicating that the effect on RBP4 gene transcription translated into reduced secreted RBP4 protein. Retinaldehyde – which is primarily considered to be a precursor for retinoic acid formation but may also exert distinct, retinoic acid-independent effects in adipose cells [42] – also down-regulated RBP4 mRNA levels in MEFs-derived adipocytes, although with a lower potency than ATRA (Figure 3c). We also assayed whether synthetic compounds that selectively bind to and activate the retinoid receptors were able to reproduce the inhibitory effect of ATRA on RBP4 gene expression (Figure 3d). Similar to exposure to 1 μ M ATRA, exposure for 48 h to 1 μ M TTNPB – a pan-agonist of all retinoic acid receptors (RARs) – reduced RBP4 mRNA levels by 60% in primary MEFs-derived adipocytes. Methoprene – a pan-agonist of the rexinoid receptors (RXRs) – also reduced RBP4 mRNA levels in these cells in a dose-dependent manner,

although with a lower potency than ATRA and TTNPB. Similar effects of TTNPB and methoprene on RBP4 gene expression were found in 3T3-L1 adipocytes (data not shown). No cytotoxicity of ATRA or the retinoid receptors agonists at the working concentrations used over that of vehicle (DMSO) was detected in the cell models used by the lactate dehydrogenase method.

Discussion

In this work, we provide novel evidence of a down-regulatory effect of retinoic acid on adipose RBP4 gene expression. This down-regulation was apparent both in adipose depots of ATRA-treated mice and, after exposure to ATRA, in two adipocyte cell model systems with different endogenous levels of RBP4 expression, namely 3T3-L1 adipocytes and primary MEFs-derived adipocytes. Down-regulation of adipose RBP4 mRNA levels by ATRA correlated with reduced RBP4 protein content in adipose tissues of ATRA-treated mice and, in the cultured adipocytes, was accompanied by a reduction in the amount of RBP4 secreted in the culture medium and reproduced by retinaldehyde and by both RAR and RXR agonists.

Previous literature on the regulation of adipose RBP4 expression by retinoids is scarce. RBP4 expression has

Fig. 3. Retinoids down-regulate RBP4 expression in primary MEFs-derived adipocytes. MEFs-derived adipocytes that had been deprived of rosiglitazone from day 6 were treated on day 8 with vehicle (DMSO) or problem compounds for 48 h, followed by total cellular RNA extraction and medium collection. **a)** Effect of ATRA on RBP4 mRNA levels. **b)** Effect of ATRA on RBP4 protein accumulation in the culture medium. Representative Western blots for RBP4 in fresh medium and in medium after 48 h incubation with cells and the indicated additions are shown at the bottom (25 μl of medium per lane). **c)** Effect of retinaldehyde (Rald) on RBP4 mRNA levels. **d)** Effect of retinoid receptors agonists on RBP4 mRNA levels. Data in the bar diagrams are expressed relative to the mean value of the vehicle-treated cells, which was set as 100%, and are the mean $\pm$ SEM of $n = 6-7$ plates per treatment condition distributed in two independent experiments (a-c) or $n = 3$ plates per treatment condition (d). T: one-way-ANOVA, effect of treatment; bar values not sharing a common letter are statistically different by LSD post-hoc comparison ($P < 0.05$). *, **, ***: different from vehicle, Student's t ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively).

been reported to be unaffected either by retinol in differentiating BFC-1β cells (a preadipocyte cell line derived from mouse BAT) [5] or by 24 h ATRA treatment in primary cultures of rat adipocytes [43]. Though species-specific differences cannot be discarded, differences between the latter report and our work may be related to differences in the duration of the treatment, because we found the down-regulatory effect of ATRA on adipocyte RBP4 mRNA levels following a relatively lengthy time-course. This suggests that ATRA may be inducing the expression in adipose cells of a protein or proteins that repress RBP4 gene transcription. Attempts to contrast this hypothesis by using an inhibitor of translation

(cycloheximide) were hindered by the fact that the inhibitor itself markedly suppressed RBP4 mRNA levels in MEFs-derived adipocytes, suggesting that relatively short-lived transcription factors are involved in basal RBP4 gene transcription in these cells. In any case, it is possible that repression of RBP4 expression is secondary to ATRA-induced metabolic/morphological changes in mature adipocytes, in which ATRA has been shown to favor remodeling toward increased oxidative metabolism and decreased triacylglycerol content [28, 44].

