All-Trans Retinoic Acid Increases Oxidative Metabolism in Mature Adipocytes

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Key Words

Vitamin A • Retinoic acid • 3T3-L1 adipocytes •
Adipocyte metabolism • Metabolic remodeling

Abstract

Background/Aims: In rodents, retinoic acid (RA) treatment favors loss of body fat mass and the acquisition of brown fat features in white fat depots. In this work, we sought to examine to what extent these RA effects are cell autonomous or dependent on systemic factors. **Methods:** Parameters of lipid metabolism and related gene expression were analyzed in differentiated 3T3-L1 adipocytes after exposure to RA or vehicle. **Results:** Treatment with RA resulted in decreased cellular triacylglycerol content and increased basal lipolysis and fatty acid oxidation rate. At the mRNA level, RA treatment led to a reduced expression of adipogenic/lipogenic transcription factors (peroxisome proliferator-activated receptor gamma, CCAAT/enhancer-binding protein alpha, rexinoid receptor alpha) and two purported suppressors of lipolysis and oxidative metabolism (CIDEA and receptor-interacting protein 140), and to

an increased expression of proteins favoring fat oxidation (peroxisome proliferator-activated receptor gamma coactivator-1alpha, uncoupling protein 2, fasting-induced adipose factor, enzymes of mitochondrial fatty acid oxidation). These changes paralleled inactivation of the retinoblastoma protein and were preceded by an early RA-induced phosphorylation of p38 mitogen-activated protein kinase. UCP1 expression was not induced. **Conclusion:** The results indicate that RA directly favors remodeling of mature 3T3-L1 adipocytes in culture toward increased oxidative metabolism.

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Introduction

Brown adipose tissue (BAT) and white adipose tissue (WAT) depots are composed mainly of brown and white adipocytes, respectively. Brown adipocytes are rich in mitochondria, have a high oxidative capacity and are specialized in the regulated production of heat (adaptive thermogenesis) by oxidation of their stored lipids and subsequent dissipation of the proton electrochemical

gradient through the activity of uncoupling protein 1 (UCP1), a brown adipocyte-specific inner mitochondrial membrane protein that behaves functionally as a proton transporter. White adipocytes are poor in mitochondria, have a low oxidative capacity, do not express UCP1 and are specialized in the storage of energy in the form of triacylglycerol and the supply of energy to the body in situations of energy deficit. Several agents/conditions known to stimulate thermogenesis in BAT promote the acquisition of BAT-like features in typical WAT depots of rodents, supporting the concept of plasticity of the adipose organ [1]. Such remodeling is of interest because there is evidence that a reduced brown adipose-like phenotype in white fat may contribute to obesity and type 2 diabetes in humans [2, 3].

Retinoic acid (RA), the carboxylic acid form of vitamin A, is a nutrient derivative with many remarkable effects on adipocyte biology affecting preadipocyte survival, embryonic stem cell commitment into the adipocyte lineage, adipogenesis of preadipocyte clonal cell lines, UCP1 expression in brown adipocytes, and the production of several adipocyte-secreted bioactive proteins (adipokines), including leptin and resistin [4, 5]. RA regulates gene expression mainly through binding to and activation of two types of ligand-modulated transcription factors: the retinoic acid receptors (RARs), which respond to both all-trans RA (ATRA) and 9-cis RA, and the rexinoid receptors (RXRs), which are thought to respond specifically to 9-cis RA [6]. In addition, direct or retinoid receptor-mediated nongenomic effects of RA leading to changes in the activity of protein kinases including protein kinase C, p38 mitogen-activated protein kinase (p38 MAPK) and extracellular signal-regulated kinase 1/2 have been described in cell systems, including adipocytes [7, 8].

Acute ATRA treatment reduces body weight and adiposity in mice independently of changes in food intake [5, 9-12]. ATRA-induced reduction of adiposity correlates with an increase in the thermogenic capacity of BAT [9, 10] and skeletal muscle [13], and with changes in the metabolic profile of WAT. Adipogenic/lipogenic capacity is reduced in WAT depots of ATRA-treated mice [11], which show signs of remodeling toward the acquisition of BAT features, including an increased capacity for oxidative metabolism, a switch from unilocular to multilocular intracellular lipid distribution and UCP1 gene expression [12]. The remodeling seen *in vivo* could be the result of the recruitment of brown precursors present in the depots, a transformation of the white adipocytes, or both, and could imply both direct effects of RA on the

adipose cells and indirect effects of the treatment. Here, we have addressed these issues by analyzing the effects of RA in differentiated murine 3T3-L1 adipocytes.

