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Remodeling of White Adipose Tissue after Retinoic Acid Administration in Mice

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A reduced brown adipose phenotype in white adipose tissue (WAT) may contribute to obesity and type 2 diabetes in humans. Retinoic acid, the carboxylic form of vitamin A, triggers in rodents a reduction of body weight and adiposity and an increased expression of uncoupling proteins in brown adipose tissue and skeletal muscle. In this study, we investigated possible remodeling effects of all-*trans* retinoic acid (ATRA) in WAT depots. Changes in the expression of genes related to thermogenesis and fatty acid oxidation and levels of phosphorylated retinoblastoma protein were analyzed in WAT depots of adult NMRI male mice acutely injected with ATRA or vehicle, together with biometric and blood parameters. Body fat loss after ATRA treatment was unaccompanied by any increase in circulating nonesterified fatty acids or ketone bodies and accompanied by increased rectal temperature. The treatment triggered an up-regulation of the mRNA levels of uncoupling proteins 1 and 2, peroxisome proliferator-activated receptor γ coactivator-1α, peroxisome proliferator-activated receptor α, muscle- and liver-type carnitine palmitoyltransferase 1, and subunit II of cytochrome oxidase in different WAT depots. Levels of phosphorylated retinoblastoma protein in WAT depots were increased after ATRA treatment. Adipocyte size was reduced, and the number of multilocular adipocytes was increased in inguinal WAT of ATRA-treated mice. The results indicate that ATRA favors the acquisition of brown adipose tissue-like properties in WAT. Understanding the mechanisms and effectors involved in the remodeling of WAT can contribute to new avenues of prevention and treatment of obesity and type 2 diabetes. (*Endocrinology* 147: 5325–5332, 2006)

THE ADIPOSE ORGAN of vertebrates contains two main tissues (1). White adipose tissue (WAT) stores energy in the form of triacylglycerol and supplies energy to the body through lipolysis and subsequent release of fatty acids into the plasma in situations of energy deficit, such as fasting. Brown adipose tissue (BAT) is specialized in adaptive thermogenesis, *i.e.* the regulated dissipation of energy as heat in response to cold and diet, through the oxidation of its stored lipids linked to the activity of uncoupling protein 1 (UCP1), a BAT-specific inner mitochondrial membrane protein capable of dissipating the proton electrochemical gradient generated by the respiratory chain during substrate oxidation, thus uncoupling oxidative phosphorylation (reviewed in Ref. 2). Brown adipocytes are rich in mitochondria, have cytoplasmic lipids arranged in numerous small droplets (multilocularity), and, aside from UCP1, express high levels of fatty acid oxidation enzymes and respiratory chain components, contributing to a high oxidative capacity. Typical white adipocytes, on the other hand, do not express UCP1, are poor in mitochondria, store lipids in a unique big droplet (unilocularity), and have a low oxidative capacity.

Whereas, in rodents, well-defined BAT depots are present throughout life, in humans, BAT depots undergo atrophy soon after birth, but the precise total amount of BAT in adult humans is not known. Together with many histological and immunohistochemical reports, it has been calculated that in visceral depots of adult lean humans there is one brown adipocyte every 100–200 white adipocytes (3). Brown adipocytes can be recruited under certain conditions and, in addition, the remodeling of mature white adipocytes into mitochondria-rich cells with high capacity for fatty acid oxidation has been described (reviewed in Ref. 4). Several agents/conditions known to stimulate thermogenesis in BAT promote *in vivo* the acquisition of BAT features in WAT depots of rodents, including cold exposure and β3-adrenoceptor agonist treatment (see Ref. 4), bezafibrate treatment (5), recombinant adenovirus-induced hyperleptinemia (6), and chronic feeding a diet enriched in n-3 polyunsaturated fatty acids of marine origin (7). Remodeling of WAT is of interest because evidence is accumulating that a reduced oxidation of lipids and a reduced brown adipose phenotype in white fat may contribute to obesity and type 2 diabetes in humans (3, 8–11).

Vitamin A is a nutrient with many remarkable effects on adipose tissue biology and the energy balance control system (reviewed in Refs. 12 and 13). Administration of retinoic acid, the carboxylic acid form of vitamin A, induces in mice the expression of UCP1 in BAT and UCP3 (another uncoupling protein family member) in skeletal muscle and triggers a

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Abbreviations: ATRA, All-*trans* retinoic acid; BAT, brown adipose tissue; CPT1, carnitine palmitoyltransferase 1; CPT1-L, liver-type CPT1; CPT1-M, muscle-type CPT1; CREB, cyclic AMP response element binding protein; LSD, least-significant diference; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; PPARα, peroxisome proliferator-activated receptor α; RAR, retinoic acid receptor; RXR, rexinoid receptor; UCP1, uncoupling protein 1; WAT, white adipose tissue.

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reduction of body weight, adiposity, and WAT lipogenic/adipogenic capacity (12). Retinoic acid treatment also results in improved glucose tolerance (14) and suppresses the expression of leptin (12) and resistin (14), two adipocyte secreted proteins (adipokines), the excess of which has been linked to insulin resistance. Retinoic acid is a well-known regulator of mammalian gene expression, mainly through activation of two types of retinoid receptors of the nuclear hormone receptor superfamily of ligand-modulated transcription factors: the retinoic acid receptors (RARs), which respond to both all-*trans* and 9-*cis* retinoic acid, and the rexinoid receptors (RXRs), which respond specifically to the 9-*cis* isomer (reviewed in Ref. 15).