In contrast with the down-regulation seen in adipose depots, ATRA treatment had no effect on RBP4 mRNA levels in liver. Previous studies on the effect of retinoids

on liver RBP4 expression reveal a complex picture. On the one hand, animal studies have shown that liver RBP4 mRNA levels do not change in response to changes in the vitamin A status (reviewed in [45]). On the other hand, it has been reported that retinol and retinoic acid induce RBP4 mRNA expression in human and mouse hepatoma cell lines [4, 46] and that, in mice, a single oral dose of retinoic acid (either ATRA or the 9-cis isomer) up-regulates liver RBP4 mRNA levels [4]. Moreover, a retinoic acid response element in the promoter region of the RBP4 gene has been described that allows transactivation of transcription by liganded RAR homodimers, RXR homodimers and, especially, RAR:RXR heterodimers in cells of hepatic origin [47]. We were unable to detect an increase in liver RBP4 mRNA levels following subcutaneous ATRA injection for 4 consecutive days in mice. It is possible that both the regimen of ATRA treatment (single *versus* repeated doses) and the way of administration (oral *versus* subcutaneous) are factors that influence effects on liver RBP4 gene transcription. In any case, our results reveal a differential regulation of liver and adipose RBP4 gene expression by ATRA that deserves further investigation and may be related to differences in the battery of retinoid receptors and transcriptional cofactors expressed in each tissue.

Treatment of adult, vitamin A-sufficient mice with ATRA led to a marked increase of RBP4 levels in serum. Adipose tissue is unlikely to be a source for this increase, because ATRA reduced RBP4 gene expression both in WAT depots *in vivo* and in cultured adipocytes and, in the cell model, this was accompanied by a reduction in RBP4 protein accumulation in the culture medium. The liver is a likely source, not only because it is the main site of RBP4 production but also because in this tissue ATRA treatment depleted RBP4 protein levels without affecting RBP4 mRNA levels, suggesting that reduction in liver RBP4 protein content reflects enhanced release into the circulation. Importantly, in support of this interpretation, previous reports have shown that retinoic acid can bind to RBP4 [48, 49] and stimulate secretion of RBP4 by cultured liver cells [50, 51], similar to retinol, whose positive effect on hepatic RBP4 secretion (in the form of holo-protein) is well established [45]. Thus, a plausible explanation for the increased serum RBP4 levels in ATRA-treated mice is that excess ATRA in liver cells is favoring the export of the retinoid bound to RBP4, potentially minimizing toxicity. This is in line with our nondenaturing PAGE results, which are consistent with a preponderance of the retinoic acid-RBP4:transthyretin

complex in serum of the ATRA-treated mice.

Since glucose tolerance and insulin sensitivity were improved after ATRA treatment ([25] and this work), our results would indicate that increased serum levels of holo-RBP4 of non-adipose origin do not cause *per se* insulin resistance in mice. Although the idea that insulin resistance is related to adipose-derived rather than liver-derived RBP4 was implicit in the seminal work by Yang et al. [6], the molecular basis for this difference remains unclear (whether or not there may be differences in the glycoprotein moiety, for instance). It is also unclear whether apo-RBP4, holo-RBP4 or both are the active species for the effect [6]. In fact, an involvement of apo-RBP4 seems more plausible, as some of the manipulations that were reported [6] to result in increased insulin resistance (such as overexpression of RBP4 in transgenic mice and injection of RBP4 in normal mice) are not to be expected to translate into differences in serum holo-RBP4 levels in the absence of changes in the vitamin A status of the animals. Moreover, a recent report showed that, in children, the serum RBP4 to serum retinol ratio more strongly correlates with components of the metabolic syndrome than serum RBP4, suggesting a specific involvement of apo-RBP4 [10].

Results in this work are compatible with adipose-derived RBP4 being a contributor factor to systemic insulin resistance in mice, as ATRA treatment down-regulated RBP4 expression specifically in adipocytes (but not in the liver) while it enhanced systemic glucose tolerance and insulin sensitivity of the animals ([25] and this work). Remarkably, ATRA treatment also suppresses the adipose expression of another insulin resistance factor of adipose origin in mice, namely resistin [25], as well as the adipose expression of leptin [25-27]. RBP4 [20], resistin [52-54] and leptin [55] are known to exert paracrine effects in WAT antagonizing insulin signaling, which in WAT is pro-adipogenic and pro-lipogenic, and ATRA is an agent that promotes body fat loss in mice *in vivo* and a metabolic remodeling of adipocytes (both *in vivo* and in cell models) toward increased oxidative metabolism [24, 28, 44]. Given the essential role of adipose cells in fat storage, from a teleological point of view the ATRA-induced down-regulation of adipose RBP4, resistin and leptin expression may be viewed as part of a security mechanism to avoid fat depletion (by increasing local insulin sensitivity in the adipocytes, and also through the CNS-mediated consequences of reduced leptinemia) in the context of an overall fat-mobilizing effect of ATRA. Agents displaying fat-mobilizing effects coupled to down-regulatory effects on the expression of insulin resistance

factors of adipose origin might be ideally suited to serve as insulin sensitizers, provided that safety issues can be guaranteed.

In summary, this work demonstrates a selective effect of ATRA inhibiting RBP4 expression specifically in adipocytes, which correlated with increased systemic insulin sensitivity in the ATRA-treated mice. These results are compatible with an involvement of adipose-derived RBP4 in the development of insulin resistance, and reinforce the concept that vitamin A vitamers may affect insulin sensitivity through effects on adipokine production.

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