Materials and Methods

Cell culture

Fetal calf serum (FCS) and fetal bovine serum (FBS) were from Linus (Madrid, Spain); Dulbecco's modified Eagle's medium (DMEM), dexamethasone, methylisobutylxanthine, and ATRA were from Sigma (St. Louis, MO). Other reagents used were of the highest purity available. 3T3-L1 cells (American Type Culture Collection, LGC Deselaers SL, Barcelona, Spain) were cultured in DMEM supplemented with 10% FCS. Two days after cells reached confluence (referred as day 0), they were induced to differentiate in DMEM supplemented with 10% FBS, 1 μ M dexamethasone, 0.5 mM methylisobutylxanthine and 1 μ g/ml insulin for 48 h, followed by 48 h in DMEM supplemented with 10% FBS and 1 μ g/ml insulin. The cells were subsequently refed every 48 h with fresh medium without insulin. Adipogenesis was monitored by morphological examination of the cells for lipid accumulation. ATRA was added at the times and doses indicated in the figure legends; control cells received an equal volume of vehicle (DMSO). No cytotoxicity of ATRA at the working concentrations used over that of DMSO was detected by the lactate dehydrogenase method [5].

Cloning and retroviral transduction: Mouse peroxisome proliferator-activated receptor γ 2 (PPAR γ 2) cDNA including an optimized Kozak sequence, a 5' SalI site and a 3' ApaI site was generated by polymerase chain reaction (PCR) using pSPORTmPPAR γ 2 (kindly provided from B.M. Spiegelman) as template and the primers: 5': 5'-AGT CGA CGC CAC CAT GGT TGA CAC AGA GAT GCC A and 3': 5'- TGG GCC CCT AAT ACA AGT CCT TGT AGA ATC. The resulting PCR product was blunt-end ligated into the EcoRV site of pBluescript II KS+ (Stratagene, La Jolla, CA) to generate pBSKmPPAR γ 2. The PPAR γ 2 sequence in pBSKmPPAR γ 2 was verified by sequencing. The pBABEmPPAR γ 2 was generated by subcloning the SalI/NotI fragment from pBSKmPPAR γ 2 into SalI/NotI of pSPORT and then blunt-end ligate the SmaI fragment of the resulting vector into the SmaI site of a modified pBABepuro retroviral vector (kindly provided by O. A. MacDougald). To generate retrovirus, Phoenix Eco packaging cells (generously supplied by Dr. G. P. Nolan) were transfected with pBABEmPPAR γ 2 or the control vector (pBABepuro) by standard calcium-phosphate method. 48 h post-transfection, filtered viral supernatants from the packaging cell line were used to infect 30-40% pre-confluent 3T3-L1 cells growing in DMEM supplemented with 10% FCS. Two days after infection, stably transduced 3T3-L1 preadipocytes were selected in medium containing puromycin. Stably transduced 3T3-L1 cells were allowed to reach confluence and, two days later (day 0), induced to differentiate as above, except that rosiglitazone (0.5 μ M) was included from day 0 on. Immunoblotting analysis of cells at day 0 confirmed robust PPAR γ 2 expression in the cells transduced with the PPAR γ 2-expressing vector and lack of

Table 1. Primers used in the PCR reactions

Gene	Primer sequence	Accession number
ACO	5'-GGGTCATGGAATCATCT TCG A-3' 5'-GAATGAACTCTTGGGTCTTGG G-3'	NM_025729
CIDEA	5'-GACAGAAATGGACACCGGG-3' 5'-GATTCCTTAACACGG CC-3'	AF041376
C/EBP α	5'-GAACAGCAACGAGTACCGGGTA-3' 5'-GCCATGGCCTTGACCAAGGAG-3'	M62362
CPT1-L	5'-ACAAATTATGTGAGTGACTGG -3' 5'-GATCCCAGAAGACGAATAGG -3'	NM_013495
FIAF	5'-TGGCAATGCCAAATT GCT C-3' 5'-GTGTCCTCACCCCCCAA T-3'	NM_020581
FAT/CD36	5'-AATTAGAACCGGGCCACGTA -3' 5'-CGCCAACTCCCAGGTACAA-3'	NM_007643
FOXC2	5'-GAAAGCGCCCCTCTCTCAG-3' 5'-TGCGGATAAGTTACCTGCGA -3'	NM_013519.1
MCAD	5'-GGATTAGGGTTTAGTTTTGAGTT -3' 5'-CCAAAGAATTTGCTTCAATAGC -3'	AW105998
PEPCK-C	5'-AGCCTCGACAGCCTGCCAG-3' 5'-CCAGTTGTTGACCAAAGGCTTT-3'	NM_011044.1
PGC1 α	5'-GTCCTCACAGAGACACTGGA-3' 5'-TGGTTCTGAGTGCTAAGACC-3'	AF049330
PPAR α	5'-CGTTTGTGGCTGGTCAAGTT-3' 5'-AGAGAGGACAGATGGGGCTC-3'	NM_011144
PPAR γ	5'-ACAGCAAATCTCTGTTTTATGC -3' 5'-TGCTGGAGAAATCAACTGTGG-3'	NM_011146
PPAR δ	5'-TCCGTCCCAAAGCGGATGA-3' 5'-GCCTCGGGCTTCCACTAC-3'	NM_011145.2
pRB	5'-GGGACATCTGAGAAGTTCCAG-3' 5'-CTGCTTTTGCATTCTGTTCG-3'	NM_009029.1
RIP140	5'-CGGCCTCGAAGGCGTGG-3' 5'-AAACGCACGTCAGTATCGTC -3'	NM_173440
RXR α	5'-GGACCGCTCCTCAGGCA-3' 5'-CTTGCAGCCCTCACAACTGTAT -3'	NM_011305
TBP	5'-ACCCTTCACCAATGACTCCTATG -3' 5'-ATGATGACTGCAGCAAATCGC -3'	XM_123010
THIO	5'-TCCAGGACGTGAAGCTAAAGC-3' 5'-CATTGCCCACGGAGATGTC -3'	NM_146230
UCP2	5'-ATACCAGAGCACTGTCTGAAGC-3' 5'-CGTCTTGACCACATCAACAGG-3'	NM_011671