The aim of this study was to investigate possible remodeling effects of retinoic acid treatment on WAT depots of mice using physiological, morphological, and molecular approaches.

Materials and Methods

Animals, treatment, and tissue collection

Twelve-week-old NMRI male mice (CRIFFA, Barcelona, Spain), fed *ad libitum* regular laboratory chow (Panlab, Barcelona, Spain; 73.4% carbohydrate-, 18.7% protein-, and 7.9% lipid-derived energy; 5 UI vitamin A/kcal) and kept at 22 C with a 12-h light/12-h dark cycle (lights on at 0800 h), received one daily sc 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 d before they were killed (five to seven animals per group). Controls were injected the vehicle (olive oil). Energy intake during the treatment period was estimated on a per-cage basis (two to three animals per cage) from the actual amount of food consumed by the animals and its caloric equivalence. Rectal temperature was measured just before the animals were killed using a digital thermometer (RS, Barcelona, Spain). The animals were killed with CO_2 and decapitated at the start of the light cycle. Blood was collected from the neck and serum was prepared and frozen at −20 C. Interscapular BAT and inguinal, epididymal, and retroperitoneal WAT depots were excised in their entirety, weighed, frozen in liquid nitrogen, and stored at −70 C.

In other experiments (as indicated), the animals were transferred to thermoneutrality (30 C) 1 wk before being injected daily either vehicle or 50 mg ATRA per kilogram of body weight for 4 consecutive days. All animal experimentation was conducted in accord with accepted standards of humane animal care, and the protocols were approved by our institutional bioethical committee.

RNA extraction and RT-PCR analysis

Total RNA was extracted from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the supplier's instructions. Changes in response to ATRA treatment in the mRNA levels of uncoupling proteins (UCP1, UCP2, and UCP3), peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), peroxisome proliferator-activated receptor α (PPARα), muscle- and liver-type carnitine palmitoyltransferase 1 (CPT1-M and CPT1-L, respectively) and subunit II of cytochrome oxidase were assessed by independent RT-PCR assays using β-actin expression as an internal control. In brief, 1 μg total RNA was denatured at 65 C for 10 min before being reverse transcribed using murine leukemia virus reverse transcriptase (MuLV RT; PerkinElmer, Madrid, Spain) in the presence of 50 pmol of random primers, in a PerkinElmer 2400 Thermal Cycler (conditions: 15 min at 20 C, 30 min at 42 C, and 5 min at 95 C). Once the reaction was completed, 5 μl of the retrotranscription medium was added to a PCR mix containing *Taq* DNA polymerase (Promega, Lyon, France), and suitable concentrations of specific primers for β-actin cDNA and target gene cDNA (experimentally determined for each tissue and target gene, see Table 1). The reaction mixture was first heated to 95 C for 2 min to denature the cDNA. This was followed by cycles of denaturation at 95 C for 15 sec, annealing at 56–60 C for 15 sec, and extension at 72 C for 30 sec, with an additional extension at 72 C for 7 min after the last cycle; cycle number and annealing temperature were optimized for each tissue and target gene (Table 1). The PCR products were separated in 2% agarose gel (Agarose D-1 Low EEO; Pronadisa, Madrid, Spain) in 0.5× Tris-borate-EDTA buffer, stained with ethidium bromide, and visualized using an image-recording system (GeneSnap; Chemigenius, Cambridge, UK). The densities of the target bands were quantified using an image processing and analyzing program (GeneTools; Syngene, Frederick, MD).

Western blotting analysis

Adipose tissue samples were homogenized in 50 mM Tris HCl buffer, pH 7.4, containing 250 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 50 mM NaF, 1 mM orthovanadate, and protease inhibitors, with a Teflon/glass homogenizer. The homogenates were centrifuged at 500 × *g* for 10 min at 4 C, and the supernatant was used for total protein quantification with the BCA protein assay kit (Pierce, Rockford, IL). Protein was fractionated by SDS-PAGE (10% polyacrylamide) and then electroblotted to a nitrocellulose membrane. Black amide B10 staining provided visual evidence for correct loading and blotting of proteins. Membranes were blocked

TABLE 1. Primers and conditions used in the PCR

Gene	Primer sequence (forward first, reverse second; 5′–3′)	Accession no.	T_a (C)	No. of PCR cycles (iWAT, rWAT, eWAT)	pmol β-Actin primers/pmol target gene primers (iWAT, rWAT, eWAT)
UCP1	GCTTGTCAACACTTTGGAAAGG GTTTTATTCGTGGTCTCCCAGC	NM_009463	57	24, 22, 27	1/20, 1/20, 1/25
UCP2	CAGTTCTACACCAAGGGCTC AGCATGGTCAGGGCACAGTG	NM_019354	56	23, 25, 23	4/10, 4/10, 4/10
UCP3	GGAGGAGAGAGGAAATACAGAGG CCAAAGGCAGAGACAAAGTGA	NM_009464	60	30, 26, 29	2/40, 1/30, 1/40
PGC-1α	GGAGGCAGAAGAGCCGTC AGGAGGGTCATCGTTTGTGG	NM_008904	60	27, 26, 27	1/5, 1/5, 1/35
PPARα	CGTTTGTGGCTGGTCAAGTT AGAGAGGACAGATGGGGCTC	NM_011144	61	26, 28, 27	1/20, 1/20, 1/20
COX-II	AAGACGCCACATCCCCTATT CTTCAGTATCATTGGTGCCCT	AF378830	58	23, 23, 23	5/3, 5/3, 5/3
CPT1-L	GCTCGCACATTACAAGGACAT TGGACACCACATAGAGGCAG	NM_013495	56	25, 25, 27	2/20, 2/20, 2/20
CPT1-M	AAGGGTAGAGTGGGCAGAGG GCAGGAGATAAGGGTGAAAGA	NM_009948	60	25, 27, 27	4/20, 4/20, 3/25
β-Actin	ACGGGCATTGTGATGGACTC GTGGTGGTGAAGCTGTAGCC	NM_007393			

T_a, Annealing temperature; COX-II, subunit II of cytochrome oxidase; eWAT, epididymal WAT; iWAT, inguinal WAT; rWAT, retroperitoneal WAT.

in PBS-Tween (for UCP1 analysis) or TBS-Tween (for phospho-retinoblastoma protein analysis) containing 5% fat-free dried milk, washed, and then incubated with the corresponding primary antibody, according to the supplier's instructions. Polyclonal antibody against UCP1 was purchased from Alpha Diagnostics (San Antonio, TX) and against phospho-(Ser 780)-retinoblastoma protein, from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated antirabbit IgG antibody (Amersham Biosciences, Buckinghamshire, UK) was used as secondary antibody. Development of the immunoblots was performed using an enhanced chemiluminescence Western blotting analysis system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Bands in films were analyzed by scanner photodensitometry and quantified using the BioImage program (Millipore, Bedford, MA).

Light microscopy, morphometric analysis, and UCP1 immunohistochemistry

WAT depots were fixed by immersion in the perfusion fixative (4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4) overnight at 4 C, dehydrated, cleared, and then paraffin-embedded so that the plane of section corresponded with the one of the wider surface. Three-micrometer-thick sections at the same level were obtained and stained with hematoxylin/eosin to assess morphology. Morphometric analysis was performed by digital acquisition of adipose tissue areas (Digital Still Camera DXM 1200 and Nikon 6000 Eclipse Microscope). The multilocular adipocytes present in those sections were counted and the number was expressed as percentage of all adipocytes in the section using a drawing tablet and the Nikon Lucia IMAGE Version 4.61 morphometric program. Unilocular cells are spherical in fresh tissue and have a rounded profile in hematoxylin/eosin-stained sections; 200 random adipocyte profiles per animal were drawn using a digital image analysis system to determine their sectional area (16).

Sections of inguinal WAT were used for immunohistochemical localization of UCP1 protein using 3% hydrogen peroxide to inactivate endogenous peroxidases followed by normal rabbit serum to reduce nonspecific staining. The sections were incubated overnight (4 C) with a sheep antirat UCP1 antibody (kindly provided by Dr. D. Ricquier, Centre National de la Recherche Scientifique, Paris, France) at a final dilution of 1:2000, and processed by the avidin-biotin complex method (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Appropriate positive controls were used to check antibody specificity.

Other parameters tested

Commercial enzymatic colorimetric kits were used for the determination of serum nonesterified fatty acids (Wako Chemicals GmbH, Neuss, Germany) and circulating levels of triacylglycerol [Triglyceride (INT)20; Sigma Diagnostics, St. Louis, MO], glucose (D-Glucose UV-method; Roche Biopharm GmbH, Darmstadt, Germany) and 3-hydroxybutyrate (β-HBA Procedure No. 310-UV; Sigma Diagnostics). Circulating levels of insulin were determined by enzyme-linked immunosorbent assay (rat-insulin ELISA; DRG Instruments GmbH, Marburg, Germany). Total lipid extraction on WAT was performed (17) and triacylglycerol content was measured in the lipid extract using the glycerol phosphate oxidase-trinder reagent kit from Sigma. Tissue DNA was determined by a fluorometric method that uses 3,5-diaminobenzoic acid (18).

Statistical analysis

Data are presented as means ± SEM. Differences between groups were assessed by one-way ANOVA; contrast between means was assessed by least-significance difference (LSD) or Student's *t* test *post hoc* comparisons. Differences between groups were considered statistically significant at $P < 0.05$.

Results

Effects of ATRA treatment on biometric and blood parameters of NMRI mice

The effects of ATRA treatment on several biometric and circulating parameters (measured in the fed state) are shown in Table 2. The treatment triggered a dose-dependent reduction of body weight and of the mass of all fat depots analyzed, but did not affect energy intake, in accordance with previous results (14). Rectal temperature was significantly increased in the animals treated with 10 and 50 mg ATRA per kilogram of body weight per day compared with vehicle-treated control animals. Circulating triacylgycerol levels were reduced in ATRA-treated mice, in accordance with previous results (14). Circulating levels of glucose, insulin, nonesterified fatty acids, and 3-hydroxybutyrate were not significantly affected by ATRA treatment. Aminotransferase activity in serum, a marker of hepatic damage, was previously shown to be unaffected by ATRA treatment under the conditions and doses used in this study (19).