expression in the cells transduced with the control vector (not shown). Where indicated, ATRA was added from day 0 on.

Real-time PCR

Reagents used for RNA extraction and analysis were from Invitrogen (Carlsbad, CA), Sigma (St. Louis, MO), Promega (Madison, WI) and Amersham (Buckinghamshire, UK). Total RNA was extracted from cells using Trizol reagent (Invitrogen) according to the supplier's instructions. cDNA synthesis was performed from 1 μ g of RNA using M-MLV reverse transcriptase (Invitrogen) and Oligo-d(T) (Sigma) as a primer. Real-time PCR assays on newly synthesized cDNA were

conducted using SYBR Green JumpStart Taq Ready Mix (Sigma) and specific primers for each gene of interest, and run on an ABI Prism 7700 real-time PCR machine (PE Applied Biosystems, Foster City, CA). The expression of selected genes was in all cases normalized to the expression of TATA-binding protein (TBP). Sense and antisense primers used were designed with specific primer analysis software Primer3 (Whitehead Institute for Biomedical Research, Cambridge, MA), and their sequences are shown in Table 1. Specificity of the primers and primer binding sites was analyzed by the ENTREZ and BLAST databases utilities (National Center for Biotechnology Information, Bethesda, MD).

Table 2. Changes in gene expression in mature 3T3-L1 adipocytes in response to a 24 h ATRA treatment: Mature 3T3-L1 adipocytes at day 8 or 10 were treated with vehicle (DMSO) or ATRA at the indicated concentrations for 24 h before harvesting and total RNA extraction. Expression of the listed genes was determined by real-time PCR, and was in all cases normalized to the expression of TBP. Data are expressed relative to the mean value of the vehicle-treated cells, which was set at 1.000, and are the mean $\pm$ SEM of $n=5$ to 10, distributed in at least two independent experiments (except PEPCK, CIDEA and FIAF data, which are the mean $\pm$ SEM of $n=3$ to 4 in a single experiment). [#] One-way-ANOVA or Student's *t*-test significance is given. Values within a row not sharing a common letter are statistically different by LSD post-hoc comparison ($P<0.05$). ACO, acyl-CoA oxidase; THIO, 3-keto acyl-CoA thiolase; other abbreviations are as introduced in the text.

mRNA	ATRA			<i>P</i> value [#]
	DMSO	1 μ M	10 μ M	
PPAR γ	1.000 $\pm$ 0.079	1.034 $\pm$ 0.167	0.920 $\pm$ 0.070	
RXR α	1.000 $\pm$ 0.083 ^a	0.723 $\pm$ 0.077 ^b	0.632 $\pm$ 0.008 ^b	$P<0.005$
C/EBP α	1.000 $\pm$ 0.067 ^a	0.564 $\pm$ 0.075 ^b	0.596 $\pm$ 0.031 ^b	$P<0.001$
CPT1-L	1.000 $\pm$ 0.096 ^a	1.395 $\pm$ 0.147 ^b	1.827 $\pm$ 0.148 ^c	$P<0.001$
MCAD	1.000 $\pm$ 0.064 ^a	1.117 $\pm$ 0.080 ^a	1.378 $\pm$ 0.092 ^b	$P<0.01$
ACO	1.000 $\pm$ 0.056	1.108 $\pm$ 0.138	1.079 $\pm$ 0.071	
THIO	1.000 $\pm$ 0.057	1.102 $\pm$ 0.118	1.089 $\pm$ 0.086	
UCP2	1.000 $\pm$ 0.149 ^a	1.322 $\pm$ 0.320 ^{ab}	1.860 $\pm$ 0.167 ^b	$P<0.05$
PGC-1 α	1.000 $\pm$ 0.084 ^a	1.111 $\pm$ 0.129 ^a	1.467 $\pm$ 0.120 ^b	$P<0.05$
RIP140	1.000 $\pm$ 0.061 ^a	0.883 $\pm$ 0.058 ^a	0.602 $\pm$ 0.059 ^b	$P<0.001$
CIDEA	1.000 $\pm$ 0.320	0.400 $\pm$ 0.064		$P=0.059$
FOXC2	1.000 $\pm$ 0.109 ^a	0.987 $\pm$ 0.109	1.063 $\pm$ 0.046	
PEPCK-C	1.000 $\pm$ 0.334 ^a	0.971 $\pm$ 0.032 ^a	2.169 $\pm$ 0.430 ^b	$P=0.056$
FAT/CD36	1.000 $\pm$ 0.116 ^a	1.304 $\pm$ 0.193 ^{ab}	1.444 $\pm$ 0.071 ^b	$P<0.05$
FIAF	1.000 $\pm$ 0.162 ^a	1.126 $\pm$ 0.074 ^a	2.920 $\pm$ 0.983 ^b	$P<0.005$
pRB	1.000 $\pm$ 0.041 ^a	0.707 $\pm$ 0.054 ^b	0.648 $\pm$ 0.035 ^b	$P<0.001$

Immunoblotting analysis

Cell extracts were obtained by addition of lysis buffer to the dishes. Lysates were boiled and treated with benzonase. Cell debris was spun down at 13000 rpm for 3 min and supernatants were used for total protein quantification using the Bradford method. Protein was fractionated by SDS-polyacrylamide gel electrophoresis and then electroblotted to a PVDF membrane. Black amide B10 staining provided visual evidence for correct loading and transfer of proteins. After blocking, membranes were incubated with primary antibody, according to the supplier's instructions. Anti-phospho-retinoblastoma protein (Ser760) was purchased from Cell Signaling Technology (Beverly, MA); anti-phospho-p38 MAPK (Thr180/Tyr182), from New England Biolabs (Ipswich, CA); and anti-retinoblastoma protein (C-15) and anti-p38 MAPK, from Santa Cruz Biotechnology (Santa Cruz, CA). Appropriate peroxidase-conjugated secondary antibodies were used (from DAKO, Carpinteria, CA and Amersham Biosciences, Buckinghamshire, UK). Development of the immunoblots was performed using an enhanced chemiluminescence western blotting analysis system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Bands in films were analysed by scanner photodensitometry and quantified using the BioImage program (Millipore, Bedford, MA).

Oxidation assay

After exposure of 3T3-L1 adipocytes to ATRA or vehicle (as detailed in the legend to Figure 3), medium was removed and cells were incubated for 4 h in fresh medium containing 0.2 mM L-carnitine (Sigma, St. Louis, MO) and 200 μ M [¹⁴C(U)] palmitate (0.1 μ Ci/ml, from Perkin Elmer, Boston, MA) in the continued presence of ATRA or vehicle. Where indicated, cells were treated with etomoxir 1 h before palmitate addition. Incubations were terminated by acidification with 6 M HCl. Acid-soluble products in 2 ml of the medium were extracted by addition of 1 ml cold 1 M HClO₄. After centrifugation (10 min, 1800 g), radioactivity in the supernatant was measured by scintillation counting.

Other parameters analyzed

For quantification of triacylglycerol content, cellular lipids were stained with Oil Red O (Sigma, St. Louis, MO), followed by extraction of the dye and spectrophotometric quantification at 510 nm. In some experiments, a commercial enzymatic colorimetric kit (Triglyceride (INT)20, Sigma, St. Louis, MO) was used to assess triacylglycerol content in phosphate-buffered saline cell homogenates. Levels of glycerol and nonesterified fatty acids (NEFA) in the culture medium were measured using enzymatic kits (from Sigma, St. Louis,

Fig. 1. A) ATRA treatment results in reduced triacylglycerol content in mature 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes at day 8 were treated with ATRA or vehicle (DMSO) for up to 5 days with replacement of medium with fresh ATRA every 24 h. At 24 h-intervals, cells were photographed and some of the plates used for cellular triacylglycerol (TG) quantification as described in the Methods section. The photographs correspond to cells treated with DMSO or the indicated concentrations of ATRA for 5 days. The bar diagram depicts the relative TG content of cells treated with DMSO (100%) or the indicated concentrations of ATRA for 3 days. B) Inclusion of ATRA from the start of the differentiation process results in reduced triacylglycerol accumulation in PPAR γ 2-transduced 3T3-L1 adipocytes. Pre-confluent 3T3-L1 cells were transduced with a retrovirus expressing PPAR γ 2 (PPAR γ 2-transduced cells) or the corresponding control (pBABE-transduced cells). Two days post-confluence (day 0), transduced cells were induced to differentiate with the standard cocktail plus 0.5 μM rosiglitazone in the continuous presence of vehicle (DMSO) or 1 μM ATRA. Photographs and relative TG content in the bar diagram correspond to PPAR γ 2-transduced cells differentiated for 9 days in medium containing either DMSO or 1 μM. Data in the bar diagrams are the mean $\pm$ SEM of $n = 4$ to 5 plates per treatment condition. ATRA, one-way ANOVA, effect of ATRA, $P < 0.005$; *, Student's t test, ATRA vs vehicle, $P < 0.001$. Adipose conversion of pBABE-transduced cells was completely prevented by 1 μM ATRA, in agreement with previous observations [14] (data not shown).