TABLE 2. Effects of ATRA treatment on biometric and blood parameters in NMRI mice

	Control	ATRA treatment			ANOVA (P value)
		10 mg/kg	50 mg/kg	100 mg/kg	
Body weight before (g)	43.1 ± 1.2	42.7 ± 0.9	42.5 ± 0.9	42.2 ± 1.1	
Body weight after (g)	44.2 ± 1.1^{a}	40.6 ± 0.8^{b}	38.7 ± 0.6^{bc}	36.8 ± 0.8^{c}	<0.001
Food intake (MJ·g animal^{-1}·d^{-1})	1782 ± 67	1715 ± 109	1703 ± 75	1690 ± 109	
iBAT mass (mg)	204 ± 14^{a}	120 ± 9^{b}	113 ± 7^{bc}	86 ± 5^{c}	<0.001
eWAT mass (mg)	984 ± 101^{a}	753 ± 107^{ab}	693 ± 71^{b}	586 ± 117^{b}	<0.05
iWAT mass (mg)	526 ± 45^{a}	431 ± 49^{ab}	374 ± 36^{bc}	278 ± 38^{c}	<0.005
rWAT mass (mg)	322 ± 41^{a}	225 ± 38^{ab}	208 ± 32^{b}	160 ± 43^{b}	<0.05
Adiposity index^{d}	4.53 ± 0.34^{a}	3.75 ± 0.44^{ab}	3.59 ± 0.33^{ab}	2.98 ± 0.49^{b}	0.068
Rectal temperature	36.7 ± 0.14^{a}	38.1 ± 0.30^{b}	38.0 ± 0.12^{b}	36.8 ± 0.10^{a}	<0.001
Serum glucose (mM)	8.79 ± 0.55	8.37 ± 0.54	8.25 ± 0.85	7.83 ± 0.59	
Serum insulin (pM)	13.09 ± 2.11	11.66 ± 2.29	8.69 ± 1.47	9.75 ± 1.75	
Serum triacylgycerol (mM)	3.03 ± 0.31^{a}	1.75 ± 0.15^{b}	1.67 ± 0.16^{b}	1.81 ± 0.22^{b}	<0.001
Serum NEFA (mEq/liter)	1.41 ± 0.16	1.51 ± 0.21	1.43 ± 0.15	1.47 ± 0.28	
Serum 3-hydroxybutyrate (mM)	0.063 ± 0.017	0.057 ± 0.003	0.124 ± 0.026	0.076 ± 0.019	

Twelve-week-old NMRI male mice received a daily sc injection of ATRA at a dose of 10, 50, or 100 mg/kg body weight during the 4 d before they were killed. Control animals were injected the vehicle (olive oil). Data are the mean ± SEM of at least 10 animals per group, distributed in two independent experiments, except rectal temperature and 3-hydroxybutyrate data, which are the mean ± SEM of a single experiment (five to six animals per group). iBAT, Interscapular BAT; eWAT, epididymal WAT; iWAT, inguinal WAT; rWAT, retroperitoneal WAT; NEFA, nonesterified fatty acids.

$^{a-c}$ Values within a row not sharing a *common letter* are statistically different by LSD *post hoc* comparison ($P < 0.05$).

^{d} Combined mass of all fat pads taken expressed as a percentage of body weight.

ATRA treatment increases the expression of brown adipocyte-specific genes and genes involved in lipid oxidation in WAT depots of NMRI mice

Changes in response to ATRA treatment of the mRNA expression levels of several genes related to thermogenesis, respiratory chain function, and fat oxidation in WAT depots were analyzed (Table 3). Genes selected included those encoding uncoupling proteins (UCP1, UCP2, and UCP3), PGC-1α, PPARα, carnitine palmitoyltransferase 1 (CPT1; both muscle- and liver-type), and subunit II of cytochrome oxidase. UCP1 is a brown-adipocyte-specific marker and the key molecular effector of BAT thermogenesis (2), and it has been suggested that uncoupling protein activity, especially that of UCP2 and UCP3, may facilitate fatty acid handling by mitochondria and hence fat oxidation (see Ref. 20). PGC-1α is a transcriptional coactivator linked to mitochondrial biogenesis, adaptive thermogenesis, respiration, and brown *vs.* white adipocyte differentiation, among other processes (reviewed in Ref. 21). PPARα is a fatty acid-activated transcription factor that enhances the transcription of genes encoding proteins involved in fatty acid oxidation (reviewed in Ref. 22), including CPT1, which catalyzes the initial reaction in the mitochondrial import of long chain fatty acids, a tightly regulated step in fatty acid use. Subunit II of cytochrome oxidase is a component of the mitochondrial respiratory chain.

UCP1 mRNA levels were significantly up-regulated after ATRA treatment in the three WAT depots analyzed; the effect was maximal (between ~1.6- and 5.5-fold increase, depending on the WAT depot considered) at a dose of 50 mg ATRA per kilogram of animal per day and diminished at 100 mg ATRA per kilogram of animal per day. UCP2 mRNA levels were significantly up-regulated in epididymal and retroperitoneal WAT (~2-fold increase in both depots at 100 mg ATRA per kilogram of animal per day), but not in inguinal WAT of ATRA-treated mice. UCP3 mRNA levels were not affected by ATRA treatment in any of the WAT depots analyzed (data not shown). The mRNA levels of PPARα, PGC-1α, CPT1-M, CPT1-L, and subunit II of cytochrome oxidase were increased in WAT depots after ATRA treatment; the effect reached statistical significance for PPARα mRNA and PGC-1α mRNA in the epididymal and retroperitoneal depots (~2- and 3-fold increase, respectively, at 100 mg ATRA per kilogram of animal per day, in both depots), CPT1-M mRNA in the epididymal depot (~4-fold increase at 100 mg ATRA per kilogram of animal per day), CPT1-L mRNA in the inguinal depot (~1.4-fold increase at 100 mg ATRA per kilogram of animal per day) and subunit II of cytochrome oxidase mRNA in the epididymal depot (~1.6-fold increase at 100 mg ATRA per kilogram of animal per day).