MO and Wako Chemicals GmbH, Neuss, Germany, respectively).

Statistical analysis

Data are presented as mean $\pm$ SEM. Statistical significance 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

ATRA repressed the expression of adipogenic/lipogenic transcription factors in differentiated 3T3-L1 adipocytes

ATRA inhibits the adipose conversion of preadipocyte cell lines by preventing the expression of

Fig. 2. ATRA increases glycerol release in mature 3T3-L1 adipocytes. A) Glycerol levels in the culture medium were measured after treatment of differentiated 3T3-L1 adipocytes at day 10 with vehicle (DMSO) or the indicated concentrations of ATRA for 24 h. Data are the mean $\pm$ SEM of $n=6$ to 9 plates per treatment condition. ATRA, one-way ANOVA, effect of ATRA, $P<0.005$. B) At time 0, culture medium in contact with the differentiated adipocytes was changed and 1 μ M ATRA or vehicle (control cells, C) added. Glycerol accumulation in the culture medium was followed by determining glycerol levels at the indicated time-intervals following ATRA/vehicle addition. Data are the mean $\pm$ SEM of triplicate plates for each time point. *, Student's t test, ATRA vs corresponding time control, $P<0.05$.

the key adipogenic transcription factors PPAR γ and CCAAT/enhancer-binding protein α (C/EBP α) [14]. Not only PPAR γ is the master regulator of adipogenesis but it is also key to lipogenesis and hypertrophy in mature adipocytes [15], likely acting as a heterodimer with RXR α [16]. We analyzed the impact of ATRA treatment on the expression of PPAR γ , C/EBP α and RXR α in differentiated 3T3-L1 adipocytes. PPAR γ mRNA levels were unaffected by exposure of the cells to up to 10 μ M ATRA for 24 h (Table 2), but were reduced by 51% after exposure to 1 μ M ATRA for 48 h ($P<0.05$). C/EBP α and RXR α mRNA levels were already down-regulated after 24 h ATRA treatment (Table 2) and the inhibition persisted after 48 h treatment.

ATRA reduced triacylglycerol content in differentiated 3T3-L1 adipocytes

Exposure of 3T3-L1 adipocytes to a single dose of ATRA (up to 10 μ M) for 24 h or 48 h did not affect the triacylglycerol content or the triacylglycerol/protein ratio of the cells. However, a 72 h ATRA treatment where fresh medium and ATRA were added every 24 h triggered a significant reduction of the intracellular triacylglycerol

content (up to 30% reduction at 10 μ M ATRA), which was also apparent from phase-contrast photographs of 3T3-L1 adipocytes treated with ATRA for 5 days under the same conditions (Figure 1A).

To evaluate the dependence of the ATRA-induced reduction of triacylglycerol content on the ATRA-induced PPAR γ down-regulation, 3T3-L1 cells were transduced with a PPAR γ 2- retrovirus expression vector. 3T3-L1 cells ectopically expressing PPAR γ 2 have been previously shown to be able to differentiate into adipocytes in the presence of ATRA provided the retinoid is first added after the cells have reached confluence [17]. This model allows the continuous presence of ATRA from the start of the differentiation process and prevents the ATRA-induced down-regulation of PPAR γ in the fully differentiated cells. Confirming previous observations [14, 17], ATRA prevented hormonally-induced adipogenesis in 3T3-L1 cells transduced with the control vector but not in 3T3-L1 cells transduced with the PPAR γ 2-expressing vector, so that less than 1% of the former (not shown) and more than 95% of the latter cells (see photographs in Figure 1B) exhibited lipid droplets after 9 days of differentiation in the continuous presence

Fig. 3. ATRA stimulates palmitate oxidation in 3T3-L1 adipocytes. A) Differentiated 3T3-L1 adipocytes at day 8 were treated with the indicated concentrations of ATRA or vehicle (DMSO) for 24 h. The medium was then changed and the cells incubated with [^{14}C (U)] palmitate for 4 h, as described in Methods. Data are the mean $\pm$ SEM of $n = 4$ to 5 plates for each treatment condition. ATRA, one-way ANOVA, effect of ATRA, $P < 0.05$. B) PPAR γ 2-transduced 3T3-L1 adipocytes differentiated for 9 days in the presence of 1 μM ATRA or vehicle (DMSO) were incubated for 4 h in fresh medium containing [^{14}C (U)] palmitate, as above. Where indicated, cells were treated with etomoxir 1 h before palmitate addition. Data are the mean $\pm$ SEM of 4 plates for each treatment condition. *, Student's t test, ATRA vs corresponding control, $P < 0.05$. In A and B, acid soluble products derived from palmitate oxidation were measured by scintillation counting.