We could clearly detect UCP1 protein by immunoblotting in the inguinal depot, where increased levels after injection of 50 mg ATRA per kilogram of animal per day for 4 consecutive days were apparent when the treatment was conducted under conditions of thermoneutrality (30 C) (Fig. 1, $P = 0.093$, Student's t test). When the animals were kept at 22 C, which is a temperature that can already elicit a thermogenic response in the mouse, high interanimal variability, especially in the control group, masked the ATRA effect (data

TABLE 3. Effects of ATRA treatment on gene expression in WAT depots of NMRI mice

	Control	ATRA treatment			ANOVA (P value)
		10 mg/kg	50 mg/kg	100 mg/kg	
Inguinal WAT					
UCP1 mRNA	100 ± 19^{ab}	88.5 ± 19^{a}	155 ± 27^{b}	93.8 ± 11^{a}	<0.05
UCP2 mRNA	100 ± 3	89.5 ± 7	89 ± 4	84.5 ± 3.4	
PGC-1α mRNA	100 ± 18	115 ± 12	179 ± 28	123 ± 31	
PPARα mRNA	100 ± 19	136 ± 37	208 ± 51	131 ± 10	
CPT1-M mRNA	100 ± 28	97.3 ± 22	160 ± 60	59.7 ± 5.3	
CPT1-L mRNA	100 ± 9.3^{a}	111 ± 6.9^{ab}	126 ± 8.5^{bc}	135 ± 6.6^{c}	<0.05
COX-II mRNA	100 ± 4.5	84.2 ± 4.6	112 ± 8.5	135 ± 33	
Epididymal WAT					
UCP1 mRNA	100 ± 32^{a}	261 ± 37^{b}	316 ± 50^{b}	236 ± 26^{b}	<0.005
UCP2 mRNA	100 ± 7.5^{a}	75 ± 5^{a}	111 ± 10^{a}	235 ± 31^{b}	<0.001
PGC-1α mRNA	100 ± 21^{a}	162 ± 19^{ab}	204 ± 21^{bc}	298 ± 52^{c}	<0.005
PPARα mRNA	100 ± 28^{a}	127 ± 17^{ab}	179 ± 29^{b}	194 ± 20^{b}	<0.05
CPT1-M mRNA	100 ± 37^{a}	136 ± 43^{a}	233 ± 71^{a}	435 ± 113^{b}	<0.01
CPT1-L mRNA	100 ± 17	133 ± 7.1	153 ± 15	161 ± 32	
COX-II mRNA	100 ± 5.3^{a}	108 ± 1.1^{a}	140 ± 8.4^{b}	156 ± 12^{b}	<0.001
Retroperitoneal WAT					
UCP1 mRNA	100 ± 47^{a}	213 ± 47^{a}	556 ± 110^{b}	257 ± 67^{a}	<0.005
UCP2 mRNA	100 ± 9^{a}	104 ± 9^{a}	138 ± 6^{b}	171 ± 15^{b}	<0.001
PGC-1α mRNA	100 ± 12^{a}	140 ± 22^{ab}	176 ± 30^{b}	270 ± 32^{c}	<0.005
PPARα mRNA	100 ± 13^{a}	185 ± 17^{b}	175 ± 24^{b}	227 ± 28^{b}	<0.01
CPT1-M mRNA	100 ± 35	143 ± 37	256 ± 74	200 ± 28	
CPT1-L mRNA	100 ± 21	134 ± 11	150 ± 17	146 ± 10	
COX-II mRNA	100 ± 1.4	97.7 ± 11	122 ± 34	129 ± 11	

Levels of the indicated mRNAs were semiquantified by RT-PCR assays in the indicated WAT depots of 12-wk-old NMRI male mice treated with vehicle (olive oil) or ATRA (one daily sc injection, at the indicated dose, during the 4 d before the animals were killed). Data are the mean ± SEM of at least five animals per group and are expressed relative to the mean value of the control, vehicle-treated, group, which was set as 100%.

$^{a-c}$ Values within a row not sharing a *common letter* are statistically different by LSD *post hoc* comparison ($P < 0.05$). COX-II, Subunit II of cytochrome oxidase.

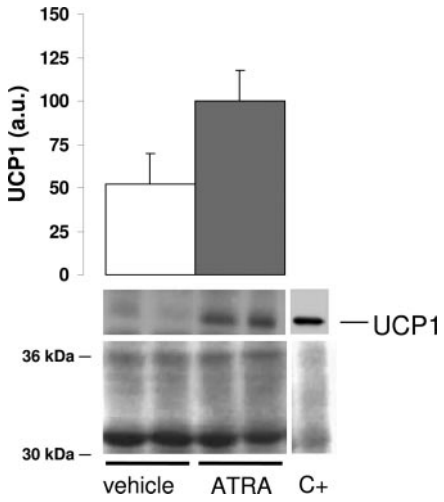

FIG. 1. Effect of ATRA treatment on UCP1 protein levels in inguinal WAT of NMRI mice. Twelve-week-old NMRI male mice preadapted to near thermoneutrality (30 C) for 1 wk were sc injected with vehicle (olive oil) or 50 mg ATRA per kilogram of body weight per day during the 4 consecutive days before they were killed. UCP1 protein levels were analyzed by immunoblotting in homogenates of inguinal WAT. Data are the mean ± SEM of five to six animals per group and are expressed relative to the mean value of the control (vehicle-treated) group, which was set as 100%. Representative Western blots are shown at the *bottom*, together with a section of the black amide B10-stained blots, showing equal loading and blotting of proteins (80 μg of total inguinal WAT protein per lane). C+, Positive control (interscapular BAT of a control animal, 5 μg of total protein).

not shown). In any case, levels of UCP1 protein in inguinal WAT of animals treated with 50 mg ATRA per kilogram of animal per day were less than 2% the levels in interscapular BAT of control animals.