of 1 μM ATRA. Nevertheless, triacylglycerol content at day 9 was 50% lower in the PPAR γ 2-transduced cells differentiated in the presence of ATRA than in the PPAR γ 2-transduced cells differentiated in its absence (Figure 1B). This result suggests that ATRA-induced reduction of triacylglycerol accumulation does not solely rely on ATRA-induced PPAR γ down-regulation, but involves additional mechanisms.

ATRA activated lipolysis in differentiated 3T3-L1 adipocytes

The effect of ATRA on basal lipolysis in differentiated 3T3-L1 adipocytes was analyzed by measuring both glycerol and NEFA release. Glycerol levels were significantly higher in the culture medium of ATRA-treated adipocytes than in that of vehicle-treated adipocytes (33.5% higher at 10 μM ATRA) (Figure 2A). Moreover, after 24 h to 48 h treatment, more glycerol had accumulated in the culture medium of adipocytes exposed to 1 μM ATRA than in that of adipocytes exposed to the vehicle (Figure 2B). However, NEFA levels in the culture medium, measured after a 10-fold concentration through

evaporation, were not increased after ATRA treatment (13.37 ± 1.29 μM NEFA in the medium from vehicle-treated cells; 13.01 ± 1.46 μM NEFA in the medium from 10 μM ATRA-treated cells; $n = 5$ -6 plates for each treatment condition).

ATRA activated fatty acid oxidation in differentiated 3T3-L1 adipocytes

Fatty acid oxidation in cells was evaluated by measuring changes in the production of acid soluble products from exogenously administered, uniformly labeled, ^{14}C -palmitate. Non-transduced 3T3-L1 adipocytes that had been exposed to ATRA (1 μM and 10 μM) or vehicle from day 8 to day 9 and PPAR γ 2-transduced 3T3-L1 adipocytes differentiated for 9 days in the continuous presence of 1 μM ATRA or vehicle were incubated for 4 h in fresh medium containing carnitine, labeled palmitate, and the same concentration of ATRA or vehicle to which they had been previously exposed. ATRA treatment triggered an increase in palmitate oxidation that was evident in the non-transduced 3T3-L1 adipocytes (1.5-fold increase at 10 μM ATRA,

Fig. 4. ATRA triggers p38 MAPK phosphorylation in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes at day 8 were treated with 10 μ M ATRA for the indicated periods of time (A) or with DMSO (control cells) or the indicated concentrations of ATRA for 2 h (B). Levels of (Thr180/Tyr182) phosphorylated p38 MAPK (P-p38 MAPK) and non-phosphorylated p38 MAPK in cell extracts were analysed by immunoblotting.

$P < 0.05$) and more marked in the PPAR γ 2-transduced 3T3-L1 adipocytes (4.5-fold increase at 1 μ M ATRA, $P < 0.05$) (Figure 3). ATRA-induced increase in palmitate oxidation in the latter cells was largely blunted by etomoxir (Figure 3B), an inhibitor of mitochondrial fatty acid oxidation.

ATRA induced gene expression changes in differentiated 3T3-L1 adipocytes consistent with increased lipid mobilization, oxidation and turnover

Treatment of differentiated 3T3-L1 adipocytes with ATRA for 24 h dose-dependently increased the mRNA levels of a collection of genes encoding enzymes, uncoupling proteins, adipokines and transcriptional regulators that may favor fat oxidation, namely liver-type carnitine palmitoyltransferase 1 (CPT1-L), medium-chain acyl-CoA dehydrogenase (MCAD), uncoupling protein 2 (UCP2), fasting-induced adipose factor (FIAF) and peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC-1 α) (Table 2). Concomitantly, the treatment reduced the mRNA levels of proteins

purportedly involved in the suppression of lipolysis and oxidative metabolism in adipocytes, namely cell death-inducing DNA fragmentation factor α -like effector A (CIDEA) and receptor-interacting protein 140 (RIP140). The mRNA levels of two proteins related to fatty acid uptake and re-esterification, fatty acid translocase (FAT/CD36) and the cytosolic form of phosphoenolpyruvate carboxykinase (PEPCK-C), were up-regulated after ATRA treatment. The mRNA levels of enzymes involved in peroxisomal β -oxidation (acyl-CoA oxidase and 3-keto acyl-CoA thiolase) and of forkhead transcription factor FOXO2, the activity of which may sensitize adipocytes to the fat mobilizing and thermogenic effects of cAMP [18], were unaffected by ATRA treatment.

We also evaluated ATRA effects on the expression of PPAR α and PPAR δ , two PPAR isoforms that transactivate genes for proteins involved in fatty acid catabolism [19]. Treatment of 3T3-L1 adipocytes with ATRA under conditions that resulted in the down-regulation of PPAR γ mRNA levels (48 h treatment) did not affect PPAR δ mRNA levels and was accompanied by a 27% increase ($P = 0.059$) of PPAR α mRNA levels.

ATRA treatment triggered inactivation of the retinoblastoma protein and an early, transient phosphorylation of p38 MAPK but failed to induce UCP1 expression in differentiated 3T3-L1 adipocytes

Down-regulation and/or inactivation (through phosphorylation) of the retinoblastoma protein (pRB) are involved in white to brown adipocyte conversion [20] and activation of p38 MAPK through phosphorylation is a critical event both in the adrenergic [21] and RA-induced [7] signaling cascades leading to UCP1 expression in brown fat. Treatment of differentiated 3T3-L1 adipocytes with ATRA for 24 h reduced pRB mRNA expression levels (Table 2) and, as previously reported in 3T3-F442A adipocytes [22], promoted pRB inactivation by phosphorylation (data not shown). In addition, ATRA treatment elicited a transient phosphorylation of p38 MAPK in 3T3-L1 adipocytes that was dose-dependent and peaked after 2 h of exposure to the retinoid, declining thereafter (Figure 4). Despite these effects, UCP1 mRNA was not induced in 3T3-L1 adipocytes after 24 h treatment with up to 10 μ M ATRA, not even upon co-treatment with rosiglitazone (1 μ M) and isoproterenol (100 nM), two other known inducers of UCP1 expression in brown adipocytes. UCP1 mRNA was also undetectable in PPAR γ 2-transduced 3T3-L1 adipocytes differentiated for 9 days in the continuous presence of ATRA (up to 10 μ M) and rosiglitazone (0.5 μ M).

Discussion

In rodents, acute ATRA treatment triggers a loss of body fat that correlates with changes in WAT depots including a reduction of adipocyte size, triacylglycerol content and lipogenic capacity and an increase in oxidative capacity [11, 12]. In this report, we present evidence that the above effects are partly attributable to direct actions of ATRA on mature white adipocytes. In particular, we show that ATRA treatment caused changes in gene expression in mature 3T3-L1 adipocytes consistent with enhanced oxidation of fatty acids at the expense of storage reflecting increased lipolysis, increased palmitate oxidation rate and, in the long-term, reduced triacylglycerol content. The changes elicited by ATRA in the mature adipocytes, though not huge, involved an internally consistent cohort of genes and biochemical parameters implicated in many of the previously reported effects of *in vivo* ATRA treatment in WAT depots of mice.

ATRA-induced gene expression changes in mature

3T3-L1 adipocytes that are in line with reduced lipogenesis included the down-regulation of the mRNA levels of PPAR γ and RXR α , which as heterodimers control genes critical for fat accretion in mature adipocytes [15, 16]. ATRA-induced changes in line with increased lipid mobilization and oxidation included the up-regulation of CPT1-L, MCAD, UCP2, PGC-1 α and FIAF, as well as the down-regulation of CIDEA and RIP140. CPT1 and MCAD are important for mitochondrial oxidation of fatty acids. UCP1 homologues such as UCP2 might indirectly facilitate the oxidation of fatty acids in mitochondria [23]; in addition, ATP depletion through increased uncoupling protein (here UCP2) activity may result in less efficient fatty acid and triacylglycerol synthesis (which are ATP-dependent processes), contributing to reduced triacylglycerol accumulation. PGC-1 α is a transcriptional coactivator of several transcription factors that positively control genes involved in mitochondriogenesis, adaptive thermogenesis, respiration and fatty acid oxidation (reviewed in [24]). FIAF is a secreted protein highly expressed in WAT whose overexpression in transgenic mice causes leanness and gene expression changes in WAT in line with enhanced lipolysis and oxidative metabolism [25]. CIDEA is a purported inhibitor of UCP1 activity and lipolysis and fatty acid oxidation in BAT of rodents [26], and its depletion leads to the stimulation of basal lipolysis in isolated human adipocytes [27]. RIP140 is a corepressor for many nuclear receptors highly expressed in WAT and has been proposed to be a major suppressor of oxidative metabolism in white adipocytes [28].