ATRA treatment favors retinoblastoma protein phosphorylation in WAT depots of NMRI mice

It has been suggested that inactivation of the retinoblastoma protein by phosphorylation may be a key event in brown adipogenesis (23), and we have shown previously that ATRA treatment favors retinoblastoma protein phosphorylation in mature 3T3-F442A white adipocytes in culture (24). We tested whether ATRA treatment affects the levels of phosphorylated retinoblastoma protein in WAT depots *in vivo* by using a specific antibody in Western blotting settings. Levels of Ser^{780} phospho-retinoblastoma protein were higher in WAT depots of ATRA-treated mice (50 mg/kg animal per day, 4 d, 30 C) than in WAT depots of control animals (Fig. 2). The effect reached statistical significance in the retroperitoneal depot, in which phosphorylated retinoblastoma protein was readily detectable only after ATRA treatment.

ATRA treatment induces morphological and tissue composition changes in inguinal WAT of NMRI mice

We conducted a morphological study comparing inguinal WAT sections of control and ATRA-treated animals (50 mg/kg animal per day, 4 d, 30 C) at the light microscopy level. The treatment triggered a significant reduction in the size of the unilocular cells (mean sectional area: controls, 1437 ± 33 μm^2; ATRA-treated, 1035 ± 86 μm^2, n = 10 distributed in two independent experiments, $P < 0.001$). In addition, the number of cells exhibiting fragmentation of the large lipid storage droplet into numerous small lipid droplets (multilocularity) was markedly increased upon ATRA treatment (Fig. 3). Although these cells resembled brown adipocytes, nearly all were negative for expression of UCP1 by immunohistochemistry. Tissue composition analysis confirmed that the reduced size of the unilocular inguinal adipocytes in ATRA-treated mice is most likely to be due to reduced lipid content, because both protein and DNA content per gram of fresh tissue increased (by 16 and 44%, respectively) whereas triacylglycerol levels per gram of fresh tissue decreased (by 18%) after ATRA treatment ($P < 0.05$, Student's t test, n = 5–6). These changes are in accordance with previously published changes in BAT composition after ATRA treatment in mice (25).

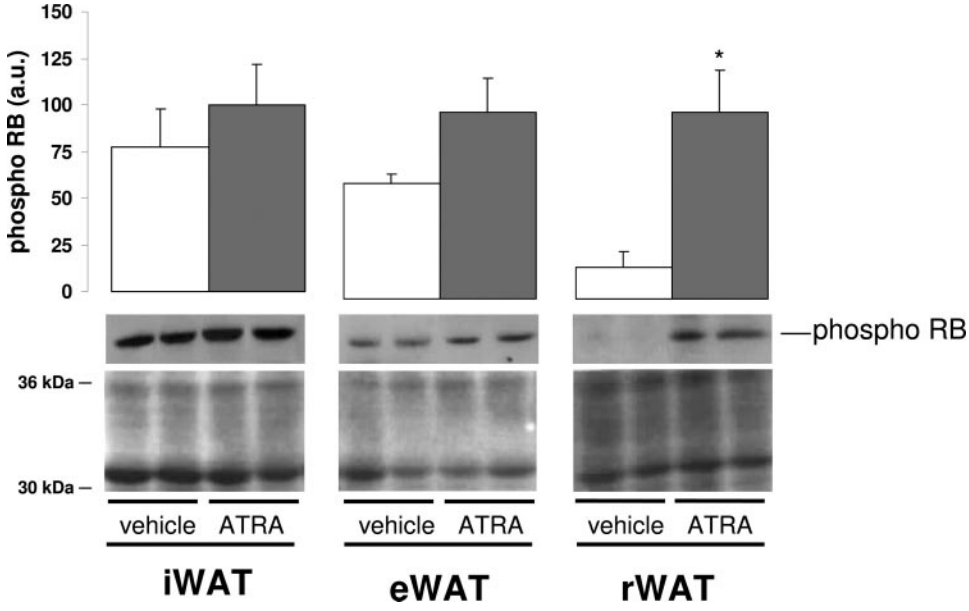

FIG. 2. Effect of ATRA treatment on phospho-retinoblastoma protein levels in WAT depots of NMRI mice. Twelve-week-old NMRI male mice preadapted to near thermoneutrality (30 C) were sc injected vehicle (olive oil) or 50 mg ATRA per kilogram of body weight per day during the 4 consecutive days before they were killed. Levels of phospho-retinoblastoma protein (phospho-RB) in WAT homogenates were analyzed by immunoblotting using an anti phospho-(Ser 780)-RB antibody. Data are the mean ± SEM of five to six animals per group and are expressed relative to the mean value of the ATRA-treated group, which was set as 100%. Representative Western blots are shown at the *bottom*, together with a section of the black amide B10-stained blots, showing equal loading and blotting of proteins (80 μg of total WAT protein per lane). Student's t test significance: *, $P < 0.01$. eWAT, Epididymal WAT; iWAT, inguinal WAT; rWAT, retroperitoneal WAT.

Discussion

In this work we report molecular and morphological data indicating an effect of ATRA favoring a remodeling of WAT toward the acquisition of BAT features, and particularly an enhanced oxidative metabolism.

ATRA treatment favored the mobilization of the adipocyte intracellular fat reserves, as indicated by the reduced mass of the adipose depots, the reduced size of inguinal adipocytes, and the reduced inguinal WAT triacylglycerol content in ATRA-treated animals compared with control animals. ATRA treatment also induced a concerted up-regulation of the mRNA levels of UCP1, UCP2, respiratory chain components (subunit II of cytochrome oxidase), and transcription factors (PGC-1α, PPARα) and enzymes (CPT1) involved in fatty acid oxidation in different WAT depots, of statistical significance for many gene-depot pairs and consistent with an increased capacity for oxidative metabolism/thermogenesis in these depots. Moreover, ATRA-induced body fat loss was not accompanied by increased circulating nonesterified fatty acids (Table 2), suggesting that fatty acids mobilized from fat stores undergo oxidation within the adipocytes or, if secreted, are rapidly taken up by other tissues for oxidation. A lack of effect of ATRA treatment on WAT UCP3 expression is in keeping with a requirement of MyoD, a master regulator of muscle cell differentiation that is selectively expressed in cells of the myogenic lineage, for retinoid-stimulated UCP3 gene transcription (26). Accordingly, up-regulated UCP3 gene expression is found in muscle (27) but not in BAT (27) or WAT (this work) of ATRA-treated mice.

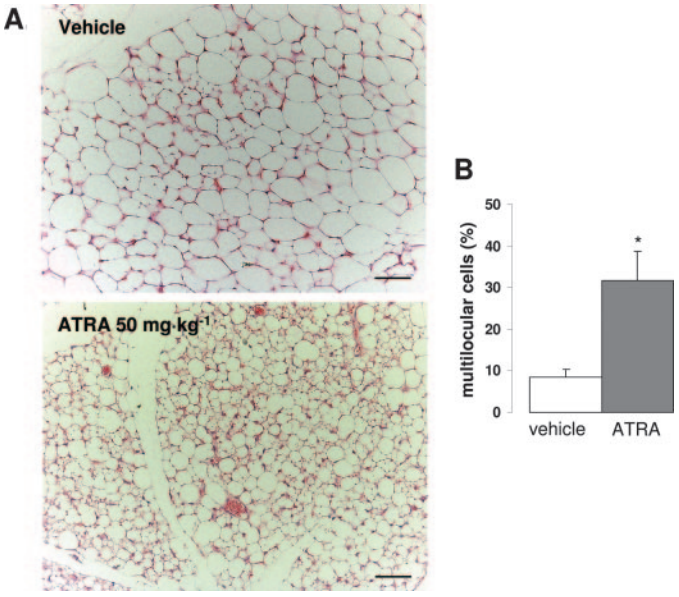

FIG. 3. ATRA treatment increases the appearance of multilocular adipocytes in inguinal WAT. Twelve-week-old NMRI male mice kept near thermoneutrality (30 C) were sc injected vehicle (olive oil) or 50 mg ATRA per kilogram of body weight per day during the 4 consecutive days before they were killed. The inguinal WAT depot was fixed, and sections were stained with hematoxylin/eosin for morphometric analysis. A, Hematoxilin/eosin representative sections of inguinal WAT of control and ATRA-treated mice, showing the appearance of multilocular adipocytes in the latter. *Scale bar*, 40 μm. B, Mean percentage of multilocular cells in inguinal WAT sections of control and ATRA-treated mice. Data are the mean ± SEM of 10 animals per group, distributed in two independent experiments. Student's *t* test significance: *, $P < 0.005$.

We have shown previously that ATRA treatment in mice reduces body weight and adiposity without changing food intake and increases the thermogenic capacity of BAT and skeletal muscle (reviewed in Ref. 12). Reduced circulating triacylglycerol levels in the face of unchanged food intake (Ref. 14 and Table 2) is also suggestive of an ATRA effect favoring net fatty acid catabolism in the liver, an aspect that is currently under investigation in our laboratory. All in all, it seems likely that ATRA favors oxidative metabolism/thermogenesis in the whole animal. Increased systemic oxidative metabolism/thermogenesis is expected to result in an elevation of body temperature and, indeed, rectal temperature was increased in mice treated with 10 and 50 mg ATRA per kilogram of body weight per day compared with control animals. The effect on rectal temperature was not seen at the highest dose tested (100 mg ATRA/kg body weight·d), which may reflect the turning on of compensatory mechanisms to avoid excessive energy waste.

Enhanced expression and activity of PPARα, which as a heterodimer with RXR transactivates a collection of genes involved in fatty acid catabolism, may be a key event in coordinating the gene program that elevates WAT catabolic capacity after ATRA treatment. CPT1 (28) and UCP1 (29) are known PPARα target genes so their increased expression in WAT depots of ATRA-treated mice may be secondary, at least in part, to ATRA-induced PPARα up-regulation. The UCP1 gene promoter contains a complex retinoic acid response element aside from a PPAR response element, and thus can also be regulated by ATRA through RAR activation, as demonstrated in brown adipocytes (30, 31). The mechanism(s) by which ATRA induces PPARα expression in WAT depots is/are not known. Of note, ATRA treatment is also likely to favor full transcriptional activity of the PPARα: RXR heterodimer in adipose depots by ensuring the provision of both fatty acid ligands of the PPARα moiety (through enhanced fat mobilization) and 9-*cis* retinoic acid, the endogenous RXR ligand, which may be produced from ATRA through isomerization. Unlike RAR:RXR and other heterodimers of RXR with nuclear receptors, PPAR:RXR are permissive heterodimers that can be activated by ligands of either partner and are synergistically activated in the presence of both ligands (32).