ATRA may favor an increase in triacylglycerol turnover in 3T3-L1 adipocytes, with stimulation of both lipolysis and fatty acid re-esterification. This is suggested by: (i) the unchanged intracellular triacylglycerol content in the face of increased lipolysis –with increased glycerol release but, importantly, no detectable increases in NEFA levels in the culture medium– observed after 24 h and 48 h ATRA treatment; (ii) the fact that the ATRA-induced reduction of intracellular triacylglycerol content became evident under conditions (long-term exposure with changes of culture medium every 24 h) hampering fatty acid re-esterification; and (iii) the fact that ATRA treatment led to an up-regulated expression of both FAT/CD36, which facilitates the uptake of long-chain fatty acids into adipocytes [29], and PEPCK-C, a key enzyme in the production of glycerol-3-phosphate (required for triacylglycerol synthesis) from precursors other than glucose or glycerol [30]. The re-uptake of lipolysis-derived fatty acids is likely to be less marked in adipocytes of

ATRA-treated mice than in ATRA-treated 3T3-L1 adipocytes, because *in vivo* (but not in the cell system) there is the possibility that fatty acids released are cleared by other tissues, leading to an effective loss of body fat as observed [5, 9-12].

Results in this study are consistent with a partially white-to-brown metabolic remodeling of 3T3-L1 mature adipocytes initiated by ATRA. However, ATRA failed to induce the expression of UCP1, the hallmark of the brown adipocyte phenotype. Synergistic interactions among several components of the UCP1 gene enhanceosome appear to control the induction of the UCP1 gene in white fat depots [31]. In our 3T3-L1 adipocytes, ATRA failed to induce the UCP1 gene despite up-regulation of PGC-1 α expression (a central coactivator of several transcription factors important for UCP1 gene expression [24]), down-regulation of RIP140 expression (a known repressor of UCP1 gene transcription [32]), down-regulation of pRB expression and promotion of pRB inactivation (an important event for brown adipogenesis and particularly UCP1 gene expression [20]) and promotion of p38 MAPK phosphorylation (an important event in signaling cascades leading to UCP1 expression in brown fat [7, 21]).

Unlike in ATRA-treated 3T3-L1 adipocytes, UCP1 gene expression was found to be induced in WAT depots of ATRA-treated mice [12]. The difference may suggest that the “ectopic” UCP1 induction seen *in vivo* reflects recruitment of brown precursors contained within WAT depots and/or the contribution of systemic factors. It is also possible that 3T3-L1 adipocytes are aberrant with respect to some signaling pathways controlling UCP1 induction *in vivo*. However, induction of mitochondrial biogenesis and PGC-1 α has been described in 3T3-L1 adipocytes following treatment with agents such as rosiglitazone and polyunsaturated fatty acids of marine origin [33-35]. Moreover, like ATRA, other agents/conditions that *in vivo* reduce body fat and promote the expression of UCP1 in WAT depots increase oxidative metabolism but fail to induce the UCP1 gene when tested on differentiated white adipocytes. This is the case of depletion of RIP140 [28, 36], bezafibrate treatment [37, 38] and adenovirus-induced hyperleptinemia [39, 40].

ATRA might exert remodeling toward increased oxidative metabolism in adipocytes through different mechanisms. ATRA treatment may favor the transcriptional activity of PPAR α :RXR and PPAR δ :RXR heterodimers on genes involved in fat oxidation by ensuring the provision of both fatty acid agonists of the

PPAR moiety (through enhanced lipolysis) and 9-cis RA, which can be produced from ATRA through isomerization [41] (PPARs act as heterodimers with RXR that can be activated by ligands of either partner and are synergistically activated in the presence of both ligands [19]). In addition, ATRA has been reported to bind to PPAR δ with high affinity activating PPAR δ -mediated transcription [42]. RAR activation can also be involved, as some of the genes up-regulated in 3T3-L1 adipocytes following ATRA treatment, in particular the PEPCK-C gene and the MCAD gene, are well established retinoid-target genes [43]. Finally, ATRA effects on p38 MAPK may also be of relevance. In keeping with previous findings in brown adipocytes [7], we show here that ATRA triggers an early and transient increase in p38 MAPK phosphorylation in 3T3-L1 white adipocytes. Phosphorylation activates p38 MAPK, and active p38 MAPK induces PGC-1 α gene transcription [21], and catalyses the activating phosphorylation of both PGC-1 α [21, 24] and PPAR α [44] and the inactivating phosphorylation of pRB [45]. Although the time course of the ATRA effect on p38 MAPK phosphorylation in our system is quite different from that of other ATRA effects, early activation of p38 MAPK might conceivably initiate events and/or trigger persistent changes that contribute to and converge in an enhancement of oxidative metabolism.

In summary, we show in this study that ATRA directly promotes a remodeling of mature 3T3-L1 adipocytes toward increased capacity for oxidative metabolism and reduced capacity for lipogenesis, and actually increases lipolysis and fatty acid oxidation rate and reduces triacylglycerol content in these cells. Knowledge of agents capable to enhance oxidative metabolism in white adipocytes and their mechanisms of action can contribute to new avenues of prevention and treatment of obesity and related metabolic disorders.

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