Enhanced expression and activity of PGC-1α may also be crucial in coordinating the gene program that elevates WAT catabolic capacity after ATRA treatment. PGC-1α enhances the transcriptional activity of PPARα on the UCP1 gene (29) and on mitochondrial fatty acid oxidation genes (33); it also coactivates other transcription factors that positively control UCP1 gene transcription, such as RARs and thyroid hormone receptor (reviewed in Ref. 21). Levels of PGC-1α are normally low in WAT and high in BAT, and forced expression of PGC-1α has been shown to induce the expression of UCP1, respiratory chain components (including subunit II of cytochrome oxidase) and fatty acid oxidation genes (including CPT1) in 3T3-L1 murine white adipocytes (21) and human sc white adipocytes (34), and to actually increase fatty acid oxidation in the latter cells (34). Moreover, different murine transgenic models of overexpression of PGC-1α in WAT

result in animals with BAT-like WAT depots, reduced adiposity and improved insulin sensitivity (see Ref. 21). ATRA might affect PGC-1α expression in WAT and other tissues through effects on cyclic AMP response element binding protein (CREB) phosphorylation; transcription of the PGC-1α gene is enhanced by phosphorylated CREB (see Ref. 21) and ATRA has been shown to induce a sustained phosphorylation of CREB in some cell systems, through activation of extracellular signal-regulated kinases 1/2 (35).

Increased levels of phosphorylated retinoblastoma protein in WAT depots of ATRA-treated mice also support a remodeling effect of ATRA on WAT depots toward the acquisition of brown fat features, because active (hypophosphorylated) retinoblastoma protein has been shown to inhibit brown adipogenesis (23). It has been suggested that absence or inactivation of retinoblastoma protein by hyperphosphorylation may provide a window permissible for brown adipocyte differentiation (23).

Cross-talk between ATRA signaling and the transcriptional machinery controlling the cellular capacity for oxidative metabolism may involve, among other mechanisms, p38 mitogen-activated protein kinase, the activity of which has been shown to be stimulated by ATRA through a nongenomic effect (36) and which has been shown to catalyze the activating phosphorylation of PPARα (37) and PGC-1α (see Ref. 21) and the inactivating phosphorylation of the retinoblastoma protein (38).

ATRA effects on lipogenic gene expression in WAT depots cannot be discarded. In fact, we previously reported 50−60% reduction in the expression levels of PPARγ in WATs of mice treated with ATRA under conditions similar to those used in this study (39). PPARγ is a PPAR isoform that is considered a master regulator of adipogenesis and lipogenic gene expression in mature adipose cells. Therefore, our results point to opposite effects of ATRA on PPARα and PPARγ expression in WAT depots, consistent with a net effect promoting fatty acid catabolism. Other previously published results, such as reduction of lipoprotein lipase activity in rodent WAT after oral retinoic acid administration (40), are also in agreement with an ATRA effect depressing WAT lipogenesis.

ATRA-induced WAT remodeling was accompanied by morphological changes, notably an increase in the number of multilocular adipocytes in the inguinal depot. Whether these multilocular cells derive from brown adipocyte precursor cells scattered within WAT, from mature white adipocytes through ATRA-induced remodeling or both has not been extensively investigated here. Even though ATRA treatment increased, to some extent, UCP1 mRNA and immunoblotting-detected protein levels in inguinal WAT, most of the multilocular inguinal adipocytes in ATRA-treated mice were negative for UCP1 by immunohistochemical analysis, pointing to dissociation between changes in morphology and UCP1 protein expression. Such a dissociation is also characteristic of β3-agonist-induced rodent WAT remodeling, where chronic treatment leads to the appearance of multilocular adipocytes with increased oxidative capacity, but most of them negative for UCP1 immunohistochemical staining (41, 42). During cold-exposure of mice, appearance of multilocular cells and increased UCP1 mRNA levels in WAT (already detected after 2 d in the cold) have been shown to precede the appearance of UCP1 protein (not seen after 2 d, but found after 10 d in the cold), suggesting that a more sustained stimulus is required for UCP1 protein expression (43).

In summary, we show in this study for the first time that ATRA, a vitamin A vitamer, favors the acquisition of BAT-like properties in WAT depots of mice, increasing oxidative capacity, multilocularity, and retinoblastoma protein phosphorylation in the context of increased fat mobilization. These effects of ATRA, together with its previously reported effects on adipokine expression and uncoupling protein expression in muscle and BAT, might contribute to the reduction of body weight and adiposity and the improvement of glucose tolerance seen in ATRA-treated mice (14, 25, 27, 39). Of note, many ATRA-sensitive parameters are found to be altered in the opposite direction in the context of obesity and insulin resistance in humans (3, 8–11), suggesting an influence of vitamin A status in these pathological conditions. Increased understanding of the mechanisms and effectors involved in the remodeling of white fat can contribute to new dietary and pharmacological avenues of prevention and treatment of obesity and type 2 diabetes.

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